Immunoregulatory Properties of Break Down Products of Human Choriogonadotropin

Immuunregulerende eigenschappen van afbraakproducten van humaan choriogonadotropine

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Dedicated to my parents and my family

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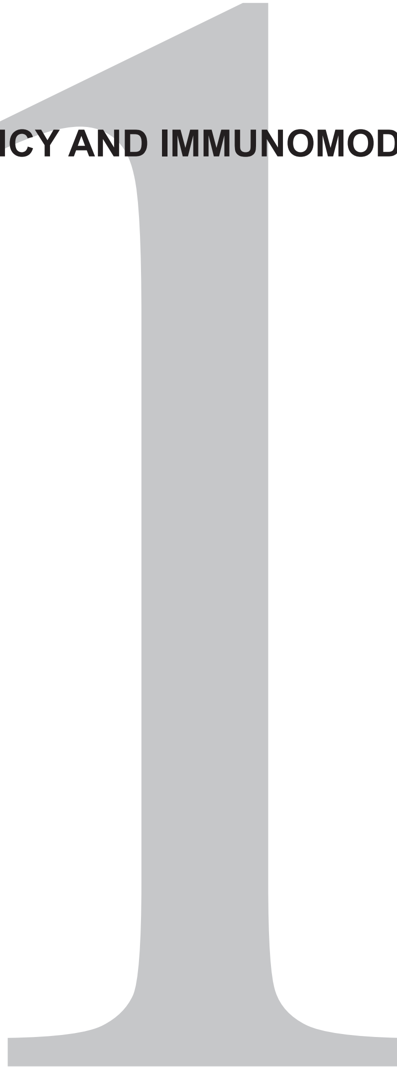

PREGNANCY AND IMMUNOMODULATION

PREGNANCY

Reproductive function in the female is cyclic. A series of functional interrelationships between the hypothalamus, the anterior pituitary, and the ovaries leads to the monthly rupture of an ovarian follicle and extrusion of an egg ("ovulation"), which is then transported to the fallopian tubes to be fertilized. Should fertilization fail to occur, menstruation ensues within 14 days and the hormonal and morphological events that led to ovulation are repeated.

Ovulation occurs around day 14 of the menstrual cycle, followed by fertilization as egg and sperm unite within 24 hours. The first three days of development occur within the fallopian tube. Upon arrival within the uterus the conceptus develops into a blastocyst (Figure 1) and begins to make mRNA for human chorionic gonadotropin (hCG), the first hormone signal from the early embryo. By day 6 after fertilization the blastocyst initiates implantation into the maternal endometrium or uterine lining. Within a few days of fertilization the blastocyst becomes a spherical structure composed of two layers. The outside layer of cells become trophoblasts and the inside of a group of cells called the inner cell mass (Figure 2A) will develop into the fetus and ultimately the baby. In addition to making hCG, the trophoblasts mediate the implantation process by attaching to, and eventually invading into the endometrium (Figure 2B). Once firmly attached to the endometrium the developing conceptus grows and continues to expand into the endometrium. One of the basic paradigms which is established even within the first week of gestation is that the embryonic/fetal cells are always separated from maternal tissues and blood by a layer of cytotrophoblasts (mononuclear trophoblasts) and syncytiotrophoblasts (multinucleated trophoblasts) (Figure 2C-F). This is critical not only for nutrient exchange, but also to protect the developing fetus from maternal immunologic attack (1).

Implantation is regulated by a complex interplay between trophoblasts and endometrium. On the one hand trophoblasts have a potent invasive capacity and if allowed to invade unchecked, spread throughout the uterus. The endometrium, on the other hand, controls trophoblast invasion by secreting locally acting factors (i.e. cytokines and protease inhibitors), which modulate trophoblast invasion. Within the placenta the syncytiotrophoblasts generate high levels of hCG which modulates cytotrophoblast differentiation towards a non-invasive hormone secreting villous-type trophoblast. The closer the trophoblasts are to the endometrium the less hCG is made, allowing the trophoblasts to differentiate into anchoring type cells (2). Trophoblasts that leave the placenta and migrate within the endo and myometrium are induced to make proteases and protease inhibitors, to further facilitate trophoblast invasion into the maternal tissues (2). Ultimately, normal implantation and placentation is a balance between regulatory gradients created by both the trophoblasts and the endometrium.

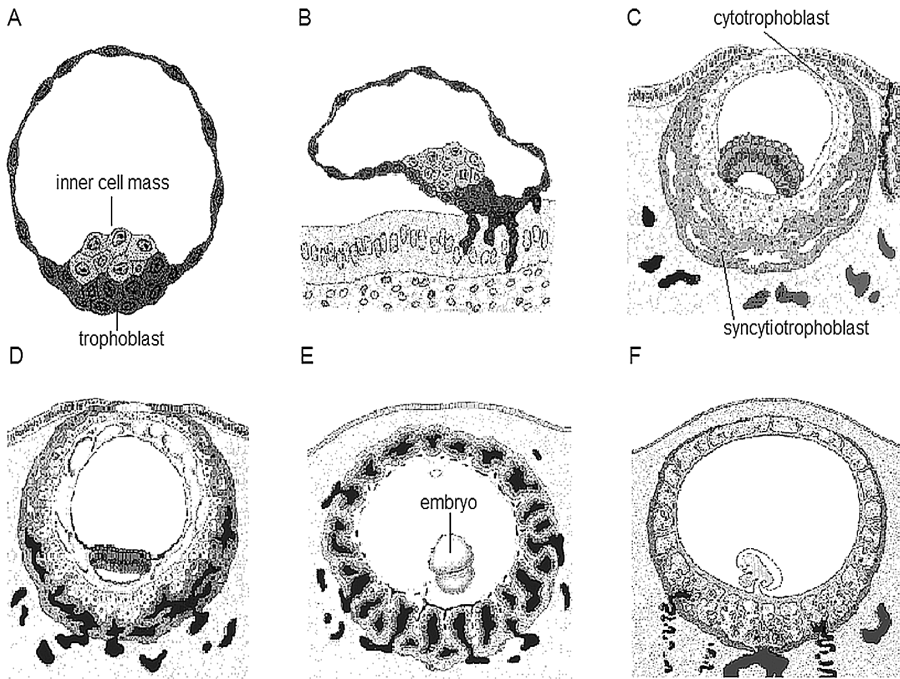

Figure 1.
(**A**) Blastocyst. By 4-5 days after fertilization the embryo has differentiated into two distinct cell types: inner cell mass (lighter cells) and trophoblast (darker cells). (**B**) Implantation. The blastocyst becomes attached to the uterine lining (endometrium) and trophoblasts begin to invade into the endometrium, beginning the process of placentation. (**C**) Implantation site. By day 9 the embryo is surrounded by two layers of trophoblasts: the inner mononuclear cytotrophoblasts and the outer multinucleated syncytiotrophoblast layer. (**D**) Implantation site: The invading trophoblasts penetrate the maternal vessels, forming pools of maternal blood which surround the growing trophoblasts. (**E**) Implantation. The embryo has begun to make an early circulatory system. The embryonic tissue and maternal blood are separated by a layer of cytotrophoblasts and syncytiotrophoblasts. (**F**). Implantation. The basic structure of the placenta has been formed with maternal blood cells being delivered to the forming placenta via spiral arteries while being drained away via uterine veins. Like the roots of a tree, the developing chorionic villi remain immersed in a space filled with nutrient rich maternal blood (Modified from Sadler TW, Langman's medical Embryology, 5th edition, Williams & Wilkins, 1985).

Interestingly, studies have shown that the endometrium of women during the menstrual cyclic secretory phase (progesterone dominant phase) also express, produce and secrete hCG hormone in the glandular and luminal epithelium (3,4). This modest local epithelial hCG synthesis by healthy secretory endometrium in connection with intensive subepithelial vascularization and stromal natural killer (NK) cell accumulation

lasts until the late secretory phase (3,4). The physiological role of this hCG secretion is to suppress cellular apoptosis in endometrial gland epithelial and stroma cells, which also explains the absence of apoptosis in the decidual epithelium during early gestation with high hCG concentrations (5). In addition, hCG can directly trigger the angiogenic activities of the endometrium for increased capillary function (6,7). Different *in vivo* and *in vitro* methods confirm the initiation of angiogenesis in response to hCG (8,9). Therefore, the modest endogenous endometrial hCG release from the gland epithelium can play a direct or indirect role in the regulation of angiogenic factors and can contribute to the initiation of microvascularization to prepare for a receptive endometrium during healthy menstrual cycle.

Another glycoprotein produced by the syncytiotrophoblast and made throughout gestation is human placental lactogen (hPL). hPL appears to regulate the lipid and carbohydrate metabolism of the mother. In addition to the hPL the syncytiotrophoblasts produce prolactin, relaxin and chorionic adrenocorticotropin. The physiological role of these hormones represents a shift from maternal to placental control. The trophoblasts also appear to produce a number of hypothalamic-pituitary hormones, including gonadotropin releasing hormone (GnRH) and corticotropin releasing hormone (CRH). In addition to the hypothalamic factors GnRH and CRH, pituitary hormones, including hCG, growth hormone (GH) and adrenocorticotropic hormone (ACTH) are also synthesized and secreted by cultured trophoblasts (10). It appears that the placenta, in addition to replacing much of the women's pituitary function during pregnancy, also replaces critical hypothalamic functions so as to maintain control and feedback loop mechanisms close to the conceptus. Table 1 outlines the various hormones and hormone-like molecules associated with human pregnancy. These hormones are produced by three different compartments (maternal, placental and fetal).

EFFECTS OF PREGNANCY ON THE IMMUNE SYSTEM

Pregnancy is a unique physiological event in which mother and child are exposed to each others human leukocyte antigens (HLA) and other molecules. How the HLA-mismatched fetus escapes rejection remains a biological enigma. Medawar was the first who discussed this problem in immunological terms. He proposed five main theories for the survival of the fetal allograft. Medawar's theories were, that the conceptus was antigenically immature; the intrauterine site was immunologically privileged; the placenta was a barrier; the pregnant mother became immunologically incompetent; or a serum blocking factor was present (11). Each theory was disproven.

To maintain a successful pregnancy, the maternal host must somehow bypass or compensate for the usual immunological processes developed to ensure recognition

Table 1. Pregnancy specific protein hormones by compartment.
GnRH, gonadotropin releasing hormone; CRH, corticotropin releasing hormone; TRH, thyrotropin releasing hormone; GHRH, growth hormone releasing hormone; hCG, human choriogonadotropin hormone; hGH, human growth hormone; ACTH, adrenocorticotropic hormone; hPL, human placental lactogen; hCT, human calcitonin; IGF-I/II, insulin-like growth factor I/II; PAPP-A, pregnancy associated plasma protein A; IGFBP-1, insulin-like growth factor binding protein 1; PP14, placental protein 14.

Maternal	Placental	Fetal
Decidual derived - Prolactin - IGFBP-1 - PP14 Corpus luteum derived - Relaxin	Hypothalamic-like (cytotrophoblast) - GnRH - CRH - TRH - GHRH - Somatostatin Pituitary like (syncytiotrophoblast) - hCG - hGH - ACTH - hPL - hCT - Oxytocin Growth Factors - Inhibine - Activin - IGF-I/IGF-II Other Proteins - Pregnancy specific β1-glycoprotein - PAPP-A	Alpha-fetoprotein

and elimination of non-self molecules (11). Therefore, the maternal immune system as a whole is to be modulated in several ways during pregnancy. A successful pregnancy is thus dependent on maternal tolerance or immune-nonreactivity to the paternal antigens inherited by the fetus. The protection begins with the trophoblast cells that define the boundary between the mother and fetus (12). These cells are derived from the outer layer of the blastocyst and, if female, have an inactivated paternal X chromosme (13). The trophoblast cell membrane is restricted in expressing the cell surface antigens that normally stimulate T-cell-dependent graft rejection responses, i.e. HLA-A, -B (class I antigens), and –D (class II antigens). Instead, trophoblast cells express a unique combination of HLA-C, HLA-E and HLA-G, which interact with uterine NK cells and decidual macrophages (14,15) to prevent NK cytotoxicity towards the trophoblast cells and to suppress CD8+ and CD4+ T cells (14,16).

Wegmann has proposed that there is a bi-directional interaction between the maternal immune system and the reproductive system ("feto-placental unit") which may result in the redirection of maternal immunity away from cell-mediated immunity and toward enhanced humoral responsiveness (17). In this model the conceptus protects itself by secreting T-helper-2 (TH2) cytokines (humoral immunity) such as interleukin-4 (IL-4), IL-5, IL-10, IL-13 and granulocyte macrophage colony stimulating factor (GM-CSF) that down-regulate T-helper-1 (TH1) cytokines (cell mediated immunity) such as IL-1, IL-2, IL-6, IL-12, IL-15, IL-18, interferon-γ (IFN-γ), and tumor necrosis factor-alpha (TNF-α) (18), which are generally harmful to the maintenance of pregnancy (19,20). Local T cells produce such cytokines, but major sites of TH2 cytokine production appear to be nonlymphoid tissues including the placental/decidual tissues, particularly the trophoblast (21). During the normal pregnancy, the TH1/TH2 activity balance is strongly shifted toward TH2 activity (so called "TH2 phenomenon"), and this has been said to play a protective role in the feto-maternal relationship. In normal pregnancy peripheral and local T cells are increased. However, peripheral T cells express TH1 cytokines, whereas T cells located in the decidua primarily express TH2 cytokines (22). Since progesterone can induce T cell differentiation towards the TH2 pathway, this has been associated with the induction of a TH2 cytokine profile *in utero* (23). Since non-haematopoietic cells such as trophoblasts and epithelial cells can also produce cytokines, it is unclear how such a TH2 bias may be induced (24,25). Progesterone may act on progesterone receptor-positive cells such as macrophages and glandular epithelial cells and/or may affect other cell types as well. Inflammation and infection can alter the balance of TH1 and TH2 cytokines causing a shift toward a TH1 predominance, that initiates and intensifies the cascade of inflammatory cytokine production involved in spontaneous abortion, preterm delivery and/or preeclampsia. The bias of immune responses to TH1 or TH2 is not limited to T-helper-cells and their responses, but also includes cells of the innate immune system such as natural killer T (NKT) cells (26). NKT cells are characterized by a fixed repertoire of T cell receptors as well as having clear NK cell attributes. There is evidence that the bias that TH cells adapt depends on the cytokine milieu created by NKT cells, which appear to play a dominant role in immunoregulation (27). Rather than discussing a TH1 or TH2 bias, which implies a key role for T-helper-cells, it is better to refer to the type 1 or type 2 bias to include the innate as well as adaptive immune responses which acknowledges the potential role of NKT cells.

Other subsets of cells involved in successful pregnancy are NK cells and macrophages. NK cells are a key component of innate immunity, particularly crucial during the early phase of immune responses against certain viruses, parasites and microbial pathogens. During early pregnancy, transformation of the non-pregnant endometrium into decidua is characterized by a dominant mature, granulated NK cell population. These uterine NK (uNK) cells in the pregnant uterus appear to retain cytolytic

activity but also strongly produce cytokines, especially IFN-γ. The interaction of NK cell receptors with HLA-A, -B and C, HLA-E and HLA-G on extravillous cytotrophoblasts is suggested to inhibit the cytotoxic activity of these cells (17,28). Moreover, uNK interactions with extravillous trophoblasts are clearly important in the precise control of decidual trophoblast invasion which, when impaired, may lead to shallow invasion and pre-eclampsia (28). In addition, the interactions of uNK cells with activated macrophages may induce NK cell cytokine production (IFN-γ and TNF-α) that leads to fetal loss (29). In a normal pregnancy resident decidual macrophages tend to produce prostaglandin E2 (PGE2), which acts to suppress cell-mediated immune responses by inhibiting antigen presenting cells (APC) production of IL-12 (27). Therefore, there must be an alternative interaction in the decidua with macrophages being replaced by trophoblasts. Indeed, trophoblasts activated by infection, potentially through Toll-like receptors (TLR) (30,31), release neutrophil chemoattractants, leading to a localized accumulation of neutrophils, which then act as antimicrobial effectors and probably also abortion effectors (32). Thus, trophoblasts are postulated to be part of the pregnancy-specific innate immune defence system (33,34).

EFFECTS OF PREGNANCY ON AUTOIMMUNE DISEASES

Since 1990, the central dogma of immunology has been that the immune system does not normally react to self. It has also become apparent that autoimmune responses are not as rare as once thought and that not all autoimmune responses are harmful. Some responses play a distinct role in mediating the immune responses in general. For example, certain forms of autoimmune response such as recognition of cell surface antigens encoded by the major histocompatibility complex (MHC) and anti-idiotypic responses against self idiotypes are considered to be essential for the diversification and normal functioning of the intact immune system (35). Apparently, an intricate system of checks and balances is maintained between the various subsets of cells of the immune system, thereby providing the individual with an immune system capable of coping with foreign invaders. In that sense, autoimmunity plays a regulating role in the immune system.

It has now also been recognized that an abnormal autoimmune response is sometimes a primary cause and at other times a secondary contributor to many human and animal diseases. Types of autoimmune disease frequently overlap, and more than one autoimmune disorder tends to occur in the same individual, especially in those with autoimmune endocrinopathies (36). Autoimmune diseases, such as systemic lupus erythematosus (SLE), diabetes, rheumatoid arthritis (RA), post-partum thyroid dysfunction and autoimmune thrombocytopenia (ATP) are characterized by autoimmune

responses directed against widely distributed self-antigenic determinants, or directed against organ- or tissue specific antigens (37). Such disease may follow an abnormal immune responses against only one antigenic target, or against many self antigens. In many instances, it is not clear whether autoimmune responses are directed against unmodified self-antigens or self-antigens that have been modified by (or resemble) any of numerous agents such as viruses, bacterial antigens and haptenic groups (36,37).

Autoimmune diseases are a heterogeneous group of disorders whose expression is influenced by multiple genes and environmental factors. Disease expression is also affected by age, gender, and reproductive status (38). For example, onset of autoimmune thyroid disease (ATD) and RA is most common in women around menopause. Both ATD and RA tend to improve during pregnancy but commonly flare up or develop initially in the postpartum period (39). In contrast to RA and ATD, the age of onset of SLE in women is highest during the reproductive years and declines markedly around the menopause. In addition, SLE, particularly the immune-complex-mediated glomerulonephritis, tends to worsen during pregnancy but may improve in the postpartum period (38,40). Similarly, antibody-mediated diseases such as ATP and autoimmune hemolytic anemia (AHA), which may be initial manifestations of SLE, frequently develop during pregnancy and may improve in the postpartum period (41,42).

Although no autoimmune disease can be considered exclusively TH1 or TH2 cytokine-driven, it is generally agreed that the clinical manifestations ATD and RA primarily reflect nonspecific macrophage and specific cell-mediated immunopathological mechanisms. TH1 cytokines, such as TNF-α and IL-1α, are most characteristically expressed at high levels in these diseases (43). In contrast, SLE, ATP, and AHA, in large part, are considered to be antibody-mediated diseases dependent on TH2 cytokines such as IL-10 (38). Thus, it can be proposed that diseases such as RA improve during pregnancy because of the suppression of non-specific and cell-mediated immunity. Moreover, RA may flare in the postpartum period because of the restoration of the capacity to produce normal, or more likely supranormal, levels of TH1 cytokines. Conversely, the development of antibody-mediated autoimmune diseases during pregnancy such as SLE and their tendency to improve in the postpartum period may represent the antithesis of the regulatory mechanisms operative in RA.

Thus, these autoimmune conditions show striking differences in the ages that are most commonly associated with their onset and their expression during pregnancy and the postpartum period. Each of these illnesses is much more common in women than men; that is, female-to-male ratios are about 19:1 in ATD, 3-4:1 in RA, and 9:1 in SLE (38). These contrasting clinical observations clearly suggest that hormones are involved in the pathogenesis of these various autoimmune diseases, although mechanisms are undoubtedly complex. The changing expression of human autoimmune diseases during pregnancy and the postpartum period is regulated, in

part, by hormonal modulation of the proinflammatory TH1 to the antiinflammatory TH2 cytokines balance (38). Further investigations are required to increase our insight into the relationship between pregnancy and autoimmune diseases expression.

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2

HETEROGENEITY IN THE BIOLOGICAL FUNCTION AND STRUCTURE OF hCG

BACKGROUND

In 1920 human chorionic gonadotropin (hCG) was the first protein hormone described and isolated from the placenta. The sole function of hCG has been then assumed to be the promotion of progesterone production by corpus luteal cells. The first pregnancy test, the rabbit test, was formulated in the 1920s and for four decades bioassays such as the rabbit test were the only practical way to measure hCG or detect pregnancy. In 1960s the first polyclonal antibodies against hCG and subsequently the competitive immunoassay was developed. This was the first rapid and sensitive test for hCG and led to the development of commercial hCG tests as seen today. In the past four decades a revolution has occurred in knowledge about this molecule through the finding that hCG is not just one biologically active molecule. Instead, hCG consists of a group of biologically important molecules having different physiological functions. Recently, several investigators such as De Medeiros, Cole and Birken extensively reviewed the function of the various biologically active forms of hCG (1-5) that we have summarized in this chapter.

CHEMISTRY AND STRUCTURE OF hCG

hCG is a member of a family of four closely related glycoprotein hormones (6). Three of the hormones, luteinizing hormone (LH), follicle-stimulating hormone (FSH) and hCG control aspects of reproduction, while thyroid-stimulating hormone (TSH) regulates metabolism. These four hormones are produced within the pituitary gland. hCG, however, is produced primarily by the trophoblasts that are destined to become placental tissue. It has been demonstrated that next to the pituitary, endometrial epithelial cells and certain trophoblastic and nontrophoblastic tumors produce hCG (7,8).

hCG has a molecular weight of approximately 38,000 Dalton with 237 amino acids organized in two dissimilar noncovalently linked subunits designated alpha (α) and beta (β) (9,10). Both subunits consist of a single polypeptide chain. The α-subunit, consisting of 92 amino acids and encoded by a single gene, is common to all four glycoprotein hormones (11,12). The ß-subunit of the four glycoprotein hormones arises from separate genes and defines hormone specificity (13,14). The β-subunit of the glycoprotein hormones displays various degrees of homology, which between hCG and LH is ~80% (6). The ß-subunit of LH contains 121 amino acids whereas β-subunit of hCG (β-hCG) contains 145 amino acids, the difference being due to a 24-amino-acid extension, the so-called C-terminal peptide (CTP) (6). Seventy percent of hCG consists of the protein chains and 30% of carbohydrate units.

The sugar branches, covalently bound to the peptide chains, are of two types: O-linked oligosaccharide containing an N-acetylgalactosamine residue linked to either a serine or a threonine residue and an N-linked oligosaccharide containing an N-acetylgalactosamine residue linked to an asparagine residue (15-17).

Six carbohydrate moieties are attached to β-hCG and two to α-hCG (Figure 1). The N-linked carbohydrate chains on α-hCG are attached to Asn52 and Asn78 and those on β-hCG to Asn13 and Asn30. Four O-linked oligosaccharides are attached to Ser121, Ser127, Ser132 and Ser138 in the CTP of β-hCG (15-17). Owing to variation in the content of terminal sialic acid, hCG displays extensive charge heterogeneity with isoelectric point (pI) values ranging from 3 to 7. β-hCG is more acidic (pI range 3–5) than α-hCG (pI range 5–8) (18,19).

α-subunit, 92 amino acids																										
M	D	Y	Y	R	K	Y	A	A	I	F	L	V	T	L	S	V	F	L	H	V	L	H	S	A	P	D
																								1		
V	Q	D	C	P	E	C	T	L	Q	E	N	P	F	F	S	Q	P	G	A	P	I	L	Q	C	M	G
4																										
																					N					
C	C	F	S	R	A	Y	P	T	P	I	R	S	K	K	T	M	I	V	Q	K	N	V	T	S	E	S
31																					52					
																			N							
T	C	C	V	A	K	S	Y	N	R	V	T	V	M	G	G	F	K	V	N	H	T	A	C	H	C	S
58																			78							
T	C	Y	Y	H	K	S																				
86						92																				

β-subunit, 145 amino acids																										
M	E	M	F	Q	G	L	L	L	L	L	L	L	S	M	G	G	T	W	A	S	K	E	P	L	R	P
																				1						
					N																	**N**				
R	C	R	P	I	N	A	T	L	A	V	E	K	E	G	C	P	V	C	I	T	V	N	T	T	I	C
8					13																	30				
A	G	Y	C	P	T	M	T	R	V	L	Q	G	V	L	P	A	L	P	Q	V	V	C	N	Y	R	D
35																										
V	R	F	E	S	I	R	L	P	G	C	P	R	G	V	N	P	V	V	S	Y	A	V	A	L	S	C
62																										
Q	C	A	L	C	R	R	S	T	T	D	C	G	G	P	K	D	H	P	L	T	C	D	D	P	R	F
89																										
					O						**O**					**O**						**O**				
Q	D	S	S	S	S	K	A	P	P	P	S	L	P	S	P	S	R	L	P	G	P	S	D	T	P	I
116					121						127					132						138				
L	P	Q																								
143		145																								

Figure 1. Amino acid sequence of hCG α-subunit and β-subunit.
Digits indicate amino acid residue positions and N and O indicate the positions of N- and O-linked oligosaccharides. Gray area indicates the residues belonging to signal peptide.

α-hCG is structured in three loops stabilized by disulfide bonds. These bonds, initially assigned between residues 7–31, 10–32, 28–60, 59–87 and 82–84 (20), were reassigned at positions 7–31, 59–87, 10–60, 28–82 and 32–84 (21,22) (Figure 2A). The last three bonds comprise the cystine knot which is a characteristic signature of polypeptide growth factors controlling cell survival, proliferation and differentiation. The two N-linked complex-type carbohydrate moieties are attached to asparagine

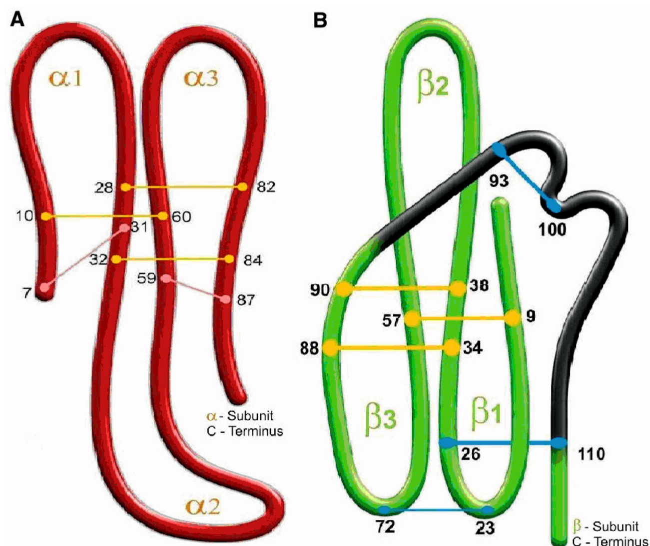

Figure 2.
(**A**) α-hCG protein core (thick red line) and assigned disulfide bonds (thin yellow and light pink line) forming three hairpin loops (α1, α2, α3). (**B**) β-hCG subunit polypeptide chain (thick green line) with its seatbelt region (thick black line) and assigned disulfide bonds. Numbers represent amino acid numbers; yellow bars, cystine knots; thick blue lines, seatbelt disulfide bonds; thin blue line, small loop disulfide bond; β1, β2, β3 loops of β-hCG (modified from Xing *et al. Protein Sci* 2001;10:226-235).

residues in the second and third loops. The β-hCG has a highly glycosylated CTP extension, rich in serine and proline, which confers the immunological and biological specificity to the whole hCG (Figure 1). The β-hCG disulfide bonds initially assigned at positions 9–90, 26–110, 34–88, 32–72, 38–57 and 93–100 (20) were further reassigned at positions 23–72, 26–110, 34–88, 38–90, 9–57 and 93–100 (21) (Figure 2B). The β-hCG molecule has two beta hairpin loops, stabilized by the disulfide bond 23–72 on one side of a central cystine knot and a long loop on the other side (22). The cystine knot is formed by a ring (residues β34–88 and β38–90 bridged by disulfide bonds) through which the β9–57 disulfide bridge penetrates. In its carbohydrate branches, hCG has asparagine units on both the α and β subunit and unique O-glycosydically linked oligosaccharide moieties on β-hCG.

BIOSYNTHESIS OF hCG

Both biosynthesis and processing of the hCG molecule resemble that of other glycoprotein hormones. Although the cell type determines the sort of oligosaccharide processing on alpha and beta subunits, the alpha–beta combination modulates the extension of this processing (23). The set of enzymes contained in the secretory cell determines the nature of the processing and composition of the oligosaccharides added to both subunits. Initially, each subunit is synthesized separately, in a non-balanced ratio, as a result of mRNA transcription of the two separate genes. The alpha subunit is usually synthesized in excess. In pregnant women, the ratio α:β subunits increase from 1.7:1.0 in the first trimester to 12.0:1.0 at term (24). The metabolic sequence, cleavage of signal peptides, assembly of the native hCG, sequential post-translational glycosylation and formation of the disulfide bonds happen at the same time that the hCG molecules are translocated from the place of synthesis in the rough endoplasmatic reticulum (RER) up to the cell surface (25).

While the α-subunit is encoded by a single gene (26), the β-subunit is encoded by a family of at least six genes arranged in tandem in a cluster on chromosome 19 (27). Initially, it was believed that only three β-hCG genes are functional genes encoding the correct β-hCG amino acid sequence. Currently it is suggested that the total amount of β-hCG gene expression rather than the expression of individual genes is important for the maintenance of normal pregnancy (28). The α and β genes *expression* may be regulated by many factors, but a key role in the control of their expression is not clear yet.

Also hCG *secretion* is under the control of a large number of factors which may act by both autocrine and paracrine mechanisms (29). On placental trophoblastic cells, these modulators interact with specific surface receptors. Upon stimulation by these

factors, α-hCG and β-hCG genes are activated by phosphorylation of a cAMP response element binding protein (CREBP) through the protein kinase C (PKC) pathway. It has been shown that α-hCG and β-hCG promoters also have binding sites for the activator protein 2 (AP-2), which suggests that this transcription factor provides a mechanism for coordinating the induction of these genes during placental cell differentiation. AP-2 might be the ultimate regulator of hCG gene expression at the promoter region (30). After transcription of the genetic message, the translation of the mRNA into the nascent α and β subunits takes place in the membrane-bound ribosomes of the RER. Each translated product is synthesized as a slightly immature peptide, named pre-α and pre-β forms, containing the specific sequences and the signal peptide extension with 24 and 20 amino acids, respectively (Figure 1). The processing of immature subunits to the mature state involves co-translational signal peptide cleavage and removal by microsomal signal peptidases while the peptide chains still reside upon the ribosomes. Just before the oligosaccharide moieties are attached to the polypeptide chain, the newly synthesized protein is released into RER channels and transported to the Golgi (31).

The association of α-hCG and β-hCG subunits form the complete dimer. Both subunits than still contain a high content of mannose. The two subunits are intimately associated with each other along much of their surfaces, each subunit having similar folds with two hairpin loops at one end and a single loop at the other (32). The assembly of hCG in the RER is made by threading the glycosylated end of α-hCG loop 2 beneath a hole formed in a disulfide latched strand of the β-subunit named seatbelt (33). The CTP of β-hCG is in contact with α-hCG in the native dimer and forms the seatbelt around the α-hCG (residues β93–110) that stabilizes the heterodimer. The final closing of the β26–110 bridge locks the seatbelt and secures the αβ dimer, preventing disassembly (34).

SECRETION OF hCG

After fertilization, the embryo produces hCG early in development. hCG is already detected at the 4- to 8-cell stage and is secreted from blastocysts by 7 days after fertilization. hCG can be detected in maternal serum near the time of implantation and it is routinely used to detect pregnancy. Serum concentrations of hCG rise rapidly after implantation with an average doubling time of about 31 h. After the first trimester, hCG levels decline before reaching a low plateau that persists throughout the remainder of pregnancy. Although the cellular mechanisms that lead to the onset of hCG gene transcription are not understood yet, it is likely that the α-hCG and β-hCG-genes are among the first embryo-specific genes to be transcribed. The mechanisms that result in

deactivation of hCG gene expression after the first trimester are also unknown.

The secretion of hCG shows spontaneous pulse-like bursts with irregular amplitudes and frequencies. The finely tuned and dynamic pattern of hCG secretion may involve an up- and down-regulation of the gonadotropin-releasing hormone (GRH) receptor (36). The process involves a serial of steps in which cells make up and release hCG, and α-hCG and β-hCG free subunits. There is no releasing factor known specific to hCG, although cAMP activates the genetic transcription of DNA sequences for synthesis of the polypeptide chains. The processing of the carbohydrate chains, their transport to the Golgi for attachment to the α and β chains, αβ combination and the release of hCG from the cell surface into the circulation are not completely clear. The primary signal for the relevant enzyme expression can be stimulated by cAMP analogs, epidermal growth factor, TNF-α, GRH, estradiol, insulin, and glucocorticoids (36,37). The principal modulators with inhibitory activity are progestational steroids and prolactin (38,39). Table 1 shows several regulating factors that seem to be involved in the hCG secretion.

hCG predominates in all normal and abnormal pregnancies. It is present at reduced levels during spontaneous abortion and ectopic pregnancy (64) and at double regular levels in Down syndrome pregnancy (65,66). hCG is also the principal molecule produced in individuals with hydatidiform moles or pregnancies comprising solely trophoblast tissue (17,67). Regular hCG, which is normal glycosylated heterodimeric hCG, is also normally produced by the pituitary gland at the time that LH peaks and in menopausal women (7,67).

Multiple variants of hCG have been detected in serum and urine samples (Figure 3). Free α-subunits of hCG and free β-subunits of hCG have been demonstrated in pregnancy serum, cancer patient serum and urine, and cancer cell line culture fluids (68,69). A β-subunit core fragment, which comprises two fragments of β-subunit, β6–40 and β55–92, held together by disulfide bonds, is found in pregnancy urine samples (70). Urine β-core fragment is used as a general tumor marker for all non-gestational malignancies (71-75). A large form of hCG dimer is synthesized by choriocarcinoma cells (17,76,77). This is hyperglycosylated hCG with 1.5 fold larger N-linked and double size O-linked oligosaccharides (17,78,79). Similar large N- and O-linked oligosaccharides have been demonstrated on the free β-hCG named hyperglycosylated free β-hCG which is produced by non-gestational cancer cells (77,80). Similar large oligosaccharides are found on the α-subunit secreted in pregnancy, free α-subunit (81). While hCG is hyperglycosylated in choriocarcinoma and gets larger oligosaccharide side chains, free α-subunit is also glycosylated in malignancy, gaining an additional O-linked oligosaccharide and called O-glycosylated free α-subunit (82).

The regular hCG and hyperglycosylated hCG degradation process involve elastase and other proteases secreted by macrophages associated with tumor tissue or present in

Table 1. Endocrine and paracrine/autocrine regulators of trophoblast hCG secretion in vitro, according to the pregnancy time (38-63).
EGF, epidermal growth factor; LIF, leukemia inhibiting factor; TNF, tumor necrosis factor; M-CSF, macrophage-colony-stimulating factor; TGF, transforming growth factor; GABA, gamma amino butyric acid; PTH, parathyroid hormone; hGH, human growth hormone.

Factor	Trimester	Effect on hCG biosynthesis	Factor	Trimester	Effect on hCG biosynthesis
hCG	Third	Stimulates	TNF-α	First	Stimulates
GnRH	First, Third	Stimulates	M-CSF	First	Stimulates
GnRH-antagonist	First	Inhibits	Leptin	Third	Stimulates
cAMP	Third	Stimulates	TGF	First	Inhibits
β adrenergic	First	Stimulates	Mifepristone	First	Inhibits
Dexamethasone	Third	Stimulates	Glycodelin A	First	Stimulates
Activin	Third	Potentiates GnRH	17β-estradiol	First	Inhibits
EGF	First, Third	Stimulates	GABA	First	Stimulates
Interleukin-1	First	Stimulates	PTH	First	Stimulates
Interleukin-6	First	Stimulates	Opioids	First	Stimulates
Inhibin	Third	Inhibits	Insulin	First	Inhibits
Progesterone	First	Inhibits	HGH	Third	Stimulates
Prolactin	Third	Inhibits	H_2O_2	Third	Stimulates
LIF	Third	Inhibits	Triiodothyronine	First	Stimulates

the circulation (83). These proteases cleave or nick hCG at β-subunit 44-45, 47-48 or 53-54 (17,83), generating a nicked hCG and a nicked hyperglycosylated hCG, a nicked free β-hCG and nicked hyperglycosylated free β-hCG (Figure 3). These enzymes progress further to cleave and release the C-terminal peptide on the nicked molecules (βCTP, β residues 93–145) generating nicked hCG missing βCTP, nicked hyperglycosylated hCG missing βCTP, nicked free β-hCG missing βCTP and nicked hyperglycosylated free β-hCG missing βCTP (17,67,83). These are 15 variants, all found in serum and urine samples in normal or abnormal pregnancies, gestational trophoblastic diseases or non-gestational malignancies (Figure 3). Of these 15 variants, only 5 are natural products made by the placenta in pregnancy or by non-trophoblastic malignancies,

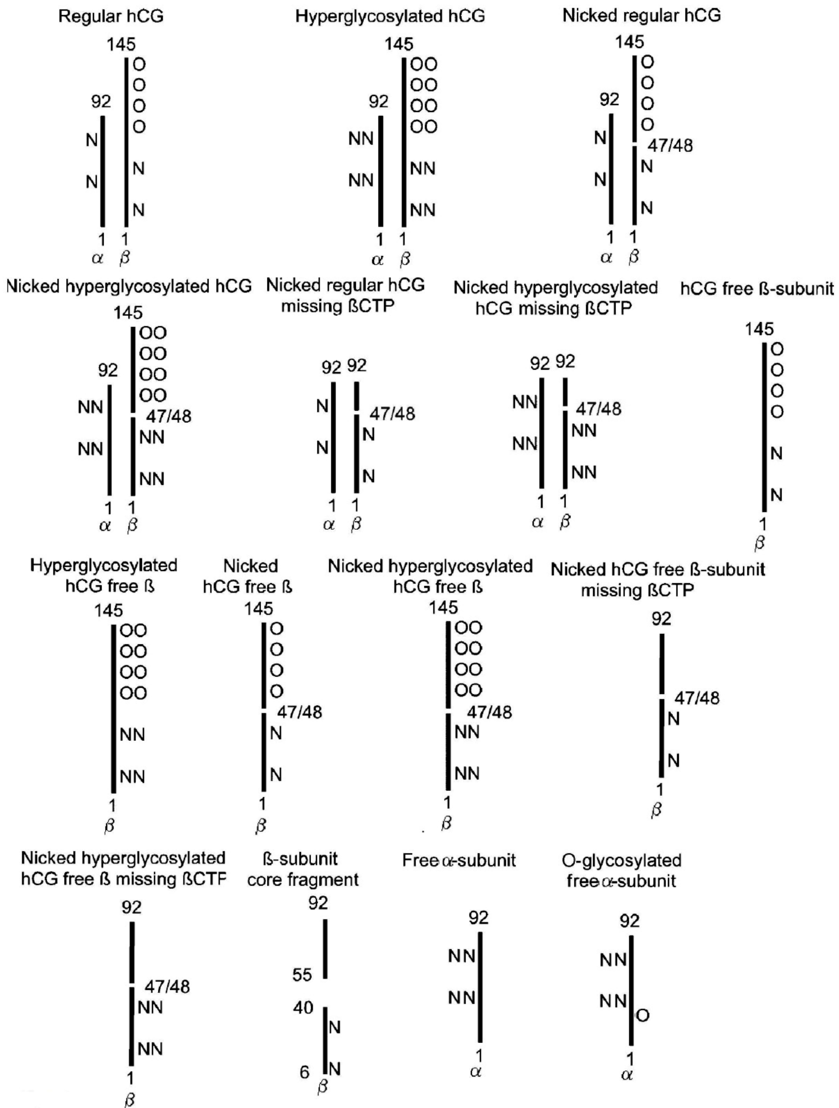

Figure 3. 15 common hCG variants present in serum and urine samples in either pregnancy, gestational trophoblastic disease or malignancy.
Numbers refer to subunit polypeptide amino acid numbers (1 and 145 in the 145 amino acid long β-subunit), O refers to O-linked and N to N-linked oligosaccharides. OO and NN refer to large or hyperglycosylated oligosaccharides. α is α-subunit and β is β-subunit. βCTP is the C-terminal segment (residues 93–145) of the regular or hyperglycosylated hCG β-subunit (from Cole LA. Reprod Biol Endocrinol. 7:1-37, 2009).

regular hCG, hyperglycosylated hCG, hyperglycosylated free β-hCG and free α-subunit and O-glycosylated free α-subunit. The other 10 variants are degradational products originating from macrophage cleavage and cleavage by proteases in the circulation and the kidney. It is also noteworthy that free α-subunit has no known biological function. Free α-subunit is considered a biological waste product (82).

In the kidney, the hCG is internalized by proximal renal tubule cells and degraded to small fragments (84), but a significant part is excreted unaltered by passage through the tubule to the collecting duct. The molecules more rich in sialic acid are eliminated more quickly (85). Desialylated hCG passes through the glomerules to the urine, or alternatively, the tubule cells take them up more quickly (86). Owing to proteolytic processing of hCG as it passes through the kidney, the urine contains a much greater variety of forms of hCG than does the blood.

The liver accumulates hCG molecules as soon as 2 h after injection of this gonadotropin. The concentration of hCG in the liver is about 5-fold lower than in the kidney (84). Removal of sialic acid increases the clearance of hCG up to 200 times (87). A large proportion of hCG does not contain the sialic acid, and these desialylated forms exposing galactose residues bind to high affinity hepatocyte receptors for galactose-terminated oligosaccharides, being further distributed in the interior of the hepatocytes (88). Native hCG is taken up primarily by Kuppfer cells in liver tissue and distributed along the sinusoides (86).

In the ovary, variable amounts of hCG molecules are internalized by granulosa/theca lutein cells (89). These cells may degrade it to small fragments (90). Initially, it was thought that the uptake of hCG was limited to the availability of specific receptors, but further studies have found that in ovarian tissue hCG can be degraded either after binding to its specific receptor or following an alternative mechanism of uptake (91). The intracellular fate of hCG in granulosa cells is not completely clear; it is probably transported to lysosomes and degraded to small molecules or fragments there (92). Human granulosa cells incorporate and degrade intact hCG to β-core fragment, even after being previously exposed to hCG *in vivo* (93). In addition, after injection of hCG, β-core fragment can be detected in follicular fluid 34–36 hours later. This suggests that either the β-core fragment accumulates in this biological compartment or represents a pool of fragments of the different gonadotropins cross-reacting with the β-core assays (94).

BIOLOGICAL ACTIVITY OF hCG

hCG and LH bind to the same specific receptor on the target cell (115). For a long time, it was believed that hCG receptors were localized and had an effect on gonadal tissues only. Many studies have characterized functional hCG/LH receptors in

several nongonadal tissues including placenta and fetal membranes, sperm, prostate, myometrium, fallopian tube, breast, skin, adrenals, thyroid, vessels, lymphocytes and the central nervous system (35,95-100).

The hCG/LH receptor is encoded by a single gene, located on human chromosome 2p21. This receptor belongs to the superfamily of G-protein-coupled seven transmembrane (TM) domain receptors (101). The gene codes for a protein receptor containing 701 amino acids structured in three distinct domains: a large N-terminal extracellular domain which binds hCG, a serpentine TM containing seven TM repeats connected by three extra- and intracellular loops (TM region) and a C-terminal tail under the cellular membrane (102).

The early secretion of hCG by the developing trophoblasts prevents its own receptor down-regulation. The extracellular domains of hCG and other gonadotropin receptors are members of the leucine-rich repeat (LRR) protein superfamily and are responsible for the high-affinity binding. α-hCG and β-hCG subunits share a receptor-binding region and an agonist activity to adjacent areas of the molecule (103). The tertiary model shows that the contact between hCG and its receptor is made by interacting residues in the curved portion of the extracellular domain of the receptor and the groove in the hormone formed by the apposition of the α-loop 2 and β-loops 1 and 3 (104).

The biological activity of hCG is dependent on the structure, proper conformational modifications in one or both subunits, and on specific regions of the protein chain and certain carbohydrate residues. Even though the ligand-binding portion of the molecule to the receptor is located on the surface of β-hCG, the amino acid residues Tyr-Tyr-His-Ly-Ser of the CTP portion of α-hCG also are important for receptor binding (105). Alpha His-94 is more involved with receptor binding and α-His-82 may be involved in the biological activation of the target cell. Proteinaceous protease inhibitors neutralize the agonist activity of hCG and reduce the binding of hCG to its receptor. Primary sequence analogies between hCG and the serine protease chymotrypsin implicate an enzymatic cascade in the hCG activation of the cell (103). The α-15–17 and α-73–75 sequences contact the second extracellular loop of the hCG receptor and promote signal transduction (101). The α-39–41 sequence in the long loop 2, which interfaces with loops 1 and 3 of the β-hCG subunit, is involved in the recognition by its receptor (107). The CTP residues α-87–92 are involved in receptor binding and biological expression (108).

There are three major biological roles for the β-hCG subunit in hCG: to carry some sites necessary for receptor binding, to induce active conformation of β-hCG, and to stabilize the hormone-receptor complex (109). There are at least two receptor-determined loops located between cysteine residues β38–57 and β93–100 in β-hCG which confer receptor specificity (105). The surface able to activate the hCG receptor include the majority of the α-hCG and the Asp-99 residue contained in loop β93–100 (111).

Although the CTP region of β-hCG is not involved in receptor binding or *in vitro* signal transduction, it is critical for the *in vivo* biological response (105). The N-terminal region of β-hCG and the C-terminal region of α-hCG also appear to be involved in receptor binding (112).

There is no general agreement regarding which receptor regions are in contact with hCG. N-terminal peptides 7–40, central peptide 102–121 and CTP 259–273 of the ectodomain seem to bind the hCG surface (104). Sequences Arg21-Pro38, Arg102-Thr115, Try253-Phe272 and Lys513-Lys583 of the extracellular domain may also be involved in receptor binding (113).

The binding of hCG to its receptor induces a conformational change in the cytoplasmic domain and generates a conventional signal transduction through the activation of the associated heterotrimeric G-protein. This leads to an increase in cAMP and the consequent activation of protein kinase A (PKA) upon activation of the adenylyl cyclase (AC) pathway (Figure 4). It also leads to an increase in intracellular calcium through the inositol triphosphate/phospholipase A2 pathway (114). Alternatively, in the endometrium, hCG induces phosphorylation of the extracellular signal-regulated kinase (ERK 1/2) in a PKA-independent manner and is involved in processes of proliferation, growth and differentiation (50). hCG receptor selectivity is given by the N-terminal three-fifth of the exodomain, which includes a specific cysteine-rich cluster (NCR), flanked by an LRR6 sequence on the surface of the hCG receptor molecule. This sequence is able to distinguish the positively-charged seatbelt loop of β-hCG, between cys10 and cys11, from other gonadotropins (115). This is why the hCG receptor is not activated by other glycoprotein hormones. The antagonist effect of trypsin, chymotrypsin and of the serum protease inhibitor protinin suggests that these inhibitors bind to the binding site of the hCG receptor or to the hCG-binding site (116).

The carbohydrate moieties of hCG have relevant roles in the biological activity of hCG. The N-linked carbohydrates on alpha, beta or both subunits have little effect on receptor binding. The removal of certain sugar residues even increases the affinity of hCG for its receptor (117). The N-linked oligosaccharides αN78, and βN30 do not play a significant role in the function of hCG, but the αN52 is crucial for signal transduction (118). The Asn13 oligosaccharide of β-hCG seems to play an important role in steroidogenesis. The mannose moieties also are essential in stimulating cAMP accumulation and steroidogenesis (119). The Asn52 on α-hCG is critical for both the cAMP response and the steroidogenesis by maintaining the stability of the dimer, for proper conformation of hCG and assuring dimer secretion (120). In addition, αAsn52 helps to position hCG in a favorable orientation for signal transduction (118).

The sialic acid content of hCG is of major significance in the receptor-binding ability and biological activity. Although the biological activity of hCG diminishes with the gradual desialylation or partial or complete removal of carbohydrate units internal to the sialic

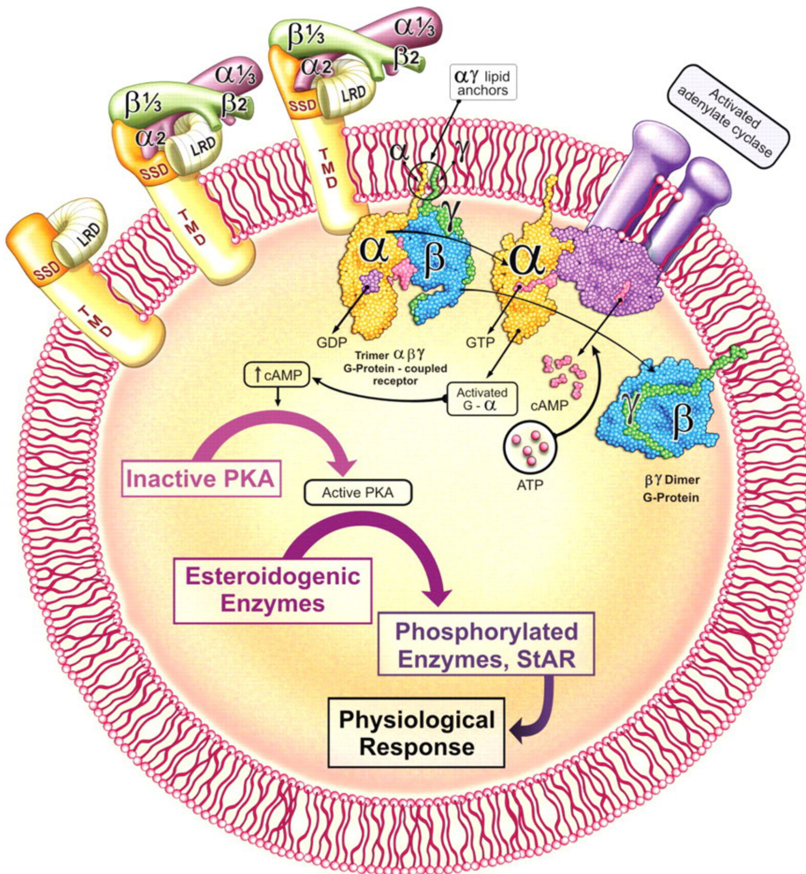

Figure 4. Model of hCG signal transduction showing the signaling specificity domain (SSD) on the extracellular surface of the transmembrane domain (TMD) and the leucine-rich domain (LRD) near the SSD–TMD complex.

The NH2- and COOH-terminal portions of the LRD contact the ends of the SSD and TMD helices. hCG binding increases the distance between the top of the SSD and the top of LRD, promotes the rotation of LRD and a gate-like movement of the LRD and creates a binding pocket for TMD rearrangement and signaling. After binding, hCG activates its receptor. Consequently, the heterotrimeric G-protein-coupled receptor is formed. GDP is released from the G-protein and is replaced by GTP. This leads to dissociation of the G-protein subunits into an α-subunit and a βγ dimer. Gα activates adenylate cyclase, which leads to an increase in intracellular cAMP levels, stimulation of PKA expression of steroidogenic acute regulatory protein (StAR), cholesterol uptake, and steroidogenic enzyme activation (P450scc, 3β-HSD, P450c17). From Moyle *et al*. J Biol Chem 279:44442-44459, 2004).

acid, the removal of certain carbohydrate residues increases the affinity of hCG for its receptor (121). The βO-linked branches, not involved in bioactivity, contribute to the longer half-life (122). Regular hCG promotes progesterone production via the corpus luteum hCG receptor. Hyperglycosylated hCG on the other had, has an autocrine rather than an endocrine function. This autocrine function is apparent in growth, invasion and tumor formation (17) via inhibition of apoptosis in cancer cells, seemingly through the transforming growth factor TGFβ-RII receptor (123). The hypothesis that some variant forms could act on other receptors as well, such as TGFβ-IIR, could explain the role of these molecules in certain abnormal pregnancy conditions and in particular cancer.

In addition to the maintenance of the corpus luteum function, hCG is involved in autocrine and paracrine regulation of EGF, TGF, and LIF for increasing placental syncytium formation (124) and in blastocyst implantation (125). It also mediates glycogenolysis in human placental villi (126), stimulates prostaglandin synthesis by placental tissue and inhibits myometrium contractility (127-132). hCG maintains maternal blood supply to support hemochorial placentation and the nutrition of the fetus (95,127-131). hCG maintains angiogenesis in the myometrial spiral arteries throughout the pregnancy (127-131). It has been shown that hCG promotes the fusion of villous cytotrophoblast cells to syncytiotrophoblast (133). The presence of hCG receptors on a variety of non-gonadal tissues suggests other functions as well. In cord blood and amniotic fluid, hCG may regulate the vascular tone (134), and attenuates the vascular response to angiotensin II (130). In its free form, β-hCG seems to inhibit or stimulate cellular growth (135). The free α-hCG is linked with prolactin secretion and control of endometrial cell differentiation (81). In addition, free α-hCG potentiates progesterone-mediated decidualization of endometrial stromal cells during normal menstrual cycles (137).

Research by multiple groups in the past few decades has dramatically changed the view on the function of hCG. The function of hCG as solely promoting progesterone production was a dogma for almost 80 years, even though hCG was only biologically active for 3 weeks of its 37 weeks of production. This made little biological sense. The view has now changed from hCG as a hormone produced by trophoblast cells that promotes progesterone production by luteal cells to hCG as a collection of a variety of molecules each with different functions. With all the new doors opening in the hCG story, everything is still far from complete.

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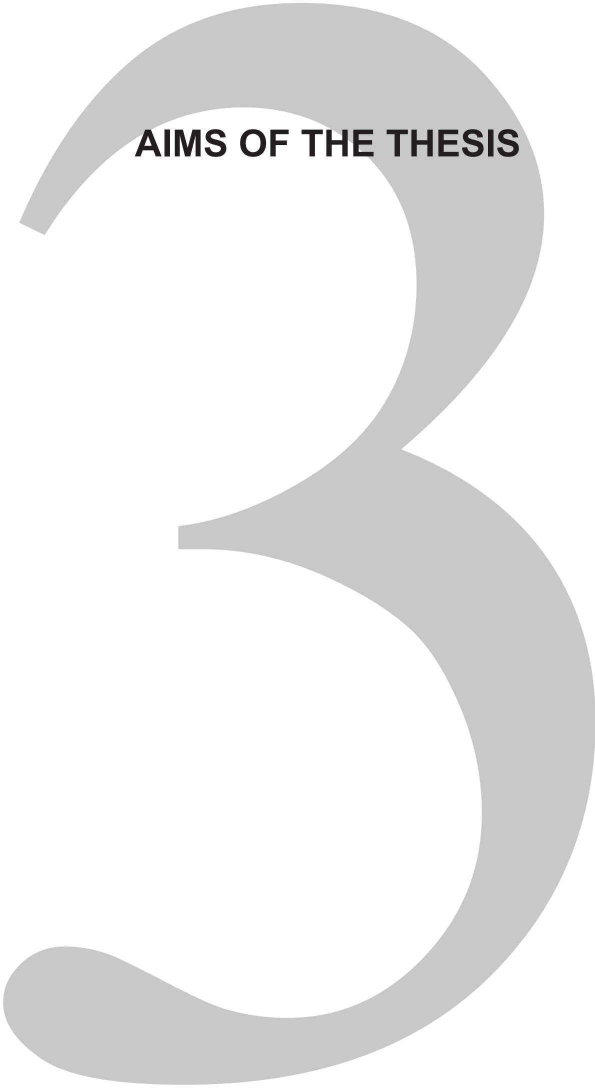


AIMS OF THE THESIS

BIOLOGICAL EFFECTS OF OLIGOPEPTIDES DERIVED FROM β-hCG LOOP-2

There are at least 15 variants of hCG that have been detected in serum and urine of normal or abnormal pregnancies, gestational trophoblastic diseases or non-gestational malignancies (1-13). Of these 15 variants, β-subunit core fragment (β-core) and nicked β-hCG are in the context of this thesis of particular interest. As discussed in chapter 2, a β-core comprises two fragments of the β-subunit, β6–40 and β55–92, held together by disulfide bonds. This form of β-subunit completely misses β-loop-2 residues 41-54 (Figure 1). In nicked β-hCG these residues are cleaved at various positions, e.g. at 43-44, 47-48, 53-54 (Figure 1). Both forms of β-hCG lose their biological function and are unable to bind to the hCG/LH receptor.

Several investigators have studied the biological effects of heterodimeric hCG and its multiple variants on gonadal tissues and/or their role in immune modulation (14-18). The smaller fragments that may liberate from the breakdown of hCG or its variants are generally considered as biological waste products. Their possible biological role has not been studied. We hypothesized that smaller fragments may liberate from β-loop-2 residues 41-54. These residues are missing in β-core, and might be further nicked as these same residues are cleaved at various positions in nicked β-hCG.

We did not restrict our analyses of β-hCG-loop-2 fragments to the fragments that are liberated from residues 40-54 according to the known cleavage sites in nicked β-hCG, but also analyzed all other possible peptides that theoretically might exist based on the primary sequence of β-loop-2. All these possible peptide-sequences were analyzed by several proteomics tools for a number of characteristics that we hypothesized might be crucial for functional activity in inflammatory processes (19). These characteristics are:

- MHC class I and class II binding, as we supposed the involvement of the antigen processing and degradation machinery in the generation of regulatory oligopeptides.
- Hydrophobicity, as we supposed the necessity of the regulatory peptides to easily traverse intracellular membranes.
- Proteolytic sites other than the ones known from nicked β-hCG.
- Homology to other putative regulatory oligopeptide sequences in endogenous proteins.

To this end the following proteomics tools were used:

- BIMAS (20) database to predict the HLA peptide binding sites.
- SYFPEITHI (20) database to predict the MHC I and II peptide binding sites.
- ExPASy ProtScale (22, 23) tools to determine the hydrophobicity.
- ExPASy Peptide Cutter (23) database to predict proteolytic sites.
- ExPASy Blasts (23) tools to determine the occurrence of oligopeptides in endogenous (host or pathogen) proteins.

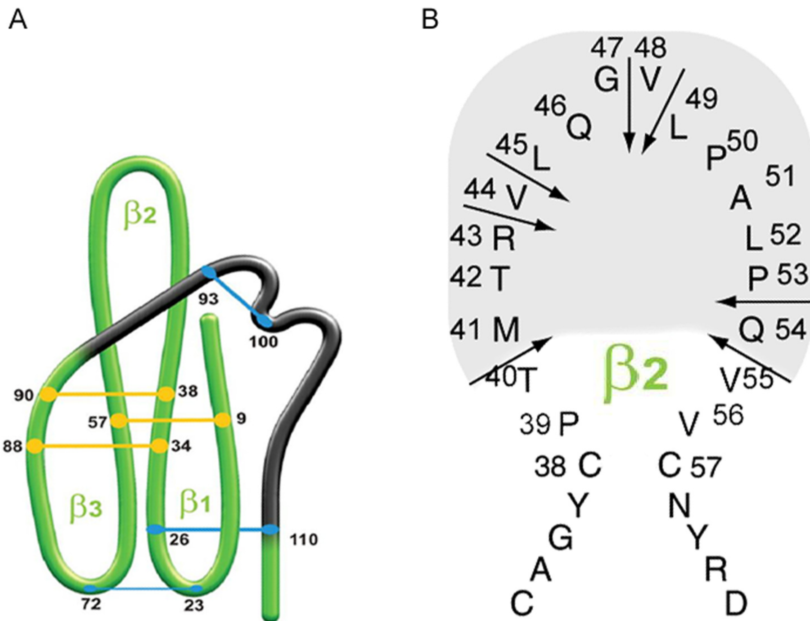


Figure 1.
A, β-hCG subunit polypeptide chain (thick green line) with its seatbelt region (thick black line) and assigned disulfide bonds. Numbers, amino acid numbers; yellow bars, cystine knots; thick blue lines, seatbelt disulfide bonds; thin blue line, small loop disulfide bond; β1, β2, β3 loops of β-hCG. **B**, shows β-hCG-loop-2 residues that are missing (residues in the gray area; MTRVLQGVLPALPQ) in β−core and shows potentially cleaved sites (43-44, 44-45, 47-48, 53-54) within this region that have been found in nicked β-hCG (modified from Xing *et al.* Protein Sci 2001;10:226-235).

Figure 2 (right). The effect of in vitro exposure of a plancental derived trophoblast cell line (ATCC; CRL 7548) to the oligopeptides LQG, LQGV, VLPALP, VLPALPQ.
MTR, MTRV and commercial hCG (Pregnyl) on proliferation. The assay was performed in triplicate by co-culturing 2 x 10^5 trophoblast cells per well in 48-well flat-bottom plates (Nalge Nunc Int., Naperville, IL, USA) either with oligopeptide (0.1 µg/ml, 1 µg/ml or 10 µg/ml) or with Pregnyl (3 IU/ml, 30 IU/ml or 300 IU/ml) in a total volume of 500 µl. Cells were incubated at 37°C in 5% CO_2 for 12, 24, 36 or 48 hours. During the final 8 hours of culture 0.5 µCi of (^{3}H) thymidine ((^{3}H)TdR) per well was added and the incorporation of (^{3}H)TdR was measured on beta-plate counter. Open symbols are data points that are significantly ($p < 0.05$) different as compared to the PBS treatment (unpaired Student's t-test). Data are given as mean ± SD and are from one representative experiment out of three.

LQG

50000
40000
30000
20000
10000
0
cpm
12 24 36 48
time (hours)
PBS 0.1 mcg/ml 1 mcg/ml 10 mcg/ml

LQGV

50000
40000
30000
20000
10000
0
proliferation (cpm)
12 24 36 48
time (hours)
PBS 0.1 mcg/ml 1 mcg/ml 10 mcg/ml

VLPALPQ

50000
40000
30000
20000
10000
0
cpm
12 24 36 48
time (hours)
PBS 0.1 mcg/ml 1 mcg/ml 10 mcg/ml

VLPALP

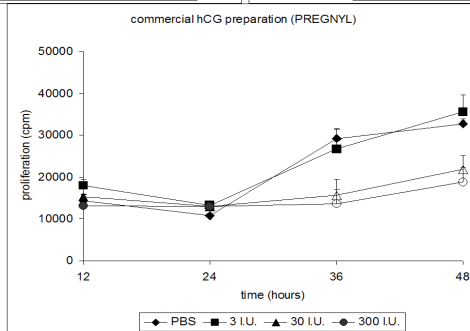
50000
40000
30000
20000
10000
0
cpm
12 24 36 48
time (hours)
PBS 0.1 mcg/ml 1 mcg/ml 10 mcg/ml

MTRV

50000
40000
30000
20000
10000
0
cpm
12 24 36 48
time (hours)
PBS 0.1 mcg/ml 1 mcg/ml 10 mcg/ml

MTR

50000
40000
30000
20000
10000
0
cpm
12 24 36 48
time (hours)
PBS 0.1 mcg/ml 1 mcg/ml 10 mcg/ml



Based upon these analyses we synthesized a number of overlapping oligopeptides from β-loop-2 residues 41-54 and from the external C-terminal flanking region namely, MTR, MTRV, LQG, LQGV, VLPALP, VLPALPQ, VVC and QVVC and tested these oligopeptides for their possible biological activity.

The effect of oligopeptides MTR, MTRV, LQG, LQGV, VLPALP, VLPALPQ and a commercial hCG preparation (Pregnyl) were tested on the proliferation of human placental-derived trophoblast cells (ATCC, CRL-7548) isolated from 5 months of gestation. The results from these experiments showed that oligopeptide LQGV significantly decreased proliferation of trophoblastic cells as did commercial hCG preparation, while oligopeptide VLPALP significantly increased the proliferation of such cells (Figure 2). The oligopeptides MTR, LQG and VLPALPQ did not affect the proliferation of trophoblastic cells (Figure 2), whereas MTRV decreased the proliferation only in the early phase of the cultures. Apparently, the oligopeptides LQGV, VLPALP and MTRV from β-loop-2 residues 41-54 affect trophoblast proliferation and thus are biologically active. Furthermore, these oliopeptides differently affect trophoblast proliferation.

It is well known that hCG maintains maternal blood flow (24) to support placentation. hCG maintains angiogenesis in the myometerial spiral arteries throughout pregnancy (25-30). It has a direct role in regulating human implantation and placentation through stimulation of vascular endothelial growth factor (VEGF) and immunomodulaton (31,32). We investigated the possible effects of the designed oligopeptides on angiogenesis in a chicken egg chorioallantoic membrane (CAM) assay. Fertilized, domestic chick eggs were incubated for 8 days and then windowed as described previously (33).

Briefly, two small holes were drilled into the eggshell. One hole was used to open the air cell and the other for injection. The oligopeptide (1μg/egg) with or without vascular endothelial growth factor (VEGF) (100 ng/egg) was injected into the egg. After 48 hours of incubation at 37°C with 32% relative humidity, the shell membrane was carefully dissected ad removed. The embryo and the blood vessels were photographed *in vivo* with the use of a microscope and the number of blood vessel branches was counted. These experiments revealed that LQG and VLPALP significantly reduced basal as well as VEGF-induced angiogenesis in the CAM assay, while LQGV significantly increased basal as well as VEGF induced angiogenesis (Figure 3). Oligopeptide VLPALPQ significantly reduced VEGF-induced angiogenesis only, whereas MTRV significantly increased basal angiogenesis (Figure 3). The oligopeptides MTR, VVC and QVVC did not affect the proliferation in the CAM assay (data not shown). These angiogenesis experiments confirmed biological activity of the several designed oligopeptides.

Pregnancy confers a transient altered immune status in the maternal host as responses are biased toward type 2 (humoral) and away from type 1 (inflammatory

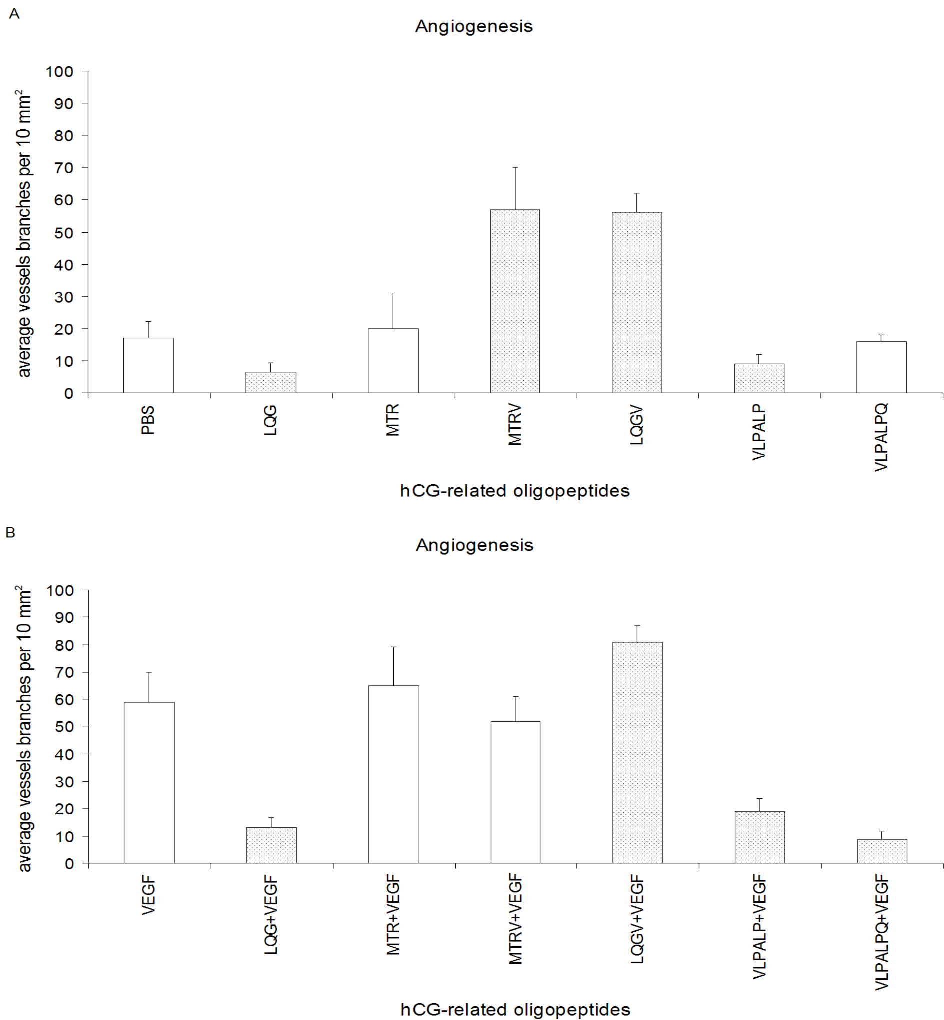


Figure 3. Effects of several hCG-related oligopeptides on basal (A) and VEGF-stimulated (B) angiogenesis in the chick chorioallantoic membrane (CAM) assay.

Results are expressed as average number of vessel branches per 10 mm^2 in high power (10 X) field of view ± SEM. For each treatment ten eggs were used and the vessel branches from four different fields per egg were counted under a dissection microscope. Light gray dotted bars show data that are significantly different from the PBS (graph A) and VEGF (graph B) controls. Data given are from one representative experiment out of three.

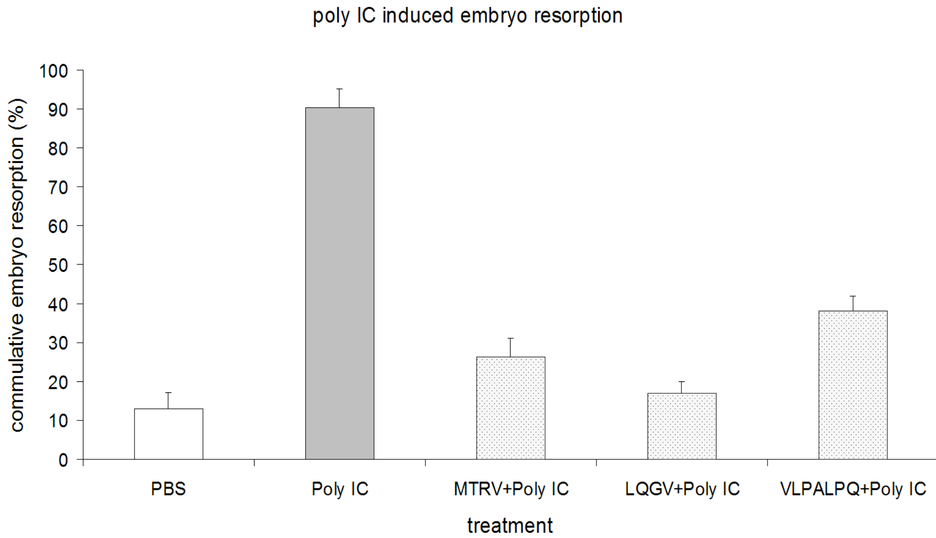


Figure 4. Influence of hCG-related oligopeptides on the cumulative poly I:C induced embryo resorption rate on day 14 of gestation in female BALB/c mice.
Ten to twelve weeks old female BALB/c mice were intraperitoneally (i.p.) injected with pregnant mare's serum gonadotropin (PMSG, Sigma-Aldrich) at a dosage of 10 IU/mouse. After 48h mice were i.p. injected with hCG at 10 IU/mouse. Each female was co-caged with a 10 to 12 weeks old male C57BL/6 mouse. The females were checked twice a day for vaginal plug and separated from the male after detection of a plug. The day of the vaginal plug was designated day 0 of pregnancy. MTRV, LQGV or VLPALPQ was i.p. injected at gestational days 3, 5, 7, 9, 11 and 13 at a dosage of 0.1 mg in 0.1 ml of PBS, whereas 0.2 mg of poly I:C in 0.5 ml PBS was i.p. injected at day 7 of gestation. Other female BALB/c mice were i.p. injected either with an equal volume of PBS instead of oligopeptide, or with poly I:C only. Poly I:C increased the cumulative embryo resorption (dark gray bars). This was significantly inhibited by each of the three hCG-related oligopeptides tested (light gray dotted bars). Data are given as mean ± SD.

and cell-mediated) phenotype (34, 35). This shift in the immune state during pregnancy is more pronounced at the maternal-fetal interface (34,35). Uncontrolled shifts in the immune state during pregnancy can have deleterious effects on the outcome of the pregnancy, and can lead to abortion. Maternal rejection of the fetus can be triggered by pro-inflammatory Th1 cytokines, such as IFN-γ and TNF-α (36). Because hCG-related oligopeptides were biologically active as shown in the trophoblast proliferation assay and in the CAM angiogenesis assay, we further tested these oligopeptides in a poly I:C-induced embryo resorption mouse model. Poly I:C, a synthetic double stranded-RNA, is known to increase the production of pro-abortive Th1 cytokines such as IFN-γ and TNF-α (37). These experiments showed that the increased poly I:C-induced embryo resorption could be significantly inhibited by multiple injection of either MTRV, LQGV or

VLPALPQ (Figure 4).

Together these studies showed that several of the designed oligopeptides that might be liberated *in vivo* from β-loop-2 residues 41-54 are biologically active *in vitro* and *in vivo*. Together these studies showed that the designed oligopeptides that might be liberated *in vivo* from β-loop-2 residues 41-54 are biologically active *in vitro* and *in vivo*.

SPECIFIC QUESTIONS TO THIS THESIS

The aim of the studies of this thesis was to increase our understanding of the biological abilities of the degradation products of the β-hCG-loop-2 peptide chain. In these studies several models were employed:

1. *Th1 mediated autoimmune disease in non-obese diabetic (NOD) mice.* The influence of commercial hCG, its lower molecular weight fractions and the effects of β-loop-2 related oligopeptides were studied on the development of type 1 diabetes and the associated Th1 response (chapter 4).
2. *LPS induced shock in BALB/c mice.* The influence of commercial hCG, its lower molecular weight fractions and the effects of β-loop-2 related oligopeptides were studied on the disease outcome. In addition, the effect of the peptides was studied on the production of macrophage migration inhibitory factor (MIF) by spleen cells, on the plasma level of liver aminotransferase, on the expression of several splenic lymphocyte and macrophage surface markers, and on the proliferative response of splenic lymphocytes (chapters 5 and 6).
3. *E.coli induced septic shock in rhesus monkeys.* We investigated whether the beneficial effect of β-loop-2 related oligopeptides on LPS induced shock in mice could be confirmed in a preclinical non-human primate model challenged with live *E. coli* bacteria. In this model the pathological features and haemodynamic parameters were studied (chapter 6).
4. *Hemorrhagic shock and resuscitation induced inflammation.* Several hCG related oligopeptides were studied for their ability to improve the disease outcome, and to modulate the proinflammatory cytokine and adhesion molecule expression in a rat model for hemorrhagic shock and resuscitation (chapter 7).
5. *Innate immune response to Listeria monocytogenes.* In this chapter the effect of the hCG-related oligopeptides was studied with regard to the immune response to and the clearance of *Listeria monocytogenes* infection in mice (chapter 8). This infection model is relevant to pregnancy since pregnant women and their babies are more vulnerable to infection with *Listeria* than non-pregnant women.

6. *Renal ischemia reperfusion injury.* We also studied the effect of hCG-related oligopeptides on renal ischemia-reperfusion injury in mice with regard to the influx and apoptosis of neutrophils, epithelial cell proliferation, serum proinflammatory cytokines and adhesion molecules (chapter 9).
7. *Solid tumor growth.* Finally we studied the influence of, especially LQGV, on tumor growth and vessel integrity in a transplantable lung tumor in mice (chapter 10).

These studies revealed a broad spectrum of activities of the hCG-related oligopeptides tested on physiological and pathophysiological processes, and suggest potential therapeutic value of these oligopeptides.

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4

INHIBITION OF DIABETES IN NOD MICE BY HUMAN PREGNANCY FACTOR

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ABSTRACT

Clinical symptoms of Th1 mediated autoimmune diseases regress in many patients during pregnancy. A prominent feature of pregnancy is the presence of human chorionic gonadotropin hormone (hCG) in blood and urine. In this report we tested the effect of clinical grade hCG (c-hCG) on the development of diabetes, a Th1 mediated autoimmune disease, in non-obese diabetic (NOD) mice. We show that treatment of NOD mice with c-hCG before the onset of clinical symptoms lowered the increased blood glucose levels, reversed the established inflammatory infiltrate of pancreatic tissue and profoundly inhibited the development of diabetes for prolonged time. c-hCG also induced profound inhibition of the functional activity (i.e. production of IFN-γ) of Th1 cells. Transfer of spleen cells from c-hCG-treated NOD mice into immuno-compromised NOD.SCID mice inhibited the development of diabetes in these otherwise non-treated mice. This shows that the treatment of the donor NOD mice with c-hCG changes the balance in the immune system. The anti-diabetic activity of c-hCG was not caused by heterodimeric hCG or its subunits. Instead, this anti-diabetic activity appeared to reside in a 100-2000 Dalton fraction of c-hCG. We called this 400-2000 Dalton fraction natural (immuno) modulatory pregnancy factor (NMPF). Although the precise nature of NMPF is not elucidated yet, we show that small peptides from loop 2 of beta hCG may account for this anti-diabetic activity.

INTRODUCTION

T cell-mediated autoimmune diseases belong to the commonest chronic diseases in industrialized countries and increase worldwide. It imposes a burden on Western economies. These diseases, including type I diabetes, Graves' disease, rheumatoid arthritis (RA) and multiple sclerosis (MS) generally have a dominant Th1 immune response in common (1,2). An adjustment of the innate and adaptive immune system during pregnancy, characterized amongst other by a Th2 cytokine profile, could maintain normal immune competence against microorganisms and also account for the clinical improvement of Th1 autoimmune disease (3-6). A better understanding of the immune modulation during pregnancy and the identification of the natural factor(s) responsible for this, could help to develop new strategies for the prevention and treatment of Th1 autoimmune diseases with less detrimental effect to the treated individual than currently used broad range immunosuppressive drugs. Since hormonal changes in pregnant women preceed and accompany immune changes, we hypothesized that hCG, an early pregnancy hormone, might account for the regression of Th1 mediated autoimmune diseases during pregnancy.

Therefore, we tested a commercial clinical grade hCG (c-hCG) preparation derived from first trimester pregnancy urine on the development of diabetes in the NOD mouse model (7). Female NOD mice spontaneously develop type I diabetes with remarkable similarity to human type I diabetes (insulin-dependent diabetes mellitus (IDDM) (8,9). The main clinical feature of this model is the elevated blood glucose level, which is caused by Th1 cell-mediated autoimmune destruction of insulin-producing β cells in the islets of Langerhans of the pancreas (10). The disease severity in NOD mice is correlated with high levels of interferon-gamma (IFN-γ), a signature cytokine for Th1 cells.

Human CG exhibits a variety of forms in serum and urine (11). Only intact hCG stimulates the LH/hCG receptor. Peptide bond cleavages result in biologically inactive hCG. Beside heterodimeric hCG, β core fragment and nicked hCG are two other major forms that are found in pregnancy serum and urine samples. Both of these forms of hCG either have peptide bond cleavages in residues 44-52 (loop 2) of β hCG or completely miss loop 2 (residues 41-54).

Several investigators have studied the biological effects of heterodimeric hCG and its variants on gonadal tissues and/or their role in immune modulation (12-14), but ignored the possible biological activity of fragments liberated from the breakdown of hCG into different variants. Loop 2 is known to be especially prone to proteases. The hCG dimer and β core fragment, uncombined α- and β hCG (free β) and nicked hCG and free β molecules (cleaved at 44-45 or 47-48) can be detected in the circulation (11). We postulate that the loop 2 region, consisting of residues 41-54 with amino acid sequence MTRVLQGVLPALPQ, which is absent in β core, can also be nicked *in vivo* and can liberate smaller fragments (oligopeptides) with biological activity. In this report we have also tested possible biological activities of two such oligopepeptides, namely LQGV and VLPALP on the development of diabetes in the NOD mouse model.

MATERIAL AND METHODS

NOD mice and BALB/c mice

All mice used in these studies were maintained in a pathogen-free facility at the Department of Immunology, Erasmus University Rotterdam, The Netherlands. NOD and NOD.SCID mice were bred in our facility. Female BALB/c mice were purchased from Harlan (Horst, The Netherlands) and were also maintained in a pathogen-free facility at the Department of Immunology. All mice were given free access to food and water. The experiments were approved by the Animal Experiments Committee of the Erasmus University Rotterdam.

Diabetes

Diabetes was assessed by measurement of the venous blood glucose level using an Abbott Medisense Precision glucometer. Mice were considered diabetic after two consecutive glucose measurements ≥11 mmol/l (200 mg/dl). Onset of diabetes was dated from the first consecutive reading.

***In vivo* treatment**

14 weeks-old female NOD mice (*n*=12) were treated with c-hCG (Pregnyl Organon, Oss, The Netherlands). Treatment was done by injecting 300 IU c-hCG diluted in PBS three times per week intraperitoneally (i.p.). Control mice were treated with PBS only. The treatment was discontinued when all PBS treated NOD mice in the experiment had developed diabetes. This was at the age of 19 weeks. Thus, the c-hCG and PBS treatments were done for five weeks. In instances of sustained hyperglycaemia of 20 mmol/l the mice were killed to avoid prolonged discomfort. The remainder mice were kept alive up to the age of 35 weeks without any further treatment. For Th polarization assessment, mice (*n*=6) were treated i.p. with 300 IU c-hCG, 10 μg lyophilized material obtained from first trimester pregnancy urine (u-hCG) or their fractions for 4 days. The u-hCG was prepared according to the procedures described below. Control mice were treated with PBS only. Spleen cells were pooled from each group and were used for $CD4^+$ T-cells preparation (see below) or were stimulated with anti-CD3 and IL-2. RPMI 1640 medium was used supplemented with 25 mM HEPES, 100 IU/ml penicillin, 50 μg/ml streptomycin, 1 mM pyruvate, 50 μM 2-ME and 10% heat inactivated FCS. All cultures were performed in triplicate or quadruplicate.

Glucose tolerance test

Glucose tolerance test (GTT) test was performed at 17 (during treatment, *n*=6) and 35 weeks of age (after treatment, *n*=6) by injecting 0.5 g/kg D-glucose intravenously (i.v.). At 3, 5, 10 and 15 minutes blood samples were collected from the tail and tested for glucose content.

Immunohistochemistry

At the age of 21 weeks 6 NOD females from the two experimental groups (PBS vs c-hCG) were killed. For detailed immunohistochemistry pancreases were removed, embedded in Tissue-tek and frozen in liquid nitrogen. Tissues were stored at –80°C until immunohistochemistry was performed. Before sectioning, microscopic slides were coated with 0.1% gelatin/0.01% chromium-alum. Thereafter, at least ten 5-μm cryosections at four non-contiguous levels (around 50 μm apart) of the stored tissue specimens were cut, dried in air overnight, and fixed with the appropriate fixatives depending on the monoclonal or polyclonal antibody used (15).

Immunohistochemistry was performed essentially as described previously (15). Pancreas cryostat sections were prepared, coded and fixed for 2 min in 2% pararosaniline. After a wash with phosphate buffered saline with 0.1% Tween-20 (Merck-Schuchardt, Hohenbrunn bei Munchen, Germany) (PBS/Tween), slides were incubated with first step antibodies for 30 min at room temperature. The antibodies used were rat RA3.6B2 for B cells, rat KT3 for T cells, rat MOMA-1 and rat $BM8^+$ for macrophages and rat ER-$MP23^+$ for dendritic-like cells. Pig anti-insulin (DAKO, Glostrup, Denmark) was used diluted 1:100 in PBS/Tween. All other monoclonal antibodies used in this study were supernatants from hamster hybridoma cultured at our department and used undiluted. Subsequently, slides were washed with PBS/Tween and incubated with peroxidase-conjugated rabbit-α-guinea pig-Ig (to detect α-insulin) or rabbit-α-rat-Ig (to detect all others) second step antibodies in the presence of 2% normal mouse serum for 30 min at room temperature. After an additional wash with PBS/Tween, slides were incubated with 0.05% (w/v) Ni-di-amino-benzidine (Ni-DAB) with 0.02% H_2O_2 and washed in water after 3 min. Finally, slides were counter stained for 3 min in nuclear fast red, dehydrated in a graded ethanol series, and mounted. For each staining run, one slide was stained with second antibody only as a control for endogenous peroxidase activity and non-specific binding of the second step, and a section of spleen was included as positive control.

Spleen cell transfer

Spleens of PBS and c-hCG treated mice were removed under aseptic conditions and single-cell suspensions were made. Erythrocytes were removed by incubating with Gey's medium for 5 minutes on melting ice. Isolated pooled spleen cells (20 x 10^6 in 0.3 ml PBS) from c-hCG treated mice (*n*=6) and control mice (*n*=6) were injected i.v. into the tail vein of 8 weeks-old female NOD.SCID mice (*n*=6).

In other experiments spleen cells were recovered from diabetic female NOD mice and stimulated *in vitro* with IL-2 (50 U/ml) along with 300 IU/ml c-hCG (Pregnyl, Organon; Profasi, Serono; APL, Wyeth Ayerst), or 10 μg/ml from HPLC purified fractions of c-hCG or hCG directly purified from pregnancy urine (u-hCG, see below), recombinant hCG (10 μg/ml) (r-hCG, Sigma, St. Louis, MO, USA), its subunits (r-α-hCG or r-β-hCG) or purified hCG (from pregnancy urine; 10 μg/ml). After 48hrs of culture, cells were collected and evaluated for their viability by trypan blue staining. Cells were washed twice with PBS and 20 x 10^6 cells were i.v. transferred into 8-wk-old female NOD.SCID mice.

***In vitro* stimulation of splenocytes**

Splenic cell suspensions (2 x 10^5 cells/well) were cultured in 96-well flat-bottom plates (0.2 ml) and stimulated with plate bound anti-CD3 (145-2C11, 25 μg/ml) and

IL-2 (50 U/ml) along with c-hCG or u-hCG or fractions derived from it. After incubation for 48hrs, supernatants were collected for cytokine analyses. RPMI 1640 medium was used supplemented with 25 mM HEPES, 100 IU/ml penicillin, 50 μg/ml streptomycin, 1 mM pyruvate, 50 μM 2-ME and 10% heat inactivated FCS. All cultures were performed in triplicate or quadruplicate.

Preparation and stimulation of purified $CD4^+$ T cells

Purified $CD4^+$ T cells from the spleen were obtained by complement depletion with antibodies to heat stable antigen (HSA), CD16/32, MHC class II (BALB/c, M5/114; NOD, M10/216) and GR-1. Cells were further purified using magnetic activated cell sorting with a cocktail of biotinylated mAbs against CD11b (M1/70), B220 (RA3 6B2), CD8 (YTS-169) and CD40 (FGK-45.5), followed by incubation with streptavidin-conjugated microbeads (Milteny Biotech, Bergisch Gladbach, Germany). $CD4^+$ T-cells used for experiments were always 90-95% purified as determined by flow cytometry.

Th polarization assay

For Th polarization assay, primary stimulation of purified $CD4^+$ cells was done by culturing 1 x 10^5 cells/well in 96-well flat-bottom plates (Nalge Nunc Int., Naperville, IL., USA). The cells were stimulated with plate-bound anti-CD3 mAb (145-2C11; 25 μg/ml) in the presence of soluble anti-CD28 mAb (37-51; 10 μg/ml) and IL-2 (50 U/ml). For differentiation of Th1 cells (Th1 polarizing condition), anti-IL-4 mAb (11B11; 10 μg/ml) and IL-12 (10 ng/ml) were added to the cultures. For differentiation of Th2 cells (Th2 polarizing condition) IL-4 (35 ng/ml) and anti-IFN-γ mAb (XMG 1.2, 5 μg/ml) were employed. Neutral (non-polarized) condition cultures contained only anti-CD3, anti-CD28 and IL-2. All doses were optimised in preliminary experiments. After 4 days of culture, the cells were washed 3 times and transferred to new anti-CD3-coated 96-well plates and restimulated in the presence of IL-2 (50 U/ml) and anti-CD28 (10 μg/ml). All cultures were performed in triplicate or quadruplicate. Forty-eight hours later, supernatants were collected and assayed for IL-4, IFN-γ, IL-10 and (total) TGF-β production by ELISA as a read-out for Th1 versus Th2 polarization.

Cytokine ELISA

Flat bottom microplates (96-wells, Falcon 3912, Microtest II Flexible Assay Plate, Becton Dickinson, Oxnard, CA, USA) were coated with capture antibody diluted in PBS (SXC-1; 1 μg/ml, 11B11 and XMG 1.2; 5 μg/ml) at 4°C for 18h. After coating, plates were washed (PBS, 0.1% BSA, 0.05% Tween-20) and blocked with PBS supplemented with 1% BSA at room temperature for 1h. After washing, samples and standards were added and incubation was continued for at least 4 hours at room temperature. Thereafter, plates were washed and biotinylated detection antibodies were added (2A5.1 for IL-10

and BVD624G for IL-4, 0.1 μg/ml; R46A2 for IFN-γ, 1 μg/ml) and incubated overnight at 4°C. After washing, streptavidin-peroxidase (1/1500 diluted, Jackson Immunoresearch, West Grove, PA, USA) was added. After 1h, plates were washed and the reaction was visualized using 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS, 1 mg/ml, Sigma, St. Louis, MO, USA). Optical density was measured at 414 nm, using a Titertek Multiscan (Flow Labs, Redwood City, CA, USA).

The amount of TGF-β was measured with commercially available ELISA kit (Genzyme Corp, Cambridge, MA, USA) according to the protocol provided by the manufacturer.

The detection limits of the various ELISA were: IL-4: 80 pg/ml, IFN-γ: 800 pg/ml, IL-10: 80 pg/ml and TGF-β: 32 pg/ml.

Purification of u-hCG from first trimester pregnancy urine

First trimester pregnancy urine (2 litres) was collected in a bottle from a healthy volunteer and was refrigerated until delivered at the laboratory within 2 days. Upon delivery, the pH was adjusted to 7.2-7.4 with sodium hydroxide and allowed to sediment for 1h at room temperature (RT). Approximately, 75% of the supernatant was decanted and the remainder close to the precipitate was centrifuged (10 min at 25000 rpm at 4°C) to remove sediment and added to the rest of the supernatant. The supernatant was filtered through 0.45 μm in a Minitan (Millipore) transversal filtration set-up. Subsequently, the filtrate (2 litres) was concentrated in an Amicon ultrafiltration set-up equipped with a YM Diapore membrane with a 10 kilo Dalton (kDa) cut-off. The final volume (250 ml) was dialysed against 2 changes of 10 litres of Milli Q water. Next the sample was further concentrated by 10 kDa cut-off in an Amicon ultrafiltration to a final volume of 3 ml. The material was further lyophilized for purification and for other experiments.

Gel permeation

c-hCG or u-hCG was fractionated by gel filtration using a fast-protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden) equipped with Superdex 75 (10mm ID x 300 mm L) gel permeation column. Superdex columns have superb resolution and reproducibility for separating monomeric and dimeric forms of proteins and peptides. The separation range of this column was 70,000 - 3000 Dalton. 50 mM of ammonium bicarbonate in combination with 4% methanol was used as a running buffer and the selectivity profile of the column was determined. FPLC fractions of c/u-hCG in 50 mM of ammonium bicarbonate in combination with 4% methanol were further analyzed by gel filtration using a high performance liquid chromatography (HPLC) system (Shimadzu Co., Japan) equipped with a macrosphere size exclusion (GPC) 60Å (7.5mm ID x 300mm L) column (Alltech, Illinois, USA). This is an appropriate column for separation of not only proteins, but also peptides. The separation range for this

column was 28,000 – 250 Dalton and the flow rate was 0.5 ml/min. External molecular weight standards were employed to calibrate the column elution positions. The eluted fractions from FPLC and HPLC columns were lyophilized for the use in experiments.

In vivo treatment with oligopeptides

Fourteen weeks-old or 6 weeks-old female NOD mice (n=5-6) were treated i.p. with 10 μg or 100 μg of oligopeptide LQGV or VLPALP three times per week for three weeks. Control mice were treated with PBS only. Up till the age of 30 weeks once in a week diabetes was assessed by measurement of the venous blood glucose level using an Abbott Medisense Precision glucometer.

In another experiment 14 weeks-old female NOD mice (n=5) were treated i.p. three times a week with 100 μg of oligopeptide LQGV or VLPALP for three weeks. After the treatment splenic dendritic cells (DC) were isolated and assessed for NF-kappa B activation.

Isolation of splenic DC

Splenic DC were isolated using MACS CD11c (N418) MicroBeads (Miltenyi Biotec), following the manufacturer's protocol. In short, single cell suspensions were obtained by incubating the spleens with Collagenase D (GibcoBRL, Life technologies). The obtained cell suspension was incubated with CD11c MicroBeads, and labelled cells were separated from the suspension using an autoMACS (Miltenyi Biotech).

Nuclear extracts

Three million cells/ml were plated in a 12 well plate (Nalge Nunc International) 2 hours before treatment with LPS (100 ng/ml). After stimulating the cells for 30 minutes, cells were collected in a tube and centrifuged for 5 minutes at 2000 rpm (rounds per minute) at 4ºC (Universal 30 RF, Hettich Zentrifuges). The pellet was washed with ice-cold TBS pH 7.4 (Tris buffered saline) once and then the pellet was resuspended in 400 μl of a hypotonic buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, protease inhibitor cocktail (Complete™ Mini, Roche)) and left on ice for 15 minutes. 25 μl 10% NP-40 was added, and the sample was centrifuged (2 minutes, 4000 rpm, 4ºC). The supernatant (cytoplasmic fraction) was discarded. The pellet, which contains the nuclei, was washed with 50 μl buffer A and then the pellet was resuspended in 50 μl buffer C (20 mM Hepes pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 10% (v/v) glycerol, 1 mM DTT, protease inhibitor cocktail). The samples were left to shake at 4 °C for at least 60 minutes. Finally, the samples were centrifuged and the supernatant (nucleic fraction) was stored at –70ºC.

Bradford reagent (Sigma) was used to determine the final protein concentration in the extracts.

Dection of p65 subunit of NF-kappa B

In nucleic extracts of splenic DC p65 subunit of NFkB was detected using TransFactor™ NFκB kit (Clontech, BD) which is an ELISA based method to detect NFκB in nuclear extracts. Wells provided in the kit were coated with DNA containing the κB site and then the binding of NFκB to the coated well was detected with a specific antibody against p65 according to the manufacturer's protocol.

Statistics

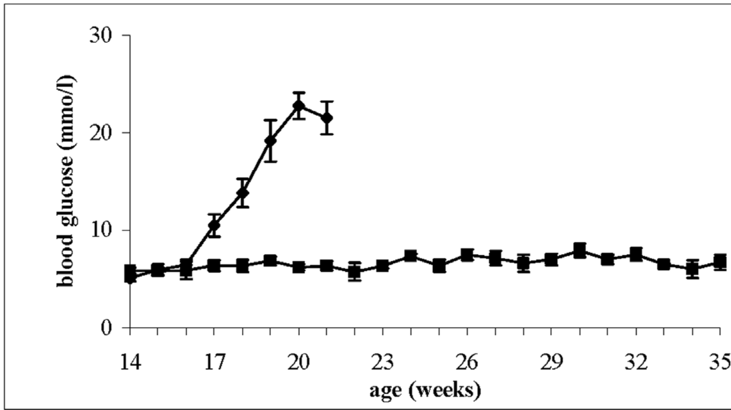
Data was analyzed by Student's T test and differences were considered significant at $p<0.05$.

RESULTS

In order to determine whether hCG treatment affects the diabetes development in NOD mice, we tested c-hCG on pre-diabetic female NOD mice. In our female NOD colony the majority of β cells (>80%) are histologically and functionally destroyed between 15 and 21 weeks of age and clinical symptoms appear between 15-21 weeks of age. c-hCG treatment of 14 weeks-old female NOD mice was done with a titrated dose of 300 IU i.p. three times per week. In the experiments reported, all PBS treated NOD control mice had developed diabetes and showed blood glucose levels over 20 mmol/l at 20 weeks of age. In contrast, c-hCG treated mice remained non-diabetic as evidenced by blood glucose levels not higher than 7 mmol/l (Figure 1A). This remained so up to the termination of the experiment at the age of 35 weeks.

Figure 1 (right). Effect of 5 weeks of c-hCG treatment (3 times a week, 300 IU c-hCG starting at the age of 14 weeks) in female NOD mice on blood glucose levels (n=12).
(**A**), Blood glucose levels of c-hCG (-◊-) and PBS (-♦-) treated NOD mice. From 17 weeks and onwards the values between the groups were significantly different. After treatment the c-hCG treated mice (n=6) were kept alive for an additional 16 weeks without any further treatment. (**B**), GTT in c-hCG and PBS treated NOD mice (n=6) at 17 (during treatment) and 35 weeks of age (after treatment). Blood glucose levels of PBS (-♦-) and c-hCG (-◊-) treated NOD mice after glucose challenge at 17 weeks of age, and of c-hCG (-■-) treated NOD mice at 35 weeks of age are shown. The values of PBS and c-hCG groups are significantly different for all data points. (**C**), Transfer of diabetes resistance from c-hCG treated NOD mice to NOD.SCID mice. Spleen cells were isolated two weeks after termination of the 5-week c-hCG or PBS treatment (n=6). Viable pooled cells were infused i.v. into 8-weeks-old NOD.SCID mice (n=6) in a dose of 20 x 10^6 cells/mouse. Transfer of splenocytes from PBS-treated NOD mice to 8 weeks-old female NOD.SCID mice consistently induced diabetes after 22 days. Blood glucose levels of NOD.SCID mice receiving splenocytes from PBS-treated NOD mice (-♦-) and c-hCG treated NOD mice (-◊-) are shown here. Data points from 4 weeks and onwards are significant by different. The results shown here are from a single experiment and representative of three independent sets of experiments.

A



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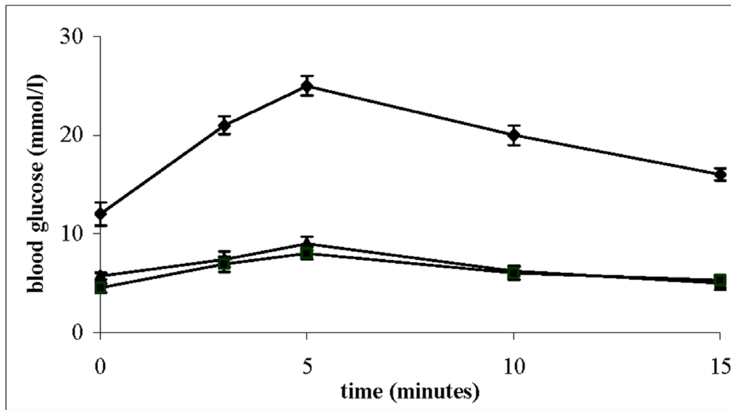

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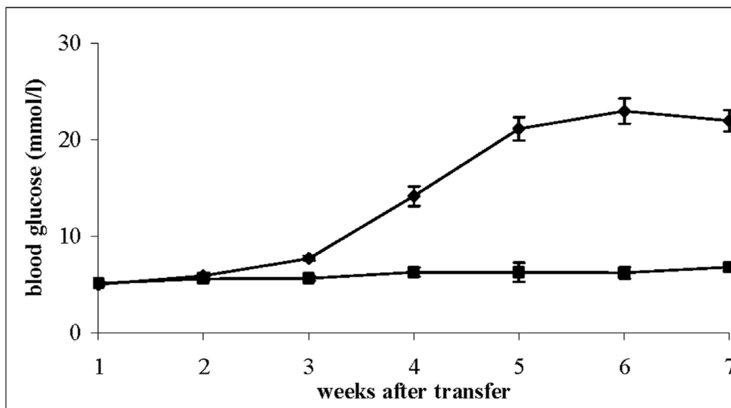


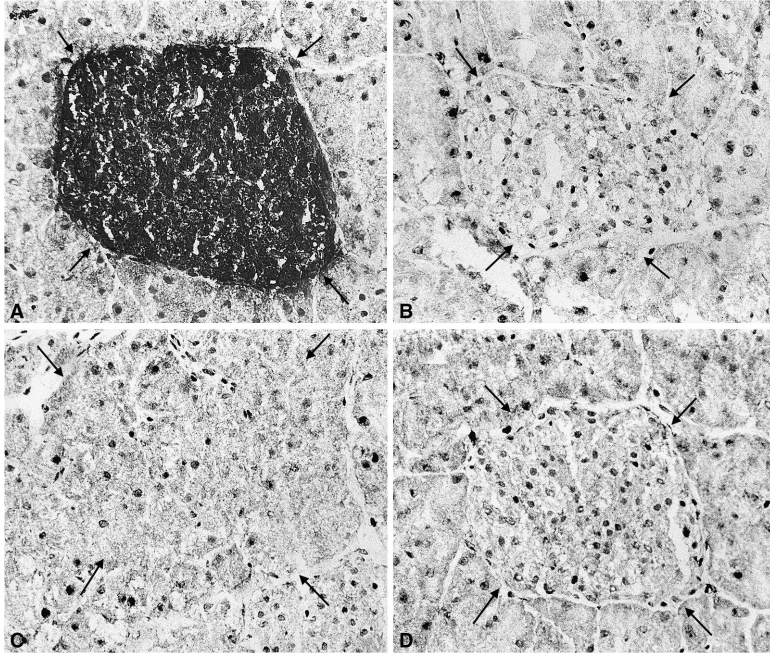
Histological examinations of the pancreatic tissue of the c-hCG treated mice at 21 weeks of age showed large insulin-producing islets and virtually no insulitis (Figure 2A-D, upper panel). Control-treated mice showed no insulin producing cells except one PBS treated mouse where only few weakly positive insulin producing cells and a massive infiltrate was found containing large numbers of T and B lymphocytes, macrophages (MOMA-1$^+$ and BM8$^+$ cells) and dendritic-like (ER-MP23$^+$) cells (partly shown in Figure 2A-D, lower panel) in the islets.

Impairment of the glucose tolerance test (GTT) is positively correlated to insulitis, but negatively correlated to the number of functional β cells (16,17). This test showed that NOD mice during the 5 weeks of c-hCG treatment and during the follow-up period till 35 weeks of age were tolerant for glucose (Figure 1B).

Splenocytes of diabetic NOD mice contain CD4$^+$ and CD8$^+$ diabetogenic T cells, which can effectively transfer the disease into immuno-compromised NOD.SCID mice (18,19). We transferred splenocytes from 21-weeks-old PBS and c-hCG treated NOD mice into NOD.SCID mice. Four weeks after transfer, all NOD.SCID mice which had received splenocytes from control (PBS) NOD mice were diabetic (Figure 1C), but mice which had received splenocytes from c-hCG treated NOD mice remained non-diabetic.

The effect of consecutively 4 days of *in vivo* c-hCG treatment on the Th1 and Th2 cell populations of NOD mice was studied in an *in vitro* Th polarization assay. For comparison BALB/c mice were similarly tested. Under Th1 polarizing conditions (thus in the presence of IL-12 and anti-IL-4), purified CD4$^+$ T-cells from c-hCG treated NOD mice showed a lower capacity to produce IFN-γ producing cells than PBS treated mice (19±2 vs 28±3 ng IFN-γ/ml). Under neutral conditions purified CD4$^+$ T-cells from c-hCG treated NOD mice also showed a significantly lower IFN-γ production as compared to PBS treated mice (Figure 3A), while no significant differences were observed between the groups in IL-4 production under neutral conditions (Figure 3B). The same was true for the effect of c-hCG treatment of NOD mice on the *in vitro* IL-4 production by purified CD4$^+$ T-cells under Th2 polarizing conditions (2 ng IL-4/ml). The IL-10 (Figure 3C) and TGF-β (Figure 3D) production by purified CD4$^+$ T-cells from c-hCG treated NOD mice *in vitro* under neutral conditions was significantly increased as compared to PBS treated NOD control mice.

Figure 2 (right). Immunohistochemistry of the pancreas of NOD mice after five weeks of c-hCG treatment.
Representative islets of PBS and c-hCG treated mice are shown. Staining of pancreatic tissue for insulin (**A**), B cells (**B**), T cells (**C**) and macrophages (**D**) of c-hCG (magn. 320x, upper panel) and PBS (magn. 128x, lower panel) treated NOD mice is shown. At least ten independent microscopic fields were examined from at least three animals per group. At least 50 islet sections were examined per mouse.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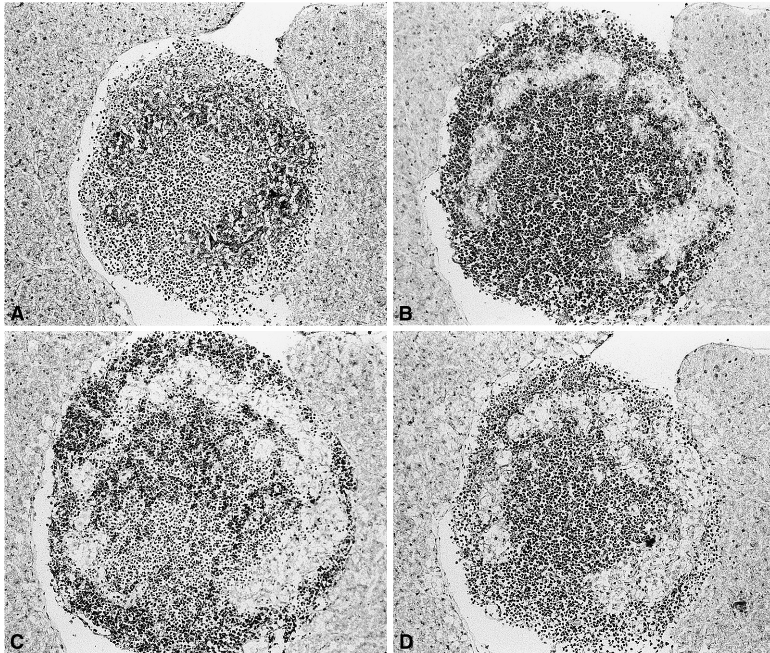
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In contrast to NOD mice, PBS and c-hCG treated BALB/c mice did not show differences in IFN-γ production under neutral conditions (Figure 3A) as well as Th1 polarizing (18±3 vs 17±3 ng IFN-γ/ml) conditions, while a significant upregulation of IL-4 production was observed under neutral conditions (Figure 3B). No significant differences in IL-4 production were observed under Th2 polarizing conditions between PBS and c-hCG treated BALB/c mice (21±4 vs 26±2 ng IL-4/ml).

hCG is a heterodimeric glycoprotein hormone composed of two non-covalently bound glycosylated α and β subunits. During pregnancy it is produced by the placenta

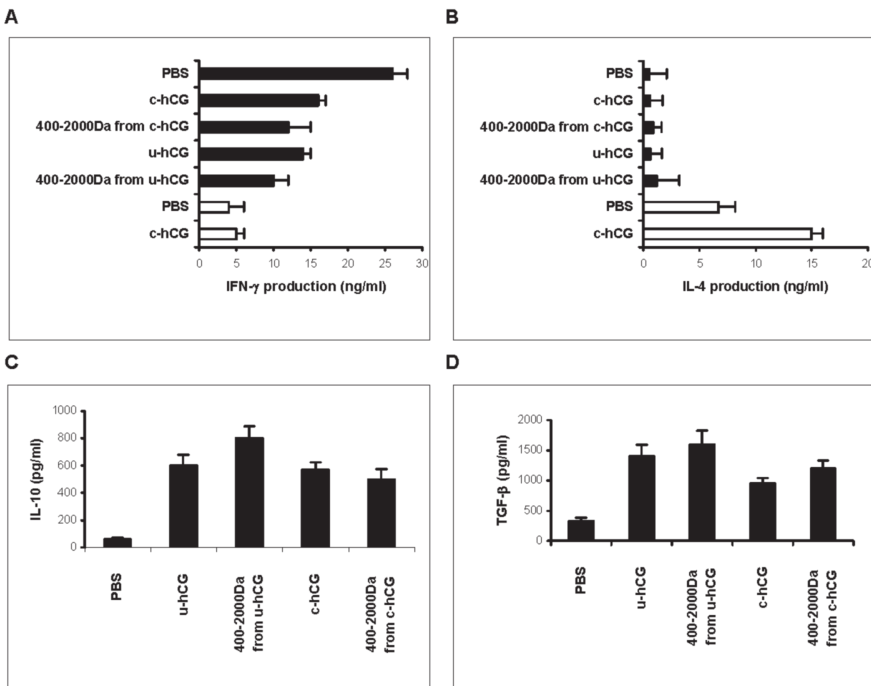


Figure 3. Effect of c/u-hCG treatment on Th polarization in NOD and BALB/c mice under neutral (non-polarizing conditions).

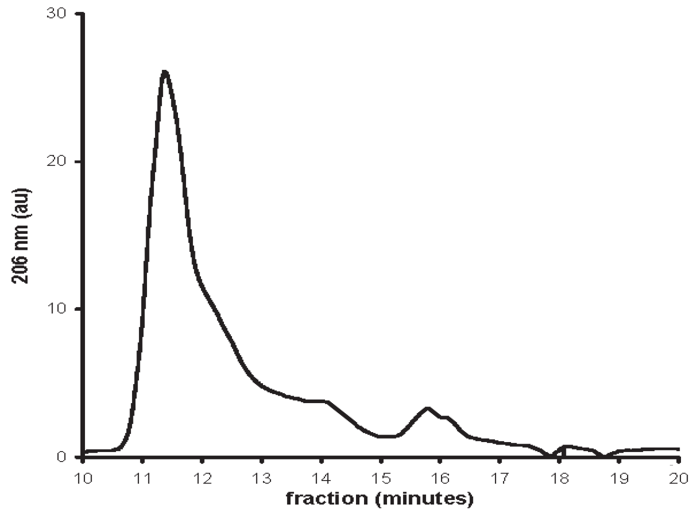
$CD4^+$ T-cells were purified from spleens of mice (n=6) treated with c/u-hCG or its 400-2000 Dalton fraction. Cells were stimulated with anti-CD3/anti-CD28 and cultured for 4 days with IL-2. Subsequently, the cells were washed and restimulated for 2 days with anti-CD3, anti-CD28 and IL-2 only. Supernatant levels of IFN-γ (**A**) and IL-4 (**B**) were measured by ELISA. Black bars correspond to $CD4^+$ T-cell cultures of NOD mice and white bars of BALB/c mice. The supernatant levels of IL-10 (**C**) and TGF-β (**D**) in these cultures are also shown. These results are from one experiment and are representative of at least three independent sets of experiments (* $p<0.05$).

and occurs in high concentration in circulation and urine in many different molecular forms such as heterodimer, free subunits, β core fragment, proteolytically cleaved forms and fragments (11,12,20,21). Commercial hCG preparations are derived from first trimester pregnancy urine and vary in their content of hCG and related molecules. To define better the active moiety in first trimester pregnancy urine (u-hCG) and in the heterodimeric hCG itself, we fractionated c-hCG and u-hCG by gel filtration using an FPLC system. Neither c/u-hCG fractions greater than 25,000 Dalton, nor r-hCG, highly purified urinary hCG, r-α-hCG and r-β-hCG inhibited the anti-CD3 stimulated IFN-γ production *in vitro* by spleen cells from diabetic NOD mice. Also the transfer of diabetes to NOD.SCID mice by such treated spleen cells from diabetic NOD mice was not inhibited (data not shown). We further fractionated c/u-hCG on a HPLC system and these fractions were also tested for their anti-diabetic effect. These experiments showed that the active moiety eluted at molecular range 400-2000 Dalton when assayed for IFN-γ inhibiting capacity and transfer of diabetes to NOD.SCID mice, employing spleen cells from diabetic NOD mice (Figure 4). This 400-2000 Dalton fraction from c/u-hCG also inhibited the development of diabetes in NOD mice, while no such inhibitory effect was observed with 400-2000 Dalton fraction from the urine of non-pregnant women (data not shown). Similar to c-hCG treatment *in vivo*, under Th1 polarizing conditions the c-hCG and u-hCG 400-2000 Dalton fractions inhibited the IFN-γ production (16±3 vs 28±3 ng IFN-γ/ml and 17±3 vs 28±3 ng IFN-γ/ml, respectively) as well as under neutral conditions by NOD $CD4^+$ T-cells (Figure 3A). The IL-4 production, however, was not affected under neutral (Figure 3B) as well as Th2 polarizing conditions (data not shown).

The effect of the 400-2000 Dalton c/u-hCG fractions on the *in vitro* IL-10 (Figure 3C) and TGF-β production (Figure 3D) was similarly enhanced when compared to PBS as found after culture with c-hCG and u-hCG.

We also tested two other c-hCG preparations from Profasi and APL for their anti-diabetic activity. Profasi and APL showed differential and only partial dose-dependent inhibitory effects (data not shown).

Beside heterodimeric hCG, β core fragment and nicked hCG are two other major forms of hCG that are found in the pregnancy serum and urine samples. Both of these forms of hCG either have peptide bond cleavages in residues 44-52 (loop 2) of β hCG or completely miss loop 2 (residues 41-54). This loop 2 region with amino acid sequence MTRVLQGVLPALPQ may liberate smaller fragments (oligopeptides) with biological activity. We designed and tested two oligopeptides from the loop 2 of β hCG, namely LQGV and VLPALP on the development of diabetes in the NOD mouse model. LQGV and VLPALP treatment of 14 weeks-old female NOD mice was done with a dose of 100 μg (total treatment dose of 900 μg per mouse) or 10 μg (total treatment dose of 90 μg per mouse) i.p. three times per week for three weeks. These experiments revealed that



Anti-diabetic activity - - - - - + + - - -
IFN-γ inhibition - - - - - + + - - -

Figure 4. Analysis of c-hCG by gel filtration using an HPLC system. GPC 60Å column was run at a flow rate of 0.5 ml/ml and 1 ml fractions were collected.
Each fraction was lyophilized and assayed for IFN-γ inhibiting capacity and transfer of diabetes to NOD. SCID mice, employing spleen cells from diabetic NOD mice. This figure shows that the IFN-γ inhibitory and anti-diabetic activity elutes in fractions that correspond to a molecular weight between 400 to 2000 Dalton.

both LQGV and VLPALP treatment significantly reduce the diabetes development after the age of 17 weeks (Figure 5).

Several studies have demonstrated that DC from NOD mice exhibit elevated levels of NF-kappa B activation upon stimulation due a hyperactive I kappa B kinase (22-24). These studies have shown that elevated NF-kappa B activation and consequently enhanced APC function are specific for the NOD genotype and correlate with the progression of insulin-dependent diabetes mellitus (25-27). These results also provided evidence for a role for NF-kappa B in regulating the APC function of DC. Therefore, we determined whether treatment of 14 weeks-old female NOD mice with either LQGV or VLPALP was associated with a decrease in NF-kappa B activity in the nuclear extracts of splenic DC by measuring the amount of *in vitro* LPS stimulated NK-kappa B p65 subunit, which is the transactivitor of numerous genes activated by NF-kappa B.

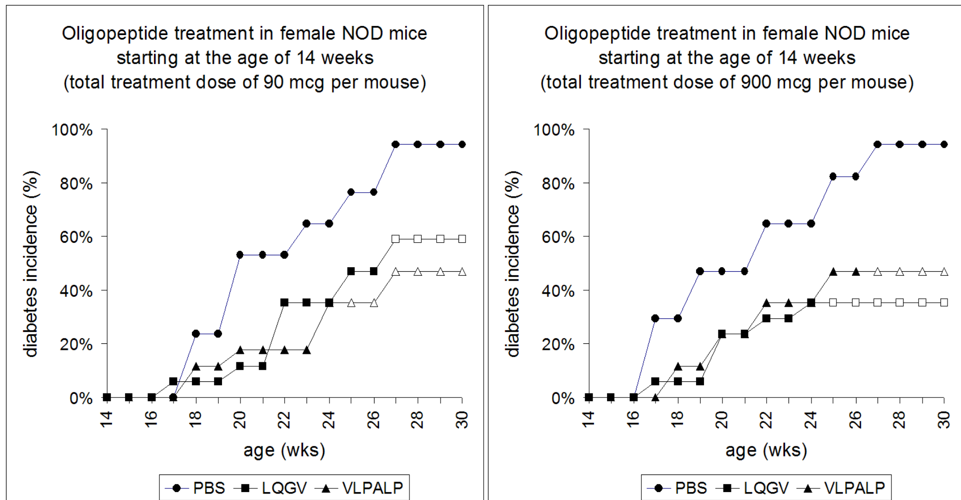


Figure 5.
Effect of 3 weeks of oligopeptide treatment of female NOD mice (10 or 100 μg oligopeptide, three times a week) with either a total treatment dose of 90 μg per mouse (**A**) or 900 μg per mouse (**B**) on diabetes incidence. Data are from three independent experiments (5-6 animals per group). Open square (□) and triangle (Δ) show age at which the difference in diabetes incidence is highly significant (p<0.0001, two-tailed Fisher's exact test) compared to the PBS group.

Figure 6 shows that *in vitro* stimulation of splenic DC from either LQGV or VLPALP treated NOD mice with LPS leads to a significantly (p<0.045) lower amount of p65 subunit of NF-kappa B in the nucleus as compared to LPS stimulated splenic DC from PBS treated female NOD mice.

DISCUSSION

The study presented here demonstrates that short-term (5 weeks) treatment of female NOD mice starting prior to the onset of the symptoms, with hCG preparations derived from first trimester pregnancy urine, inhibits the development of diabetes. This was supported by GTT testing, showing that the treated NOD mice were tolerant for glucose during 5 weeks of c-hCG treatment and during the follow-up period till 35 weeks of age. This and histochemical analysis indicated the presence of sufficient numbers of insulin-producing β-cells. The treatment even reversed established inflammatory infiltrates of the pancreas as evidenced by histology. Previous studies of our department have shown (15) that in our NOD colony at the age of 14 weeks the mice have abundant infiltrates

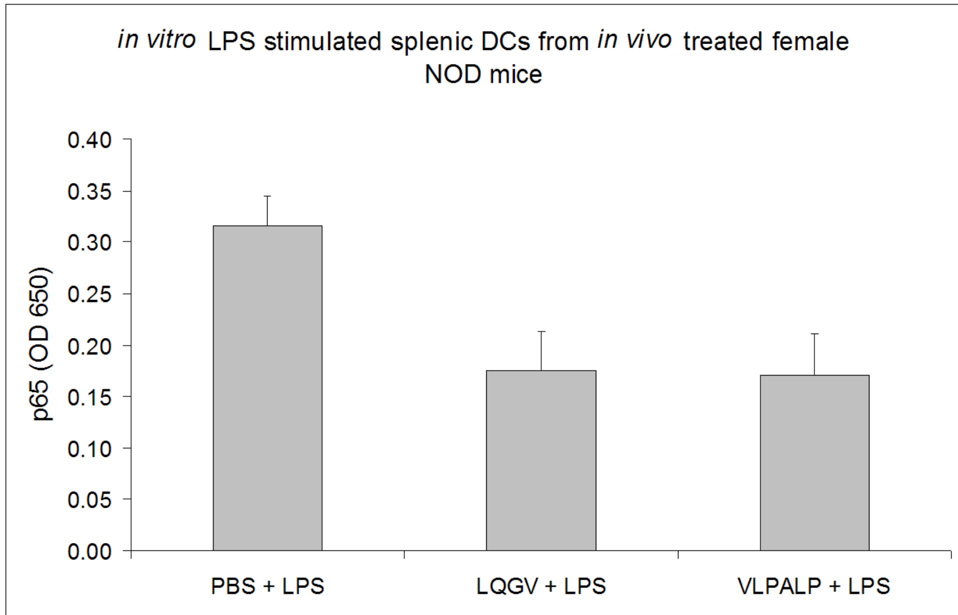


Figure 6.
Effect of 5 weeks of oligopeptide treatment (three times a week, 100 μg oligopeptide) starting at the age of 14 weeks on NFκB activation measured *in vitro* as amount of LPS (100 ng/ml) stimulated p65 subunit present in the nucleic extract of isolated splenic DC from female NOD mice (5 mice per group).

and severe insulitis (stage 4/5) in their pancreas correlating with the start of clinical symptoms of diabetes. These infiltrates consist of T and B lymphocytes, macrophages and dendritic-like cells. Others have demonstrated that short-term low-dose anti-CD3 treatment can also induce a complete remission of the diabetes when applied to adult NOD females within 7 days of full-blown diabetes (28). However, in the latter studies the remission was not associated with the disappearance of the insulitis.

Splenocytes of diabetic NOD mice contain diabetogenic T cells, which can transfer the disease into immuno-compromised NOD.SCID mice (18,19). Splenocytes from NOD mice treated with c-hCG for five weeks and from c-hCG-treated NOD mice 16 weeks after termination of the c-hCG treatment were not able to induce diabetes in NOD.SCID mice. This shows the fast and prolonged inhibitory effect of c-hCG-treatment on diabetogenic NOD cells. *In vivo* and *in vitro* c-hCG treated diabetogenic NOD

splenocytes produced low levels of IFN-γ, which correlated with the inability of these cells to transfer the disease to NOD.SCID mice.

Further investigation also revealed the direct or indirect effect of c/u-hCG treatment on $CD4^+$ T-cells of NOD mice. Such *in vivo* treated purified $CD4^+$ T cells showed lower IFN-γ production levels under Th1 polarizing and neutral conditions. This not just implicates intrinsic changes in T cells to develop into Th1 cells, but also suggests a smaller pool of IFN-γ producing Th1 cells *in vivo* due to the c/u-hCG treatment. No significant differences were observed between the groups in IL-4 production under Th2 polarizing and neutral conditions. Instead, upregulation of IL-10 and TGF-β production was observed under neutral conditions, showing that *in vivo* c/u-hCG treatment inhibited the IFN-γ production and the preferential outgrowth of Th1 cells by inducing increased levels of anti-inflammatory cytokines.

Splenocytes from c/u-hCG treated BALB/c mice did not show differences in IFN-γ production in the Th polarization assay as compared to control mice, but did show a significant upregulation of IL-4 production under neutral conditions. This excludes the possibility of a general inhibitory effect of c/u-hCG treatment on cytokine production and substantiates the immunomodulatory effect of the treatment.

Several groups have reported anti-Kaposi’s Sarcoma (KS) and anti-HIV activity of some hCG preparations (29). This was not due to the native hCG heterodimer. In these studies the active moiety was thought to be β-core or an as yet unidentified hCG associated factor. The mechanism of action was proposed to be the selective induction of apoptosis or direct cytotoxic effects on the tumor cells (30). No infiltration of the tumor with mononuclear cells was observed, leading to the conclusion that the anti-KS activity could not be due to an immune-mediated response (31).

As evident by HPLC, our 400-2000 Dalton bioactivity from pregnancy urine and thereof derived hCG preparations eluted at a position which is far removed from the elution positions of hCG (38 kDa), β-hCG free subunit (>15 kDa) and β-core (> 10 kDa). In addition, neither c/u-hCG fractions greater than 2000 Dalton, nor r-hCG, highly purified urinary hCG, r-α-hCG and r-β-hCG showed inhibition of IFN-γ production and anti-diabetic activity. This argues against the possibility that functional heterodimeric hCG, its subunits or β-core could be responsible for the immunomodulatory and anti-diabetic effects. We named this 400-2000 Dalton bioactivity Natural (immuno)Modulatory Pregnancy Factor(s) (NMPF). Heat (30 minutes at 56°C) and protease enzyme (elastase, pronase) treatment abrogated the immunomodulatory effect of NMPF suggesting a peptide nature of the factor(s). Our data excludes that the mechanism through which NMPF, with a proven molecular weight between 400 and 2000 Dalton, exerts its anti-diabetic effect and the c-hCG associated component that exerts the anti-KS/anti-HIV effect (30) are the same.

We are not yet able to define the exact structure of peptide nature component(s) in

NMPF that is responsible for immunoregulatory effect. We designed various peptides from the primary structure of β hCG loop 2 since this structure is known to be especially prone to proteases. We postulate that the loop 2 region consisting of residues 41-54 with amino acid sequence MTRVLQGVLPALPQ, which is absent in β core and is cleaved at various positions in nicked hCG, can liberate *in vivo* smaller fragments with biological activity. We synthesized several partly overlapping oligopeptides from the amino acid 41-54 sequence. In this report we show the effect of LQGV and VLPALP on the development of diabetes in NOD mice. Both oligopeptides were able to reduce the incidence of diabetes in NOD mice at a dose of 10 µg oligopeptide per treatment (total dose: 90 µg). A dose of 100 µg oligopeptide per treatment (total dose: 900 µg) did not significantly increase the proportion of NOD mice that did become diabetic. In contrast to these oligopeptides, NMPF fully inhibited the development of diabetes suggestive of the presence of multiple oligopeptides in NMPF. This might imply that a cocktail of oligopeptides from loop 2 might be able to fully inhibit the development of diabetes in NOD mice as NMPF did.

Various studies have described a defective NF kappa B regulation in NOD DC (22-27). DC derived from NOD mice are more sensitive to various stimuli. This might be consistent with a role of DC in the development of diabetes in NOD mice. In this study we have shown that the reduction of diabetes incidence in NOD mice due to the treatment with LQGV and VLPALP is associated with lower *in vitro* LPS induced NF-kappa B activity in splenic DC. Whether there is a direct or indirect effect of these oligopeptides on NF-kappa B activation remains unknown and will be center of focus in future studies. Various investigators have demonstrated that DC are able to transfer experimental autoimmune diseases such as thyroiditis, encephalomyelitis and type 1 diabetes with great efficiency and play an important role in the regulation of the autoimmune response early in the disease pathogenesis (32,33). Treatment of female NOD mice with LQGV and VLPALP at 6 weeks of age did not affect the diabetes incidence (data not shown). Interestingly, histological examinations of the pancreatic tissue of the NMPF and oligopeptide (LQGV and VLPALP) treated mice at 23 weeks of age showed small insulin-producing islets with virtually no insulitis (data not shown) suggesting the induction of a regenerative process due to the treatment.

Recently, several studies have reported the reversal of type 1 diabetes in NOD mice by the recovered host beta cells (34-36). In these studies the beta cells recovered from the immune attack and proliferated to restore the beta-cell mass, showing that the dampening of the autoimmune attack, coupled with the recovery of residual host islets, underlies the cure in mice. Beta-cell mass has also been shown to increase under several other circumstances, including pregnancy. Mice in which the insulin receptor in the liver cells has been knocked out respond by increasing beta-cell mass by factor 10 (37). Therefore, it is possible that NMPF and loop 2 oligopeptide have effect

on beta-cell proliferation and regeneration in addition to the effect on immune system. Consequently, if we can harness the endogenous capacity of beta cells to proliferate and can combine this ability with a more effective blunting of the autoimmune attack in humans, it may well be possible to devise new treatments for type 1 diabetes. In this respect the oligopeptides from loop 2 of beta hCG are interesting candidates.

In summary, our data shows that a low molecular weight fraction from first trimester human pregnancy urine, commercial hCG preparations and particular oligopeptides from loop 2 of beta hCG can prevent diabetes development in NOD mice and possibly other Th1 mediated autoimmune diseases. Further studies into the mechanism(s) of action through which these small factors exert their actions are in progress.

ACKNOWLEDGMENTS

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INHIBITION OF SEPTIC SHOCK IN MICE BY AN OLIGOPEPTIDE FROM THE β-CHAIN OF HUMAN CHORIONIC GONADOTROPIN HORMONE

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SUMMARY

Human chorionic gonadotropin (hCG) is a heterodimeric placental glycoprotein hormone required in pregnancy. In human pregnancy urine and in commercial hCG preparations (c-hCG) it occurs in a variety of forms including breakdown products. Several reports have suggested modulation of the immune system by intact hormone, but such effects of breakdown products have not been reported. In the accompanying paper in this issue we report that a 400-2000Da fraction from c-hCG and from human pregnancy urine inhibits Th1 mediated diabetes in NOD mice. The active component(s) were called natural (immuno)modulatory pregnancy factor(s) (NMPF). In this report we show that a single treatment with the same low molecular weight NMPF-fraction up to 24 hours after high dose lipopolysaccharide (LPS) injection inhibited septic shock in mice. This counteracting effect of NMPF paralleled the down regulation of the effects of LPS on the production of macrophage migration inhibitory factor (MIF) by spleen cells, on the plasma level of liver aminotransferase, and on the expression of several splenic lymphocyte and macrophage surface markers.

Based on the primary structure of the β-chain of hCG we designed a synthetic hexapeptide Valine-Leucin-Proline-Alanine-Leucine-Proline (VLPALP) and showed it to have the same protective effects as the 400-2000Da NMPF-fraction. These results indicate a new strategy for the treatment of septic shock and the potential of therapeutic use of this synthetic oligopeptide.

INTRODUCTION

hCG is heterodimeric glycoprotein hormone produced in pregnancy by the placental trophoblast. It maintains the steroid secretions of the corpus luteum and thereby maintains the lining of the uterus for development of the embryo after its implantation (1). Clinical symptoms of Th1 mediated autoimmune diseases regress in many patients during pregnancy (2,3). In the accompanying paper (4) we showed for the first time the inhibition of Th1 mediated autoimmune diabetes in NOD mice by a fraction from hCG preparation that contains one or more 400-2000 Dalton natural (immuno) modulatory pregnancy factors (NMPF). It is postulated that this NMPF, possibly in concert with other immunomodulatory factors, accounts for an adjustment of the immune system during pregnancy. This adjustment is not only leading to clinical improvement of Th1 autoimmune diseases, but also to the maintenance of normal immune competence against microorganisms and suppression of the Th1-mediated immune response to the paternal antigens of the developing fetus.

In the present paper we investigated whether the 400-2000Da NMPF was not only

able to suppress the ongoing insulitis and to reverse the development of diabetes in NOD mice (4), but also septic shock in mice. We used high-dose endotoxin shock in BALB/c mice as a model of acute inflammation and human septic shock syndrome (5,6). In this model intraperitoneal (i.p.) injection of a high dosis of lipopolysaccharde (LPS) triggers an acute inflammatory response characterized by the rapid increase of the plasma level of liver aminotransferase and the serum level of proinflammtory cytokines such as macrophage migration inhibitory factor (MIF) (7). We show that the 400-2000Da NMPF-fraction from hCG is able to prevent and even to suppress mortality in mice due to LPS-induced septic shock.

hCG occurs in various forms in blood and urine during pregnancy (1,8). Apart from its heterogeneity of carbohydrate content, nicks at various regions of hCG, particularly in the β-chain, are responsible for these different forms. We designed a synthetic oligopeptide (i.e. VLPALP, corresponding to residues 48-53) from the primary structure of the β-chain of hCG with similar anti-shock activity in the endotoxin shock model as the 400-2000Da NMPF-fraction.

MATERIAL AND METHODS

Mice, endotoxin shock model, and hCG-treatment

BALB/c mice were purchased from Harlan (Horst, The Netherlands) and maintained as described in the accompanying paper (4). For the induction of sepsis, 8-12 weeks old female BALB/c mice (*n*=10) were injected i.p. with 8 mg/kg LPS (E. coli 026:B6; Difco Laboratories, Detroit, MI, USA). The experiments were approved by the Animal Experiments Committee of the Erasmus University Rotterdam.

To test the effect of NMPF on LPS-induced septic shock, hCG-preparations from several sources were used: commercial hCG preparation (c-hCG; Pregnyl Organon, Oss, The Netherlands, Lot numbers 209893, 235863, 248455), hCG purified from first trimester pregnancy urine (u-hCG), the 400-2000Dalton fractions of c-hCG and u-hCG, recombinant hCG (r-hCG; Sigma, St. Louis, MO, USA), its subunits (r-α-hCG and r-β-hCG; Sigma, St. Louis, MO, USA), highly purified urinary hCG and the synthetic oligopeptide VLPALP designed from β-chain of hCG. BALB/c mice were treated with either a dose of 300 IU c-hCG, 5 mg/kg u-hCG, 0.5 mg/kg 400-2000Dalton NMPF-fraction from c-hCG or u-hCG, 5 or 15 mg/kg NMPF peptide (VLPALP) or 5 mg/kg r-hCG or its subunits (r-α-hCG or r-β-hCG). This treatment was done either two hours or 24 hours after the LPS injection. Control groups were treated with PBS instead of LPS, and/or PBS instead of hCG/NMPF.

Purification and dose titration of preparation of u-hCG from first trimester human pregnancy urine and of the 400-2000Da NMPF-fractions was done according to the method described in the accompanying paper (4).

Semi-quantitative sickness measurements

Mice were scored for sickness severity using the following criteria. Score 1: percolated fur, but no detectable behaviour differences compared to untreated control mice; score 2: percolated fur, huddle reflex, responds to stimuli (such as tap on cage), just as active during handling as untreated control mice; score 3: slower response to tap on cage, passive or docile when handled, but still curious when alone in a new setting; score 4: lack of curiosity, little or no response to stimuli, quite immobile; score 5: laboured breathing, inable or slow to self-right after being rolled onto back (moribund); score 6: dead.

Cytokine assay

Spleen cells were isolated from treated BALB/c mice 24 hours after the LPS shock induction as described previously (4). Splenic cell suspensions were cultured (2 x 10^5 cells/well) in 96-well flat-bottom plates (0.2 ml) and stimulated with LPS (10 μg/ml). RPMI 1640 medium was used supplemented with 25 mM HEPES, 100 IU/ml penicillin, 50 μg/ml streptomycin, 1 mM pyruvate, 50 μM 2-ME and 10% heat inactivated fetal calf serum. All cultures were performed in triplicate or quadruplicate. After incubation for 12hrs, supernatants were collected for MIF analysis.

The amount of MIF was measured with commercially available ELISA kit (Chemicon International, Temecula, CA, USA) according to the protocol provided by the manufacturer. The detection limit was 800 pg/ml.

Analysis of liver enzymes

Hepatocyte damage was assessed 24 hours after LPS administration by measuring plasma enzyme activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using commercial kits (Merck, Darmstadt, Germany) on an automated analyzer (ELAN-Analyzer; Eppendorf, Hamburg, Germany).

Flow-cytometric analysis

Spleen cells were isolated from treated BALB/c mice 24 hours after the LPS shock induction and used for the analysis of cell surface markers. Splenic cells (2 x 10^5) were resuspended in PBS containing 1% BSA and 0.1% sodium azide (PBS-BSA-azide). For the staining of surface antigens, spleen cells were incubated with FITC or PE-conjugated mAb against CD3, B220, NK1.1, MHC-II and F4/80 (all obtained from PharMingen, San Diego, CA, USA). After washing twice with PBS-BSA-azide, the cells were resuspended and analyzed on FACScan (Becton Dickinson, San Jose, CA, USA). 10 μl propidium iodide (0.2 mg/ml) was added to evaluate the viability of the cells. In all samples the viability was higher than 94%. 10^4 events were collected and the expression of the markers analyzed using CellQuest software (Becton Dickinson).

Statistics

Data was analyzed by Student's T test and differences were considered significant at $p<0.05$.

RESULTS

The model for LPS-induced septic shock syndrome in BALB/c mice was standardized by testing various doses of E. coli 026:B6 LPS i.p. Every 12 hours the survival and sickness scores were assessed. For the experiments reported here, we used a treatment of 8 mg LPS per kg body weight (BW), which was lethal for all BALB/c mice by 60 hours. The signs of sickness were apparent in all LPS-treated mice, but the kinetics and severity were different between the mice. In order to quantitate the observed differences, we used a semi-quantitative sickness scoring system that was standardized in several shock experiments using large numbers of BALB/c mice (*n*=30). In these experiments, mice i.p. injected with 8 mg LPS/kg BW achieved a sickness score (see section Material and Methods) of about 2 within 12 hours. At the time point of 24 hours most of the BALB/c mice had reached the sickness score of 3 or 4. Thereafter the mice gradually reached a sickness score of 5 or died (score 6). At the 60 hours time point all LPS-injected mice were dead (score 6).

Treatment of BALB/c mice with c-hCG (300 IU) or u-hCG (5 mg/kg) 2 hours after LPS (8 mg/kg BW) injection increased the survival rate to 80% as compared to 0% of the PBS treated mice, while the 400-2000Da fraction purified from c-hCG or u-hCG (0.5 mg/kg) prevented mortality and reduced the sickness score in all mice (Figure 1A). The maximum sickness scores observed in c/u-hCG and the 400-2000Da fraction treated groups during the experiment were 3 and 2, respectively. The score 3 was observed in all (10/10) c-hCG treated mice at 24 hours after LPS-injection, and the score 2 was observed in all (10/10) 400-2000Da NMPF-fraction treated mice at 24 hours after LPS-injection. In the hours thereafter the mice steadily recovered, so that at 84 hours after the shock induction all treated mice had a sickness score not higher than 1. Two days later all mice had completely recovered. Similar hCG treatments using highly purified urinary hCG, r-hCG, r-α-hCG and r-β-hCG were ineffective (data not shown).

HCG occurs in various forms during pregnancy. It is known that hCG and its uncombined subunits, especially β-hCG, are prone to proteolytic cleavage resulting in different forms of "nicked" hCG (8). In order to determine whether such fragments or other breakdown products of hCG could be responsible for the above described anti-shock activity, we designed a synthetic oligopeptide with the amino acid sequence of valine-leucine-proline-alanine-leucine-proline (VLPALP) from the primary structure of β-hCG and tested it for its anti-shock activity in the same model. Similar to the c/u-hCG

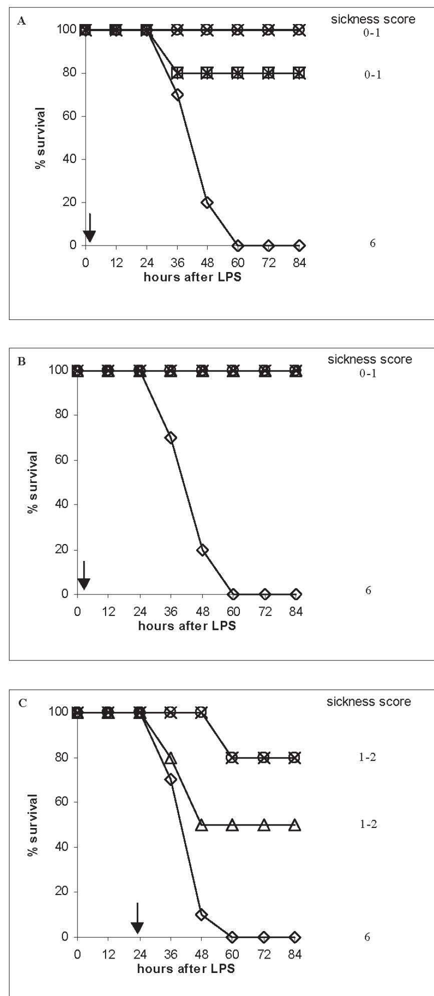

Figure 1. Effect of commercial hCG preparation (c-hCG), hCG from first trimester pregnancy urine (u-hCG), 400-2000Da c-hCG (c-hCG fraction), 400-2000Da u-hCG (u-hCG fraction) and VLPALP on the survival of BALB/c mice subjected to LPS-induced septic shock.
(A), Survival percentages and sickness scores (at 84 hours) of PBS (◇), c-hCG (□), u-hCG (⁎), c-hCG fraction (x), and u-hCG fraction (o) treated mice. Treatment was performed 2 hours (arrow) after the LPS injecton. **(B)**, Survival percentages and sickness scores (at 84 hours) of PBS (◇), c-hCG fraction (x), u-hCG fraction (o) and VLPALP (Δ) treated mice. Treatment was performed 2 hours (arrow) after the LPS injection. **(C)**, Survival percentages and sickness scores (at 84 hours) of PBS (◇), c-hCG fraction (x), u-hCG fraction (o) and VLPALP (Δ) treated mice. Treatment was performed 24 hours (arrow) after the LPS injection. The results shown are from a single experiment and representative of at least three independent sets of experiments (each group: n=10).

fractions, treatment of BALB/c mice with VLPALP (5 mg/kg BW) two hours after LPS (8 mg/kg BW) injection completely inhibited mortality due to septic shock. The maximum sickness score observed in the VLPALP-treated mice was 2 (at 24 hours) decreasing to score 1 (at 60 hours), with complete recovery of the majority of the mice at 84 hours (Figure 1B).

Since different pathophysiological processes are involved in septic shock syndrome we determined whether the NMPF fractions and VLPALP have the ability to inhibit the septic shock in the later stages of the disease as well. Therefore, we postponed the 400-2000Da NMPF-fraction and the VLPALP-treatment till 24 hours after the septic shock induction by LPS. In these experiments the same dose of NMPF fraction (5mg/kg) was also effective in reducing the mortality, although this delayed treatment was not equally effective as treatment at 2 hours after LPS-injection. After this delayed treatment we found an 80% survival (Figure 1C); most of the mice showed improved sickness scores already 24 hours after the delayed NMPF treatment. In this set-up of delayed NMPF treatment, VLPALP was able to reduce the mortality to 50% provided a threefold higher dose (15 mg/kg) was used as in the experiments employing VLPALP-treatment 2 hours after LPS-injection. In this experiment the sickness scores of the mice of all 4 experimental groups ranged from 3 to 5 at 24 hours after LPS-injection, which, in both NMPF fraction treated groups and the VLPALP-treated group, decreased to 1 or 2 after 84 hours. At 96 hours after LPS-injection all mice had completely recovered.

Overwhelming inflammatory responses are essential features of septic shock and play a central role in the pathogenesis of the tissue damage, multiple organ failure, and death induced by sepsis (9). Cytokines, especially macrophage migration inhibitory factor (MIF), once released, induce the expression of an array of proinflammatory mediators by macrophages and activated T cells, which strongly promote the characteristic inflammatory response (7). Therefore 24 hours after the shock induction, we isolated splenocytes from BALB/c mice, which were treated 2 hours after LPS (8 mg/kg BW) injection with NMPF fraction or VLPALP. These splenocytes were *in vitro* restimulated with LPS (10 μg/ml) and after 12 hours of culture the MIF levels were determined in the supernatants. These experiments revealed that the *in vivo* treatment with either NMPF fraction or VLPALP significantly inhibited the MIF production as compared to PBS-treated mice subjected to high dose LPS-administration (Figure 2A). We did not find significant differences in MIF levels produced by LPS-stimulated splenocytes obtained from mice not previously injected with LPS, but only treated with NMPF fractions or VLPALP as compared to PBS (Figure 2B).

Consistent with the clinical improvement induced by NMPF fraction or VLPALP-treatment, and the MIF release reducing effects of these treatments, NMPF fraction and VLPALP reduced the plasma levels of both ALT and AST liver enzymes in the LPS-injected mice (Figure 2C,D). The plasma levels of these enzymes in mice treated with

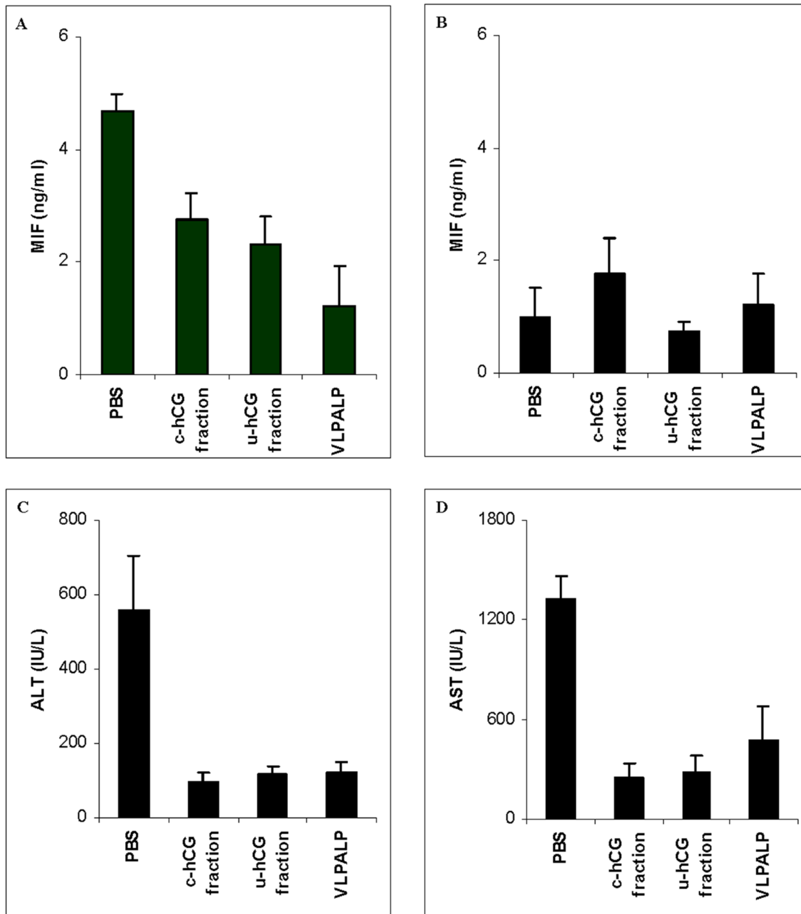

Figure 2. Effect of 400-2000Da NMPF-fraction from c-hCG (c-hCG fraction) and u-hCG (u-hCG fraction), and VLPALP on the in vitro MIF production by spleen cells and on the plasma aminotransferase levels of septic shock mice.
(A) MIF levels in cultures of splenocytes isolated from LPS-injected mice 24 hours after LPS-injection. The mice were treated with PBS, c-hCG fraction, u-hCG fraction or VLPALP 2 hours after LPS injection. **(B)** MIF levels in cultures of splenocytes isolated from PBS-injected control mice 24 hours after PBS-injection. The mice were treated with either PBS, c-hCG fraction, u-hCG fraction or VLPALP 2 hours after PBS-injection (i.e. 22 hours before harvest of the spleen cells). **(C)** Plasma alanine aminotransferase (ALT) and **(D)** plasma aspartate amniotransferase (AST) levels of mice 24 hours after LPS-injection and treated (at 2 hours after LPS-injection) with either PBS, c-hCG fraction, u-hCG fraction or VLPALP. The results presented are from a single experiment and representative of at least three independent sets of experiments (n=6).

NMPF fraction or the oligopeptide VLPALP only (and not previously with LPS) were not significantly different as compared to PBS-treated mice (data not shown).

Activation of cellular immune responses requires the expression of major

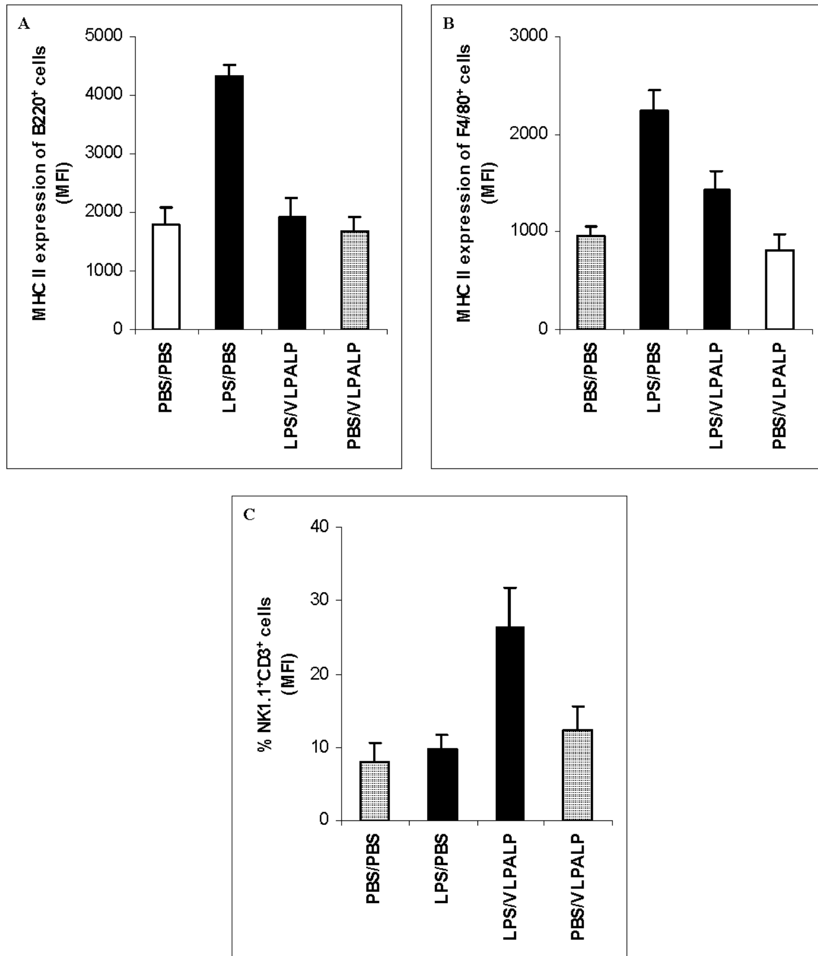

Figure 3. Effect of VLPALP (and PBS as control) on the expression of several surface markers on spleen cells of BALB/c mice i.p. injected with LPS or PBS as a control. VLPALP (and PBS as control) injection was done 2 hours after the injection of LPS (and PBS as control).
(A), Mean fluorescence intensity (MFI) of MHC II on B220$^+$ cells; **(B)** MFI of MHC II on F4/80$^+$ cells; and **(C)** percentage of NK1.1$^+$CD3$^+$ cells. Solid bars represent LPS-injected mice; hatched bars represent PBS-injected mice. These results are from one experiment and are representative of at least three independent sets of experiments, (n=6).

histocompatibility complex (MHC) molecules on cells of the innate (macrophages) and adaptive (B cells) immune systems (10). In sepsis the upregulation of MHC class II (MHC II) molecules on macrophages and B cells is a hallmark of the overactivated immune system (11). Therefore, we also examined the influence of VLPALP-treatment on MHC II expression on F4/80$^+$ (macrophage marker) and B220$^+$ (B cell marker) cells during septic shock. In these experiments the LPS-induced MHC II expression on both splenic cell populations was significantly decreased in mice treated with VLPALP (Figure 3A,B). Mice treated with VLPALP alone (and not previously with LPS) did not show a significant change in MHC II expression. Remarkably, mice treated with LPS and VLPALP showed an increased percentage of splenic NK1.1$^+$CD3$^+$ cells (Figure 3C). Treatment of mice with LPS or VLPALP alone did not show such difference ($p=0.44$ and $p=0.15$, respectively) as compared to PBS treated mice (Figure 3C).

DISCUSSION

In pregnancy the immune system is tightly regulated in order to maintain the co-existence of mother and fetus (12). Many women experience less disease severity or even remission of Th1 mediated autoimmune diseases during pregnancy. This epiphenomenon has been attributed at least in part to a shift from a “Th1” to a “Th2” state of the immune system during pregnancy (12), leading to view that pregnancy is associated with a state of immunosuppression. Considering that pregnant women in general do not show an increased susceptibility to infections, this supposed immunosupression must be quite selective in order to provide for the required immunological homeostasis between mother and fetus. We have shown in the accompanying paper (4) that a 400-2000Da NMPF fraction from pregnancy urine and commercial hCG preparation inhibits Th1-mediated diabetes in NOD mice. The data presented in the present report show that the same NMPF fraction is also capable of inhibiting an acute inflammatory response as is the case in high-dose LPS-induced septic shock in mice.

The pathophysiology of septic shock shares characteristics of systemic inflammatory response syndrome (SIRS) and multisystem organ failure (MOF) involving bacterial products, immunocompetent cells, soluble mediators, and cell-cell interactions between blood cells and endothelium (9,12). In the high-dose LPS model for septic shock in mice, hCG-derived NMPF, including the synthetic peptide VLPALP, were found to be capable of inhibiting the disease severity as well as mortality. This effect was not only apparent from the clinical symptoms, but also from the inhibition of the *in vitro* MIF production and the inhibition of the plasma aminotransferase levels and the cellular characteristics in the spleen. The LPS-induced increase of plasma aminotransferase level is indicative for liver damage which plays a crucial role in septic shock (6).

Several reports have suggested immunosuppressive and antiproliferative effects of hCG *in vitro* (13,14). In these experiments the main sources of the intact hormone were pregnancy urine, commercial hCG preparations and recombinant hCG. In view of the anti-diabetic (4) and the anti-shock effects of the 400-2000Da NMPF fraction of hCG and the anti-shock effect of the synthetic oligopeptide VLPALP, it is possible that the previously reported immunosuppressive and antiproliferative effects were due to hCG break down products.

It is of interest that the low molecular weight NMPF fractions and VLPALP were also able to decrease the clinical symptoms and mortality due to septic shock when treatment was given as late as 24 hours after LPS-administration. Apparently the active component(s) interfered with the inflammatory process and the tissue damage underlying the clinical symptoms. Preliminary data also shows the inhibition of exotoxin shock induced by toxic shock syndrome toxin (TSST-1) by NMPF (data not shown), which indicates that the anti-shock activities of NMPF are not limited to endotoxin shock. These properties fulfill essential requirements for any anti-sepsis agent, as in patients treatment is always started after the onset of the clinical symptoms of septic shock.

The down-regulation of MHC II expression both on macrophages and activated B cells may not only be beneficial in controlling acute inflammatory responses, but likely also in chronic inflammatory immune reactions, particularly in autoimmunity in which MHC II expression plays a critical role in the presentation of autoantigens to T cells (10). This suggests that VLPALP might also be effective in the prevention and possibly the suppression of autoimmunity. In Figure 3 we showed that NMPF peptide upregulated the percentage of $NK1.1^{+}CD3^{+}$ cells. These so-called natural killer-T cells (NKT cells) are a subpopulation of T cells that share some characteristics with NK cells (15,16). NKT cells are phenotypically, functionally and developmentally heterogeneous. In general three distinct subsets ($CD4^{+}$, $CD8^{+}$, and $CD4^{-}CD8^{-}$) are known. These subpopulations are differentially distributed in a tissue-specific fashion (16). We do not know yet to which subset(s) the VLPALP-induced NKT cell subpopulation belongs. NKT cells are believed to be involved in immune responses ranging from suppression of autoimmunity to tumor rejection (16,17).

Most interesting is the observation that the 400-2000Da NMPF fractions and VLPALP did not affect the splenic MIF-production, the plasma aminotransferase levels, the MHC II expression on splenic macrophages and B cells, and the proportion of $NK1.1^{+}CD3^{+}$ double positive cells in the spleen of otherwise untreated mice. This strongly suggests that NMPF do not have a general immunosuppressive effect, but correct disturbances of the immune homeostasis as occurs after high dose LPS-injection and during the development of diabetes in NOD-mice (4). This warrants studies on the therapeutic potential of VLPALP and related NMPF in inflammation, autoimmunity, transplantation, allergy and related conditions, including pre-eclampsia and tumor rejection.

In summary, our data shows that a low molecular weight fraction from first trimester human pregnancy urine and the novel synthetic oligopeptide VLPALP designed from β-hCG can inhibit septic shock in BALB/c mice. Moreover, we showed for the first time that hCG fragments can regulate the immune balance and therefore might be of potential value to correct dysregulation of the immune system in a variety of immune and inflammatory diseases.

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MITIGATION OF SEPTIC SHOCK IN MICE AND RHESUS MONKEYS BY HUMAN CHORIONIC GONADOTROPIN-RELATED OLIGOPEPTIDES

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ABSTRACT

The marked improvement of several immune-mediated inflammatory diseases during pregnancy has drawn attention to pregnancy hormones as potential therapeutics for such disorders. Low molecular weight fractions derived from the pregnancy hormone human chorionic gonadotropin (hCG) have remarkable potent immunosuppressive effects in mouse models of diabetes and septic shock. Based on these data we have designed a set of oligopeptides related to the primary structure of hCG and tested these in models of septic shock in mice and rhesus monkeys. We demonstrate that mice exposed to lipopolysaccharide (LPS) and treated subsequently with selected tri-, tetra-, penta- and hepta-meric oligopeptides (i.e. MTR, VVC, MTRV, LQGV, AQGV, VLPALP, VLPALPQ) are protected against fatal LPS-induced septic shock. Moreover, administration of a cocktail of three selected oligopeptides (LQGV, AQGV and VLPALP) improved the pathological features markedly and nearly improved haemodynamic parameters associated with intravenous Escherichia coli-induced septic shock in rhesus monkeys. These data indicate that the designed hCG-related oligopeptides may present a potential treatment for the initial hyperdynamic phase of septic shock in humans.

INTRODUCTION

In immunological terms, pregnancy is an improbable symbiosis of two major histocompatibility complex (MHC)-incompatible individuals. Pregnant women are eminently capable of combating infections and often produce antibodies against paternal alloantigens of the fetus, demonstrating that they are fully immunocompetent. Nevertheless, a remarkable improvement of several immune-mediated inflammatory diseases, such as rheumatoid arthritis and multiple sclerosis, is often observed during pregnancy (1,2). These well-known, albeit poorly understood, features are suggestive for a selective modulation of the immune system in such a way that harmful immune processes to mother and fetus are suppressed, while beneficial immune processes are not affected. Certain hormones that are produced exclusively during pregnancy, such as oestriol or human chorionic gonadotropin (hCG), have been held responsible for the selective modulation of adverse immunological functions (2–4).

Human chorionic gonadotropin (hCG) is a heterodimeric glycoprotein produced by trophoblasts (5). Its main hormonal function is to preserve the lining of the uterus, which is needed for the healthy development of the implanted embryo (5). Activity-guided purification of commercial hCG preparations has shown that oligomeric degradation products represent a significant part of the immunosuppressive activity in immune-mediated inflammatory disease models (6,7), but they lack the endocrine effects of

the complete protein. These capacities make hCG-derived oligopeptides a potentially interesting source of therapeutics for immune-mediated inflammatory diseases.

The aim of the current study was to test whether such oligopeptides related to hCG can also reduce the severity of lipopolysaccharide (LPS)-induced and Escherichia coli-induced septic shock. Contamination of the bloodstream with bacteria or bacterial endotoxins (e.g. LPS) can cause massive activation of complement and the release of proinflammatory cytokines, such as tumour necrosis factor (TNF)-α(8). Irrespective of the origin (infectious or non-infectious), this leads to life-threatening haemodynamic changes, such as tachycardia and hypotension culminating in multi-organ failure. Without adequate treatment, septic shock can be lethal within a few days after onset.

Sepsis is the second leading cause of death in intensive care units (ICUs) in the United States (9,10) and is responsible for about 10% of overall deaths annually (11). There is a high unmet need for a safe and effective treatment of septic shock, particularly to prevent the irreversible multiple organ failure stage.

For the present study we designed a set of hCG-related oligopeptides all related to loop 2 of the β-chain, namely MTR, MTRV, LQG, LQGV, VLPALP, VLPALPQ and VVC, as well as an alanine variant of LQGV, namely AQGV. This selection was based on known nick sites in this loop (12–14). The oligopeptides were first tested in a murine LPS infusion model for attenuation of septic shock. The mouse study yielded three peptides that rescued 100% of the mice from death after 48 h, namely AQGV, LQGV and VLPALP. These three oligopeptides were tested subsequently as a cocktail for the rescue of rhesus monkeys from septic shock induced by infusion of living *E. coli*. Administration of a single dose of the oligopeptide cocktail delayed the manifestation of septic shock symptoms, reduced pathomorphological changes markedly in the gastrointestinal tract and nearly normalized the haemodynamic parameters.

Collectively, the reported data show that the tested oligopeptides are well tolerated and display promising therapeutic effects in two relevant animal models of septic shock.

MATERIAL AND METHODS

Animals

Mice. Female 8–16-week-old BALB/c mice were used. The mice were bred under specific pathogen-free conditions according to the protocols described in the Report of the Federation of European Laboratory Animal Science Association’s (FELASA) working group on animal health (15). The mice were bred and used in the facilities for experimental animals of the Erasmus MC. The mice had access to pelleted food (Hope Farms, Woerden, the Netherlands) and sterilized water *ad libitum*.

Rhesus monkeys. Eight healthy female rhesus monkeys (*Macaca mulatta*) were purchased from the purpose-bred breeding colony at the Biomedical Primate Research Centre. Individual data of the monkeys are given in Table 1. Prior to inclusion in the experiment, monkeys received a physical health check and were tested for haematological, serological and microbiological abnormalities. Only monkeys that were declared healthy by the institute's veterinarian staff were entered into the experiment.

Table 1. Individual rhesus monkey data and treatment regimen.

Treatment group	Animal ID	Gender	Date of birth	Date experiment	Age (years)	Weight (kg)
I Control;	Ri429	F	15-01-96	28-11-01	5.8	5.6
termination after 8 h	Ri274	F	24-02-98	24-08-05	7.5	4.9
(mean age 7.1 yr)	Ri8012	F	01-08-98	20-09-06	8.1	5.8
II Oligopeptide cocktail;	Ri459	F	21-06-96	28-11-01	5.4	5.4
termination after 8 h	Ri7046	F	03-07-98	20-09-06	8.2	5.7
(mean age 7.1 yr)	Ri11152	F	07-11-98	27-09-06	7.8	5.3
III Oligopeptide cocktail;	Ri 427	F	17-06-96	28-11-01	5.4	4.8
recovery allowed	C 152	F	14-02-95	24-08-05	10.5	6.2
(mean age 8.0 yr)						

Oligopeptides

Selection was based on either the known preferential cleavage sites or known nick sites of the sequence MTRVLQGVLPALPQVVC ($aa_{41–57}$) of loop 2 (Figure 1) of the β-subunit of hCG (12–14,17). Oligopeptides selected for the *in vivo* studies reported in this publication were the most effective in pilot studies, i.e. MTR ($aa_{41–43}$), MTRV ($aa_{41–44}$), LQG ($aa_{45–47}$), LQGV ($aa_{45–48}$), AQGV (alanine replaced oligopeptide of LQGV; $aa_{45–48}$), VLPALP ($aa_{48–53}$), VLPALPQ ($aa_{48–54}$) and VVC ($aa_{55–57}$). Oligopeptides were synthesized (Ansynth BV, Roosendaal, the Netherlands) using the fluorenylmethoxycarbonyl (Fmoc)/tert-butyl-based methodology with a 2-chlorotritylchloride resin as the solid support.

LPS-induced acute septic shock in mice

Female 8–12-week-old BALB/c mice (n = 5–6 per group) were injected intraperitoneally (i.p.) with a lethal dose of LPS (8 mg/kg; *E. coli* 026:B6; Difco Laboratories, Detroit, MI, USA). This procedure leads to 100% death within 84 h. Mice were treated subsequently with either a single i.p. dose of phosphate-buffered saline (PBS) or oligopeptide (5 mg/kg) in PBS at 2 h or 24 h post-LPS challenge. The optimal oligopeptide dose for this model has been defined in a previous study (7). Survival of the mice was monitored

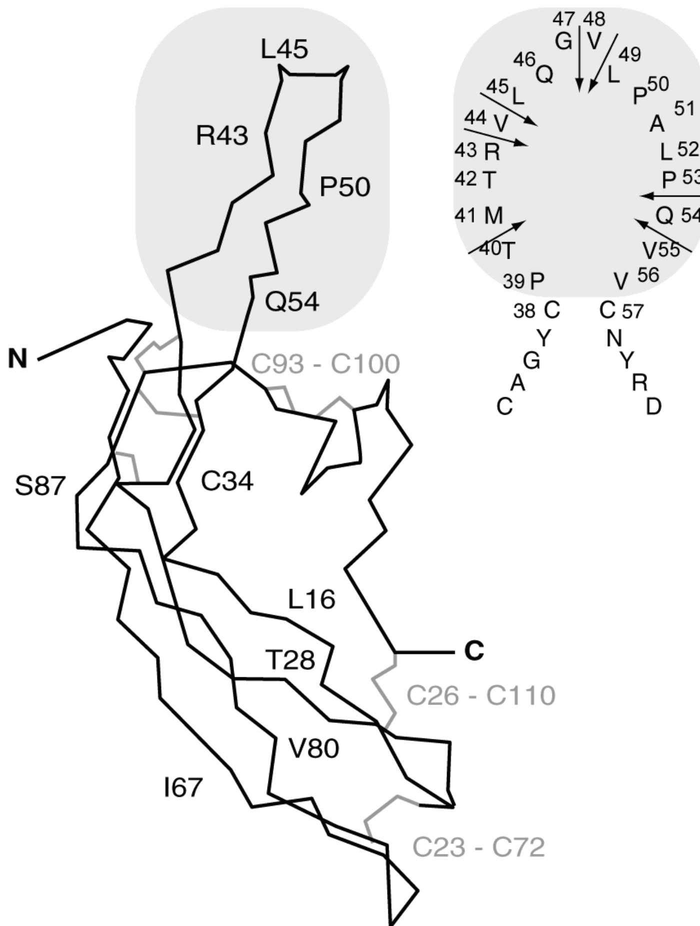

Figure 1. Structure of β-human chorionic gonadotropin (hCG) with loop 2 and the amino acid sequence of loop 2 indicated.
Adapted from Lapthorn et al. (16). Arrows point to the preferential cleavage sites in loop 2 ($aa_{41–57}$).

for 84 h at least three times per day. Mice were scored in a semi-quantitative fashion (scores 1–6) for sickness severity, as described previously (7).

LPS and CD3-induced proliferation of splenocytes

Sixteen-week-old female BALB/c mice (*n* = 5 per group) were injected i.p. with either LQGV or AQGV (5 mg/kg) or the equivalent volume of solvent (PBS). After 1 h of treatment the mice were killed and spleens were isolated. Five spleens from a group were pooled and splenocytes were isolated. For T cell stimulation splenocytes were

cultured ($2•5 \times 10^5$ cells/well) in 96-well flat-bottomed plates (0•2 ml) in triplicate and stimulated with anti-CD3 (145-2C11, 10 mg/ml) in combination with interleukin (IL)-2 (Biosource, CA, USA; 40 U/ml). For B cell and macrophage stimulation, splenocytes were cultured (5×10^5 cells/well) in 96-well round-bottomed plates (0•2 ml) in triplicate and stimulated with 10 mg/ml of LPS (*E. coli* 026:B6; Sigma; 69H4022) or with PBS. Splenocytes were then incubated at 37°C in 5% CO_2 for 12, 24, 36 or 48 h. During the final 8–12 h of culture 0•5 mCi of (^{3}H)-thymidine ((^{3}H)TdR) per well was added and the incorporation of (^{3}H)TdR was measured on a beta-plate counter.

E. coli-induced acute septic shock in adult rhesus monkeys

Bacteria. The *E. coli* strain used for septic shock induction was purchased from the American Type Culture Collection (ATCC) (*E. coli* 086a: K61 serotype; ATCC 33985). In a control experiment the strain had proved equally sensitive to complement-mediated lysis in fresh human and rhesus monkey serum (data not shown).

Prior to the experiment a fresh *E. coli* culture was set up in brain heart infusion (BHI) culture broth. The *E. coli* strain was cultured for 1 day, harvested and washed five times to remove free endotoxin. Just prior to infusion into the monkey a sample of the bacteria suspension was collected to assess the concentration and viability. To this end, serial dilutions of the *E. coli* stock were plated on BHI agar and cultured overnight at 37°C. The colonies on each plate were counted and the numbers of colony-forming units (CFU)/ml were calculated. The body weight measurement on the day of the experiment was used to calculate the *E. coli* dose (1010 CFU/kg). The *E. coli* stock was suspended in pyrogen-free isotonic saline (NPBI, Emmer Compascuum, the Netherlands) adjusted to the required concentration, and kept on ice until infusion. Just prior to infusion the total dose was resuspended in a volume of 50 ml isotonic saline of room temperature.

Preparation of the animals. Monkeys were fasted overnight prior to the experiment. On the morning of the experiment the monkeys were sedated with ketamine hydrochloride (AST Pharma, Oudewater, the Netherlands) and transported to the operation chamber. Monkeys were placed on their side on a temperature-controlled heating pad to support body temperature. Body core temperature was monitored using Ohmeda Excel 210 SE anaesthesia equipment (Datex-Ohmeda, Hoevelaken, the Netherlands). Monkeys were intubated orally and allowed to breathe freely. The femoral or cephalic vein was cannulated and used for infusion of isotonic saline, live *E. coli*, antibiotics and the oligopeptide cocktail. Fluid loss was compensated by infusing isotonic saline containing 2•5% glucose (Fresenius, ‘s-Hertogenbosch, the Netherlands) at a rate of 3•3 ml/kg/h. To avoid discomfort to the monkeys they were kept anaesthetized using O2/N2O/isoflurane inhalation anaesthesia during the two *E. coli* infusions and a 6-h observation period following *E. coli* challenge. The monkeys were kept under constant veterinary inspection during the complete time-course of the experiment.

Induction of septic shock. A 1-h time-period was included to monitor baseline values of heart rate and blood pressure prior to infusion of *E. coli*. Septic shock was induced by infusion of a fatal dose (1010 CFU per kg body weight) of live *E. coli* over a period of 2 h followed immediately by intravenous (i.v.) administration of the bacteriostatic agent Enrofloxacin (dose: 9 mg/kg; Enrofloxacine (Baytril 2•5%); Bayer, Mannheim, Germany) to kill surviving bacteria and to synchronize shock induction. In two independent (dose titration) studies with the chosen *E. coli* strain, a dose of 1010 CFU/kg body weight-induced acute shock leading to death about 8 h after start of the infusion (unpublished observations). Thirty minutes after the start of *E. coli* infusion the monkeys received a single i.v. bolus injection (1 ml per kg body weight) of oligopeptide cocktail containing LQGV, AQGV and VLPALP at a concentration of 5 mg/ml each; the dose was extrapolated from initial studies in mice (7). The peptide solution was injected into the i.v. cannula via which isotonic saline was infused continuously. For experimental reasons explained in the Results section, monkey Ri427 was allowed to recover after 8 h and received no further treatment. Monkey C152 was allowed to recover after 8 h and received a second treatment at this time-point and a third treatment at 24 h.

Pathology

Post-mortem examination of all monkeys was conducted immediately after the monkeys were killed. The monkeys underwent gross necropsy in which the abdominal and thoracic cavity was opened and internal organs were examined *in situ*. Pictures were made of the internal organs of some of the monkeys. Tissues of all organs were preserved in neutral aqueous 4% solution of phosphate-buffered formaldehyde and processed subsequently for histopathological examination by a pathologist.

Cytokine production

Cytokine levels in plasma were analysed using Cytometric Bead Array™ (CBA; BD Biosciences, San Diego, CA, USA). TNF-α, IL-1β, IL-6 and IL-8 were detected using the human inflammation CBA kit. Tests were performed according to the manufacturer’s instructions and known to have cross-reactivity with rhesus monkey. The results were expressed as pg/ml. The limits of detection were as follows: 2•6 pg/ml TNF-α, 7•2 pg/ml IL-1β, 2•5 pg/ml IL-6, 3•6 pg/ml IL-8.

Statistics

Data were analysed by two-tailed Fisher’s exact test and unpaired Student’s *t*-test. Data differences were considered significant at $P < 0•05$.

Ethics

The protocols of the mouse studies have been reviewed and approved by the

Animal Research Ethics Committee of Erasmus MC (Rotterdam, the Netherlands). The protocols of the rhesus monkey studies have been reviewed and approved by the Animal Research Ethics Committee of the Biomedical Primate Research Centre (Rijswijk, the Netherlands).

RESULTS

Effects of hCG-related oligopeptides on LPS-induced mortality in mice

Intraperitoneal injection of LPS in BALB/c mice induced a fatal septic shock leading to 100% mortality within 84 h. Several synthetic oligopeptides related to loop 2 of the hCG β-chain (Figure 1) had a marked protective effect on the morbidity and mortality induced by the LPS administration. In the first series of experiments a single dose (5 mg/kg body weight) of the individual oligopeptide or PBS as control was administered 2 h after the LPS injection. At the 48 h time-point all (17 of 17) LPS-injected mice receiving PBS were dead (Figure 2a, PBS). Oligopeptides LQGV and the alanine-substituted derivative AQGV were equally effective as VLPALP in rescuing mice (17 of 17) from LPS-induced septic shock (Figure 2a). In a previous study (7) the latter peptide was found to prolong survival time beyond 72 h when administered 2 h after a lethal challenge of LPS. In the second series of experiments a single dose of the individual peptides was given 24 h after the LPS administration. Here, five of the eight oligopeptides prevented mortality completely (Figure 2b). Of all eight oligopeptides tested in this model, only AQGV rescued all (17 of 17) mice from septic shock when administered early (at 2 h) as well as late (at 24 h) after LPS-injection.

Signs of sickness were apparent in all LPS-treated mice, but the course and severity differed between individual animals. In order to quantify the observed differences, a semiquantitative sickness scoring system was used, with severity scores ranging from 0 to 6. In these experiments, mice injected i.p. with 8 mg LPS/kg body weight reached a sickness score of 2 or 3 within 14 h. At 24 h, most of the mice had reached a sickness score above 4. Thereafter the mice progressed gradually to a sickness score of 5 or had succumbed (score 6). Injection of LQGV, VLPALP or AQGV 2 h after LPS injection reduced the average sickness scores to less than 2 at 84 h after LPS injection (Table 2). The other oligopeptides were less effective. Although LQGV, VLPALP and AQGV all rescued mice from the fatal outcome of septic shock and reduced sickness scores, AQGV treated mice had significantly ($P < 0•01$) lower sickness scores at 48 h after LPS injection than LQGV- and VLPALP-treated mice. Treatment of BALB/c mice with either MTR, MTRV, VLPALPQ, VVC or AQGV at 24 h after LPS injection rescued all mice from septic shock. Here, mice treated with AQGV also had significantly ($P < 0•01$) lower sickness scores 48 h after LPS injection compared to mice treated with MTR,

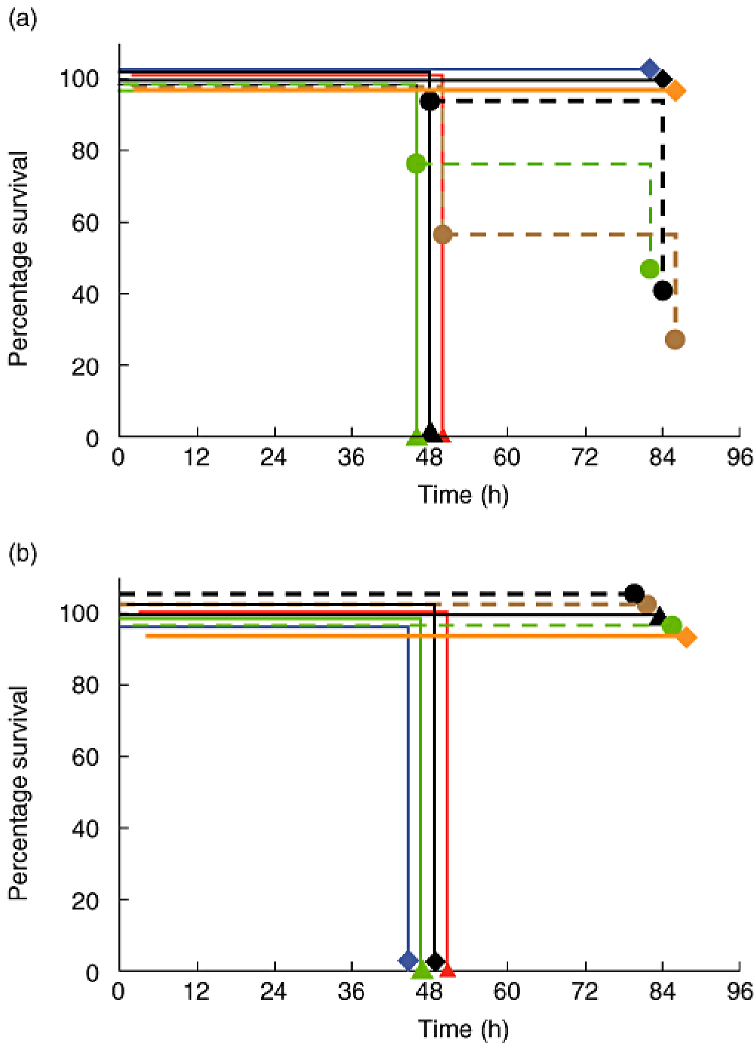

Figure 2. Survival of mice with lipopolysaccharide (LPS)-induced septic shock after treatment with a single dose of different human chorionic gonadotropin (hCG)-related oligopeptides.
Mice were treated with the following oligopeptides (5 mg/kg body weight i.p.): phosphate-buffered saline (PBS) (red triangle) MTR ($aa_{41–43}$; black circle, dashed line), MTRV (aa_{41-44}; brown circle, dashed line), LQG ($aa_{45–47}$; green triangle), LQGV ($aa_{45–48}$; blue diamond), AQGV (alanine replaced oligopeptide of LQGV; $aa_{45–48}$; orange diamond), VLPALP ($aa_{48–53}$; black diamond), VLPALPQ ($aa_{48–54}$; black triangle) and VVC ($aa_{55–57}$; green circle, dashed line). Mice were treated either 2 h (a) or 24 h (b) after administration of LPS intraperitoneally. Cumulative data are presented from three independent experiments (five to six animals per group/experiment). The survival percentages at 84 h were highly significant (*P* = 0•000001, two-tailed Fisher's exact test) for peptides LQGV, AQGV and VLPALP at 2 h and for peptides MTR, MTRV, VLPALPQ, VVC and AQGV compared to the PBS group.

Table 2. Average sickness scores of mice with lipopolysaccharide (LPS)-induced septic shock after treatment with a single dose of different human chorionic gonadotropin (hCG)-related oligopeptides.

Treatment	Average sickness scores after time interval (hr)*									
	2 h after LPS induction					24 h after LPS induction				
	0	14	24	48	84	0	14	24	48	84
PBS	0.0	2.8	4.3	-†	-	0.0	3.0	4.5	-	-
MTR	0.0	2.5	3.0	4.2	2.3	0.0	2.8	4.3	3.8‡	3.0‡
MTRV	0.0	2.7	4.0	4.0	3.5	0.0	3.0	4.8	3.3‡	3.2‡
LQG	0.0	3.0	4.7	-	-	0.0	2.7	4.7	-	-
LQGV	0.0	2.5	3.0	2.7‡	1.2	0.0	2.8	4.2	-	-
VLPALP	0.0	2.5	3.2	2.8‡	1.7	0.0	2.5	4.0	-	-
VLPALPQ	0.0	3.2	4.5	-	-	0.0	3.2	4.5	2.7	2.7‡
VVC	0.0	2.8	4.2	4.4	3.7	0.0	3.3	4.7	2.7	2.8‡
AQGV	0.0	2.0	2.3	1.7	1.0	0.0	2.7	4.2	1.8	1.5

*Mice were treated with the various oligopeptides (5 mg/kg body weight intraperitoneally (i.p.)) 2 h or 24 h after administration of LPS i.p. (n = 6). † Indicates that none the mice had survived at that time-point. ‡ Indicates that the sickness scores are significantly higher than the AQGV-treated group (P < 0•01).

Mice were scored for sickness severity as described previously (7) using the following criteria: score 1: percolated fur, but no detectable behaviour differences compared with untreated control mice; score 2: percolated fur, huddle reflex, responding to stimuli (such as tap on cage) and just as active during handling as untreated control mice; score 3: slower response to tap on cage, and passive or docile behaviour when handled, but still curious when alone in a new setting; score 4: lack of curiosity, little or no response to stimuli, and defect mobility; score 5: laboured breathing and impaired righting reflex; score 6 was defined as death. The results illustrated are from a single experiment and representative of at least three independent sets of experiments (each group: n = 6). PBS: phosphate-buffered saline.

MTRV, VLPALPQ and VVC. Remarkably, AQGV not only prevented mortality but also reduced the average sickness score from 4•2 at the time of treatment to 1•5 60 h later. Two days later all mice that were rescued from septic shock death by the oligopeptide treatment had recovered and had a sickness score of 1 or no longer had any signs of sickness. The other oligopeptides were less effective in reducing sickness scores (MTR, MTRV, VLPALPQ, VVC) and preventing mortality (LQG, LQGV, VLPALP).

LPS and CD3-induced proliferation of splenocytes

The effect of LQGV and AQGV on systemic immune activation was assessed by measuring splenocyte proliferation in response to a T cell stimulator (anti-CD3 and IL-2) and a B cell and macrophage stimulator (LPS).

Splenocytes derived from LQGV and AQGV-treated mice had a significantly (P < 0•05) reduced *in vitro* proliferative response to CD3/IL-2 after 24 h of stimulation (Figure 3). Splenocytes derived from AQGV-treated mice also showed a significantly (P < 0•05) reduced *in vitro* proliferative response to LPS (Figure 3). Both LQGV and AQGV treatment were associated with a significantly (P < 0•05) reduced basal proliferation level (data not shown).

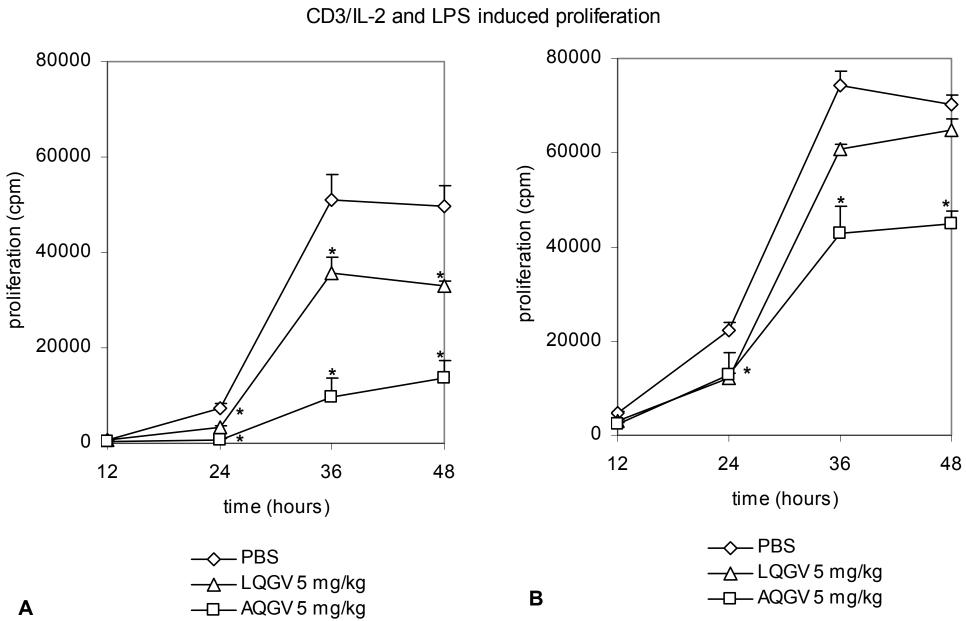

Figure 3. The effect of *in vivo* treatment of BALB/c mice with the oligopeptides LQGV and AQGV (5 ml/kg) on (A) the *in vitro* CD3/IL-2, and (B) the LPS-induced splenocyte proliferation. Both oligopeptides significantly (*$P < 0•05$) reduced *in vitro* the capacity of the splenocytes to proliferate upon CD3/IL-2 stimulation. However, splenocytes derived from AQGV peptide-treated mice also showed significantly (*$P < 0•05$) reduced *in vitro* proliferative response to LPS after 24 h of stimulation, while no significant differences in *in vitro* proliferative response of splenocoytes derived from LQGV peptide-treated mice to LPS were found. The results presented are from a single experiment and representative of at least three independent sets of experiments ($n = 5$).

Treatment of *E. coli*-induced septic shock in rhesus monkeys

The three most promising oligopeptide candidates from the septic shock experiments in mice – AQGV, LQGV and VLPALP – were selected for evaluation in a preclinical model of *E. coli*-induced septic shock in rhesus monkeys.

Control animals (group I, consisting of monkeys numbered Ri429, Ri274, Ri8012) received 0•9% sodium chloride solution starting at 30 min after initiation of the infusion of the *E. coli*. These monkeys were not allowed to recover from anaesthesia and were killed 8 h after start of the infusion. Three other monkeys (group II, consisting of monkeys numbered Ri459, Ri7046, Ri11152) received a bolus injection of a cocktail containing the three selected oligopeptides (LQGV, AQGV, VLPALP; 5 mg/kg for each of the oligopeptides) dissolved in 0•9% sodium chloride solution 30 min after initiation

of the *E. coli* infusion. Throughout the experiment the clinical condition of the monkeys was monitored. At 6 h the veterinarian responsible was asked whether the clinical condition would allow recovery from anaesthesia. Although this was approved in two of the three treated monkeys, these were nevertheless killed to compare the organ histopathology with the control monkeys. Two additional monkeys (group III, numbered Ri427, C152), which also received a bolus injection of the oligopeptide mix 30 min after initiation of the *E. coli* infusion, were allowed to recover from the anaesthesia at the veterinarian's consent on the basis of the clinical condition, and remained under constant supervision.

The clinical observations made by the veterinarian are summarized in Table 3. In the saline-treated control monkeys (group I) the septic shock crisis started 30 min after start of the *E. coli* infusion (= time-point 'post-*E. coli*'). The condition of the animals worsened rapidly and displayed the typical signs of a septic shock crisis, characterized by highly unstable blood pressure and oxygen saturation (data not shown), disturbed electrocardiogram (ECG) output and several other abnormal clinical parameters (Table 3). The condition of the monkeys treated with the oligopeptide cocktail (groups II and III) was much more stable, except in monkey Ri11152. During the first 8 h of the experiment, four of five peptide-treated monkeys had a stable heart rate (data not shown), normal ECG and normal or depressed respiration (Table 3); however, depressed respiration was deep but regular. The outlier peptide-treated monkey Ri11152 displayed signs of a transient crisis which was far less serious than in the saline-treated control monkeys (Table 3). This monkey was deemed unfit for recovery from anaesthesia and was killed at 8 h.

Monkey Ri7046 showed only mild signs of septic shock, but although deemed fit for recovery it was killed at 8 h post-*E. coli* infusion. Monkey Ri459 was in good clinical condition 8 h post-*E. coli* infusion, but was nevertheless killed to allow pathological examination and comparison with control animals. The monkeys in group III (Ri427 and C152) were allowed to recover from anaesthesia and remained free of septic shock symptoms for 33 h and 36 h, respectively, and succumbed with serological evidence of organ failure. From this group, Ri427 had not received additional oligopeptide treatment while C152 had received two additional injections at 8 h and 24 h post-*E. coli* infusion.

Taken together, three of three saline treated-monkeys developed severe septic shock within 6 h after *E. coli* infusion. In contrast, three of five monkeys treated with the peptide cocktail remained completely devoid of septic shock symptoms during the initial observation period of 8 h. The two monkeys that were allowed to recover from anaesthesia (Ri427 and C152) finally developed signs of multiple organ failure. In the one oligopeptide cocktail-treated monkey that showed clinical signs of septic shock, these were far less serious than in the saline-treated control monkeys.

Table 3. Clinical observations during the first 8 h after induction of septic shock in rhesus monkeys.

Treatment	Animal ID	Clinical observation	Veterinarian
Group I PBS; termination after 8 h	Ri429	At 8 h the animal showed faecal vomiting and convulsions. No pulse and arrhythmia. An abnormal ECG. During the last hours the animal displayed forced respiration and decreased blood clotting as indicated by continued bleeding after blood extraction.	No recovery allowed
	Ri274	A progressively decreasing heart rate and blood pressure was observed. The animal also developed arrhythmia. The oxygen saturation could hardly be measured and at the end decreased rapidly. The animal became increasingly unstable.	No recovery allowed
	Ri8012	After administration of the *Esherichia coli* there was a rapid development of oedema above they eyes and also in the lungs. In the final phase the animal displayed forced respiration and bronchi at auscultation indicative of oedema of the lungs. Abnormal ECG. The animal became increasingly unstable.	No recovery allowed
Group II Peptide cocktail; termination after 8 h	Ri459	The animal had a good pulse and a normal ECG. The heart sounded normal and lungs sounded clean. Although the animal displayed depressed respiration, the respiration was deep and regular.	Recovery allowed
	Ri7046	The animal remained stable during the observation period. The animal developed mild oedema after infusion of *Escherichia coli*. The animal showed mild depressed respiration, but it was deep and regular.	Recovery allowed
	Ri11152	Initial stable clinical parameters became less stable after infusion of *Escherichia coli.* After administration of Enrofloxacin a stable increased heart frequency was measured but blood pressure became difficult to assess. In the later phase of the disease oedema was diagnosed after auscultation necessitating repositioning of the animal on his side so that the animal could breath more freely. In the final phase the lungs sounded normal but an abnormal ECG was measured.	No recovery allowed
Group III Peptide cocktail; recovery allowed	Ri 427	The animal had a good pulse and a normal ECG. The heart sounded normal. The left lung displayed a slight murmur but overall sounded good. This animal had a normal regular respiration.	Actually recovered
	C 152	The animal had a good pulse and a normal ECG. The heart sounded normal and the lungs sounded clean at 8 h. This animal had a normal respiration and stable clinical parameters.	Actually recovered

ECG: electrocardiogram; PBS: phosphate-buffered saline.

Effects of hCG-related oligopeptide treatment on gross pathology and histology of vital organs

Lungs. Gross examination at necropsy of the saline-treated monkeys (Ri274, Ri429 and Ri8012) revealed dark red lungs. Microscopically prominent pulmonary oedema, vascular congestion and multi-focal areas of extravasated red blood cells (haemorrhages) were observed (see Figure 4A). In the oligopeptide-treated monkey (Ri11152) that developed septic shock the lung had a similar haemorrhagic appearance. In the other two oligopeptide-treated animals (Ri459, Figure 4B; and Ri7046, which had mild septic shock-related clinical symptoms) that were euthanized the macro- and microscopic changes were reduced markedly and moderately, respectively.

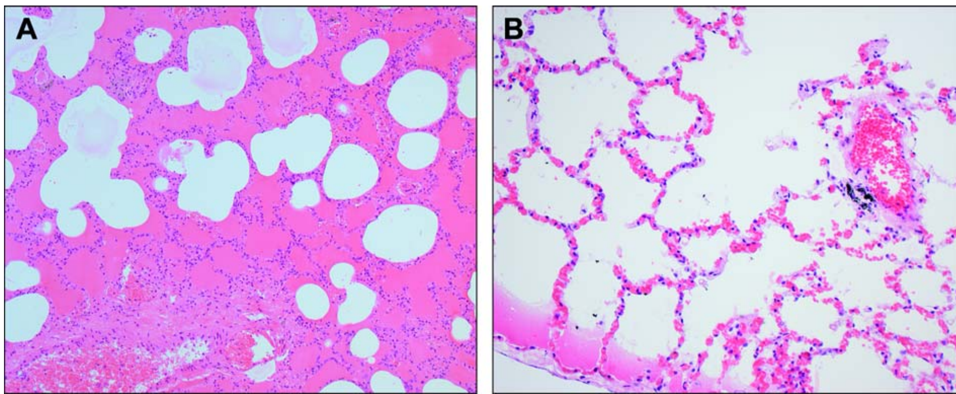

Figure 4. Reduced lung pathology treatment with oligopeptide cocktail.
The picture shows control monkey Ri 429 (**A**) and the oligopeptide-treated monkey Ri 459 (**B**). The marked difference in pulmonary oedema, vascular congestion and haemorrhages is easily visible.

Gastrointestinal tract. Macroscopically, the wall of the small and large intestine of saline-treated monkeys Ri274 and Ri8012 (Figure 5A) was moderately thickened (oedematous) with multi-focal to coalescing bright red areas present predominantly on the intestinal mucosa accompanied by scant greenish liquid faecal material in the lumen. Microscopically, the intestinal mucosa of these monkeys exhibited moderate vascular congestion, multi-focal areas of haemorrhage and mild to moderate oedema with mildly increased numbers of multi-focal lymphoplasmacytic infiltrates (Figure 6C). Compared to the saline-treated monkeys, the intestinal mucosa of the oligopeptide-treated monkeys Ri459, Ri7046 and Ri11152 showed similar but milder gross lesions (Figure 5C,E). Microscopically, monkey Ri11152 (Figure 6D) exhibited only mild vascular congestion and oedema with few small haemorrhagic foci.

In the stomach of the saline-treated monkeys Ri429, Ri274 and Ri8012 (Figure 5B) we observed severely affected gastric mucosa, exhibiting marked oedema, congestion

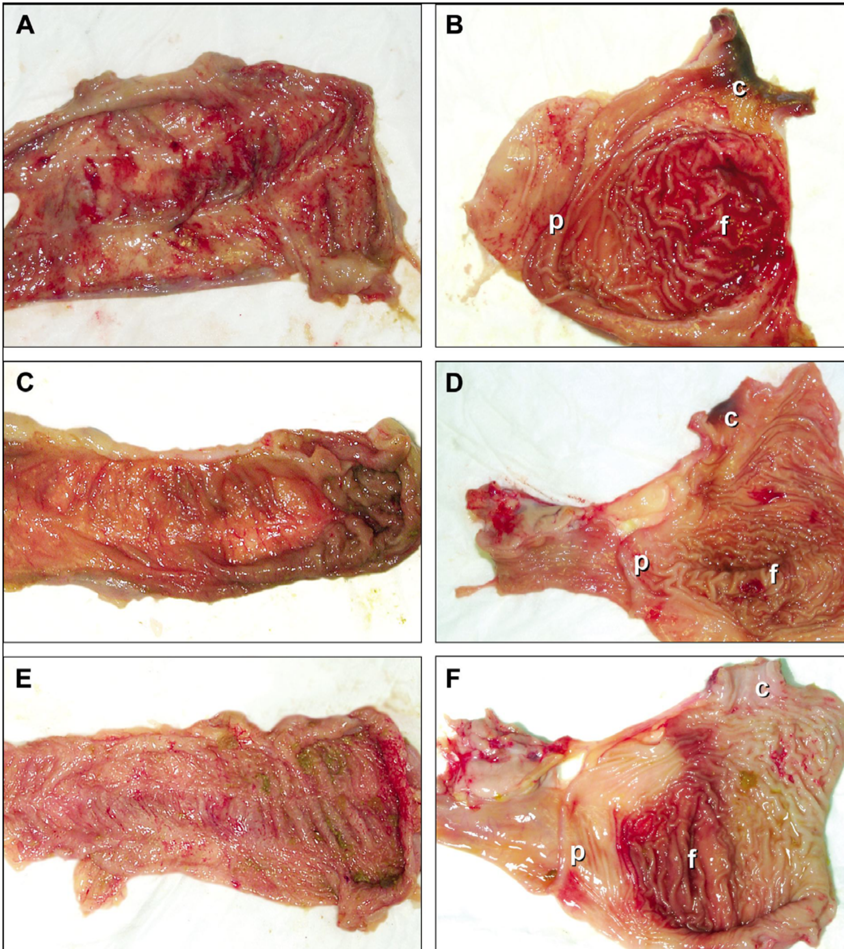

Figure 5. Diminished septic shock-related gross pathological findings after treatment of rhesus monkeys with a cocktail of three human chorionic gonadotrophin (hCG)-related oligopeptides. *Escherichia coli*-related septic shock was induced in eight rhesus monkeys. Six animals were killed at 8 h in order to evaluate the effect of treatment with hCG-related oligopeptides at the level of histopathology after the acute phase of septic shock relative to phosphate-buffered saline (PBS)-treated control monkeys. A significant difference was observed between tissues from control monkeys (e.g. Ri8012; **A**, **B**) and treated monkeys (e.g. Ri7046: **C**, **D**; Ri11152: **E**, **F**). The gastric mucosa of control monkey Ri8012 (**B**) exhibits marked oedema, congestion and haemorrhage of the cardia (c) and fundus (f) and multiple petechial haemorrhages in the pylorus (p), while the treated monkeys (e.g. Ri7046; **D** and Ri11152; **E**) have milder lesions in the cardia and pylorus. Furthermore, the intestinal wall of the large intestine of control animals (e.g. Ri8012; **A**) exhibits multi-focal to coalescing areas of haemorrhage and oedema, while treated animal Ri7046 (**C**) shows milder lesions and treated animal Ri11152 (**F**) exhibits only scattered petechial haemorrhagic foci.

and haemorrhage of the cardia and fundus and multiple petechial haemorrhages in the pylorus. Microscopically, these alterations were consistent with marked vascular congestion of the gastric mucosa and submucosa, vasodilatation in the lamina propria of the gastric mucosa (Figure 6A), multi-focal haemorrhages, marked oedema and occasional sloughing (necrosis and loss) of gastric epithelium (degeneration and loss of few parietal and chief cells), accompanied by multi-focal lymphoplasmacytic infiltrates.

The oligopeptide-treated monkeys Ri7046 and Ri11152 showed only minimal gross lesions. Monkey Ri7046, compared to the control monkey Ri8012, showed milder lesions: mild congestion and few small haemorrhagic foci in the cardia and pylorus (Figure 5D). Monkey Ri11152 exhibited only minimal to mild vascular congestion and oedema with few petechial haemorrhagic foci (Figure 5F). In the cardia of monkey Ri274 (data not shown) only few petechial haemorrhages were found. Microscopically, these gross lesions were comprised of scattered congested blood vessels, few foci of extravasated red blood cells and mild oedema.

Liver. The liver of the oligopeptide-treated and control monkeys was unremarkable macroscopically. Microscopic abnormalities in the hepatic parenchyma of saline-treated monkeys Ri429, Ri274 and Ri8012 (Figure 6E) consisted of moderate to marked sinusoidal and intravascular neutrophilic granulocytosis, multi-focal congested vessels and multi-focal pericentral haemorrhages. The only observed microscopic alterations in the liver of oligopeptide-treated monkeys Ri459, Ri7046 and Ri11152 (Figure 6F) were occasional sinusoidal neutrophils and few apoptotic hepatocytes.

Other organs. The myocardium of the control monkeys Ri429, Ri274 and Ri8012 exhibited multi-focal degeneration of myocardiocytes and focal haemorrhage in Ri274. On the other hand, no significant abnormalities were present in the myocardium of the oligopeptide-treated monkeys Ri459, Ri7046 and Ri11152 (data not shown). Similarly, the pancreas of the control-treated monkeys Ri429, Ri274 and Ri8012 exhibited mild interlobular oedema and random degeneration of acinar cells, while no significant findings were present in the pancreas of the oligopeptide treated monkeys Ri459, Ri7046 and Ri11152 (data not shown).

Overall, the most serious pathomorphological changes related to septic shock were observed in the saline-treated control monkey Ri8012. Similar but less severe changes were observed in the two other monkeys from the control group. The least severe abnormalities were observed in the oligopeptide-treated monkeys Ri459, Ri7046 and Ri11152. These findings indicate a marked beneficial effect of the AQGV–LQGV–VLPALP oligopeptide cocktail on the course of this experimentally induced septic shock.

Effect of hCG-related oligopeptide treatment on the cytokine burst in rhesus monkeys

The effect of the AQGV–LQGV–VLPALP oligopeptide treatment was also evaluated

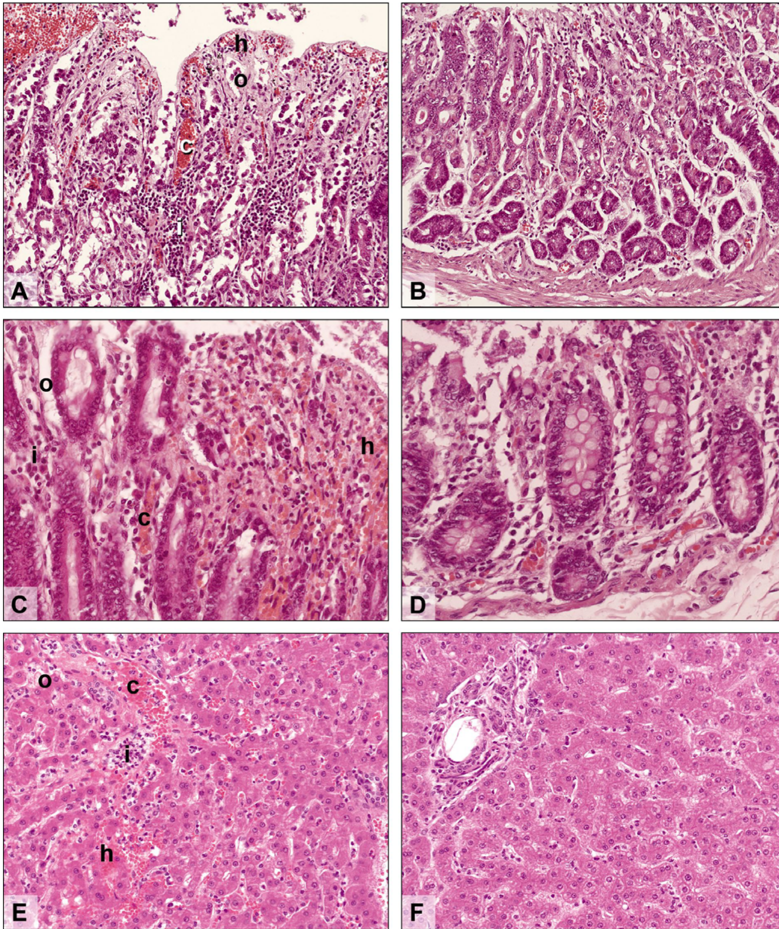

Figure 6. Decreased severity of septic shock-related microscopic lesions after treatment of rhesus monkeys with a cocktail of three human chorionic gonadotropin (hCG)-related oligopeptides. Microscopic examination of tissues of control monkey Ri8012 (**A, C, E**) demonstrates marked vascular hyperaemia, vasodilation, multi-focal haemorrhages, marked oedema, mildly increased number of multi-focal lymphoplasmacytic infiltrates and sloughing (necrosis and loss) of epithelium in the lamina propria of the gastric mucosa (**A**) while the treated monkey Ri11152 (**B**) displays significantly minimized changes. The large intestinal mucosa in control monkey Ri8012 (**C**) exhibits moderate vascular dilation, active mucosal congestion, multi-focal areas of extravasated red blood cells (haemorrhage), mild to moderate oedema and mildly increased lymphoplasmacytic infiltrates, while the treated monkey Ri11152 (**D**) exhibits mild vascular hyperaemia and oedema with few small haemorrhagic foci. The hepatic parenchyma of control monkey Ri8012 (**E**) shows variable number of multi-focal sinusoidal neutrophils (periportal, random sinusoidal and intravascular), multi-focal hyperaemic blood vessels, multi-focal haemorrhage and oedema, while microscopic hepatic alterations of the treated monkey Ri11152 (**F**) are restricted to the presence of occasional sinusoidal neutrophils.

on the plasma levels of proinflammatory cytokines of the control and oligopeptide-treated monkeys. *E. coli* infusion was associated with an increase of IL-1β, TNF-α, IL-6 and IL-8 plasma levels over time in both control and oligopeptide-treated monkeys. The plasma levels of these proinflammatory cytokines did not differ markedly between the oligopeptide group and the untreated monkeys (Figure 7).

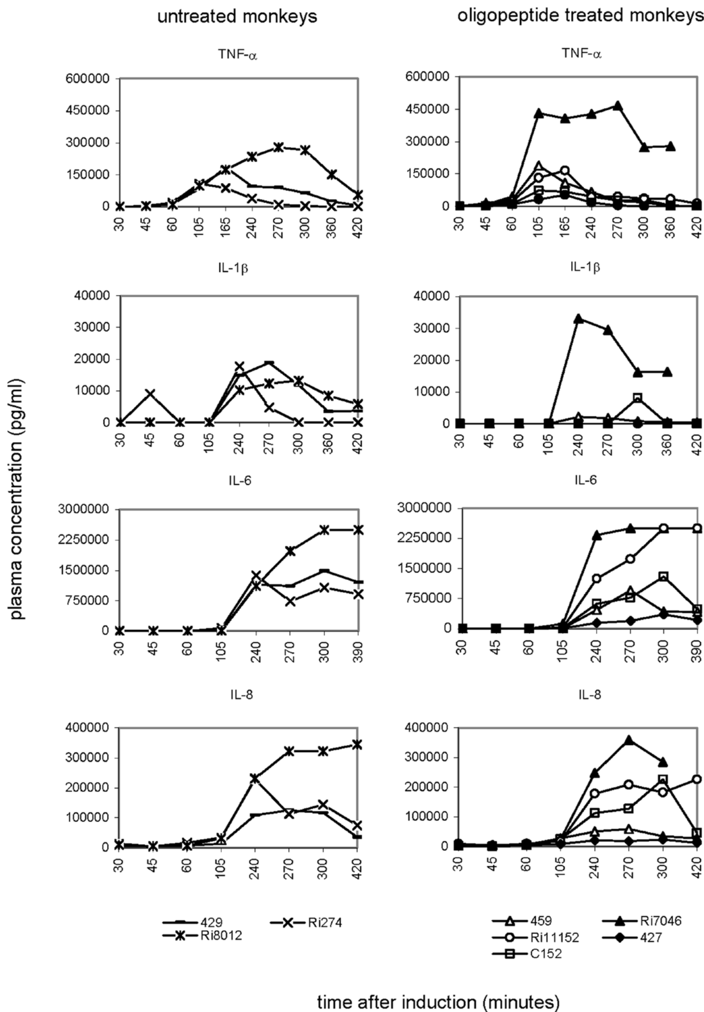

Figure 7. Plasma levels of inflammatory cytokines.
Escherichia coli infusion of rhesus monkeys was associated with an increase of interleukin (IL)-1β, tumour necrosis factor-α, IL-6 and IL-8. The plasma levels of these proinflammatory cytokines did not differ markedly between the oligopeptide-treated monkeys and the untreated monkeys.

DISCUSSION

In previous studies the immunomodulatory activity of the pregnancy hormone hCG in LPS-induced acute septic shock and in diabetes in non-obese diabetic (NOD) mice was mapped to a 400–2000 Dalton fraction in pregnancy urine (6,7). It was hypothesized that the active fraction contained oligopeptides derived from the sequence MTRVLQGVLPALPQVVC (residues 41–57) of loop 2 of the hCG-β chain (17). 'Missing' of loop 2 from the β-subunit (as in hCG β-core fragment) and 'nicking' of the β-subunit in the loop 2 region of the molecule, specifically between residues 44–49, can both reduce the biopotency of hCG. Cleavage of the peptide bonds in this area of the molecule also reduced biopotency and immunochemical recognition by monoclonal antibodies directed to the heterodimeric hormone (12–14,18,19). Based on known preferential cleavage sites in loop 2 several oligopeptides were synthesized, including MTR, MTRV, LQG, LQGV, VLPALP and VLPALPQ. In addition, the peptide VVC from the flanking COOH-side and the alanine substitution variant AQGV were synthesized. In the current study the eight synthetic oligopeptides were tested for their suppressive effect on LPS-induced septic shock in mice (Table 2). The results showed that all mice that were treated at 2 h post-LPS with LQGV, VLPALP or AQGV survived beyond 48 h, while all mice in the saline-treated control group had already succumbed from fatal septic shock within 24 h. Remarkably, the mice could even be rescued from fatal septic shock when treatment with either one of the oligopeptides MTR, MTRV, VLPALPQ, VVC or AQGV was started as late as 24 h post-LPS, with sickness scores higher than 4•0 (Table 2). Of the evaluated oligopeptides, only AQGV was fully effective in reducing the severity and mortality of septic shock at early (i.e. 2 h post-LPS) as well as late (i.e. 24 h post-LPS) administration. The different *in vivo* efficacy of AQGV and LQG was mirrored by a different *in vitro* immunosuppressive capacity. While AQGV reduced the *in vitro* proliferative response of splenocytes to LPS as well as CD3/IL-2, LQGV only reduced the proliferative response of splenocytes to CD3/IL-2. These data suggest that the suppressive effect of AQGV targets macrophages/monocytes, B cells and T cells and therefore possibly affects early and late mechanisms of septic shock in which different cell types and pathological pathways are active (20,21). Although treatment with LQG, LQGV or VLPALP (5 mg/kg) at 24 h after LPS administration failed to reduce mortality, VLPALP reduced mortality to around 50% after administration of a threefold higher dose (15 mg/kg) at 24 h post-LPS (7). This shows that detailed dose–response studies are needed for a full insight into the ability of the various hCG-related oligopeptides to inhibit LPS-induced shock in mice early and/or late after LPS administration.

Based on the observed beneficial effects of hCG-derived oligopeptides in the mouse septic shock model we chose to evaluate this new treatment principle in a septic shock model in deeply sedated rhesus monkeys induced by infusion of live *E. coli*. We selected

the three most effective peptides in the mouse studies, namely AQGV, LQGV and VLPALP, and administered them as an i.v. cocktail. With regard to the primary end-point, all three monkeys that received mock treatment with saline succumbed from fatal septic shock within 8 h after the *E. coli* infusion. Four of five monkeys that were treated with the oligopeptide cocktail were protected completely against septic shock. Haemodynamic parameters, such as heart rate, blood pressure and SpO2, varied considerably between individual animals, but confirmed the clinical diagnosis of suppressed septic shock by oligopeptide treatment.

Two oligopeptide-treated monkeys without marked clinical symptoms were allowed to recover to assess whether the single treatment also protected them from late-onset shock symptoms. As one of these monkeys had to be killed with clinical signs of septic shock at 33 h post-*E. coli*, the second monkey received an additional infusion of the oligopeptide cocktail at 8 and 24 h post-*E. coli*. However, the additional two oligopeptide doses failed to rescue this monkey from multi-organ failure at 36 h, as could be concluded from the high serum levels of ASAT and ALAT, indicating liver damage, of creatinine and urea reflecting kidney dysfunction, as well as the non-selective markers LDH and lactate over the final 20 h (data not shown).

Evaluation of the secondary end-point, histological assessment of septic shock-related damage to vital organs, showed markedly less severe pathological changes in the three oligopeptide-treated monkeys that displayed no (Ri459) minor (Ri7046) or moderate clinical signs of septic shock (Ri11152) compared to the three saline-treated control monkeys. Despite the variability in primary and secondary end-point parameters that were obtained from a limited number of investigated cases, the overall data set warrants the conclusion that administration of an oligopeptide cocktail consisting of AQGV, LQGV and VLPALP protects rhesus monkeys against the clinical and pathological consequences of acute septic shock syndrome. Importantly, these results were established without any further supportive treatment. Despite the strong protective effect of the oligopeptide cocktail during the acute clinical crisis, monkeys that were allowed to recover from anaesthesia nevertheless succumbed within the following 24 h with clinical symptoms of septic shock. Moreover, at autopsy of the monkeys that were left alive and killed 1 day later with clinical signs of shock, the same pathological changes of septic shock syndrome as in the control monkeys could also be observed. This late complication of severe septic shock may be due to the translocation of bacteria from the damaged intestines.

To gain insight into the protective effect observed in the monkey model we measured TNF-α, IL-1β, IL-6 and IL-8 plasma levels. The plasma levels of these cytokines are elevated strongly during the initial 24 h after septic shock induction, and reduction of systemic levels and inhibition of these cytokines has been shown to be associated with improved survival in some animal models (22). While we have observed that LQGV or

AQGV treatment in a rat model of severe haemorrhagic shock reduced TNF-α and IL-6 serum levels (23), we observed similar plasma profiles for TNF-α, IL-1β, IL-6 and IL-8 in oligopeptide-treated and untreated rhesus monkeys. These data suggest that the oligopeptide treatment had no profound effect on the production or release of these proinflammatory cytokines in this model, suggesting that suppression of the systemic storm is not a probable explanation for the beneficial effect of the oligopeptide cocktail. However, the data on plasma cytokine levels should be interpreted with caution, as considerable variation was observed between individual monkeys. Moreover, the data were collected from a small number of monkeys used, which differed in genetic background and age. Hence, we cannot exclude completely the possibility that the oligopeptides affect systemic cytokine release in this model.

Gender-specific differences in immune responses after shock occur and have been attributed to the different immunomodulatory effects of male and female steroid hormones (24). In our study we used non-pregnant female mice and monkeys, but did not check for the oestric cycle or female sex hormone levels. Nevertheless, we consider it unlikely that female sex hormones have influenced the different outcome between peptide and non-peptide treated animals, as these peptides have been found to inhibit haemorrhagic shock-associated inflammation in male rats (23).

Thus far it is unclear by which molecular mechanism the remarkable pleiotropic beneficial effects of hCG-related oligopeptides in different models of acute shock are mediated. Recently we reported that male rats treated with either LQGV or AQGV alone, at 30 min after the onset of severe haemorrhagic shock, displayed not only reduced plasma levels of TNF-α and IL-6, but also reduced E-selectin, TNF-α and IL-6 mRNA transcript levels in the liver. LQGV treatment was also associated with a significant reduction of neutrophil accumulation in the liver (23). Endothelial activation, characterized by increased expression of adhesion molecules (E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)) and subsequent tissue infiltration by neutrophils is an early event in septic shock and a crucial step towards the development of organ failure (20,25,26). E-selectin blockage has been shown to protect against neutrophil-induced tissue injury during endotoxaemia in mice (27). Considering these data and our previous findings (23), we hypothesize that reduced E-selectin expression upon oligopeptide treatment may have contributed to the lower inflammatory cell accumulation and reduced organ damage, as was observed in the rhesus monkey study presented here.

Gene expression analysis in several rodent models of inflammatory disease confirmed that treatment with LQGV or AQGV reduces gene expression for proinflammatory cytokines and adhesion molecules (Khan et al. in preparation). This suggests that the immune regulatory effect of the hCG-related regulatory oligopeptides in rodents may, at least in part, be driven by the regulating gene expression. It is as yet unknown,

however, whether the oligopeptides act by binding to surface-expressed or cytosolic receptors. Preliminary data exclude that LQGV, AQGV and VLPALP act via binding to the luteinizing hormone (LH) receptor as they do not bind to the LH receptor nor do they interfere with the binding of hCG to the LH receptor (data not shown). Due to their small size and low molecular weight, these oligopeptides might easily pass the cell membrane (28) and interfere with signalling cascades or interact with regulatory sequences or transcriptional complexes.

In conclusion, the results of the current study show marked beneficial effects of some synthetic oligopeptides (3–7 amino acids) related to the primary structure of the pregnancy hormone hCG in experimental models of acute septic shock in two different species. No adverse effects of the hCG-related oligopeptides were observed in these two species. Therefore these oligopeptides may have therapeutic value for this often-fatal condition.

A Phase Ia multi-dose safety trial with AQGV (EA-230) in man has been completed (29), in which subjects received three times daily i.v. infusions of EA-230 for 3 days. No significant adverse events were found to be associated with the use of EA-230. Also a Phase Ib trial – designed as a double-blind, randomized, single-dose, placebo-controlled LPS challenge trial with EA-230 – has been completed (30). The primary aim of this trial was to determine whether EA-230 administration attenuates the inflammatory response induced by LPS infusion into healthy volunteers. In the trial one group each of 12 subjects received LPS by injection, followed 30 min later by either administration of EA-230 or placebo. Pharmacokinetic analysis of the study subjects showed not just a reduction of blood C-reactive protein levels, but also reduction of several proinflammatory cytokines (IL-6, IL-8 and TNF-α) compared to the placebo group. In addition, the treatment also reduced the rise of body temperature and the drop in white blood cell counts that are induced typically by LPS. Correspondingly, the data revealed an increase in the anti-inflammatory cytokine IL-10. The outcome of these studies has been considered successful, because they suggest that EA-230 may reduce a systemic inflammatory response elicited by endotoxin. This LPS study was selected to create an artificial proxy for an actual clinical scenario. These study results, combined with the results of Phase Ia single-dose trials, provide the basis for the development and implementation of more comprehensive Phase II human trials on EA-230.

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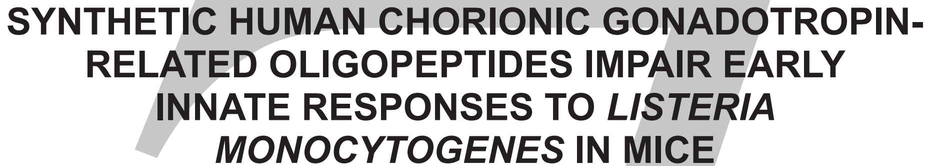

SYNTHETIC HUMAN CHORIONIC GONADOTROPIN-RELATED OLIGOPEPTIDES IMPAIR EARLY INNATE RESPONSES TO *LISTERIA MONOCYTOGENES* IN MICE

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ABSTRACT

Background: Synthetic human chorionic gonadotropin (hCG)-related oligopeptides are potent inhibitors of pathogenic inflammatory responses induced by in vivo lipopolysaccharide exposure or hemorrhagic shock-induced injury. In this study, we tested whether hCG-related oligopeptide treatment similarly altered inflammatory responses and innate host defenses in mice during experimental *Listeria monocytogenes* infection.

Methods: Mice were infected with *L. monocytogenes* and treated with hCG-related oligopeptides (LQGV, VLPALP, or AQGV) or phosphate-buffered saline. Subsequently, mice were analyzed for bacterial loads, cytokine and chemokine responses, and inflammatory cell infiltrates in target organs.

Results: Oligopeptide administration increased bacterial numbers in the spleen and liver at 6 h after infection. Simultaneously, CXCL1/KC and CCL2/MCP-1 plasma levels as well as neutrophil numbers in the spleen, blood, and peritoneal cavity decreased. In contrast, at 18 h after infection, systemic tumor necrosis factor α, interleukin 12 p70, interleukin 6, and interferon γ levels increased statistically significantly in oligopeptide-treated mice compared with controls, which correlated with increased bacterial numbers.

Conclusion: These data show that treatment with hCG-related oligopeptides (LQGV, VLPALP, and AQGV) inhibits early innate immune activation by reducing initial chemokine secretion following infection. This leads to bacterial overgrowth with subsequent enhanced systemic inflammation. Our data underscore the importance of early innate immune activation and suggest a role for hCG-derived oligopeptides at the placenta that increases the risk of *L. monocytogenes* infections.

INTRODUCTION

Listeria monocytogenes is an intracellular bacterium with a predilection for causing invasive infection in individuals with compromised host defenses (1). It is also a model pathogen used for understanding innate and adaptive immune responses to bacterial infection (1). Innate immune receptors, such as Toll-like receptors and nucleotide oligomerization-binding domain–like receptors, mediate the earliest recognition of *L. monocytogenes* components and trigger signaling pathways that induce specific chemokine and cytokine responses (2–4). Chemokines are critical for directing cellular recruitment to infected tissues, and cytokines, such as interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), and interleukin 12 p70 (IL-12p70) are crucial for eradicating *L. monocytogenes* through activation of NK cells and organ-

specific phagocytes (5, 6). Accordingly, mice that lack key chemokines, cytokines, or their cognate receptors and downstream signaling proteins are highly susceptible to *L. monocytogenes* infection (7–11).

Pregnancy is one condition that increases the risk of invasive listeriosis (1). Pregnancy transiently biases immune responses of the maternal host toward a type 2 (humoral) and away from a type 1 (cell-mediated) phenotype (12). This shift is most pronounced at the maternal-fetal interface, but it may also modulate systemic immunity to some extent. Consequently, pregnancy has deleterious effects on the outcome of infections such as leishmaniasis, malaria, toxoplasmosis, and listeriosis (13–16). In addition, autoimmune diseases such as systemic lupus erythematosus, in which the principal pathology is autoantibody production, tend to flare up during pregnancy, whereas rheumatoid arthritis is ameliorated in the maternal host (17).

The pregnancy hormone human chorionic gonadotropin (hCG) exerts endocrine and immunosuppressive functions (18). Recently, we demonstrated that hCG induces dendritic cells to differentiate toward a tolerogenic phenotype (19). In addition, hCG was observed to decrease IFN-γ production and prevent the onset of autoimmune diabetes in nonobese diabetic mice (20). Remarkably, the antidiabetic effect was not due to native hCG but resided in a 400–2000 Da peptide fraction that likely originated from proteolytic degradation of loop 2 of β human chorionic gonadotropin (β-hCG) (21). On the basis of preferential cleavage sites within loop 2 of β-hCG, we developed and tested the activity of synthetic oligopeptides in experimental inflammation (22–24). These studies demonstrated that the oligopeptides LQGV and AQGV (alanine substitution) reduced proinflammatory cytokine levels, reduced adhesion molecule expression, and diminished granulocytic infiltration into the liver following hemorrhagic shock and into the kidneys following ischemia and reperfusion (22, 23). In addition, the oligopeptide VLPALP reduced lipopolysaccharide (LPS)–induced mortality as effectively as did the 400–2000 Da hCG-derived peptide fraction (25).

To gain more insight into the effects of hCG-related oligopeptides on innate immune responses during infection with live bacteria, we tested the extent to which hCG-related oligopeptides altered host defenses in experimental *L. monocytogenes* infection in mice. Our data suggest that treatment with hCG-related oligopeptides impairs innate immune responses against *L. monocytogenes* by inhibiting chemokine responses and subsequent cellular recruitment to infected organs. This results in delayed bacterial clearance and more aggressive bacterial propagation. These data underscore the importance of early activation of innate immune responses following *L. monocytogenes* infection.

MATERIALS AND METHODS

Animals

Specific pathogen-free C57BL/6 mice (Jackson Laboratory and Harlan) were 8–16 weeks of age when used in experiments and allowed food and water ad libitum. Experiments were approved by the local animal care and use committees.

Bacteria

Inocula of live *L. monocytogenes* (strain EGD) and heat-killed *L. monocytogenes* (HKLM) were prepared as described elsewhere (26).

L. monocytogenes infection model

Mice were infected by intravenous injection of 2.0–5.5 $\log_{10}$ colony-forming units (CFUs) of wild-type *L. monocytogenes* or 7.0 $\log_{10}$ CFUs of the *L. monocytogenes* *Δhly* mutant DP-L2161 (27). The hCG-related oligopeptides VLPALP, LQGV, and AQGV (GL Biochem Shanghai) were dissolved in phosphate-buffered saline (PBS) and intraperitoneally injected (50 mg/kg body weight) starting 24 h before infection and continued every 24 h thereafter. PBS-treated mice served as controls. Mice were killed at fixed time points.

Tissue collection and evaluation of bacterial CFUs

Peritoneal lavage was performed with ice-cold PBS. Liver, spleen, and bone marrow were isolated aseptically at necropsy. Blood was collected from the submandibular vein into ethylenediaminetetraacetic acid-containing tubes (Greiner Bio-one), and plasma was obtained by centrifugation (3000 rpm for 10 min), immediately frozen, and stored at −80°C until assayed. Liver and spleen were divided into sections for evaluation of bacterial CFUs or for flow cytometric analysis. Sections were weighed and homogenized, and bacterial CFUs were quantified as described elsewhere (26). CFUs of bacteria in blood, peritoneal washings, and bone marrow cell suspensions were determined similarly. Results are expressed as mean (± standard error of the mean) $\log_{10}$ CFUs of bacteria per gram of tissue, per milliliter of blood, per milliliter of peritoneal fluid, or per femur.

In vitro stimulation of splenocytes

Uninfected mice were injected intraperitoneally with PBS or LQGV (50 mg/kg); after 18 h, splenocytes were isolated and cultured at a density of 10^6 cells/mL in Roswell Park Memorial Institute medium containing antibiotics and 5% fetal calf serum. Cells were stimulated with HKLM (10^3–10^8) or LPS (100 ng/mL), and supernatants were collected after overnight incubation for cytokine level measurements.

Evaluation of cytokines and chemokines in plasma and culture supernatant

Levels of TNF-α, IL-6, IL-12p70, and CCL2/MCP-1 were determined in plasma by means cytometric bead array (BD Biosystems), as described elsewhere (22). CXCL1/KC level in plasma and TNF-α and IL-6 levels in culture supernatant were determined by means of enzyme-linked immunosorbent assay according to the manufacturer's instructions (R&D Systems Europe). Results are expressed as mean (± standard error of the mean) in units of picograms per milliliter.

Flow cytometric analysis

Flow cytometric analysis was performed on blood, peritoneal lavage, bone marrow, and spleen cell suspensions, as described elsewhere (26). Briefly, cells were incubated with monoclonal antibody directed against Ly6C (ER-MP20 (28)), Ly6G (1A8), and CD11b (M1/70; BD PharMingen) for 30 min; washed 3 times with a solution of PBS, 0.5% bovine serum albumin, and 20 mmol/L sodium azide; and fixed with 1% paraformaldehyde (weight per volume). Monocytes were defined as CD11b-positive, Ly6C-positive, and Ly6G-negative. Neutrophils were defined as CD11b-positive, Ly6C-positive, and Ly6G-positive. Flow cytometric analysis was performed on a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using Flow-Jo software (version 7.5.4; TreeStar).

Statistical analysis

Statistical analysis was performed using SPSS software (version 15; SPSS). Differences between groups were analyzed using the Kruskal-Wallis statistical test. If this resulted in $P < .05$, the Dunn multiple comparison posttest was performed, and a result of $P < .05$ was considered statistically significant. Correlation coefficients were determined with the Pearson correlation analysis with significance set at $P < .05$.

RESULTS

Association between hCG-related oligopeptide treatment and increased bacterial numbers after lethal infection

To assess the effects of LQGV, VLPALP, and AQGV during *L. monocytogenes* infection, oligopeptide-treated mice and control mice were infected with 4 $\log_{10}$ CFUs of bacteria (1-3 median lethal doses), and then bacterial loads in target organs were measured.

In the spleens of PBS-treated mice, the mean bacterial load increased from 4.2 $\log_{10}$ CFUs of bacteria at 6 h after infection to 7.4 $\log_{10}$ CFUs at 72 h after infection. By comparison, for each oligopeptide, CFUs of bacteria were statistically significantly

higher in oligopeptide-treated mice than those in control mice at 6 h after infection. Moreover, at each time point, splenic CFUs were statistically significantly higher in the LQGV-treated mice ($P < .01$) and AQGV-treated mice ($P < .05$) compared with the PBS-treated mice (Figure 1A).

There was a mean of 3.8 $\log_{10}$ CFUs detected in the livers of PBS-treated mice at 6 h after infection, which increased to a mean of 7.6 $\log_{10}$ CFUs at 72 h after infection As also found in the spleen, CFUs of bacteria in the liver were statistically significantly higher in oligopeptide-treated mice ($P < .05$) at 6, 18, and 72 h after infection than in PBS-treated mice (Figure 1B).

Bacteria were not detected in blood and bone marrow of PBS-treated mice until 48 and 72 h after infection, respectively. In contrast, bacteremia was noted by 18 h after infection in oligopeptide-treated mice, with LQGV-treated mice always showing the highest bacterial load in the blood (Figure 1C). In bone marrow, bacteria were present at 18 h after infection in LQGV-treated mice and by 48 h after infection in VLPALP-treated mice and in AQGV-treated mice. LQGV-treated mice and AQGV-treated mice had the highest bacterial loads in bone marrow at 72 h after infection (Figure 1D).

Effect of LQGV treatment during sublethal challenge with wild-type *L. monocytogenes* and infection with avirulent *L. monocytogenes* mutants

Because LQGV treatment consistently produced the highest bacterial loads during lethal wild-type infection, subsequent experiments tested the effect of this oligopeptide in 2 different models of sublethal infection. First, mice were infected with 2.0 $\log_{10}$ CFUs

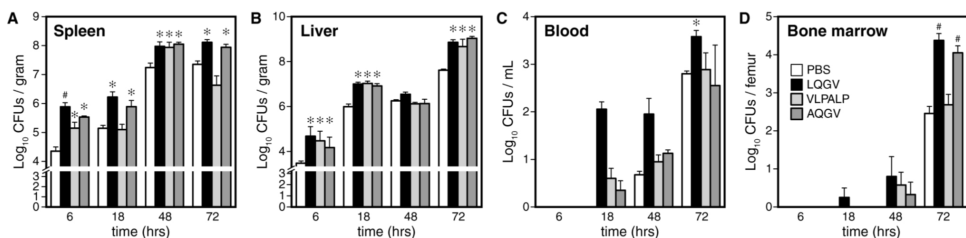

Figure 1. Increased bacterial loads as a result of human chorionic gonadotropin–related oligopeptide treatment after lethal Listeria monocytogenes challenge.
Phosphate-buffered saline (PBS; control) or one of the oligopeptides (VLPALP, LQGV, or AQGV) was administered intraperitoneally daily starting 24 h before infection with 4 $\log_{10}$ colony-forming units (CFUs) of bacteria. Mice were killed at the indicated time points, and CFUs of bacteria in tissues were quantified. Results are presented as CFUs of bacteria in the spleen ($\log_{10}$ CFUs/g) (A), liver ($\log_{10}$ CFUs/g) (B), blood ($\log_{10}$ CFUs/mL) (C), and bone marrow ($\log_{10}$ CFUs/femur) (D) from the indicated treatment groups at 6, 18, 48, and 72 h after infection. Results shown are from 1 of 3 representative experiments (n = 4 mice/group). $^*P < .05$ and $^\#P < .01$ compared with PBS-treated mice.

of wild-type *L. monocytogenes* (0.01 median lethal doses), and bacterial loads in organs were quantified as above. As also found during lethal infection, LQGV treatment resulted in statistically significantly increased bacterial loads in the spleen ($P < .01$) and liver ($P < .01$) at 72 h after infection compared with PBS treatment. Furthermore, 3.0 log_{10} CFUs of bacteria were detected in bone marrow from LQGV-treated mice, whereas bone marrow from PBS-treated mice remained sterile (Figure 2A). In a second set of experiments, mice were infected with 7.0 log_{10} CFUs of avirulent *Δhly L. monocytogenes* mutants that do not produce listeriolysin O or escape phagosomes and are eliminated rapidly. As found above, there were greater numbers of bacteria in the spleens and livers of LQGV-treated mice at 6 and 18 h after infection, compared with PBS-treated mice (Figure 2B). Collectively, these data suggest that LQGV impairs host defenses against bacterial infection in general, rather than acting specifically against mechanisms triggered by bacteria that access the cytosol.

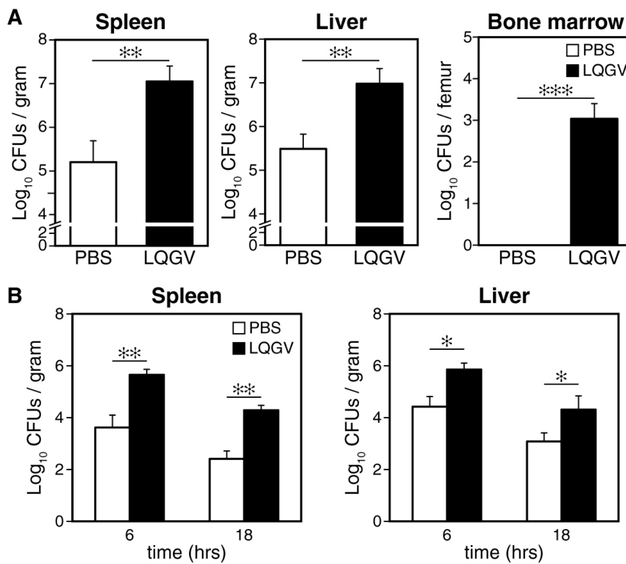

Figure 2. Increased bacterial loads as a result of LQGV treatment following a sublethal *Listeria monocytogenes* challenge or infection with avirulent *Δhly L. monocytogenes mutants*.
(A) Results for infection with wild-type *L. monocytogenes*. Phosphate-buffered saline (PBS) or LQGV was administered intraperitoneally daily starting 24 h before infection with 2 log_{10} colony-forming units (CFUs) of wild-type *L. monocytogenes*. CFUs of bacteria in spleen, liver, and bone marrow were quantified at 72 h after infection. **(B)** Results for infection with *Δhly L. monocytogenes* mutants. PBS or LQGV was administered intraperitoneally daily starting 24 h before infection with 7 log_{10} CFUs of *Δhly L. monocytogenes* mutants. CFUs of bacteria in spleen and liver were measured at 6 and 18 h after infection (n = 4 mice/group). * $P < .05$ and ** $P < .01$ compared with PBS-treated mice.

Decrease of in vitro cellular responsiveness to HKLM and LPS by LQGV treatment in uninfected mice

Next, we tested the extent to which LQGV treatment impaired the ability of cells to generate proinflammatory cytokines. For this, uninfected mice were treated with PBS or LQGV. After 18 h splenocytes were isolated and stimulated in vitro overnight with varying amounts of HKLM or 100 ng/mL LPS. The results showed that HKLM induced dose-dependent production of TNF-α and IL-6 by splenocytes from both groups of mice (Figure 3). However, splenocytes from LQGV-treated mice produced statistically significantly less TNF-α and IL-6 than did cells from control mice ($P < .05$). Similar results were obtained when splenocytes were stimulated in vitro with LPS (Figure 3).

Inhibition of early neutrophil accumulation during *L. monocytogenes* infection by oligopeptide treatment

Subsequent experiments tested the degree to which treatment with LQGV, VLPALP, or AQGV impaired cellular recruitment following infection. For this, we analyzed neutrophil and monocyte numbers in the spleen, blood, and peritoneal cavity of PBS-treated or oligopeptide-treated mice at steady state and after infection. The percentages of neutrophils in the blood, spleen, and peritoneal cavity were increased over steady state at 6 h after infection in PBS-treated mice. Interestingly, treatment with each oligopeptide resulted in lower neutrophil counts in each compartment at 6 h after infection as well as at 18 h after infection, with the exception of the peritoneal cavity (Figure 4A and 4B).

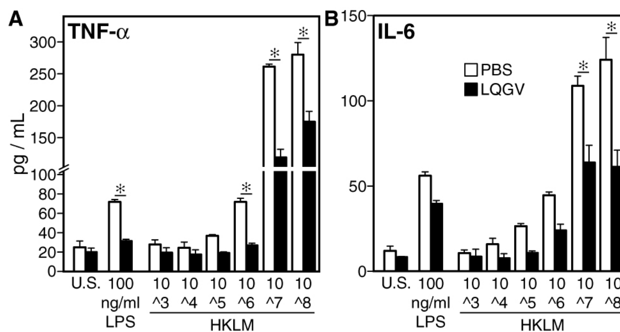

Figure 3. Impaired production of proinflammatory cytokines displayed by splenocytes from LQGV-treated mice.
Uninfected mice were treated with phosphate-buffered saline (PBS) or LQGV. After 18 h splenocytes were isolated and subsequently cultured in vitro overnight with increasing amounts of heat-killed *Listeria monocytogenes* (HKLM) or 100 ng/mL lipopolysaccharide (LPS). Concentrations of tumor necrosis factor α (TNF-α) **(A)** and interleukin 6 (IL-6) **(B)** in culture supernatants were measured by means of enzyme-linked immunosorbent assay. Data depicted are from 1 of 3 representative experiments ($n = 4$ mice/group). U.S., unstimulated. * $P < .05$ for LQGV-treated cells compared with PBS-treated cells.

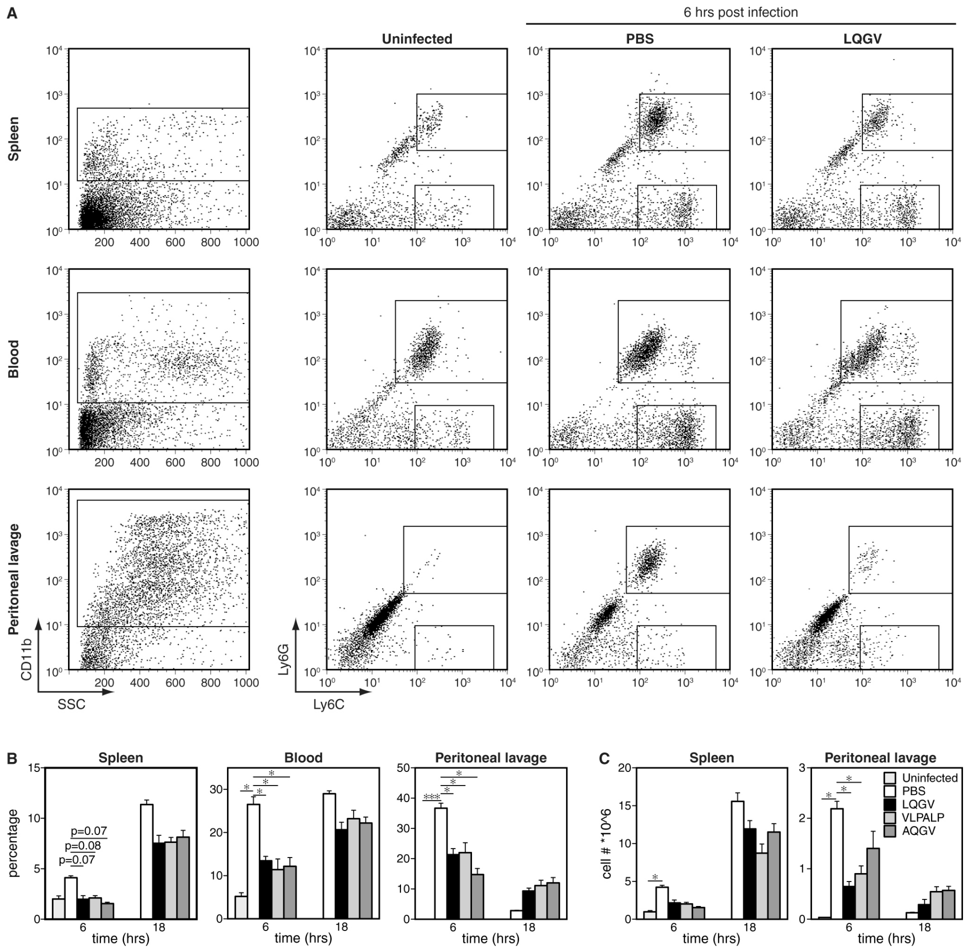

Figure 4. Inhibition of the initial mobilization of neutrophils by synthetic human chorionic gonadotropin-related oligopeptides during *Listeria monocytogenes* infection.
(A) Dot plots showing representative flow cytometric profiles from the spleen, blood, and peritoneal cavity from mice 6 h after infection with 4 $\log_{10}$ CFUs of *L. monocytogenes* and from uninfected mice. CD11b-positive cells were selected, and monocytes and neutrophils were analyzed on plots of Ly6C vs Ly6G. Monocytes were defined as CD11b-positive, Ly6C-positive, and Ly6G-negative. Neutrophils were defined as CD11b-positive, Ly6C-positive, and Ly6G-positive. Neutrophil (*upper box*) and monocyte (*lower box*) windows are shown. **(B)** Quantification of percentages of neutrophils in spleen, blood, and peritoneal lavage. **(C)** Neutrophil numbers in spleen and peritoneal cavity. Data depicted are from 1 of 3 representative experiments with similar findings (n = 4 mice/group) and are given as mean (± standard error of the mean). * P < .05 for oligopeptide-treated groups compared with phosphate-buffered saline (PBS)-treated control group or for PBS-treated mice compared with uninfected mice.

At 18 h after infection, the percentage and absolute numbers of neutrophils in the peritoneal lavage decreased in all mice compared with the percentage and absolute numbers at 6 h after infection, but this decrease was not as marked in oligopeptide-treated mice as in PBS-treated mice (Figure 4B and 4C). In contrast to the results with neutrophils, monocyte populations were not altered in any of the oligopeptide-treated groups (data not shown). These data suggest that the previously demonstrated increased in bacterial loads in target organs could be due to inhibition of neutrophil accumulation.

Alteration of systemic cytokine and chemokine levels upon hCG-related oligopeptide treatment during *L. monocytogenes* infection

Next, we tested whether reduced mobilization of inflammatory cells in vivo correlated with inhibition of systemic proinflammatory chemokine and cytokine levels. At 6 h after infection, plasma concentrations of CCL2/MCP-1 and CXCL1/KC increased statistically significantly in PBS-treated mice compared with uninfected mice (CCL2/MCP-1 concentration, 148 vs 20 pg/mL, respectively ($P < .05$); CXCL1/KC concentration, 1086 vs 215 pg/mL, respectively ($P < .05$)) (Figure 5). Although oligopeptide-treated mice also showed increased CCL2/MCP-1 levels at 6 h after infection, both CCL2/MCP-1 and CXCL1/KC levels were statistically significantly lower in oligopeptide-treated mice than the levels found in PBS-treated mice ($P < .05$). Increased concentrations of TNF-α, IL-12p70, IL-6, and IFN-γ were first detected in plasma at 18 h after infection in all mice, and they were statistically significantly higher in oligopeptide-treated mice ($P < .05$) than in PBS-treated mice (Figure 5). At 18 h after infection, CCL2/MCP-1 and CXCL1/KC levels further increased in all mice to comparable levels. These data demonstrate that the elevation of systemic levels of chemokines precedes the elevation of systemic levels of proinflammatory cytokines and that administration of LQGV, VLPALP, or AQGV initially inhibits the early chemokine response.

Determination of chemokine and cytokine plasma levels by bacterial load

To explain the apparent contradiction between depression of early chemokine levels but enhanced proinflammatory cytokine levels following oligopeptide treatment, we hypothesized that increased levels of proinflammatory cytokines at 18 h after infection were related to increased bacterial loads in target organs at this time point. To test this hypothesis, mice were infected with increasing amounts of bacteria (4 $\log_{10}$, 5 $\log_{10}$, and 5.5 $\log_{10}$ CFUs), and cytokine and chemokine plasma levels were measured at 18 h after infection. The results revealed a statistically significant positive correlation between increasing amounts of infecting bacteria and higher plasma levels of TNF-α, IL-12p70, IL-6, IFN-γ, CCL2/MCP-1, and CXCL1/KC (Figure 6A) as well as greater bacterial loads in vivo (Figure 6B). These data support a causal relationship between bacterial load and the concentration of inflammatory mediators in plasma. Moreover,

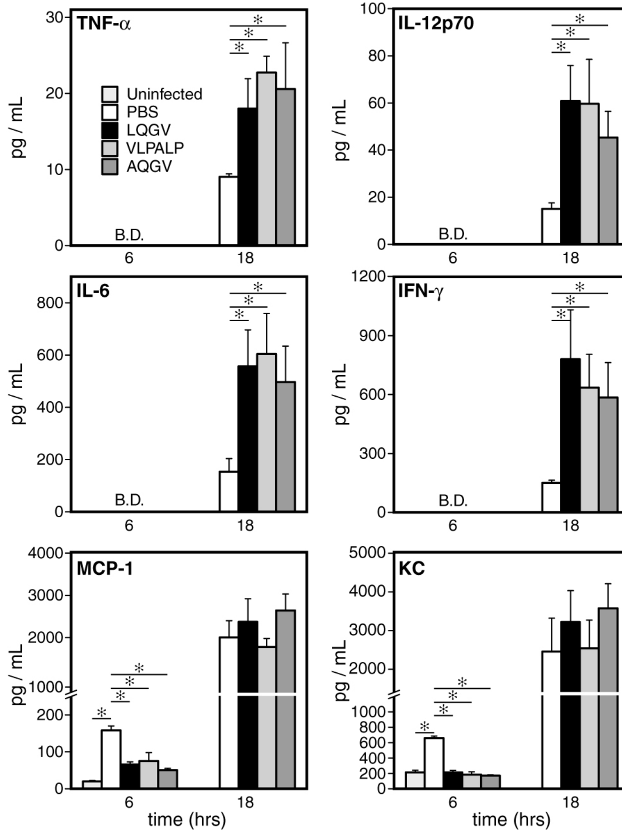

Figure 5. Massively increased levels of cytokines as a result of treatment with LQGV, VLPALP, or AQGV following lethal challenge with *Listeria monocytogenes*.
Phosphate-buffered saline (PBS), VLPALP, LQGV, or AQGV was administered intraperitoneally daily starting 24 h before infection with 4 log_{10} CFUs of L. monocytogenes. Mice were killed at fixed time points. Plasma levels of tumor necrosis factor α (TNF-α), interleukin 12 p70 (IL-12p70), interleukin 6 (IL-6), interferon γ (IFN-γ), CCL2/MCP-1, and CXCL1/KC in different experimental groups were determined at 6 and 18 h after infection Data depicted are from 1 of 3 representative experiments with similar findings (n = 4 mice/group). * $P < .05$ for oligopeptide-treated groups compared with PBS-treated control group or for PBS-treated mice compared with uninfected mice. B.D., below detection limit.

statistically significant correlations between TNF-α, IL-12p70, IL-6, and IFN-γ plasma levels and splenic CFUs were observed at 18 h after infection in LQGV-treated mice, VLPALP-treated mice, and AQGV-treated mice. In contrast, this correlation did not hold for CCL2/MCP-1 and CXCL1/KC (Figure 6C). These data indicate that the increased cytokine levels in plasma at 18 h after infection in LQGV-treated mice, VLPALP-treated mice, and AQGV-treated mice are caused by the increased bacterial load at this time point.

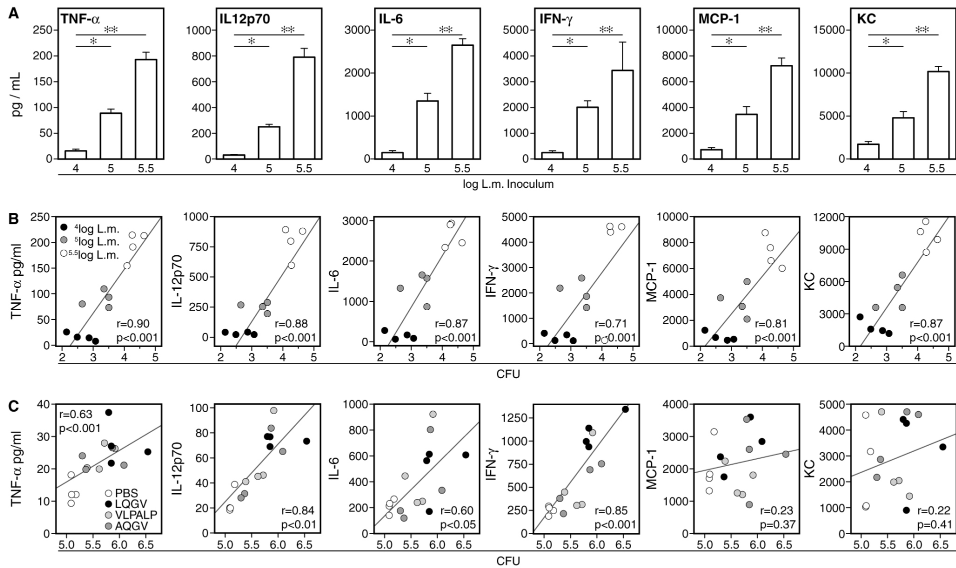

Figure 6. Positive correlation between systemic cytokine and chemokine levels and bacterial load.
Mice were infected with 4 log10, 5 log10, or 5.5 log10 colony-forming units (CFUs) of wild-type *Listeria monocytogenes*. Mice were killed at 18 h after infection, and plasma levels of tumor necrosis factor α (TNF-α), interleukin 12 p70 (IL-12p70), interleukin 6 (IL-6), interferon γ (IFN-γ), CCL2/MCP-1, and CXCL1/KC were determined. **(A)** Mean (± standard error of the mean) plasma concentrations of TNF-α, IL-12p70, IL-6, IFN-γ, CCL2/MCP-1, and CXCL1/KC determined from groups of 4 mice. Statistical differences between groups of mice are shown (* $P < .05$; ** $P < .01$). **(B)** Correlation analysis between plasma cytokine and chemokine concentrations and CFUs of bacteria in spleens of mice infected with different inocula. Statistical significance was determined by means of Pearson correlation analysis. **(C)** Correlation analysis between plasma levels and splenic CFUs in different experimental groups determined at 18 h after infection. Data depicted are from 1 of 3 representative experiments (n = 4 mice/group). PBS, phosphate-buffered saline; L.M., *L. monocytogenes*.

DISCUSSION

Innate immune responses against *L. monocytogenes* are characterized by the early mobilization of neutrophils and monocytes, as well as by the production of proinflammatory cytokines and chemokines (29). In this study we show that hCG-related oligopeptides inhibit innate immune activation during *L. monocytogenes* infection, which results in overwhelming bacterial propagation and excessive inflammation. In addition, we demonstrate that a systemic chemokine response manifested by increased plasma concentrations of CCL2/MCP-1 and CXCL1/KC precedes similar

elevations of proinflammatory cytokines. These observations are in line with other studies that showed MyD88-independent production of CCL2/MCP-1 in the spleen within 6 h of infection (30). Inhibition of this early response leads to a more aggressive infection, as was also found in our studies (30). These data underscore the importance of the magnitude and rapidity with which innate immune activation follows *L. monocytogenes* infection.

Neutrophil migration to infected foci is important for controlling bacterial growth locally and for preventing bacterial dissemination (31). In this study we show that treatment with hCG-related oligopeptides reduced neutrophil accumulation in the blood, spleen, and peritoneal cavity after *L. monocytogenes* infection. Reduced neutrophil recruitment is likely related to diminished CXCL1/KC production, as reflected by decreased plasma levels and splenic messenger RNA levels (data not shown) at 6 h after infection. Consequently, bacterial numbers increased more rapidly in the spleen and liver and were detected earlier in the blood and bone marrow than in control mice. These data are in line with previous studies that demonstrated that both reduced neutrophil migration and neutrophil depletion lead to overwhelming bacterial propagation in *L. monocytogenes* infection and in cecal ligation and puncture models (13, 32, 33). CCL2/MCP-1 regulates monocyte recruitment during the initial stage of inflammation (34). We observed decreased CCL2/MCP-1 levels upon treatment with hCG-related oligopeptides, but we found no statistically significant effect on monocyte recruitment. This finding may be explained by preserved expression of other monocyte-attracting chemokines, such as CCL7/MCP-3 (35).

Neutrophil recruitment to infected organs, IFN-γ production by NK cells, and TNF-α and IL-6 production by monocytes, macrophages, and TNF-α– and inducible nitric oxide synthase–producing dendritic cells are all essential features of the innate immune response against *L. monocytogenes* (29). In this study we found that LQGV treatment of uninfected mice impaired the ability of splenocytes to produce TNF-α and IL-6 in vitro in response to HKLM or LPS stimulation. This finding suggests that LQGV interferes with innate immune receptor-mediated activation, although the mechanisms involved are not yet clear. In contrast to decreased cytokine production in vitro, IFN-γ, TNF-α, IL-6, and IL-12p70 plasma levels increased dramatically when mice were treated with hCG-related oligopeptides prior to *L. monocytogenes* infection. These increased plasma levels correlated statistically significantly with splenic bacterial numbers, suggesting a causal relationship between bacterial load in target organs and the magnitude of the systemic inflammatory response. This notion was confirmed experimentally by infecting mice with increasing doses of *L. monocytogenes* as well as by in vitro data from HKLM stimulation of splenocytes. LQGV clearly impaired the ability of splenocytes to produce TNF-α and IL-6 upon HKLM stimulation. Nevertheless, both TNF-α and IL-6 levels increased markedly when cells were stimulated with greater amounts of HKLM,

and they exceeded cytokine levels produced by splenocytes from PBS-treated mice stimulated with a lower dose of HKLM. This mimics the in vivo situation of oligopeptide-treated mice having a higher bacterial burden than did PBS-treated mice and supports the notion that the increased cytokine levels detected in vivo in oligopeptide-treated mice are due to greater bacterial loads. In sublethal *L. monocytogenes* infection, LQGV treatment also was associated with increased bacterial numbers in the spleen and liver, as well as with greater bacterial numbers in the bone marrow. The latter finding is significant because the bone marrow was sterile in control mice. This finding underscores the strong immunosuppressive capacity of LQGV in mice. These data show that early innate immune activation is important for the initial infiltration of phagocytes at the sites of infection to control *L. monocytogenes* growth locally, a response that is fully active within the first 6 h after infection (36). This rapid response also is essential for limiting bacterial dissemination to other organs or compartments, such as the bone marrow.

An alternative explanation for increased bacterial numbers following oligopeptide treatment could be a reduced capacity of resident effector cells to kill bacteria immediately following infection. Although we cannot fully exclude this possibility, we consider it to be less likely, because after cellular invasion, virulent bacteria escape from phagosomes in a listeriolysin O-dependent fashion, then replicate intracellularly and infect neighboring cells (37, 38). *L. monocytogenes Δhly* mutants lack listeriolysin O and are easily eradicated by the immune system (27). Our results showed decreasing CFUs of *Δhly* mutants in the spleen and liver of LQGV-treated mice, similar to results seen in the spleen and liver of PBS-treated mice, but with delayed kinetics. This finding suggests that LQGV treatment does not significantly impair bacterial internalization and killing by effector cells. More rapid bacterial replication following treatment with hCG-related oligopeptides therefore is likely due to decreased chemokine production and subsequent reduced neutrophil recruitment, although formally we cannot exclude the possibility that LQGV interferes with bactericidal activities. Our data are in line with other studies that demonstrated that treatment with hCG preparation also reduces chemokine production during thioglycolate-induced inflammation (39).

Pregnant women are prone to placental infection with *L. monocytogenes*, which typically occurs during later stages of pregnancy (1, 29, 40). During the third pregnancy trimester, increased nicked β-hCG and β-core fragment levels occur in urine and plasma, compared with the first trimester (41). At present we are unable to detect hCG-derived oligopeptides in blood or urine. However, the relative amounts of nicked β-hCG and β-core fragments can be detected (42), which provide an indirect estimate for the concentration of hCG-derived oligopeptides. We postulate that high amounts of smaller fragments, including LQGV and VLPALP, may be liberated from the loop 2 region during the third trimester of pregnancy. This not only could cause local immunosuppression at the placenta, which is needed to prevent fetal allograft rejection,

but also could make the host more prone to placental *L. monocytogenes* infection later in pregnancy. Interestingly, the placentas of humans and guinea pigs are much more susceptible to infection with *L. monocytogenes* compared with mouse placentas (43–45). The explanation for differential susceptibility to *L. monocytogenes* infection is unclear, but studies have shown evidence of structural similarities between human and guinea pig placentas, whereas the mouse placenta is more different (43, 46, 47). Alternatively, a placental immunological environment in humans and in guinea pigs that is more permissive for growth of *L. monocytogenes* than that in mice could explain this differential susceptibility. Interestingly, β chorionic gonadotropin (β-CG)-derived oligopeptides could contribute to such an immunological environment in humans and guinea pigs, because both species produce the β-CG subunit and share 63% homology, whereas mice lack the β-CG gene and thus do not produce β-CG. Clearly, more studies on animal models of pregnancy with *L. monocytogenes* infection are needed to unveil the precise role of CG-related oligopeptides during pregnancy.

Elsewhere, we reported that LQGV and AQGV attenuated life-threatening inflammation and organ damage associated with hemorrhagic shock and renal ischemia and reperfusion (22, 23). In addition, we showed that VLPALP treatment reduced mortality upon LPS injection (25). Although the exact mechanism of action of LQGV, VLPALP, and AQGV are not yet known, our current study suggests that suppressive effects on innate immune responses have contributed to beneficial outcomes found in these models. These data show that hCG-related oligopeptides have strong immunosuppressive activity, which provides the underlying foundation for phase 1 trials as currently performed in humans.

In summary, we demonstrate that a chemokine response precedes the proinflammatory cytokine and cellular response in *L. monocytogenes* infection. This chemokine response is essential for rapid cellular recruitment to prevent further progression of infection. Furthermore, this study reveals that the synthetic hCG-related oligopeptides LQGV, VLPALP, and AQGV inhibit early innate immune responses with subsequent increased bacterial propagation. Our data underscore the importance of early innate immune activation following bacterial infection and suggest that hCG-derived oligopeptides at the placenta could increase host susceptibility to certain infections, such as with *L. monocytogenes*.

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SYNTHETIC OLIGOPEPTIDES RELATED TO THE β-SUBUNIT OF HUMAN CHORIONIC GONADOTROPIN ATTENUATE INFLAMMATION AND LIVER DAMAGE AFTER (TRAUMA-) HEMORRHAGIC SHOCK AND RESUSCITATION

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ABSTRACT

Severe hemorrhagic shock followed by resuscitation induces a massive inflammatory response, which may culminate into systemic inflammatory response syndrome, multiple organ dysfunction syndrome and finally death. Treatments that effectively prevent this inflammation are limited so far. In a previous study, we demonstrated that synthetic oligopeptides related to the primary structure of *human chorionic gonadotropin* can inhibit the inflammatory response and mortality that follow high-dose lipopolysaccharide-induced inflammation. Considering this powerful anti-inflammatory effect, we investigated whether administration of similar synthetic hCG-related oligopeptides (LQGV, AQGV, LAGV), during hemorrhagic shock, were able to attenuate the inflammatory response associated with this condition. Hemorrhagic shock was induced in rats for 60 minutes by blood withdrawal until a mean arterial pressure of 40 mmHg was reached. Thirty minutes after induction of hemorrhagic shock, rats received a single injection with one of the hCG-related oligopeptides (LQGV, AQGV or LAGV) or 0.9% NaCl solution, as control. Treatment with LQGV, AQGV or LAGV prevented systemic release of TNF-α and IL-6 and was associated with reduced TNF-α, IL-6 and E-selectin mRNA transcript levels in the liver. LQGV treatment prevented neutrophil infiltration into the liver and was associated with reduced liver damage. Our data suggest that hCG-related oligopeptides, in particular LQGV, have therapeutic potential by attenuating the life threatening inflammation and organ damage that is associated with (trauma-) hemorrhagic shock and resuscitation.

INTRODUCTION

Severe hemorrhagic shock is caused by massive blood loss, which cannot be compensated for by the body without treatment. The primary treatment of hemorrhagic shock is focused on controlling bleeding and restoring intravascular volume to improve tissue perfusion. Many patients with severe hemorrhagic shock who are successfully resuscitated develop an inflammatory response, which may culminate into systemic inflammatory response syndrome (SIRS) and finally multiple organ dysfunction syndrome (MODS) (1). In addition, approximately 40% of patients with hemorrhagic shock develop sepsis as a result of increased gut permeability and development of compensatory anti-inflammatory syndrome (1, 2). Sepsis and MODS are the leading causes of death in critically ill patients on the intensive care unit all over the world with approximately 50% mortality (3).

The inflammatory response following hemorrhagic shock and resuscitation is characterized by increased expression of adhesion molecules, such as E-selectin and

intracellular adhesion molecule-1 (ICAM-1), on endothelial cells and hepatocytes (4). Up-regulation of these adhesion molecules facilitates tissue infiltration by neutrophils, resulting in cell-mediated organ injury (5). Furthermore, increased levels of cytokines, such as TNF-α, IL-1β, IL-6 and IL-10, are found systemically and locally in liver, lungs and intestine (6-8). These cytokines, mainly produced by immune cells, affect organ integrity directly or indirectly through induction of secondary mediators, such as thromboxanes, leukotrienes, and complement (9, 10).

The last decade, research has focused on reducing systemic and local inflammatory responses with therapeutic agents that neutralize cytokine activity or inhibit inflammatory mediator production. However, in the case of hemorrhagic shock such treatments require initiation before the onset of shock to achieve an effect (11-13). Clearly, this is impossible in most clinical settings. Therefore, therapies that efficiently inhibit the inflammatory response when initiated after hemorrhage-induced shock are more relevant. Studies on such treatments are limited (14), but are highly needed.

During pregnancy, the maternal immune system tolerates the fetus by reducing cell-mediated immune responses while retaining normal humoral immunity. Also, clinical symptoms of cell-mediated autoimmune diseases regress in many patients during pregnancy (15). Most likely a specific hormonal environment is responsible for modulating the immune system during pregnancy (15). The hormone *human chorionic gonadotropin* (hCG) is secreted by placental syncytiotrophoblasts during human pregnancy. Human CG preparations exhibit not only endocrine effects, but also immunosuppressive activity (16). We found that hCG preparations inhibited the onset of autoimmune type-I diabetes in nonobese diabetic mice (17). This anti-diabetic/anti-inflammatory activity was not due to the heterodimeric hCG nor to its α- or β-subunits, but resided in a peptide fraction of 400-2000 Dalton, which likely originates from proteolytic cleavage of loop 2 of the hCG β-subunit (17-19). Subsequently, we successfully demonstrated that synthetic oligopeptides, related to the primary sequence of loop 2 of the hCG β-subunit, inhibit inflammation, disease severity, and mortality in high-dose lipopolysaccharide-induced SIRS (18, 19). Considering this powerful effect of synthetic hCG-related oligopeptides on inflammation, we hypothesized that the administration of such oligopeptides after induction of hemorrhagic shock could inhibit the inflammatory response associated with this condition. To this end, we used LQGV, which is part of the primary structure of loop 2 of the β-subunit of hCG, and two alanine replacement variants, namely AQGV and LAGV.

Using a rat model of (trauma-) hemorrhagic shock and resuscitation, we demonstrate that either LQGV, AQGV or LAGV, administered after the induction of hemorrhagic shock, significantly prevented TNF-α and IL-6 release into the plasma and attenuated the increase in TNF-α, IL-6 and E-selectin mRNA transcript levels in the liver. In addition, LQGV treatment significantly prevented neutrophil accumulation in the

liver, which correlated with decreased organ damage as reflected by reduced lactate dehydrogenase and aspartate aminotransferase plasma levels.

MATERIALS AND METHODS

Animals

Adult male specific pathogen-free Wistar rats *(Harlan CPB, Zeist, The Netherlands)*, weighing 350–400g were used. Rats were housed under barrier conditions at 25°C with a twelve-hour light/dark cycle, and were allowed food and water *ad libitum*. The experimental protocol was approved by the Animal Experiments Committee under the Dutch Experiments on Animals Act and adhered to the rules laid down in this national law that serves the implementation of “Guidelines on the protection of experimental animals” by the Council of Europe (1986), Directive 86/609/EC.

hCG-related synthetic oligopeptides

The hCG-related oligopeptides (LQGV, AQGV and LAGV) were synthesized by Ansynth Service B.V. *(Roosendaal, The Netherlands)* and dissolved in 0.9% NaCl at a concentration of 5 mg/ml.

Surgical procedures

Rats were food deprived overnight before the start of the experiment, but were allowed water *ad libitum*. Rats were anesthetized using a mixture of N_2O/O_2/isoflurane *(Pharmachemie B.V., Haarlem, The Netherlands)*. Body temperature was continuously maintained at 37.5°C by placing the rats on a thermo controlled ‘half-pipe’ *(UNO, Rotterdam, The Netherlands)*. Endotracheal intubation was performed, and rats were ventilated at 60 breaths per minute with a mixture of N_2O/O_2/isoflurane. Polyethylene tubes *(PE-50, Becton Dickinson; St. Michielsgestel, The Netherlands)* were flushed with heparin and placed via the right carotid artery in the aorta and in the right internal jugular vein. A 5 cm midline laparotomy was performed and a supra pubic catheter was inserted to monitor urine production.

Experimental procedures

After an acclimatization period of 15 minutes, the rats were randomized into five different groups (eight rats per group): 1) sham, 2) hemorrhagic shock (HS), 3) hemorrhagic shock with LQGV treatment (HS/LQGV), 4) hemorrhagic shock with AQGV treatment (HS/AQGV) and 5) hemorrhagic shock with LAGV treatment (HS/LAGV). Hemorrhagic shock was induced by blood withdrawal, reducing the circulating blood volume until a mean arterial pressure (MAP) of 40 mmHg was reached. This level of hypotension was

maintained for 60 minutes. Thirty minutes after the induction of hemorrhagic shock, rats received a single intravenous (IV) bolus injection of 5 mg/kg bodyweight of either LQGV, AQGV, LAGV or 0.9% NaCl solution. The peptides and dosage were based on previous studies, in which we performed dose-escalation experiments (20). Sixty minutes after induction of hemorrhagic shock, rats were resuscitated by four times their shed blood volume over a period of 30 minutes to normalize the MAP, and monitored for another 120 minutes after which they were sacrificed (Figure 1A). The rats received no heparin before or during the experiment. Sham animals underwent the same surgical procedure as the hemorrhagic shock animals, but without blood withdrawal and administration of oligopeptide.

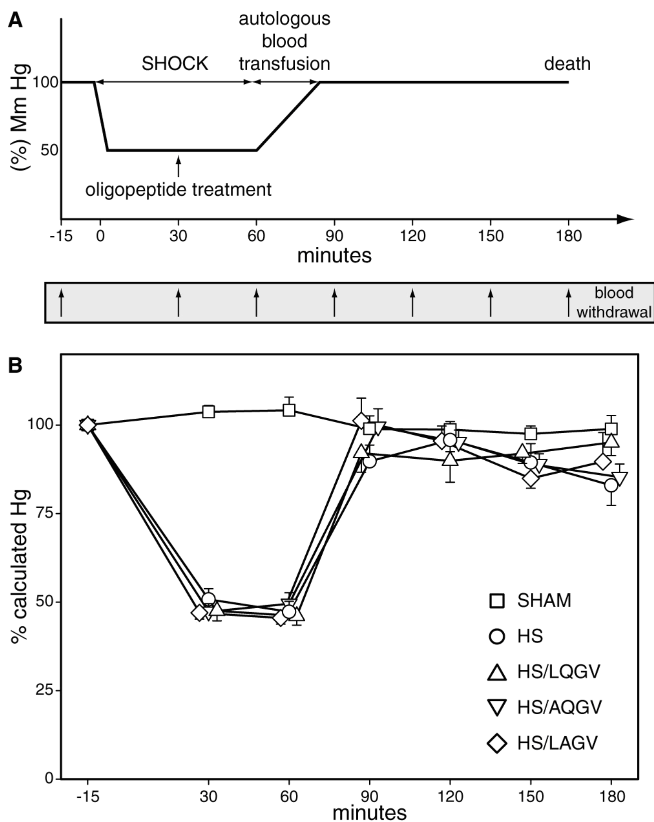

Figure 1.
(A) Schematic representation of the experimental design of induction of hemorrhagic shock in rats. **(B)** The measured blood pressure in mmHg was recalculated in percentages to standardize the experiment and to compensate for animal differences.

Measurements of mean arterial pressure

During the experiments, MAP was continuously measured using transducers *(Becton Dickinson, Breda, The Netherlands)* that were connected in line to an electronic recorder *(Hewlett Packard, 78354-A, Cheshire, USA)*.

Plasma collection and storage

Arterial blood was obtained 15 minutes before and 30, 60, 90, 120, 150 and 180 minutes after onset of hemorrhage (Figure 1A). After blood withdrawal, leukocyte numbers were determined using a coulter counter *(Beckman Coulter, Mijdrecht, The Netherlands)* and corrected for the hematocryte. Approximately, 0.3 ml of blood was placed into mini collect tubes *(Greiner, Bio-one, Alphen aan den Rijn, The Netherlands);* plasma was obtained by centrifugation (1500 rpm; 5 min), immediately frozen, and stored at -80°C until assayed.

Tissue collection and storage

Liver, lungs, ileum and sigmoid were surgically removed at 180 minutes after hemorrhagic shock induction, snap-frozen, and stored at -80°C until assayed.

Evaluation of cytokines in plasma

TNF-α, IL-6 and IL-10 plasma levels were determined by ELISA *(R&D Systems, Abingdon, UK),* according to the manufacturer's instructions.

Evaluation of mRNA levels by real-time quantitative (RQ)-PCR

RNA was isolated using the Qiagen RNeasy kit *(QIAGEN, Hilden, Germany)*, according to the manufacturer's instructions. *TNFA* (encoding TNF-α), *IL6* (encoding IL-6), *IL10* (encoding IL-10), *SELE* (encoding E-selectin), and *ICAM1* (encoding ICAM-1) gene expression levels were determined by RQ-PCR using an Applied Biosystems 7700 PCR machine *(Foster City, CA, USA)*. The expression levels of these genes were quantified by normalization against the mRNA levels of the household gene *GAPDH*. Primers and probes used are available upon request.

Immunohistochemical analysis

Cryo-sections (6 μm) were fixed in acetone/0.05%H_2O_2 for five minutes and subsequently air dried for 10 minutes. Neutrophils were visualized by staining for myeloperoxidase (MPO). Hereto, sections were incubated overnight at 4°C with a mouse-anti-rat MPO monoclonal antibody *(Hbt, Uden, The Netherlands)*. Subsequently, sections were incubated for 60 minutes at room temperature with a goat-anti-mouse-Horse Radish Peroxidase (HRP)-labeled monoclonal antibody (*Dako B.V., Glostrup, Denmark)*. For visualization of HRP activity, 3-amino-9-ethylcarbazole substrate

(*Sigma Co., St Quentin Fallavier, France)* dissolved in 50mM sodium acetate/0.02% hydroxyperoxide was used. Sections were embedded in Kaisers Gycerol/gelatin *(Boom B.V., Meppel, The Netherlands)*. Numbers of MPO positive cells were counted per high power field (HPF) at a magnification of 200x. Per section a total of 15 HPF were analyzed. Per organ a total of three consecutive sections, each separated 18μm from each other, were analyzed.

Blood biochemical analysis

Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were determined at the Erasmus MC diagnostic facility according to standard procedures.

Statistical analysis

Data are presented as the mean values ± standard deviation (SD) of the eight rats per group. Statistical analysis was performed using SPSS version 11 software *(SPSS Inc., Chicago,Ill)*. Intergroup differences were analyzed with Kruskal-Wallis statistical test. If Kruskal-Wallis statistical testing resulted in a $p < 0.05$, a Dunn's Multiple Comparison test was performed and a $p < 0.05$ was considered statistically significant.

RESULTS

Induction of hemorrhagic shock

Rats were rapidly bled, within 10 minutes, to a MAP of 40 mmHg, which was successfully maintained for 60 minutes in all four experimental groups (Figure 1B). No change in MAP was observed in sham treated rats (Figure 1B). Sixty minutes after hemorrhagic shock, rats were resuscitated to induce organ reperfusion, which was associated with a normalization of urine production (data not shown). These data indicate that shock was induced equally in all four experimental groups and was followed by successful organ reperfusion. Heart rates in all four experimental groups increased immediately after induction of hemorrhagic shock and returned to normal after resuscitation. Over time, heart rate slowly increased in all four experimental HS groups (data not shown).

Oligopeptide treatment prevents the release of pro-inflammatory cytokines into plasma

Before induction of hemorrhagic shock, TNF-α plasma levels were comparable in all five groups (~15-24 pg/ml) (Figure 2). In the HS group, TNF-α levels started to increase 30 minutes after induction of hemorrhagic shock. These levels were significantly increased

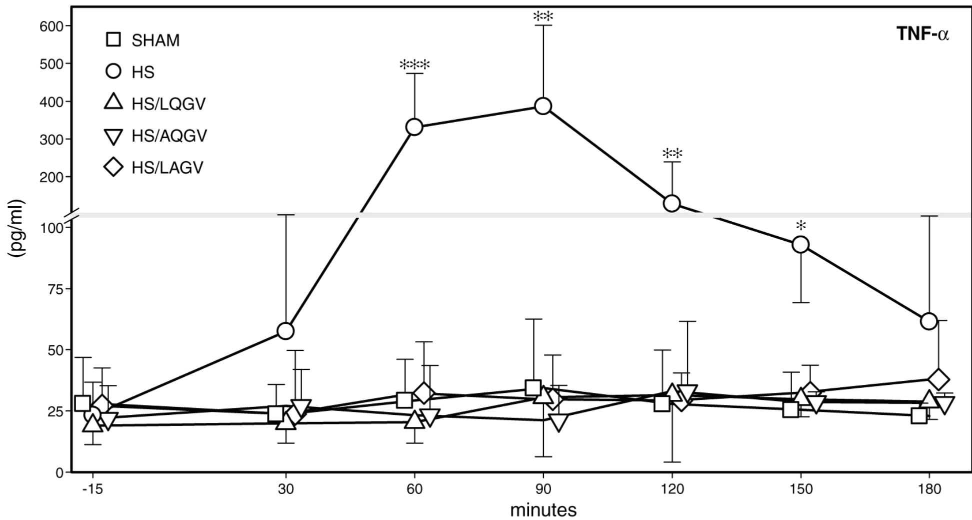

Figure 2.
TNF-α plasma levels in different experimental groups determined at 15 minutes before and 30, 60, 90, 120, 150 and 180 minutes after the onset of hemorrhagic shock. Data are presented as the mean of eight rats per group ± SD.

after 60 minutes as compared to the sham group (331 pg/ml vs 29 pg/ml; p < 0.01). TNF-α levels reached a maximum of 384 pg/ml after 90 minutes in the HS group, after which levels declined again but continued to remain increased compared to the sham group (Figure 2). In contrast, none of the oligopeptide-treated HS groups showed an increase in TNF-α plasma levels during the experiment (Figure 2). In this model of hemorrhagic shock, IL-6 levels are known to increase at a later time-point than TNF-α (21). Therefore, we determined IL-6 levels in blood samples collected 120, 150 and 180 minutes after the onset of hemorrhagic shock. In the HS group, IL-6 plasma levels were significantly increased as compared to the sham group at 120 minutes (2003 pg/ml vs 331 pg/ml; $p < 0.001$), at 150 minutes (2444 pg/ml vs 333 pg/ml; $p < 0.001$) and at 180 minutes (2940 pg/ml vs 343 pg/ml; $p < 0.001$) (Figure 3). Although, IL-6 levels tended to increase in the HS/oligopeptide treated rats as compared to sham treated rats, this never reached significance. Treatment with oligopeptides significantly diminished the release of IL-6 into plasma as compared to the non-treated hemorrhagic shock group ($p < 0.05$; Figure 3). IL-10 was undetectable in plasma of all groups throughout the experiment (data not shown). These data demonstrate that treatment with a single dose of either LQGV, AQGV or LAGV, after induction of hemorrhagic shock, significantly attenuated the increase in TNF-α and IL-6 into plasma.

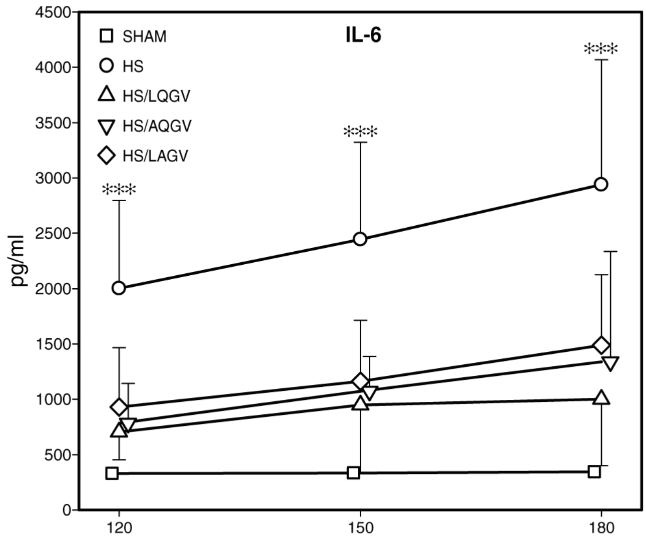

Figure 3.
IL-6 plasma levels in different experimental groups determined at 120, 150 and 180 minutes after the onset of hemorrhagic shock. Data are presented as the mean of eight rats per group ± SD.

Oligopeptide treatment is associated with a decrease in TNF-α and IL-6 mRNA transcript levels in the liver

We also analyzed the TNF-α and IL-6 mRNA transcript levels in liver, lungs, ileum and sigmoid tissues at 180 minutes after the onset of hemorrhagic shock. In the liver, TNF-α transcript levels were significantly increased in the HS group as compared to the sham group ($p < 0.001$). Oligopeptide treatment was associated with reduced TNF-α transcript levels in the liver as compared to non-treated HS rats, with only HS/LQGV showing a significant reduction ($p < 0.05$; Figure 4A). In the HS group, IL-6 transcript levels in the liver were increased ~83 times as compared to the sham group ($p < 0.001$; Figure 4B). None of the oligopeptide treated groups showed a significant increase in IL-6 transcript levels as compared to the sham group. LQGV and AQGV treatment was associated with significantly lower IL-6 transcript levels as compared to the HS group ($p < 0.05$; Figure 4B). Although, IL-10 was undetectable in plasma, IL-10 transcript levels were increased in the livers of the HS group as compared to the sham group, which approached significance ($p = 0.08$). Although not significant, LQGV treatment was associated with decreased IL-10 transcript levels as compared to the non-treated HS group (data not shown). In lungs, ileum and sigmoid tissues no differences could

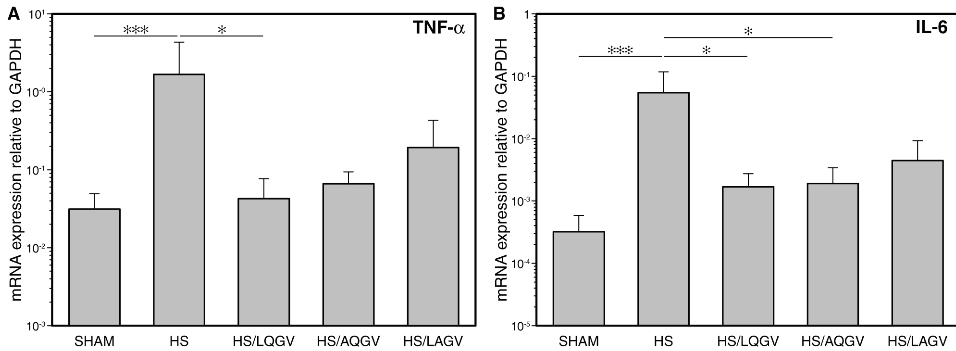

Figure 4.
Relative expression of TNF-α **(A)**, IL-6 **(B)** mRNA transcripts in the liver, 180 minutes after the onset of hemorrhagic shock. Transcript levels are normalized to the expression level of *GAPDH*. Data are presented as the mean of eight rats per group ± SD.

be detected between the various groups for TNF-α, IL-6 and IL-10 transcript levels (data not shown). These data imply that oligopeptide treatment after shock induction significantly attenuated the increase in TNF-α and IL-6 transcript levels in the liver.

Oligopeptide treatment is associated with a decrease in E-selectin mRNA transcript levels in the liver

In the HS group, the ICAM-1 transcript level was significantly increased in the liver as compared to the sham group ($p < 0.001$; Figure 5A). ICAM-1 transcript levels in the liver tended to decrease in the oligopeptide treated groups as compared to the non-treated HS group. The E-selectin transcript level in the liver of the HS group was significantly increased as compared to the sham group ($p < 0.001$). LQGV and AQGV treatment was associated with significantly lower E-selectin transcript levels in the livers as compared to the non-treated HS-group ($p < 0.05$; Figure 5B). These data demonstrate that LQGV and AQGV treatment after shock induction significantly attenuated the increase in E-selectin transcript levels in the liver, while ICAM-1 transcript levels were down regulated to a lesser extent.

LQGV treatment prevents neutrophil accumulation in the liver

In the HS group, the number of neutrophils in the liver was significantly increased as compared to the sham group ($p < 0.05$; Figure 6A). LQGV treatment significantly ($p < 0.05$) prevented this neutrophil accumulation, while AQGV and LAGV treatment did not prevent neutrophil accumulation in the liver ($p < 0.05$; Figure 6).

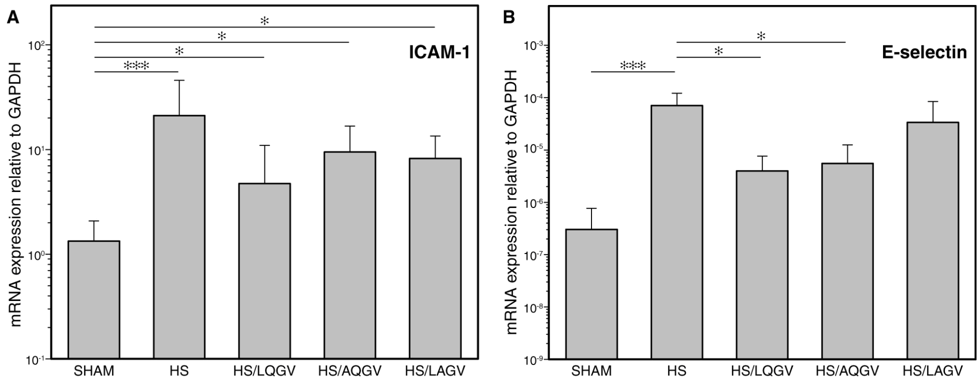

Figure 5.
Relative expression of E-selectin **(A)** and ICAM-1 **(B)** mRNA transcripts in the liver, 180 minutes after the onset of hemorrhagic shock. Transcript levels are normalized to the expression level of *GAPDH*. Data are presented as the mean of eight rats per group ± SD.

LQGV treatment attenuates organ damage

ALT, AST and LDH plasma levels were significantly increased in the HS group as compared to the sham group ((ALT; $p < 0.01$; Figure 7A), (AST; $p < 0.01$; Figure 7B) and (LDH; $p < 0.01$; Figure 7C)), while LQGV treatment significantly ($p < 0.05$) attenuated this rise in AST and LDH. AQGV and LAGV treatment did not affect ALT, AST and LDH plasma levels as compared to the untreated HS group.

DISCUSSION

In this study we used a rat model of (trauma-) hemorrhagic shock to test the therapeutic capacity of three synthetic hCG-related oligopeptides (LQGV, AQGV or LAGV). We demonstrate that a single administration of either LQGV, AQGV or LAGV, 30 minutes after shock induction, markedly prevents TNF-α and IL-6 release into plasma and diminishes the increase of TNF-α, IL-6 and E-selectin mRNA transcript levels in the liver. In addition, LQGV treatment significantly prevented neutrophil accumulation into the liver, which coincided with lower AST and LDH plasma levels.

Hemorrhagic shock followed by resuscitation is characterized by a massive production of pro-inflammatory cytokines, such as TNF-α and IL-6, by immune cells (10). Despite improvement in treatment strategies, (trauma-) hemorrhage patients may still develop an inflammatory response that can lead to sepsis, MODS and

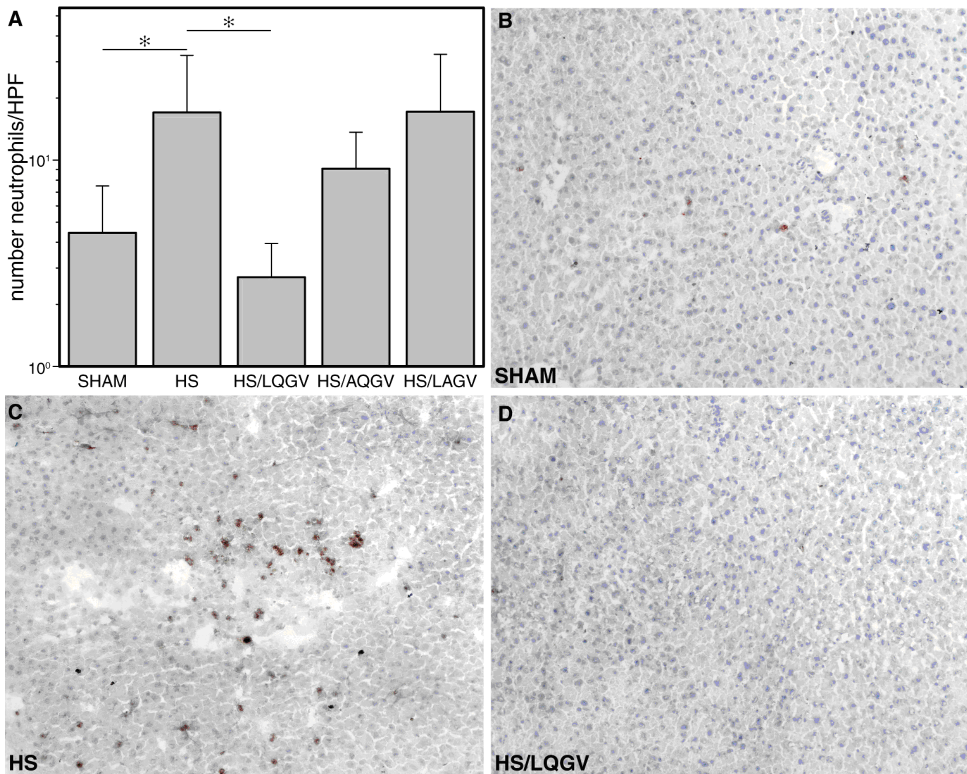

Figure 6.
(A) Number of neutrophils per HPF (magnification 200x) in the liver 180 minutes after the onset of hemorrhagic shock. Data are presented as the mean of eight rats per group ± SD.
Representative examples of livers from sham **(B)**, HS **(C)** and HS/LQGV **(D)**, 180 minutes after the onset of hemorrhagic shock.

finally death. In our model of hemorrhagic shock and resuscitation, we observed an inflammatory response, as reflected by significantly increased levels of TNF-α and IL-6 in plasma. TNF-α is a key mediator of the innate immune system that is crucial for the generation of a local protective immune response against infectious or non-infectious agents (22). However, uncontrolled TNF-α production is lethal, as it induces tissue damage and promotes the production of secondary pro-inflammatory mediators, such as IL-6 (23).

Experimental treatment strategies aimed at neutralizing bioactive cytokines, especially monoclonal antibodies against TNF-α, have been successfully applied in several inflammatory disorders, including Crohn's disease and rheumatoid arthritis (24, 25). However, clinical studies using monoclonal antibodies against TNF-α

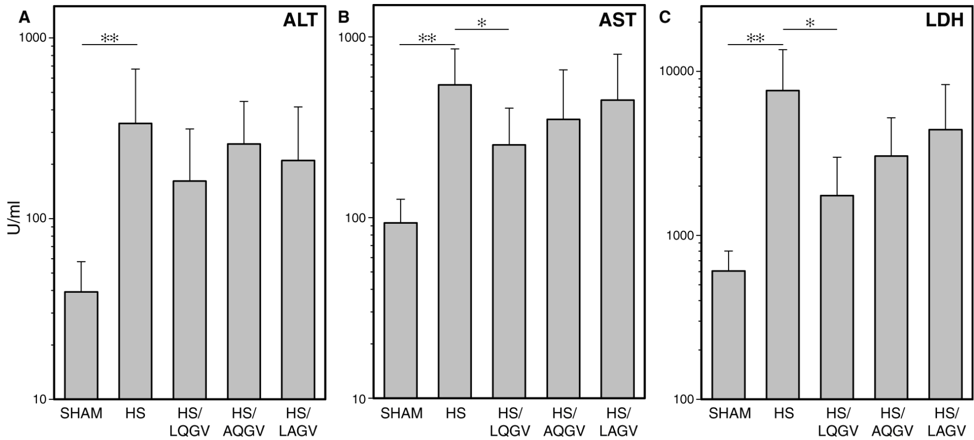

Figure 7.
Plasma levels of ALT **(A)**, AST **(B)** and LDH **(C)** 180 minutes after the onset of hemorrhagic shock. Data are presented as the mean of eight rats per group ± SD.

showed no improvement in trauma-patients (26). IL-6 is a highly pluripotent cytokine, which facilitates neutrophil infiltration into organs, thereby contributing to cell-mediated organ damage (27). In our model of (trauma-) hemorrhagic shock and resuscitation, oligopeptide treatment was associated with significantly decreased levels of TNF-α and IL-6 in plasma. Reducing TNF-α and IL-6 plasma levels is of clinical importance, because high systemic levels of TNF-α and IL-6 correlate with poor outcome and decreased survival in patients with severe trauma and infection (28). We found local TNF-α and IL-6 production in the liver after hemorrhagic shock and resuscitation, which was reduced upon oligopeptide treatment, in particular with LQGV. In lungs, ileum and sigmoid, we found no effect of hemorrhagic shock on the transcript levels of TNF-α and IL-6. Trauma-hemorrhage has been recognized to induce acute lung injury/inflammation in humans and animals (29, 30). In our model we detected no increase of TNF-α, IL-6, E-selectin and ICAM-1 transcript levels in the lungs three hours after hemorrhagic shock, suggesting that a pulmonary inflammatory response was not (yet) evident. We have found that hCG-related oligopeptides efficiently inhibited SIRS and mortality that was induced upon LPS administration, which is an inflammatory model characterized by involvement of several organ systems, including the lungs (18, 19). Therefore, although we cannot conclude it from the current study, we expect that hCG-related oligopeptides can prevent hemorrhagic shock-induced pulmonary inflammation.

IL-10 is an anti-inflammatory cytokine that reduces cell-mediated immune responses and pro-inflammatory cytokine production following hemorrhagic shock (8). We were unable to detect IL-10 in plasma during the time frame of the experiments. However,

local IL-10 production in the liver was detected, since IL-10 transcripts increased in the HS group. LQGV treatment was associated with decreased IL-10 mRNA levels as compared to the non-treated HS group. Although this did not reach statistical significance, we propose that this may be of biological relevance since high IL-10 levels are associated with a high incidence of infection, MODS and increased mortality in (trauma-) hemorrhage patients (31).

Neutrophils induce organ damage and enhance inflammation by the release of oxygen radicals, proteolytic enzymes and cytokines (32, 33). Neutrophil infiltration into organs is an early event of hemorrhagic shock and resuscitation, and neutrophil depletion has been shown to prevent hemorrhagic shock induced inflammation and organ damage (5, 34). These data indicate a central role for neutrophils in the pathophysiology of hemorrhagic shock and resuscitation. Leukocyte migration from blood into organs requires the consecutive events of rolling and sticking to activated endothelial cells, followed by diapedesis and chemotaxis (35). Among these processes, selectin-mediated rolling is indispensable for initiation of leukocyte transmigration and inflammation (36). In line with this, L- or E- selectin blockage, using monoclonal antibodies, reduced liver infiltration by neutrophils as well as inflammation and organ damage following hemorrhagic shock (37). In our experiments, treatment with LQGV or AQGV significantly decreased E-selectin transcript levels in the liver. Furthermore, LQGV treatment prevented neutrophil accumulation in the liver and was associated with lower AST and LDH plasma levels after hemorrhagic shock and resuscitation. These data suggest that oligopeptide treatment, in particular LQGV, after hemorrhagic shock and resuscitation diminishes the expression of adhesion molecules, thereby inhibiting tissue infiltration by neutrophils and subsequent organ damage and systemic inflammation.

In this model, LQGV, originating from the primary sequence of loop 2 of the β-subunit of hCG, was the most effective oligopeptide for every parameter determined. Alanine replacement in this sequence reduced the biological activity. We cannot exclude that the decreased TNF-α and IL-6 mRNA levels, upon oligopeptide treatment, that we observed in the liver are the result of a diminished cellular infiltrate. However, the decrease in E-selectin mRNA, which is only expressed by endothelial cells, indicates that the tested oligopeptides also interfere with mechanisms that regulate expression/ activation of genes involved in inflammation and immunity. So far it is unclear what the underlying mechanism is by which these oligopeptides exert their effects. It is possible that they use yet unidentified receptors. However, we cannot exclude the possibility that, due to their small size and molecular weight, they penetrate the cell membrane (38) and exert their action either by interfering with signaling cascades or the transcriptional machinery. Therefore, we do not exclude that different oligopeptides have different modes of action. Studies are in progress to reveal how these hCG-related oligopeptides

exert their action.

In summary, we demonstrated that administration of a synthetic hCG-related oligopeptide (LQGV, AQGV or LAGV) after the induction of severe hemorrhagic shock significantly attenuated the pro-inflammatory response both systemically and locally in the liver. Treatment with LQGV prevented neutrophil infiltration into the liver and subsequent liver damage. These data suggest that these oligopeptides, in particular LQGV, have therapeutic potential and may reduce the morbidity and mortality associated with hemorrhagic shock and resuscitation.

ACKNOWLEGDMENTS

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AMELIORATION OF RENAL ISCHEMIA-REPERFUSION INJURY BY SYNTHETIC OLIGOPEPTIDES RELATED TO HUMAN CHORIONIC GONADOTROPIN

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ABSTRACT

Background: We have previously reported that small synthetic oligopeptides related to human β-chorionic gonadotropin (β-hCG) can reduce inflammation. Here we investigated whether such oligopeptides can reduce renal ischemia reperfusion injury in the mouse.

Methods: Ten different oligopeptides were administered 1 minute before induction of renal ischemia and 1 minute before reperfusion.

Results: Survival at 72 hours post reperfusion was significantly higher in mice treated with oligopeptides MTRV, LQG, VLPALPQ or AQGV as compared to placebo treated mice. Some oligopeptides were more effective than others. AQGV completely prevented mortality and best preserved kidney function. Next, AQGV was tested in a dose-escalating study in a range of 0.3 to 30 mg/kg. A survival gain was observed with all doses. Improvement of kidney function was observed from 1 mg/kg. Highest survival and best preserved kidney function was observed at 3 and 10 mg/kg.

Upon treatment with AQGV, a significantly lower influx of neutrophils was found, apoptosis was decreased, whereas tubular epithelial cell proliferation was significantly increased at 24 hrs post-reperfusion. Serum levels of TNF-α, INF-γ, IL-6 and IL-10 were significantly decreased at 24 hours post reperfusion. E-selectin mRNA levels in kidneys were significantly decreased at 6 hours post-reperfusion. AQGV did not reduce mortality when treatment was started after reperfusion.

Conclusions: This study shows that small oligopeptides related to the primary structure of β-hCG, especially AQGV, are promising potential drugs for preventing the development of renal ischemia-reperfusion injury.

INTRODUCTION

Inflammation plays a major role in the pathophysiology of renal ischemic injury (1). The initial ischemic injury results in up-regulation of adhesion molecules on activated endothelium and release of cytokines, reactive oxygen species (ROS) and eicosanoids. Leukocytes, recruited by chemokines and pro-inflammatory cytokines, potentiate injury by generating more ROS and eicosanoids, thereby further enhancing inflammation.

Human chorionic gonadotropin (hCG) is a hormone produced during pregnancy by placental trophoblasts (2), but is also produced by the pituitary gland and leukocytes in non-pregnant females and males (3,4). It consists of an α- and β-chain. In human pregnancy urine and in commercial hCG preparations hCG occurs in a variety of forms, including breakdown oligopeptide products. During pregnancy, the urine contains increasing proportions of nicked hCG and hCG β-core fragments (5). Nicked hCG has

peptide bond cleavages in loop 2 of the β-chain between residues 44 and 52, whereas hCG β-core completely lacks the β-chain loop 2, which consists of amino acid residues 41-54.

We have previously shown that the 400 – 2000 Dalton peptide fraction of pregnancy urine, but not of normal female or male urine, is able to inhibit the development of diabetes in NOD mice, whereas fractions greater than 2000 Dalton, including hCG, did not have this activity (6). We then reported that the synthetic hexapeptide VLPALP, which is part of the primary structure of the hCG β-chain loop 2, reduced mortality in a murine model of lipopolysacharide (LPS) induced systemic inflammatory response syndrome (7,8).

Based on these findings, and known preferential cleavage sites of the hCG β-chain loop 2 (5,9-12), we selected six different synthetic oligopeptides (MTR, MTRV, LQG, LQGV, VLPALP and VLPALPQ), which are part of the primary structure of loop 2 of the hCG β-chain, as well as four alanine variants of LQG and LQGV (AQG, LAG, AQGV and LAQV) (Figure 1), and tested these in a murine model for their capacity to reduce renal I/R injury.

MATERIALS AND METHODS

Experimental design

The experimental protocol was approved by the Animal Experiments Committee under the Dutch Experiments on Animals Act and adhered to the rules laid down in this national law that serves the implementation of “Guidelines on the protection of experimental animals” by the Council of Europe (1986), Directive 86/609/EC.

Ten different hCG-related oligopeptides (MTR, MTRV, LQG, LQGV, VLPALP, VLPALPQ, AQG, AQGV, LAG and LAGV) were evaluated for their capacity to reduce ischemia-reperfusion induced renal injury as compared to mice treated with phosphate-buffered saline (PBS). Five mg/kg body weight of oligopeptide or PBS in a volume of 0.1 mL was administered intravenously (i.v.) 1 minute before clamping the kidney, and 1 minute before releasing the clamp.

Subsequently a dose-escalating study was performed with AQGV. The AQGV was given in doses of 0.3, 1, 3, 10, and 30 mg/kg in a volume of 0.1 mL and was administered i.v. 1 minute before clamping, and 1 minute before releasing the clamp. Possible toxic side effects were studied by careful observation of control and peptide treated mice for signs of discomfort.

Contra-lateral kidney samples were obtained for further analysis. At 24, and 72 hr post-reperfusion, mice were sacrificed and clamped kidneys were harvested and snap frozen for further analysis. Serum urea levels were measured to determine kidney

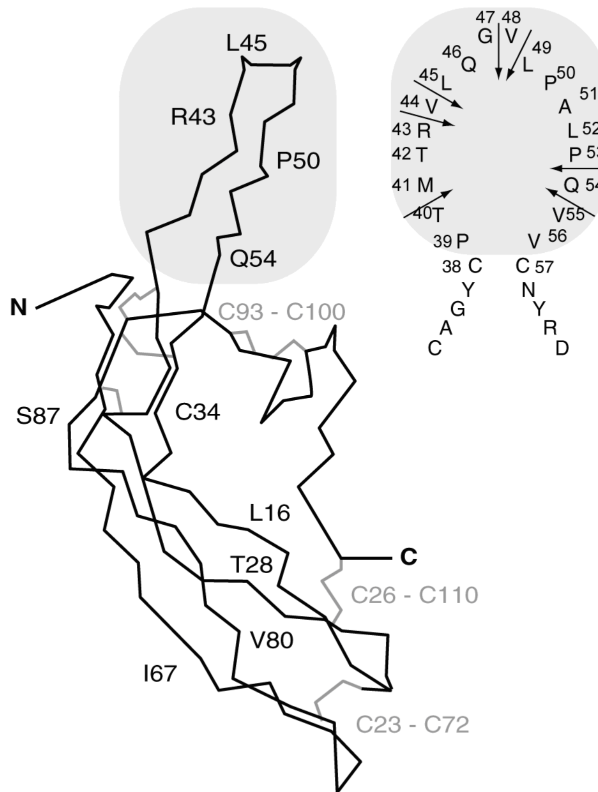

Figure 1. Structure of β-hCG with loop 2 and the amino acid sequence of loop 2 indicated. Adapted from Lapthorn *et al*. (35). Arrows indicate the preferential cleavage sites in loop 2.

function. Infiltrating cells were analysed using immunohistochemistry. In all groups survival was assessed and analyzed by Kaplan-Meier analysis.

In an additional experiment AQGV was given in a dose of 5 mg/kg BW in a volume of 0.1 mL and administered i.v. 1 minute before clamping, and 1 minute before releasing the clamp. At 6 and 24 hr post-reperfusion, mice were sacrificed and blood was obtained for cytokine measurements in serum. From the 6hr post-reperfusion group the clamped kidney was harvested for determination of mRNA expression levels.

Furthermore, survival experiments were performed in which mice received PBS or AQGV (5mg/kg BW) at 12 and 24 hours, or at 6 and 12 hours post-reperfusion.

Mice

Male C57BL/6JOlaHsd mice of 12-16 weeks of age were obtained from Harlan (Horst, The Netherlands). Mice were kept under standard laboratory conditions (temperature 20-24ºC, relative humidity 50-60%, 12 h light/12 h dark) and were allowed free access to food (Hope Farms, Woerden, The Netherlands) and water.

Ischemia model

Mice were anaesthetized by isoflurane inhalation. Anaesthesia was maintained using a mixture of N_2O/O_2/isoflurane. Blood was collected by retro-orbital

puncture. Body temperature was maintained by placing the mice on heating pads. Following a midline abdominal incision, the left renal pedicle was localized and clamped for 25 minutes using an atraumatic micro-vascular clamp. After inspection for signs of ischemia, the wound was covered with PBS soaked cotton and the animal was covered with a tin foil insulation sheet. After release of the clamp, restoration of blood-flow was inspected visually and a contra-lateral nephrectomy was performed. The abdominal wound was closed in two layers, and mice were given 0.5 ml PBS subcutaneously.

Oligopeptides

Selection was based on either the known preferential cleavage sites or known *in vivo* nick sites of the sequence MTRVLQGVLPALPQ (aa_{41-54}) of loop 2 of the β-subunit of hCG (14, 16-19). Selected oligopeptides were MTR (aa_{41-43}), MTRV (aa_{41-44}), LQG (aa_{45-47}), AQG and LAG (alanine replaced oligopeptides of LQG), LQGV (aa_{45-48}), AQGV and LAGV (alanine replaced oligopeptide of LQGV), VLPALP (aa_{48-53}), VLPALPQ (aa_{48-54}). Oligopeptides were synthesized (Ansynth BV, Roosendaal, The Netherlands) using the fluorenylmethoxycarbonyl (Fmoc)/tert-butyl-based methodology with a 2-chlorotritylchloride resin as the solid support. Oligopeptides were dissolved in PBS at a concentration of 1 mg/ml and stored at -20°C in small aliquods.

Functional measurements

Serum urea and creatinine values were measured using a kinetic urease method where the decrease in NADH adsorbance is measured photometrically, using an ELAN multianalyser (Eppendorf-Merck, Germany).

Immunohistochemistry

Primary antibodies used were rat-anti-mouse CD4, CD8, CD45, neutrophils, macrophages, CD54 (Serotec, Oxford, UK). Antibodies were diluted in PBS/5% BSA solution. Primary antibody was applied on for 30 min at RT and slides were subsequently incubated with a mixture of goat-anti-rat IgM+IgG (H+L) alkaline-phosphate conjugated

antibody (Southern Biotech, Birmingham, USA) for 30 min at RT. Enzyme detection was performed using Naphthol AS-MX, New Fuchsine, sodium-nitrite and levamisol mixture in Tris-HCl pH8 as a substrate for 30 min at RT in the dark.

Formalin-fixed-paraffin sections (3 μm) were used for Ki-67 staining. Slides were deparaffinized and rehydrated and boiled for antigen retrieval in a 0.01M sodium citrate solution for 30 min in a microwave-oven. Endogenous peroxidase was blocked with a 0.03% H_2O_2 solution. The sections were incubated overnight at 4°C with rat-anti-mouse Ki-67 primary antibody (Dako Cytomation, Glostrup, Denmark) and subsequently incubated for 30 min at RT with rabbit-anti-rat IgG conjugated with HRP secondary antibody (Dako Cytomation, Glostrup, Denmark). Enzyme detection was performed using DAB as a substrate. Slides were rinsed in tap water, counterstained with haematoxylin and rinsed with tap water again. As a negative control the primary antibody was omitted. Positive cells were counted in 10 high power fields (400X) using a semi quantitative scoring system as follows: 0: no positive cells, 1: 1-10 cells, 2: 11-30 cells, 3: 30-60 cells, 4 > 60 cells.

Measurement of apoptotic cells

Formalin-fixed-paraffin sections were stained for apoptotic cells by TUNEL staining using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore, USA) according to the manufacturers instructions. Positive cells were counted in 10 fields at a magnification of 400x .

Cytokine measurements

TNF-α, IFN-γ, IL-6, IL-10, IL-12, and MCP-1 were measured using a commercially available cytometic bead array (CBA) (BD Biosciences, San Jose, CA, USA) and a BD FACSArrayTM Bioanalyzer (BD Biosciences). Analysis of the data was performed using FCAP ArrayTM software (BD Biosciences). Assay sensitivity was 2.5 pg/ml.

Real-time quantitative (RQ)-PCR analysis

Sections of kidney were homogenized and RNA was isolated using the Qiagen RNeasy kit (Qiagen, Hilden, Germany). In total 1μg of RNA was reverse transcribed and RQ-PCR using an AppliedBiosystems 7700 PCR machine (Foster City, CA, USA) was performed as described previously (13). In all 6h samples the mRNA transcript levels of TNF-α, IFN-γ, IL-6, IL-10, IL-12, MCP-1, and the adhesion molecules E-selectin and ICAM-1 were determined. Transcript levels of these genes were quantified by normalization against ABL.

Statistical analysis

Survival data were compared by log-rank analysis. Other data were analysed using

ANOVA, followed by a Mann-Whitney – U test. Calculations were performed using SPSS v11.0 for Windows. A p value ≤0.05 was considered statistically significant. Data are presented as mean values ± standard error of the mean.

RESULTS

Effect of hCG-related oligopeptide treatment on survival

25 minutes of warm renal ischemia and contra-lateral nephrectomy resulted in a survival of 50% in the control group at 3 days post-reperfusion (Table 1). The groups treated with oligopeptides MTR, LQGV, VLPALP, AQG, LAG and LAGV (5 mg/kg), had survival rates not significantly different from controls. Treatment with LQG led to a significant better survival (90%), while treatment with oligopeptides MTRV, VLPALPQ or AQGV totally prevented mortality.

Table 1. Effect of various hCG-related oligopeptides (5 mg/kg) on the survival of mice subjected to ischemia-reperfusion damage.

	Survival		
Treatment	**24h**	**72h**	**p**
PBS	90%	50%	
MTR	100%	60%	ns
MTRV	100%	100%	< 0.05
LQG	100%	90%	< 0.05
LQGV	100%	80%	ns
VLPALP	100%	70%	ns
VLPALPQ	100%	100%	< 0.01
AQG	100%	70%	ns
AQGV	100%	100%	< 0.01
LAG	100%	70%	ns
LAGV	90%	90%	ns

Effect of hCG-related oligopeptide treatment on kidney function

Treatment of mice with oligopeptide MTRV, AQGV or LAGV provided significant ($p<0.05$) functional protection against renal I/R injury at both 24 and 72 h, as measured by serum urea levels (Figure 2). Although treatment with LQG resulted in significantly decreased serum urea at 24 hours post-reperfusion ($p<0.05$), at 72 hours no significant beneficial effect was found. While treatment with VLPALPQ did not cause a significant

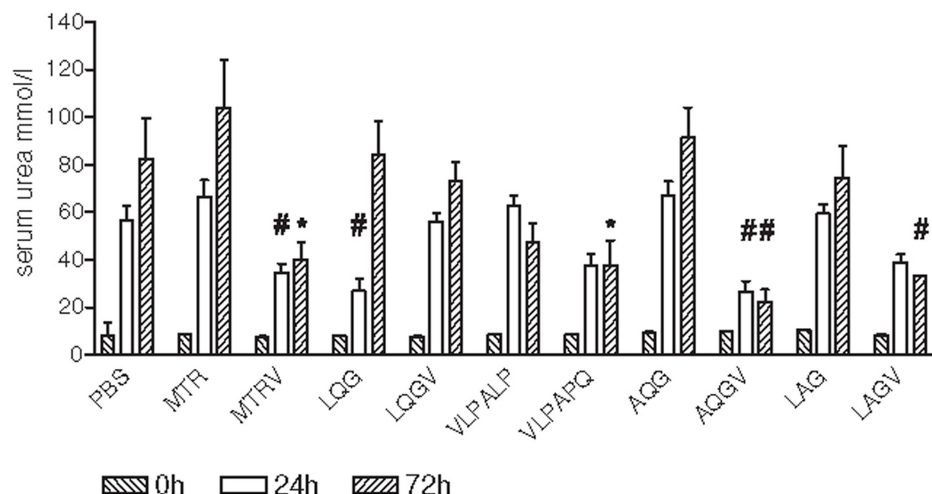

Figure 2. Renal function as reflected by serum urea levels.
Pre-operative values and 24 and 72 hours post reperfusion values in the different oligopeptide treated groups were compared to PBS-treated controls. Treatment with MTRV and AQGV significantly reduced renal function loss both at 24 hours and 72 hours after ischemia reperfusion injury. Treatment with LQG reduced serum urea levels at 24 hours. Treatment with VLPALPQ and LAGV showed significantly reduced serum urea levels at 72 hours. *$p<0.05$ and #$p<0.01$ (n=10 animals / group).

decrease in serum urea at 24 hours, at 72 hours it was significantly decreased as compared to the control group ($p<0.05$). Treatment with AQGV provided the most powerful protection against renal ischemia-reperfusion injury at both 24 hours ($p<0.01$) and 72 hours ($p<0.01$).

Effect of different doses of AQGV (0.3 – 30 mg/kg) on survival

Because AQGV showed the most powerful protection against warm renal I/R injury, we determined the optimal dose of this oligopeptide in a dose escalating study. Therefore, AQGV was administered in doses ranging from 0.3 to 30 mg/kg, and compared to mice treated with PBS. A survival rate of 60% was seen in the control group (Table 2). Although treatment with 0.3, 1 and 30 appeared to result in a survival benefit, no significant difference could be measured (80%, 90%, and 80%, respectively). The doses of 3 and 10 mg/kg totally prevented mortality ($p<0.05$).

Table 2. Effect of different doses of AQGV on the survival of mice subjected to ischemia-reperfusion damage.

Treatment (mg/kg)	Survival		
	24h	72h	p
PBS	100%	60%	
0.3	100%	80%	ns
1.0	100%	90%	ns
3.0	100%	100%	< 0.05
10	100%	100%	< 0.05
30	100%	80%	ns

Effect of different doses of AQGV (0.3 – 30 mg/kg) on kidney function

Treatment of mice subjected to renal I/R damage with 1, 3, 10 and 30 mg/kg AQGV resulted in significant reduction of serum urea levels at 72 hours ($p<0.05$). A dose of 3 mg/kg resulted in best preservation of kidney function, with return to normal function already observed at 72 hrs ($p<0.01$). With 0.3 mg/kg no significant benefit was observed (Figure 3). Creatinine values confirmed these data but in our model did not show the same level of responsiveness to the injury as urea.

Effects of AQGV on cellular infiltration, apoptosis and proliferation

To study the mechanism underlying the protective effect of AQGV, we investigated the cellular infiltrate and proliferation in the kidneys of mice treated with 5 mg/kg AQGV. At both 24 and 72 hr post reperfusion the neutrophil influx was significantly decreased in the AQGV-treated group (p=0.03 and p=0.022, respectively) (Figure 4A). Additional staining for CD4+, CD8+ cells and macrophages revealed no differences between the two groups (data not shown). TUNEL staining identified apototic cells which were localized mainly in the tubular epithelium (Figure 4B, middle and lower panels). The number of TUNEL-positive cells was significantly lower in AQGV treated animals 24 hours after reperfusion (Figure 4B). Ki-67 staining showed a significantly higher proliferative activity of renal tubular epithelial cells in AQGV-treated mice at 24 hours (Figure 4C). At 72 hours this difference had disappeared.

Effects of AQGV on serum cytokine levels and renal mRNA transcript levels

Using the bead-array we determined serum cytokine levels at 6 and 24 hr post-reperfusion. MCP-1 was below the detection limit in all samples. No differences in serum TNF-α, IFN-γ, IL-6, IL-10, and IL-12 levels were observed at 6hrs post-reperfusion. At 24 hrs post-reperfusion the levels for all cytokines were decreased upon AQGV treatment,

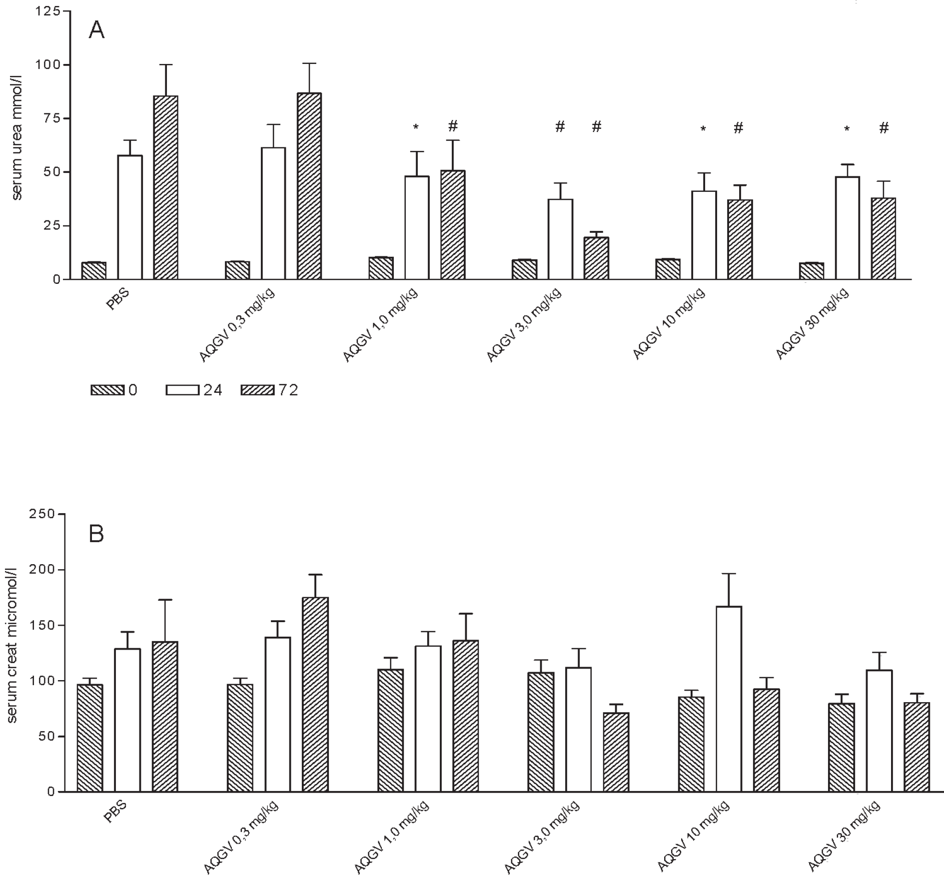

Figure 3. Renal function as reflected by serum urea (A) and creatinine (B).
Values are shown pre-operative, and after 24 and 72 hours in groups treated with AQGV in a dose escalation study (0.3 – 30 mg/kg), and compared to a PBS-treated control group. Treatment with AQGV in a dose from 1 mg/kg up to 30 mg/kg significantly reduced renal function loss after renal ischemia-reperfusion injury. The dose of 3 mg/kg was the most potent. *p<0.05 and #p<0.01 (n=10 animals / group).

with IL-6, IL-10, IFN-γ (p< 0.05), and TNF-α (p< 0.01) being significantly lower (Figure 5A).

AQGV treatment showed no effect on inflammatory cytokine mRNA levels 6 hrs post-reperfusion (data not shown). AQGV treatment did result in a significant (p< 0.05) down regulation of renal E-selectin, but not ICAM-1 mRNA expression at 6 hr post-reperfusion as compared to PBS treated mice (Figure 5B).

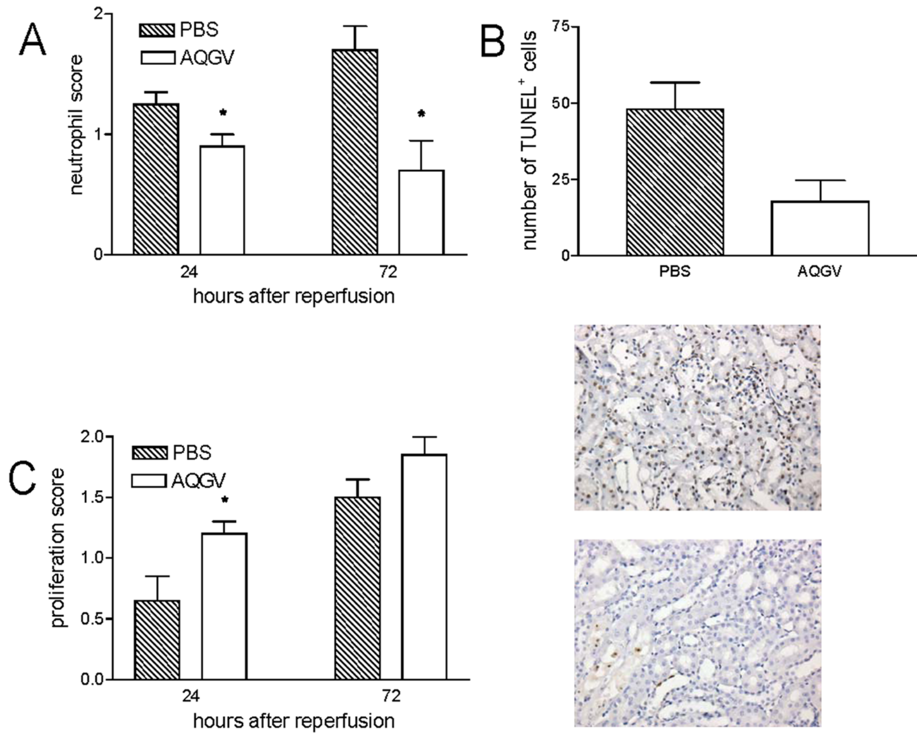

Figure 4.
A. Renal neutrophil influx as assessed by immunohistochemical staining. AQGV treatment reduced neutrophil infiltration after 25 minutes of renal warm ischemia as assessed at 24 and 72 hours post-reperfusion. Data are expressed in a semi-quantitative way as described in the Material and Methods section. *$p<0.05$ (n=10 animals / group). **B**. AQGV treatment significantly reduced the number of apoptotic cells in the kidney 24 hours after renal ischemia reperfusion injury. *p=0.01 vs PBS treated controls at 24 hours (n=6 - 10 animals / group, upper panel). Middle and lower panels: representative photomicrographs of TUNEL stained control-, and AQGV treated kidneys respectively, 24 hours after reperfusion (200x). **C**. Proliferation as assessed by Ki-67 immunohistochemistry. AQGV treatment significantly enhanced cellular proliferation at 24 hours after renal ischemia-reperfusion injury. Although a higher trend of proliferation was seen at 72 hours as well, no statistically significant difference was found. Data are expressed in a semi-quantitative way as described in the Material and Methods section. *$p<0.05$ (n=10 animals / group).

Effect of post-reperfusion AQGV treatment on survival

AQGV treatment given either at 12 and 24 hr post-reperfusion or at 6 and 12 hr post-reperfusion did not improve survival (~50%) as compared to the control group (data not shown).

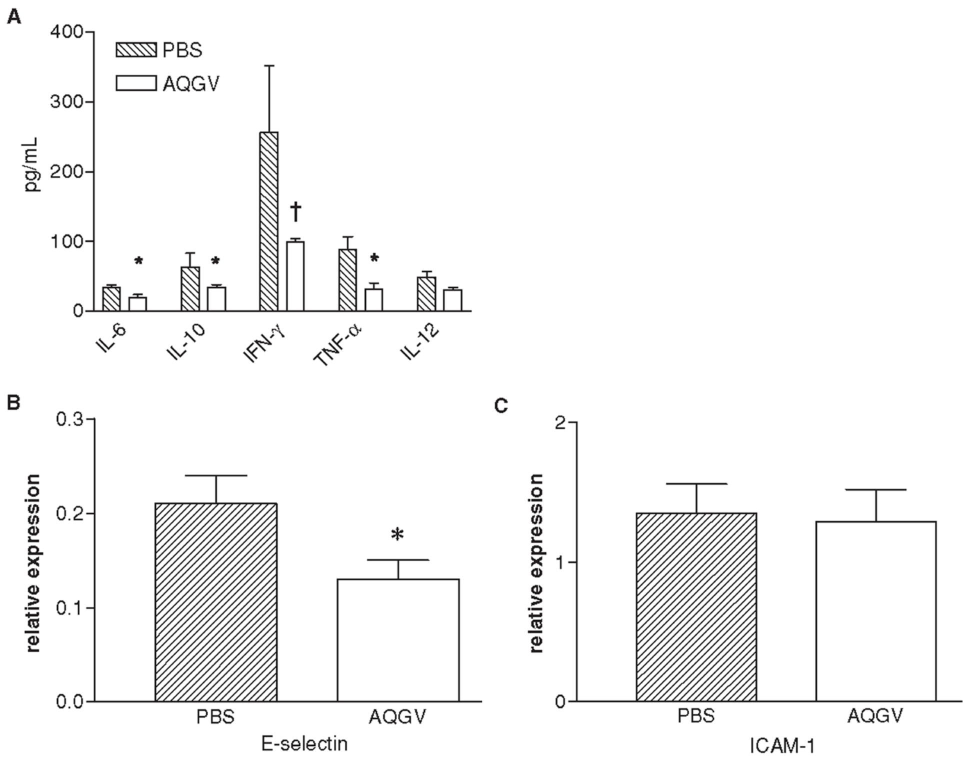

Figure 5.
A. Treatment with AQGV reduces serum cytokine levels at 24 hours after renal ischemia reperfusion injury. *p<0.05, †p<0.01 (n=5 per group). **B**. Treatment with AQGV reduces renal E-selectin (left panel) but not ICAM-1 (right panel) mRNA levels at 6 hours after renal ischemia reperfusion injury. Data are presented as mean value ± sem. *p<0.05 (n=6 animals / group).

DISCUSSION

We investigated whether treatment with synthetic oligopeptides, consisting of 3 to 7 amino acids, based on the primary structure of hCG, was able to reduce warm ischemia-reperfusion injury of the kidney. We demonstrate for the first time that oligopeptides as small as three or four amino acids can significantly reduce mortality seen after severe renal I/R injury and improves kidney function as measured by serum urea levels. Especially AQGV showed superior results in enhancing survival and preservation of kidney function after 25 minutes of renal ischemia. A dose of 3-10 mg/kg proved to be

the most potent with regard to reducing mortality as well as preserving kidney function. Furthermore, up to 30 mg/kg, no toxicity was observed. Also in rats, dogs and a human phase I study no harmful side effects of single and repeated AQGV administration were found. Data of these studies will be published elsewhere (manuscript in preparation).

Both natural hCG and commercial hCG preparations have been investigated for their role on the immune system, because of their putative immunomodulating role during pregnancy in protecting the fetus from rejection (14).

Our previous work (6) shows that short-term treatment of female NOD mice, with a hCG preparation purified from first trimester pregnancy urine, starting prior to the onset of hyperglycemic symptoms, inhibits the development of type I diabetes. Interestingly, however, the anti-diabetic activity of the used hCG preparation did not reside in the heterodimeric hCG molecule, or its subunits, but in a 400-2000 Dalton fraction.

Subsequently, we showed in a model of LPS-induced systemic inflammatory response syndrome in mice that treatment with this low weight molecular fraction was capable of inhibiting the septic shock morbidity as well as mortality (7). The same beneficial effect was obtained with the synthetic oligopeptide VLPALP, which sequence is part of loop 2 of the β-chain of hCG (7). Recently, we showed that hCG-related oligopeptides reduce inflammation and liver injury in a rat model of hemorhagic shock and resuscitation (15).

During pregnancy, hCG occurs in a variety of forms and breakdown products in serum and urine, including intact hCG, α- and β-subunits, nicked hCG, hCG β-core fragment, and smaller peptide fragments. Both nicked hCG and the β-core subunit consist of a β-chain with a defective loop 2. This loop, consisting of the amino acid residues 41-54, is absent in β-core subunit, and is cleaved in nicked hCG (9-12). Since the immunomodulatory activity of hCG resided in the low molecular weight fraction, we hypothesised that *in vivo* liberated breakdown products, such as those originating from the proteolytic cleavage of peptide bonds between amino acid residues 41-54, may have significant biological activity (8). Based on known preferential cleavage sites(5,9,10,12), we tested synthetic oligopeptides MTR, MTRV, LQG, LQGV, VLPALP, VLPALPQ and, based on alanine replacement mapping, the LQG and LQGV analogs AQG, LAG, AQGV and LAGV (Figure 1). Of these oligopeptides, MTRV, LQG, VLPALPQ and AQGV appeared able to reduce mortality and decline in kidney function induced by warm renal ischemia-reperfusion injury, AQGV being the most effective (Table 1 and Figure 1).

Cell migration plays an important role during the initial phase of renal I/R injury. Up-regulation of adhesion molecules on endothelial cells, induced by locally produced pro-inflammatory mediators, is amongst the first changes observed after renal I/R injury and is central to the pathogenesis of ischemic acute kidney injury (1,16). Subsequently leukocytes become activated by local pro-inflammatory factors, thereby

facilitating adherence to endothelial cells and subsequent renal tissue infiltration (1). Sequestered neutrophils induce parenchymal damage, followed by cytokine production by resident renal cells and infiltrating cells, which promotes further tissue damage (1,17). It has been demonstrated that renal mRNA expression of the early adhesion molecule E-selectin peaks within 6 hours post-reperfusion, with neutrophils infiltrating in parallel. E-selectin blockage with the selectin specific ligand sPSGL has been shown to inhibit renal neutrophil infiltration after I/R and to preserve kidney function (18). In mice treated with AQGV we observed decreased E-selectin mRNA levels 6 hrs post-reperfusion and decreased renal neutrophil infiltration at 24 hours post-reperfusion. Apoptotic cell death, an important determinant of cellular damage in ischemic kidneys (19) was also significantly reduced in AQGV treated mice. Additionally, serum levels of the inflammatory cytokines TNF-α, INF-γ, IL-6, and IL-10 were significantly decreased 24 hours post-reperfusion upon AQGV treatment. This data is indicative of decreased renal injury and fits with the preservation of kidney function we observed upon AQGV treatment. The lower levels of systemic cytokines observed upon AQGV treatment may be a reflection of reduced formation as well as better renal clearance of these cytokines (20). The lower serum cytokine levels likely contribute to the decreased mortality by preventing systemic inflammation and subsequent complications in these animals (21).

Our data indicate that AQGV treatment protects against renal I/R injury by interfering with early E-selectin upregulation, thereby reducing neutrophil influx, parenchymal damage and possibly cytokine production. So far it is unclear what the molecular mechanism of action is by which AQGV exerts its effects. It is possible that AQGV mediates its effect by an as yet unidentified receptor. However, we cannot exclude the possibility that, due to the small size and molecular weight, AQGV penetrates the cell membrane (22) and exerts its action either by interfering with signaling cascades or the transcriptional machinery. E-selectin is expressed *de novo* on endothelial cells after transcriptional induction by pro-inflammatory agents (23). Whether AQGV inhibits the local production of pro-inflammatory mediators that induce E-selectin or directly interferes with the intracellular signalling cascade involved in activating E-selectin transcription is not clear so far. In contrast to E-selectin, ICAM-1 expression was not altered by peptide treatment. The transcription factor HMGA1 is required for optimal activation of E-selectin gene transcription while it has no role in activating ICAM-1 transcription (24,25) Therefore it is possible that AQGV interferes specifically with pathways required for E-selectin transcriptional activation.

Although previous work revealed a pathophysiologic role of the T-cell as mediator of ischemic ARF (26,27), we did not find a significant difference between the AQGV and placebo treated mice in numbers of CD4+, CD8+ T-cells or macrophages. Our data fit with the observation that RAG-1 deficient mice (lacking both T- and B-cells) are not

protected from renal I/R injury (28).

Ki-67, a marker for cellular proliferation, is part of a nuclear protein complex expressed in the G1, S, G2 and M phases of the cell cycle in proliferating cells (29,30). Mice treated with AQGV showed significantly increased numbers of Ki-67 positive renal tubular epithelial cells at 24 hr post-reperfusion, reflecting enhancement of the regenerative process (31). The increase in proliferation is likely facilitated by a reduction in inflammation-induced tissue injury, since high levels of pro-inflammatory cytokines have been shown to suppress regeneration of ischemically damaged kidneys (32).

AQGV treatment, at a dose of 5 mg/kg BW, given at either 12 and 24 hr post-reperfusion or at 6 and 12 hr post-reperfusion was not associated with improved survival. Although we cannot formally exclude that these post-reperfusion treatment regimens improved kidney function, it appears that in the currently used model AQGV only prevents the onset of renal ischemia reperfusion injury. This may indicate that AQGV inhibits the activation of pathophysiological pathways involved in renal ischemia reperfusion injury, but is unable to reverse these pathways once activated. However, since we do not exclude that higher doses of AQGV given post-reperfusion do reverse renal-ischemia reperfusion injury, detailed dose-response studies are warranted to gain full insight into the renoprotective effect of AQGV, and other hCG-related oligopeptides.

In conclusion, this study shows that treatment of mice with 5 mg/kg of either one of the hCG-related oligopeptides MTRV, LQG, VLPALPQ or AQGV shortly before and immediately after renal pedicle clamping can significantly reduce mortality and ameliorate kidney injury in a model of warm ischemia reperfusion injury. Of the various oligopeptides evaluated, AQGV appeared to be the most potent one. The renoprotective effect of AQGV was associated with decreased renal E-selectin transcripts, decreased renal neutrophil infiltration, reduced numbers of apoptotic tubular epithelial cells and a reduction of systemic levels of TNF-α, IFN-γ, IL-6 and IL-10. This data implies that AQGV interferes with the early renal inflammatory response induced by I/R and as such prevents parenchymal damage and organ dysfunction. These new renoprotective oligopeptides show great promise for preventing the development of renal ischemia-reperfusion injury and may well be used in clinical situations where renal I/R is forseeable, such as semi-elective surgeries including kidney transplantation, cardiac surgery, and abdominal aorta surgery. So far, phase IA and phase 1B studies with AQGV (EA-230) have been successfully completed and phase II studies are underway (33,34).

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INHIBITION OF TUMOR GROWTH BY THE β-hCG RELATED SYNTHETIC OLIGOPEPTIDE LQGV

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ABSTRACT

Human chorionic gonadotropin (hCG) preparations have been recognized to inhibit the growth of Kaposi's sarcoma *in vitro* and *in vivo*, an activity demonstrated to reside in an as yet unidentified hCG-associated factor (HAF) (1-3).

We found previously that synthetic oligopeptides related to the loop-2 sequence MTRVLQGVLPALPQ of the hCG-β chain exerted biological activity in several different experimental models (4-7). Here we show that one of these oligopeptides, LQGV, inhibits the growth of Lewis Lung Carcimoma (LLC) in an *in vivo* transplantable LLC model in mice. Targeting of tumor vasculature by low-molecular-weight vascular-disrupting agents is considered a novel potential therapy to treat cancer (8). Our data imply that LQGV may exert its anti-tumor activity at least partly by specific disruption of the tumor vessel integrity, which is different from the anti-tumor activity as previously described for HAF (1-3). Our data suggest that the hCG-β chain related oligopeptide LQGV is a promising small molecule to treat cancer, either alone or in combination with cytotoxic drugs.

Human chorionic gonadotropin (hCG) is a dimeric glycoprotein hormone, which is especially produced during human pregnancy by the placental syncytiotrophoblasts (9). Besides endocrine functions, recombinant hCG and urinary hCG preparations exert other actions such as immunosuppression (10-13). While complete hCG may facilitate tumor progression (14) it has also been shown that β-hCG prevents the onset of breast cancer (15). Furthermore, several reports demonstrated anti-Kaposi's sarcoma activity of hCG preparations *in vivo* and *in vitro* (1,3,16). This anti-tumor activity was not due to the heterodimeric hCG nor its α- or β-subunit, but was ascribed to a yet unidentified

Table 1. Tumor size and response rates 21 days after tumor transplantation.

			Response Rate			
Treatment	**Avg volume day 21**	**Responses (CR plus PR)**	**CR**	**PR**	**NC**	**PD**
PBS	1600 mm^3	0/6	0	0	0	6
LQGV (5 mg/kgBW)	270 mm^3	3/6	2	1	2	1
AQGV (5 mg/kgBW)	1030 mm^3	2/6	0	2	1	3
LAGV (5 mg/kgBW)	1600 mm^3	2/6	1	1	1	3

PD = progressive disease (increase of tumor volume > 25% within 7 days); NC = No change (tumor volume remains equal) (-25% to +25%) to initial volume); PR = partial remission (tumor volume decreased 25-90%); CR = complete remission (tumor volume less than 10% of initial volume).

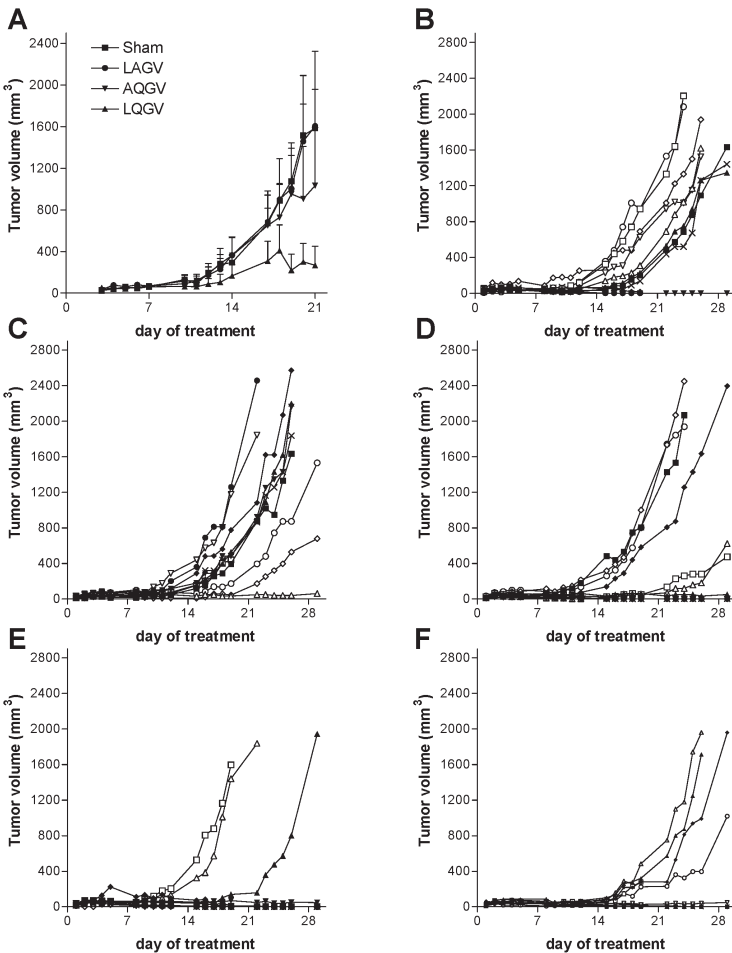

Figure 1.
Effect of β-hCG related synthetic tetrapeptides on tumor growth.
A. Anti-tumor activity screening for the tetrapeptides LQGV, AQGV and LAGV. After tumor transplantation, mice (*n*=6 per group) were i.p. injected every other weekday with either the tetrapeptide LQGV, AQGV or LAGV at a dose of 5mg/kg BW. Control animals received PBS as control. Tumor volume was measured and mean values are shown. Error bars indicate standard deviation. LQGV, which is related to the primary sequence of loop-2 of β-hCG, reduced tumor growth while its alanine replacement variants AQGV or LAGV only revealed marginal or no effect on tumor growth, respectively. **B-F.** Dose escalation study with LQGV showing all individual mice per experimental group. After tumor transplantation, mice (*n*=11 per group) were i.p. injected every other weekday with the tetrapeptide LQGV at a concentration of either 1.7 (C), 5 (D), 17 (E) or 50 (F) mg/kg BW. Control mice (B) received PBS. At day 22 post-tumor transplantation LQGV treatment at a dose of 17 mg/kg BW was associated with a significantly ($p < 0.002$) reduced tumor volume as compared to control.

hCG-associated factor of peptide nature (1,2,16).

We found that hCG preparations inhibited diabetes in NOD mice as well as LPS-induced inflammation(6,17). Comparably also these activities were not inflicted by the heterodimeric hCG nor its α- or β-subunit, but resided in a 400-2000 Dalton fraction of peptide nature, which likely originates from proteolytic degradation of the hCG β-subunit (4,6,17). Subsequently we demonstrated that synthetic oligopeptides related to the primary sequence of β-hCG loop-2 inhibited inflammation and tissue damage following shock induced by LPS or hemorrhage/resuscitation (5,6) .

Considering the suggestion that the anti Kaposi's sarcoma activity was due to an hCG-associated factor of peptide nature, and our observation that synthetic oligopeptides related to the primary structure of β-hCG exert powerful biological activity, we tested the synthetic oligopeptide LQGV, which is related to the primary structure of loop 2 of β-hCG, as well as its alanine replacement variants AQGV and LAGV, for tumor growth inhibiting activity.

First the ability was tested of the oligopeptides LQGV, AQGV, and LAGV to inhibit growth of the transplantable Lewis Lung Carcinoma (LLC) in mice. Treatment with LQGV (5 mg/kg BW) induced a delay in tumor growth with an average tumor volume of 270 mm^3 after 21 days (Figure 1A). The alanine replacement variants appeared less effective; AQGV did delay tumor growth but to a lesser extent than LQGV, while LAGV had no effect (Figure 1A). All mice receiving PBS control demonstrated progressive tumor growth, which reached on average a tumor volume of 1600 mm^3 in 21 days (Figure 1A). Although at a dose of 5 mg/kg the LQGV treated mice did not show a significant tumor growth reduction compared to the controls, the tumor response rate of mice treated with LQGV was significantly lower as compared to control mice ($p< 0.015$) with two mice showing complete tumor remission and one mouse showing partial remission (Table 1). These results indicate that administration of the tetrapeptide LQGV resulted in tumor growth delay and tumor control for at least three weeks.

Table 2. Response rates 26 days after tumor transplantation.

Treatment	PD	TR	RR
PBS	8	3	27 %
1.7 mg/kg	9	2	18 %
5 mg/kg	4	7	64 %
17 mg/kg	3	8	73 %
50 mg/kg	4	7	64 %
170 mg/kg	4	7	64 %

PD = Progressive Disease; TR = Tumor Response; RR = Response Rate

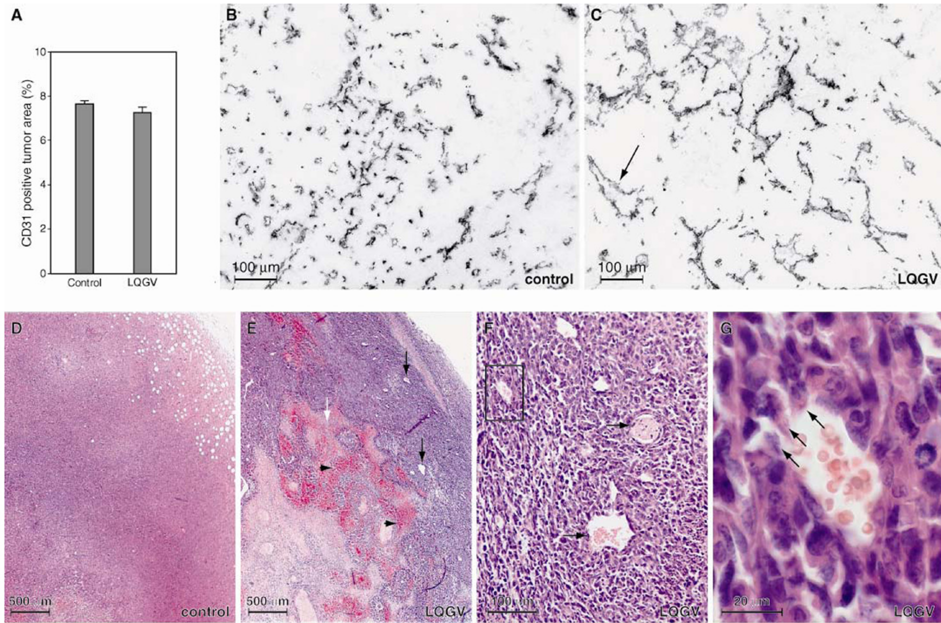

Figure 2.
Effect of the β-hCG related synthetic tetrapeptide LQGV on tumor associated vasculature.
A. Immunohistochemical staining for CD31 was performed to visualize tumor vasculature. Semi-quantitative analysis of CD31 staining revealed no differences in the level of tumor vascularization between LQGV (17 mg/kg BW) or control mice. **B.** Representative CD31 staining (magnification 16x) of tumor tissue from a control mouse. **C.** Representative CD31 staining (magnification 16x) of tumor tissue from an LQGV treated mouse. **D.** Hematoxylin eosin staining (magnification 2.5x) of viable rim of tumor tissue from a control mouse. **E.** Hematoxylin eosin staining (magnification 2.5x) of viable rim of tumor tissue from an LQGV (17mg/kg BW) treated mouse. Black arrows indicate dilated vessels, white arrow indicates necrotic area. Hemorrhages are indicated by arrowheads. **F.** Hematoxylin eosin staining (magnification 16x) of viable rim of tumor tissue from an LQGV (17mg/kg BW) treated mouse, arrows indicate dilated and destructed vessels. The boxed area is magnified (100x) in **G,** showing a dilated vessel with destroyed endothelial lining indicated by arrows.

To establish the optimum dose for LQGV to inhibit tumor growth, a dose escalation study was performed up to day 26. This revealed that the maximum tumor response was established by LQGV administration of 17 mg/kg BW (Table 2 and Figure 1B-F). At this dose significant tumor growth reduction was observed compared to the control mice at 22 days ($p< 0.002$). Above 50 mg/kg BW no further improvement of tumor response was observed (data not shown).

We examined the tumor vascularization of the three progressively growing tumors despite LQGV treatment (17 mg/kg BW) and compared them with those of the control

mice. Immunohistochemical analysis using the endothelial marker CD31 (PECAM-1) revealed no difference in the level of vascularization between tumor tissue from control mice and tumors from the LQGV treatment group (17 mg/kg BW) (Figure 2-C). Closer examination of the CD31 staining did however reveal larger and more irregularly lined vessels after LQGV treatment (Figure 2C). This was supported by hematoxylin-eosin staining of tumor tissues from LQGV treated mice, revealing vessel and endothelial abnormalities, such as dilated vessels as well as disrupted endothelial lining (Figure 2D-G). In addition, LQGV treatment was associated with necrotic areas and hemorrhages as reflected by extravasation of erythrocytes into the viable rim of the tumor (Figure 2E), which is likely the result of the observed loss of endothelial integrity.

The development of tumor associated vasculature (TAV) is essential for the growth of solid tumors, as studies using anti-angiogenic agents clearly demonstrated that inhibition of vascular growth reduces tumor development (18,19). Folkman therefore suggested inhibition of angiogenesis as a promising approach in cancer therapy [20]. In line with this, destruction of already established TAV by vascular-disrupting agents has also been shown to improve tumor outcome (8,21).

Our data suggest that LQGV interferes with TAV integrity and possibly development, which may explain its tumor growth inhibiting effect in this model. Whether LQGV exerts a direct effect on TAV or whether its action is due to secondary mediators is unclear so far. We have observed that LQGV significantly increases basel as well as VEGF-induced numbers of vessels branches in the chicken chorioallantoic membrane (CAM) assay. Therefore, it is possible that interference of LQGV with TAV integrity in combination with an increase in vascularity contribute to the anti-tumor effect. Indeed, recently Noguera-Troise *et al*. have shown that increase of Notch activity results in decreased tumor vascular density, whereas blockade of this activity results in markedly increased vessel density (22). Paradoxically, this increased vascularity seemed to be non-productive and resulted in decreased tumor growth, even for tumors that are resistant to anti-VEGF therapy (21). Importantly, no toxic side effects and vessel abnormalities in other organs were observed upon LQGV treatment. In addition, the LQGV treatment was without effect on liver and kidney parameters, leukocyte and platelet count and had no effect on animal weight and behavior (data not shown).

Upon LQGV treatment (17 mg/kg BW) outgrowth of only 3 out of 11 transplanted tumors occurred, suggesting that LQGV especially interferes with early tumor development. Inflammation is an important component of tumorigenesis, for instance pro-inflammatory cytokines within the microenvironment of an early neoplastic process drive tumor growth by enhancement of tumor cell proliferation, survival and angiogenesis (23-25). For instance, TNF-α-/- mice have been shown to develop skin tumors less frequently upon exposure to 12-O-tetradecanoylphorbol-13-acetate (TPA) (26) while endogenous TNF-α enhances the malignant character of LLC

in mice (26). Previously, we found that LQGV reduced systemic and local levels of pro-inflammatory cytokines such as TNF-α and IL-6 and expression of adhesion molecules such as ICAM-1 and E-selectin after hemorrhagic shock resuscitation, suggestive of anti-inflammatory activity (5). Besides being an endothelial cell-specific glycoprotein that mediates rolling and arrest of leukocytes to endothelium during inflammation, E-selectin is also suggested to play a role in angiogenesis (27-29). Possibly, LQGV treatment reduced tumor associated cytokine expression and E-selectin expression on endothelial cells of the developing TAV, thereby reducing (out)growth of the transplanted LLC fragments.

Previously, an hCG-associated factor of peptide nature was identified and shown to exhibit anti-Kaposi's sarcoma activity due to induction of apoptosis and inhibition of cell growth (1,2). These authors stated (1,30) that certain β-hCG peptides (of which no sequences were revealed but which may be released *in vivo* during pregnancy) inhibited growth of Kaposi's sarcoma cell lines both *in vivo* and *in vitro*. During pregnancy, besides intact hCG, nicked hCG and hCG β-core fragments occur in serum and urine. Both nicked hCG and hCG β-core contain a β chain with a defective loop 2, or with β-core completely lacking this loop (residues 41-54; MTRVLQGVLPALPQ) (4). Here we demonstrate that the synthetic tetrapeptide LQGV, a sequence present within hCG β-loop 2, inhibits the growth of LLC in mice. Although the mechanism of action requires further elucidation it appears that the anti-tumor effect of LQGV is at least partly due to TAV disruption. TAV disruption results in increased permeability and shutdown of these vessels leading to secondary tumor-cell death (8). Although LQGV on its own had a profound effect on tumor growth, it may be that combination therapy of LQGV with cytotoxic agents may be beneficial for augmented accumulation of the latter at the tumor site.

From an immunological point of view it is attractive to interfere with pathological conditions, such as tumor growth, with small molecules of only four amino acids as these are not expected to elicit an immune response, which typically requires peptides of at least 8 amino acids in length (31).

METHODS SUMMARY

LLC tissue cubes (~2 x 2 x 2 mm) were subcutaneously transplanted in C57BL/6 mice (age 8 wks; Harlan; Horst, The Netherlands). The oligopeptides (Ansynth BV, Roosendaal, The Netherlands) LQGV, related to the primary sequence of the hCG-β loop 2, and the alanine replacement variants AQGV and LAGV were tested for their ability to interfere with LLC tumor growth. Every other weekday the mice were injected i.p. with one of the oligopeptides (5 mg/kg BW) or PBS as negative control. Every

weekday, tumor diameter (including skin) was measured in 2 dimensions and tumor volume was calculated. Tumor volume changes were classified as: progressive disease (PD: volume increase >25% within 7 days), no change (NC: volume equals (-25% to +25%) the initial volume), partial remission (PR: volume decrease 25% – 90%), and complete remission (CR: volume <10% of initial volume). Mice were removed preliminary if a tumor diameter was > 20 mm, in case of ulcerations or drastic weight loss. Mice were checked daily for treatment related side effects.

A dose escalation study was performed with LQGV. To determine treatment toxicity, blood levels of ALAT, ASAT, BUN and CREAT, white blood cell count and platelet count were measured 7 days before, and 8 and 19 days after tumor transplantation. Upon experiment termination, tumor, liver, lung, spleen and kidney were removed. Formalin fixed tissue sections were stained with hematoxylin-eosin. Frozen tumor samples were sectioned and every fifth section, up to six sections per tumor, was stained for CD31 (BD Biosciences Pharmingen™, San Diego, U.S.A.) to visualize vasculature. CD31 expression was quantified using nine digital recordings (magnification of 16x) from every section and analyzed with Image Tool (*http://ddsdx.uthscsa.edu/dig/itdesc.html*). Averages and SEM were calculated from all slides per group. Statistics, Mann-Whitney U Test, was performed using SPSS v11.0 for Windows 2000. The experimental protocol was approved by the local Animal Experiments Committee.

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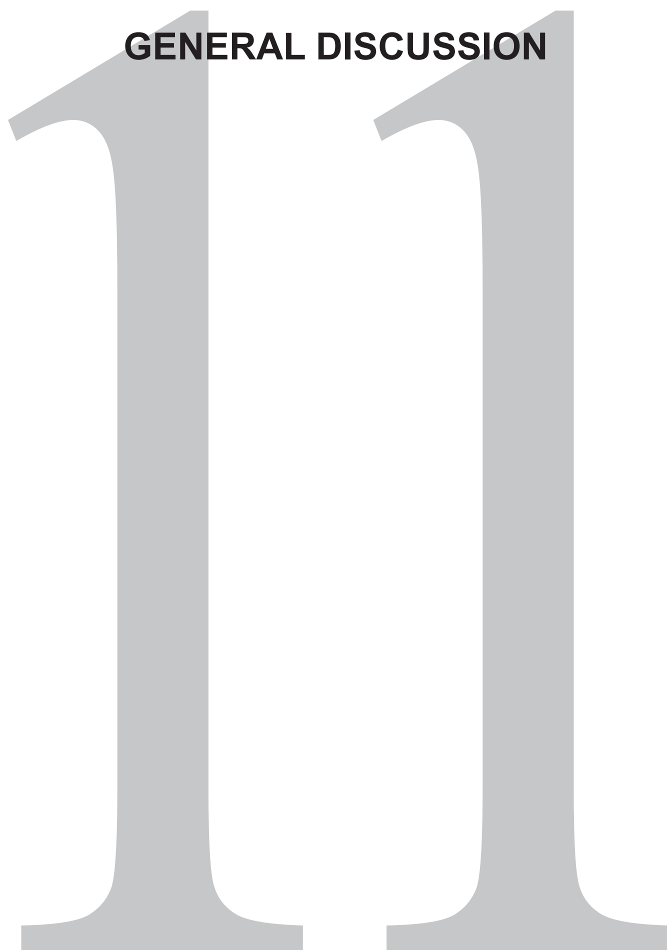

GENERAL DISCUSSION

We designed a number of oligopeptides based on the primary structure of loop-2 of β-hCG, notably MTR, MTRV, LQG, LQGV, VLPALP and VLPALPQ. In the experimental studies of this thesis we evaluated the putative regulatory influence of these oligopeptides on several physiological and pathophysiological processes. In some of these studies these oligopeptides were compared to commercial hCG or a low molecular weight (400-2000D) fraction thereof (NMPF). To this end we employed animal disease models that all share the involvement of inflammatory processes: diabetes, septic shock, haemorrhagic shock, ischemia reperfusion-related kidney failure, and a particular mouse model of transplantable lung cancer.

hCG-related oligopeptides and inflammation

In female NOD mice the individual oligopeptides inhibited the development of Th1 mediated diabetes only partially, whereas a commercial hCG-preparation and NMPF completely inhibited the development of this type of diabetes (chapter 4). In this model the treatment with the oligopeptides LQGV and VLPALP was associated with a reduction of NF-kB activity in splenic dendritic cells.

As these oligopeptides could not prevent or inhibit the disease in all experimental animals, we suggest that multiple inflammatory pathways are involved in diabetes development in this model. In contrast to this chronic Th1 mediated autoimmune disease model, both LQGV and VLPALP, as well NMPF, did inhibit LPS induced acute shock in all female BALB/c mice involved in the experiments (chapters 5 and 6). This inhibition of LPS induced shock was associated with lower MIF production by spleen cells, a less increased plasma level of liver aminotransferase, an increased percentage of NK1.1 positive T-cells, reduced LPS-induced MHC-II expression on B-cells and macrophages, and a reduced proliferative response of splenic cells *in vitro*.

The observation that NMPF, in contrast to LQGV and VLPALP, did inhibit the development of diabetes in all NOD mice tested, makes sense because *in vivo* multiple oligopeptides, with different characteristics, might be liberated from the β-hCG-loop-2 (chapter 2) and thus might dampen various pathophysiological processes involved in the β-cell destruction leading to diabetes. Therefore, in future experiments various oligopeptide mixtures should be evaluated for their therapeutic value in chronic Th1 mediated diabetes models.

In order to determine the critical amino acids required for the *in vivo* activity of LQGV, the most effective oligopeptide in most studies, various alanine replacement variants were synthesized: AQGV, LAGV, LQAV and LQGA. All these four alanine replacement variants were tested in the acute LPS-induced shock model in mice. A single treatment with AQGV oligopeptide appeared the most effective in inhibiting the mortality. AQGV oligopeptide was at least equally effective as LQGV in this model. This was found independent of whether the peptide was administered in an early or in

a late stage of LPS-induced shock development. LAGV, LQAV and LQGA were less effective in the LPS-induced shock model (data not shown).

The evaluation of the anti-shock activity of the oligopeptides applied in mice was extended to *Escherichia coli* induced hyperinflammation and septic shock in a non-human primate model, namely in fully anaesthetized rhesus monkeys (chapter 6). Control female rhesus monkeys did not survive the first 8 hours of *E.coli* intravenously (i.v.) induced septic shock, while rhesus monkeys treated with a mixture of three oligopeptides each effective in mice (LQGV, VLPALP, AQGV), survived the septic shock during 8 hours after *E.coli* administration. In this study a single injection of this mixture of oligopeptides markedly improved the pathological features and nearly completely improved the hemodynamic parameters associated with the *E. coli*-induced septic shock without any supportive therapy. However, in this model the mixture of oligopeptides did not significantly decrease the proinflammatory serum levels of TNF-α, IL-1β and IL-6. This might be due to the individual variability among the animals, the limited stability of the measured cytokines, the variability in clearance rates by the liver, and differences in the induction of innate immune responses by whole bacteria (*E.coli*) as compared to LPS.

A Phase 1A multi-dose safety trial with AQGV (EA-230) in man has been completed since then (1,2; Appendix I) and no significant adverse events were found to be associated with the use of EA-230. Also a Phase 1B trial - designed as a double-blind, randomized, single dose, placebo-controlled LPS challenge trial with EA-230 in human volunteers - has been completed (1,3; Appendix I). Pharmacokinetic analysis of the study subjects showed not just a reduction of blood C-reactive protein (CRP) levels, but also reduction of several pro-inflammatory cytokines (IL-6, IL-8 and TNF-α) as compared to the placebo group. The treatment also significantly reduced the rise of body temperature and clinical complaints, and the drop in white blood cell counts that are typically induced by LPS. This clearly shows that EA-230 reduces the systemic inflammatory response elicited by endotoxin in humans. According to the website of Exponential Biotherapies Inc., currently a Phase 2 study is in progress to test the safety and efficacy of EA-230 in reducing kidney damage in patients undergoing on-pomp cardiovascular surgery (4).

Human sepsis is an intrinsically complex disease. Populations of patients are heterogeneous with respect to the inciting cause of their disease, the co-morbid conditions that define its course, and the acute severity of their initial presentation (5-7). Septic patients undergo various stages of disease and different subgroups have different characteristics (5-7). Moreover, in each stage of the disease there is a potential imbalance between inflammation versus anti-inflammation, coagulation versus anti-coagulation, oxidant versus anti-oxidant levels and apoptotic versus anti-apoptotic processes (5-7). Therefore, although it is recognized that

inflammatory cytokines play a major role in the pathogenesis of sepsis, anti-inflammatory therapy alone generally does not lead to therapeutic success (7).

In some cases, where harmful effects of leukocytes outweigh their efficiency in killing microorganisms, inhibiting strategies may be beneficial. This seems to be the case in e.g. bacterial meningitis where rapid sequestration of neutrophils in the brain can cause irreversible tissue injury and death (8). However, inhibiting leukocyte function does not seem to be beneficial in cases where control of local or systemic infection is crucial for reducing mortality (9). Increasing neutrophil numbers and function during sepsis, especially in severely immunocompromised patients, is associated with an overall favorable outcome (9). The results of our *Listeria monocytogenes* study have shown that inhibition of early immune activation by LQGV, AQGV and VLPALP leads to bacterial overgrowth with subsequent enhanced systemic inflammation (chapter 7). The present possibilities and insights into oligopeptide treatment of septic shock are far from sufficient yet. Therefore, further studies are needed.

We also tested the hCG-related oligopeptides in a severe hemorrhagic shock (HS) model in male rats (chapter 8). HS followed by resuscitation induces a massive inflammatory response, which culminates in a systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome and, finally, death. A single treatment with oligopeptide LQGV significantly reduced the inflammatory parameters associated with HS, including the systemic release of TNF-α and IL-6. The other oligopeptides tested, AQGV and LAGV, were equally effective in reducing most of the parameters investigated. Furthermore, LQGV significantly reduced the TNF-α, IL-6, ICAM-1 and E-selectin mRNA transcript levels in the liver. LQGV treatment also prevented neutrophil infiltration into the liver and was associated with reduced liver damage. Thus, the reduction of the systemic inflammatory parameters in this model was associated with a reduced inflammatory response in the liver.

Together these studies show that several β-hCG-loop-2 related oligopeptides, including the alanine-replacement variant AQGV, can inhibit acute systemic inflammatory responses. To determine whether β-hCG-loop-2 related oligopeptides have the capacity to also influence the local acute inflammatory response in an organ, we tested several β-hCG-loop-2 related oligopeptides in an ischemia-reperfusion induced acute renal failure model in male mice (chapter 9). MTRV, LQG, VLPALPQ and AQGV significantly reduced the mortality in this model as compared to placebo treated mice. AQGV even completely prevented mortality and largely preserved the kidney function. AQGV also significantly lowered the influx of neutrophils, decreased neutrophil apoptosis in this model and increased tubular epithelial cell proliferation. In addition, AQGV decreased the serum levels of TNF-α, INF-γ, IL-6 and IL-10, and decreased the E-selectin mRNA levels in kidneys.

Together these studies show that the designed oligopeptides can inhibit the following inflammation related parameters: proinflammatory cytokines (TNF-α, IL-6, MIF), MHC-II expression on B-cells and macrophages, E-selectin expression, neutrophil infiltration, proliferation and organ damage. The oligopeptides tested can also stimulate regeneration in acute systemic as well as in local inflammation models. In these models the nature of the inflammatory responses were either different or were partially overlapping. Individual hCG-related oligopeptides might each inhibit a particular spectrum of inflammatory responses. The value of the inhibition of that particular spectrum for the effective treatment of inflammatory pathophysiology may vary between different models. By extrapolation it can be expected that similar differences may be found if such peptides would be applied in patients suffering from different inflammatory disorders.

The common part of the immune system that is affected by at least some of the anti-inflammatory oligopeptides is the innate immune system. This appears from our study on the effect of LQGV, VLPALP and AQGV on *Listeria monocytogenes* infection in mice (chapter 7). These oligopeptides were found to inhibit the very early innate immune activation required for the clearance of bacteria. This explains why treatment with these oligopeptides promoted the overgrowth of bacteria in this model. This is consistent with the observations that especially pregnant women, newborn babies and patients with a compromised innate immune system are susceptible to *Listeria monocytogenes* infection (10-12).

hCG has been extensively studied for its function in regulating placental angiogenesis and endothelial integrity (13,14). It has critical functions in trophoblast differentiation and in fetal nutrition through myometrial spiral artery angiogenesis (14). Our initial experiments with LQGV in the chicken chorioallantoic membrane (CAM) assay showed significantly increased basel vessel branching as well as VEGF-induced vessel branching (chapter 3). In addition, we have shown that LQGV inhibits the growth of Lewis Lung Carcinoma (LLC) in an *in vivo* transplantable LLC model in mice. In this model LQGV exerted its antitumor activity at least partly by stimulating the tumor vascularity in combination with disrupting the tumor associated vascular (TAV) integrity (chapter 10).

TAV integrity has emerged as a critical step promoting local tumor progression and metastatic spreading (15,16). Solid tumors show typical signs of inflammation and are infiltrated by many leukocyte populations, i.e. neutrophils, eosinophils, basophils, monocytes/macrophages, dentritic cells, natural killer (NK) cells and lymphocytes (17-19). This suggests an important role of inflammation in promoting or suppressing tumor growth. LQGV reduces systemic and local levels of proinflammatory cytokines such as TNF-α and IL-6 and expression of adhesion molecules such ICAM-1 and E-selectin (chapter 8 and 9). It may be that LQGV treatment also reduced tumor associated cytokine expression and adhesion molecules expression on endothelial cells of the developing tumor associated vascularity and thereby reduced tumor (out)growth.

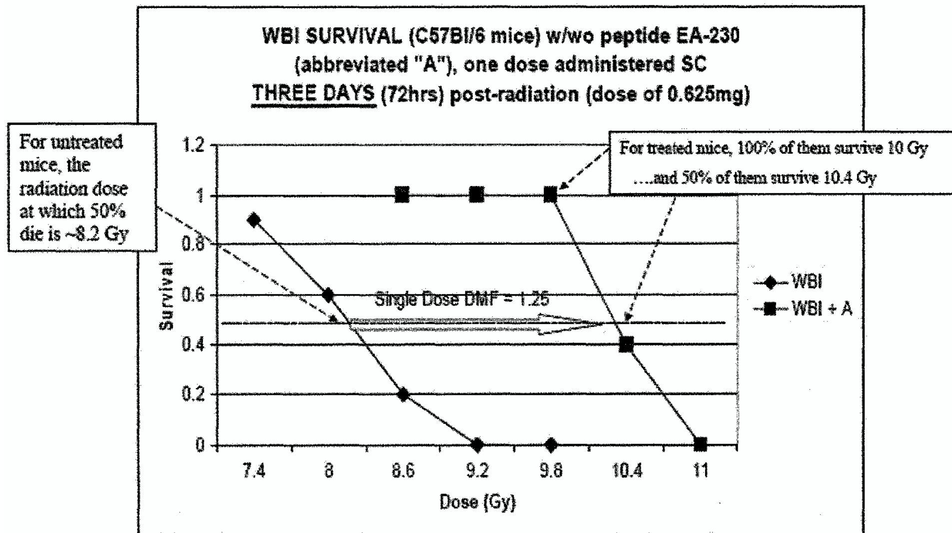

Figure 1. Treatment of mice with a single subcutaneous (s.c.) injection of AQGV (EA-230; 0.625 mg/mouse) 3 days after increasing doses of whole body irradiation (WBI) ranging from 7.4 till 11 Gy.
Treatment with AQGV led to a dose sparing of 1.25, i.e. AQGV-treated mice could accommodate 1.25 times more irradiation to show the same LD50. The test is called the Dose Reduction Factor (DRF) test, which is defined as the ratio between the LD50 of the treated group to the LD50 of the control group. An acceptable DRF value is 1.20. This means that to pass the test, at day 30 after WBI a candidate drug must have the LD50 at a radiation dose that is at least 20% higher (an increase by a factor of 1.20) than the LD50 dose for the control animals. If e.g. the LD50 for the control animals is 8.2 Gy than the LD50 for a candidate drug should be at least 20% higher, which in this case would mean a dose of 10.4 Gy. AQGV passed this test and showed a DRF of 1.25. Data from Appendix II.

Recently, it has been shown that blockade of Notch activity results in markedly increased vessel density with non-productive vascularity which in turn induces hypoxia and thereby inhibits tumor growth (20,21). It may well be that this also holds for LQGV inhibition of LLC growth. We recently started studies to determine the effect of β-hCG-loop-2 oligopeptides on Notch activity. Although LQGV on its own in the LLC mouse model already had a profound effect on tumor growth, it may be that combination therapy of LQGV and cytotoxic agents has a synergistic therapeutic effect.

AQGV and regeneration

Our experiments from 2001 had already revealed that LQGV has the ability to reduce gamma irradiation (TBI) induced morbidity in mice (22). In subsequent studies the 30-day survival (LD50) was determined for groups of mice that received increasing doses of TBI up to 11 Gy and were treated with AQGV (0.625 mg/mouse s.c.) or placebo (23; Appendix II). The groups of mice that received AQGV at day 3 after TBI showed a dose sparing of 1.25, i.e, they could accommodate 1.25 times more irradiation for LD50 (Figure 1).

TBI-induced death of mice is typically caused by infection and/or massive hemorrhage predominantly occurring between day 10 and 20. If mice are free of specific pathogens, sepsis is not an issue and thrombocytopenia is the dominant determinant of the irradiated animal's fate. Therefore, we have applied 5 Gy TBI to groups of mice, and administered either 25 mg/kg (0.625 mg/mouse) of AQGV or the diluent i.p 3 days post-TBI*. At day 13, at a moment that irradiated mice are faced with a nadir in their systemic blood leukocyte and platelet counts, we killed the mice and determined (a) spleen cellularity; (b) the femoral bone marrow cellularity; (c) the femoral bone marrow content of a variety of hemopoietic progenitors (CFU-G, CFU-M, CFU-GM, CFU-Meg, BFUe, and a series of CFU-Mix type colony forming cells).

Mice treated with AQGV at 3 days post-TBI showed considerable regeneration of hemopoietic progenitors and the less primitive stem cell subsets in the femoral marrow. Also in the spleen an increased hemopoietic activity was found (Ploemacher, Khan, Dik *et al.*, unpublished observations). These stem cell compartments were significantly expanded as compared to the other 5 Gy irradiated groups.

From previous work of Ploemacher c.s. we know that repopulation of the stem cell pool following irradiation starts relatively late and takes months to arrive at levels that do not exceed 70-80 percent of the pre-irradiation levels (24-26). In the first days post-TBI there is extensive repair and apoptosis and necrosis in all damaged cell systems, including the stem cell compartment (26,27). Directly following 5 Gy of gamma irradiation up to day 4, the stem cell compartment is reduced down to 0.2 - 1.5 per cent of the control size (28-34). It is only in the remaining 10 days after administration that AQGV could have delivered the *therapeutic* effect on the mice. It is therefore remarkable that AQGV was able to fully restore levels of neutrophils and platelets within this period.

Other agents used to treat irradiation damage, e.g. thiols and bacterial cell wall products, can be applied between 3 days before to about 30 minutes after irradiation in order to obtain a radioprotective effect on cell survival (35). Our data, however, shows that AQGV does not inhibit irradiation-induced apoptosis or necrosis, but stimulates the regeneration of functional end cells from stem cells that survived the TBI. Thus, in our model *AQGV is not a radioprotective agent* (also because it was not present at the time of irradiation), *but a regeneration stimulating agent*. This regenerative signal seems

to stimulate all irradiation surviving stem cell compartments, progenitors (CFU-C) and maturing cells, up to the level of the functional end cells. This makes AQGV a unique compound in radiation biology.

The regeneration-stimulating effect is not limited to the bone-marrow compartment. Our experiments from 1998 had already revealed that NMPF, a low molecular weight hCG fraction which presumably contains β-hCG-loop-2 derived oligopeptides, has the ability to regenerate severely damaged insulin producing islets in the pancreas of diabetic female NOD mice (chapter 4) and to enhance the repair of the *in vivo* cellular damage due to UVB radiation in female BALB/c mice (36). In ischemia-reperfusion experiments in male C57BL/6 mice, AQGV treatment led to significantly increased numbers of Ki-67-positive renal tubular epithelial cells, reflecting enhancement of the regenerative process in acute renal failure (chapter 9). Furthermore, LPS-induced shock experiments in female BALB/c mice showed that several of the oligopeptides used can rescue mice from death even when treatment was performed at the time (e.g. 24 hr after LPS-injection) when multiple organ damage had already occurred and the mice were in the state of shock (chapter 6).

The potential clinical application of AQGV for stimulating regeneration may not be limited to the post-irradiation situation following nuclear incidents or terrorist acts, but may be extended to the treatment of hematopoietic insufficiency, especially thrombocytopenia, and to clinical stem cell transplantation where conditioning may involve either chemotherapy or radiotherapy, or both.

Putative mechanisms underlying the anti-inflammatory, proliferation-modulating and regeneration-stimulating activities of hCG-related oligopeptides

Notch signaling is involved in the regulation of cell-fate decisions by modulating cellular differentiation, proliferation and survival in a wide range of organs and tissues from insects to humans (37). The mammalian Notch receptor family comprises four members: Notch1, Notch2, Notch3 and Notch4 (38,39). The Notch ligand family consists of 5 members: Jagged1, Jagged2, Dll1, Dll3 and Dll4 (39). Notch expressed on the cell surface as a heterodimer is the mature, ligand-accessible form of the receptor (39). In the absence of ligand binding, heterodimeric Notch receptors are inactive. When Notch ligand binds to the Notch receptor on an adjacent cell, a series of proteolytic cleavages occurs, resulting in the release of the Notch intracellular domain (NotchIC) that subsequently translocates into the nucleus (39,40). In the absence of nuclear NotchIC, the transcription factor C protein binding factor 1 (CBF1) binds to the DNA sequence 5'-(C/T)GTGGGAA-3' within Notch target gene promoters and represses transcription (38). When Notch signaling is activated, nuclear NotchIC binds to CBF1 and, following recruitment of the nuclear protein Mastermind-like (MAML), resulting in the formation of a ternary complex that functions as a transcriptional activator (39,41). MAML

recruits the histone acetyltransferase protein p300, resulting in histone acetylation and conversion of the local chromatin structure to a form amenable to active transcription. This results in the transcription of various Notch target genes including those belonging to the HES (Hairy/Enhancer of Split) and HEY (Hairy/Enhancer of split related with YRPW motif) families (42). Both the HES and HEY families function as transcriptional repressors (42).

Notch receptors and ligands are expressed in embryonic vasculature and are required for its remodeling (43). hCG has been shown to act on stromal fibroblasts during the window of uterine receptivity to inhibit apoptosis and to enhance differentiation through the activation of Notch1 (44). Under these conditions activation of Notch1 provides a temporary survival signal and inhibits apoptosis by upregulating anti-apoptotic genes (45,46). This suggests that hCG and Notch1 coordinately regulate decidualization by preventing apoptosis of endometrial stromal fibroblasts, averting uterine sloughing, and promoting cell survival and differentiation into the decidualized phenotype, which is critical for the maintenance of pregnancy (46).

Notch and TLR pathways have also been shown to cooperate to activate canonical Notch target genes, including the transcriptional repressors HES and HEY, and to increase the production of canonical TLR-induced cytokines TNF, IL-6 and IL-12 (47). Cooperation by these pathways to increase target gene expression is mediated by the Notch-pathway component and transcription factor RBP-J (recombination signal binding protein for immunoglobulin kappa J region), which also contributes to lethality after endotoxin injection (47). TLR- and Notch-induced Hes1 and Hey1 attenuate IL-6 and IL-12 production (47). This HES- and HEY-mediated feedback inhibitory loop is abrogated by interferon-γ (IFN-γ), which blocks TLR-induced activation of canonical Notch target genes by inhibiting Notch2 signaling and downstream transcription (47). Importantly, Notch ligand transcripts are also upregulated by endogenous NF-kB activiation through TLR (48). This demonstrates that NF-kB can trigger the Notch signaling pathway in neighboring cells by inducing the production of its ligands. This highlights the interplay between the Notch and NF-kB signaling pathways in the immune system. Together Notch, TLR, NF-kB and IFN-γ can modulate specific effector functions in macrophages, implicating a role of Notch signaling in innate immunity.

The dosage of Notch signaling determines its type of effect (49). In a leukemic T-cell line, low or high concentrations of Notch1IC promote or inhibit NF-kB transactivation, respectively. *Ex vivo* culture of cord blood progenitor cells with a low concentration of immobilized Dll1 promotes the survival of $CD34^+$ cells, while a high concentration of Dll1-induced Notch activation inhibits the generation/survival of $CD34^+$ cells (50). Similarly, the level of Notch signaling within the thymic micro environment can determine the differentiation fate of hematopoietic precursor cells (51). Interestingly, microenvironmental factors may also influence the level of Notch

signaling. For example, hypoxia increases Notch1 protein expression in neuroblastoma cells (52) and estrogen induces Notch1 and Jagged1 mRNA expression in MCF7 breast cancer cells (53). Moreover, the relative expression of Notch signal modulators such as Fringe (Frng) and Deltex (Dx) influence the intensity and ligand dependence of the Notch signal (54,55). Notch signaling can also influence cell growth and differentiation without an influence on cell fate decisions. There are several examples in *Drosophila* and *Caenorhabditis elegans* wherein activation of the Notch signaling pathway can induce cell-cycle arrest, promote cellular proliferation, or even promote apoptosis (56-58).

Alternatively, activation of Notch signaling can protect cells from apoptosis (59,60). Interestingly, *Drosophila* is acutely tolerant to low O_2 environment and withstands 3-4 h of total O_2 deprivation without showing any evidence of cell injury (61,62). Recently, it has been shown that Notch pathway genes play an important role in this hypoxia resistance and the same genes can also be beneficial in mammalian cell resistance to hypoxia (63). Thus, the pleiotropic effects that Notch signaling can exert on progenitor cells is determined in part by the strength and duration of the Notch signal delivered, as well as by the development context of the cell. These findings highlight the importance of precise Notch signal regulation for proper cellular development.

Most hematopoietic cytokines promote either the survival or the differentiation or both of hematopoietic stem cells *ex vivo* (64). Wnt, Notch and Hedgdhog signaling pathways have been implicated in self-renewal and proliferation *in vivo* (65). In irradiated adult zebrafish, Notch activity has been shown to increase the number of multilineage hematopoietic precursor cells followed by the accelerated recovery of all mature blood cell lineages (66). Notch signaling is crucial to the development of a fetus and has been also implicated in the development of cytokine producing NK cells, regulatory T cells, lymphocytes development, vasculogenesis and angiogenesis and hematopoiesis (66-71). This all defines the Notch pathway as critical for controlling homeostasis, cell fate and signal integration in development.

Based on the present data we predict that hCG-related peptides such as LQGV and AQGV have direct or indirect effects on Notch and particular other evolutionary old developmental pathways. If this turns out to be true, then LQGV, AQGV and comparable oligopeptides will support regeneration processes in clinical situations not limited to irradiation damage and innate immunity related cellular damage, but also in wound healing, revascularization, repair of central nervous system and peripheral nerve damage, ventilator induced lung injury, etc.

hCG-related oligopeptides and pregnancy

Pregnancy is suggested to represent a controlled state of inflammation with a prominent role for the innate immune system (72). There are two distinct immunological

interfaces in pregnancy: locally in the decidua between maternal immune cells and fetal trophoblasts; and systemically between circulating maternal immune cells and the syncytiotrophoblasts that form the placental villous surface. This syncytiotrophoblast layer releases proinflammatory cytokines such as TNF-α, IL-1β and IL-6 and the pregnancy hormone hCG (73). TNF-α and IL-1β receptors are involved in the hCG release from trophoblasts by stimulating IL-6 release, while hCG in turn stimulates the production of IL-6 (74).

hCG produced by trophoblasts enters into the maternal blood and interacts with maternal immune cells. At an early stage the inflammatory condition is predominant at the implantation site. Later in pregnancy this is extended to the maternal circulation (74). There is now strong evidence that in normal pregnancy, compared with the non-pregnant state, not only Th2 cytokine levels are raised, but also the levels of particular Th1 cytokines. This is in contrast to the previously reported decreased Th1 levels due to a decreased Th1/Th2 ratio (75). This systemic inflammatory response in normal pregnancy is characterized by leukocytosis, increased NK cell numbers in the decidua, increased monocyte priming, increased phagocytic activity, and the production of pro-inflammatory cytokines, including IL-6, IL-1β, TNF-α and MIF (76). It has been suggested that these inflammatory changes can be as strong as those observed in patients with sepsis, but surprisingly do not seem to harm the mother and fetus in any way (75). This all supports the notion that the dominant immune interaction during pregnancy involves the innate rather than the adaptive immune system (72,75). This is consistent with our previous results in mouse models that hCG stimulates innate immunity (77,78). Since the inflammatory changes during pregnancy are under tight control, this suggests that pregnancy-specific factors, including the hCG-level (Figure 2), help to control this inflammatory response. Uncontrolled inflammatory responses are dangerous not only for the fetus, but also for the mother, such as during preeclampsia (75,76).

On the basis of our results we propose that particular oligopeptides from β-hCG-loop-2 are among such factors that have opposite effects to hCG, e.g. inhibiting the innate immunity and preventing overstimulation of innate immunity by factors such as hCG. Based on the observation that these oligopeptides affected various basic physiological processes, including immune responses, hematopoiesis, angiogenesis, proliferation and regeneration in different models, we predict that these peptides on the molecular level affect well conserved signaling pathways and molecular targets. As discussed above, the Notch signaling pathway is a likely candidate target, and subject of our further studies. This all implies that hCG itself contains regions with oligopeptide sequences that control the biological function of itself. This may explain why hCG is extensively degraded *in vivo,* especially after the first 3 months of pregnancy.

This breakdown is likely related to the extent of hyperglycosylation of the hCG-molecule. It is striking that the extent of hyperglycosylation of hCG decreases from week

3 after the last menstruation period onwards, and that at week 10 of gestation hCG is hardly hyperglycosylated anymore (Figure 2). This kinetics of loss of hyperglycosylation of hCG increasingly facilitates the proteolytic breakdown of hCG during pregnancy.

We conclude that breakdown products of hCG as small as three or four amino acid residues not only play a central role in hCG-physiology and pregnancy success, but likely also constitute an active component in the regulation of inflammatory circuits and homeostasis. This is supported by the observation that hCG also occurs in non-pregnant individuals (79,80).

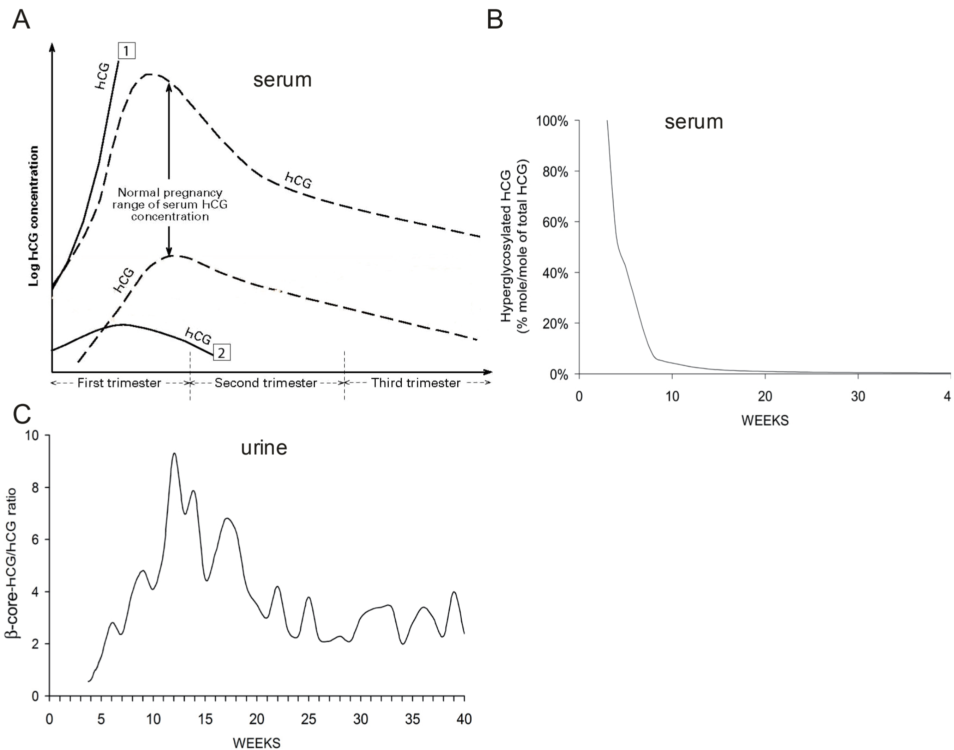

Figure 2. The kinetics of glycosylated and non-glycosylated hCG in serum and of β-core in urine during pregnancy.

A. hCG concentrations in serum during pregnancy. The high baseline hCG concentration (1) with markedly shortened doubling time suggests multiple pregnancy or a trophoblastic tumour. The low baseline hCG concentration suggests the presence of an unviable or ectopic pregnancy or threatened miscarriage. **B**. Hyper-glycosylated hCG in serum during pregnancy. C. β-core hCG in urine during the pregnancy. Modified from Cole *et al.*, 2009 (13,120) and Tran, 2006 (121).

Oligopeptides derived from other proteins than hCG; future prospects

The data presented in chapter 2 in combination with our experimental studies show that proteases are not just non-specific degradative molecules but, at least in the case of hCG, can liberate small oligopeptides with a most relevant physiological activity. In this context, it is interesting that several parasites (such as *Trypanosoma brucei brucei*) and bacteria (such as *Bacillus anthracis*) bear similar sequences in particular proteins (81,82). It might be that, after penetration of the epithelium, these microorganisms release such peptide sequences into their surroundings to suppress the local inflammatory reaction of the host, in order to make their life comfortable.

The identified hCG-related regulating small oligopeptides may cross cellular membranes without requiring membrane-bound receptors (83). Sequences of oligopeptides up to 5 amino acids overlap for 100% between human, bacterial and viral proteins (84). Therefore they are not recognized by the immune system and immunological side effects are highly unlikely. Numerous small peptides exist in endocrine and neurocrine systems that are cleaved out from larger polypeptide precursors, i.e. gonadotropin-releasing hormone (GnRH), oxytocin, antidiuretic hormone (ADH), β-endorphin, vasoactive intestinal peptide (VIP), pro-opiomelanocortin (POMC; a precursor for adrenocorticotropic hormone (ACTH), melanocyte stimulating hormone (MSH), β-endorphin, lipotropins), thyrotropin-releasing hormone (TRH), substance P (SP), neurokinin A (NKA), neuropeptide Y (NPY), insulin growth factors (IGFs) and somatostatin. It may be that hCG-related oligopeptides have overlapping functional homology with other endogenous small peptides and at physiological or supra-physiological doses affect various endocrine and neurocrine axes and their feedback systems.

Gene expression analyses and other studies taught us that these oligopeptides have a distinct regulating effect on the expression of a variety of genes involved in inflammatory pathways and immunity (81). In view of the central role of inflammatory pathways in a variety of pysiological and pathophysiological processes (85-88), this may explain the broad therapeutic activity of the identified hCG-related regulating oligopeptides in the disease models tested.

We are convinced that the case of regulation of gene expression by hCG-related small oligopeptides is not unique in biology (89). Where it was generally thought that the smallest breakdown products of proteins have no specific biological function on their own, our results show that the body may utilize the normal process of proteolytic breakdown to generate physiologically important compounds. So far ~500 human genes encoding proteases/peptidases have been identified (90). This stresses the importance of regulation of biological processes by proteases/peptidases.

Many peptide components have been identified in tissues/organs and body fluids from different species (91-98) as well as in blood cells and their culture

supernatants (99-103). Several of these peptides are hemoglobin derived, are between 2-35 amino acids in length (103), and exhibit a wide variety of biological activities (e.g. analgesic, cytolytic, proliferative/antiproliferative, ACE inhibition, enhancement of antibody production, neuronal or hormonal) (104-118).

Many natural oligopeptides can be found by computer analysis of fragments of bovine hemoglobin molecules (111). In this analysis 125489 hits were found. Of them 128 dipeptide fragments also occur among natural non-hemoglobin fragments (111). Hemoglobin fragments sharing natural non-hemoglobin oligopeptides consist of 2 to 14 amino acid residues. Oligopeptides containing the same hemoglobin fragment can have different functions and demonstrate a wide spectrum of biological activities (111). A characteristic feature of the natural non-hemoglobin oligopeptides sharing sequences with hemoglobin is the fact that the majority of them were neuropeptides and hormones (111). It has also been shown that tetrapeptides of non-hemoglobin origin are included in all these natural hemoglobin oligopeptides (111,112). This demonstrates that hemoglobin most likely acts not only as a carrier for oxygen transport, but also as a source of peptide fragments with regulatory functions which differ from those of the parent molecule. Small peptides derived from other proteins suggest the same (94-96).

This data demonstrates that naturally occurring proteolytic breakdown products, even down to the length of 2-6 amino acids and derived from proteins with known functions, exert powerful biological activity. Therefore these peptides are likely to play a role in regulating biological processes. This notion is strengthened by profound differences in peptide content between different organs of the same animal (104,106), while the peptide content of specific organs appears to be conserved between individuals and amongst species (93,105). This implies the existence of tissue and organ specific peptide contents, suggestive of conserved effector functions of specific peptides amongst individuals and species. Thus the concept emerges that tissue and organ specific proteins are degraded by peptidases, resulting in a large group of (small) peptides that represent the tissue or organ specific peptide pool or peptidome (105). This peptide pool might represent the final class of bioregulators resulting from the sequel genome, transcriptome, proteome, peptidome (105,106,108). In line with this, changes in peptide pools have been observed under pathological conditions (91,96,98,102,117,118). Whether such changes are causal to or the consequence of disease is so far unknown. Nevertheless, we cannot exclude that peptide pool changes influence the biological functioning of cells, tissues, organs and finally organisms.

We have generated antiproliferative oligopeptides based on the primary structure of several other human proteins than hCG and found them to inhibit the cell cycle gene pCDG and the auxin responsive markergene DR5::GUS involved in proliferation in a plant model (119; Appendix 2), suggesting that small oligopeptides are part of a

conserved regulatory system in biology. We predict that further research on the dissection of proteins will unlock (i) a wealth of small regulatory oligopeptides; (ii) that normally occurring regulatory peptides play a crucial role in physiological and pathophysiological processes; and (iii) that this represents a novel level of regulation that will find its way into biological and biomedical research in the coming years. Needless to say, should this be true, we foresee many new potential pharmaceutical molecules, which may correct pathological conditions and restore homeostasis.

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SUMMARY

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Pregnancy is a unique physiological event. The unborn child is in immunological terms foreign to the mother, since it has half of its genetic material from its father. From the immunological point of view there are commonalities between the unborn child and a foreign transplant. While without enough immunosuppressive therapy a foreign transplant is rejected, the unborn child undisturbingly develops within its mother's womb. Interestingly, many women who suffer from autoimmune disease (like rheumatoid arthritis, multiple sclerosis and Crohn's disease) are less affected by their disease during pregnancy. It seems that the natural suppression of their immune responses against the unborn child has a side effect which suppresses their autoimmune disease as well. However, there is no generalized immunosuppression of the maternal immune system during pregnancy since, generally, pregnant women are not more prone to infections than non-pregnant women. Apparently, the immune system enables the immunological acceptance of unborn.

We hypothesized that human chorionic gonadotropin (hCG) hormone ('pregnancy hormone') might account for the immunosuppression during pregnancy. hCG is one of the first hormones that are produced in pregnancy. We tested the effect of a commercially available hCG preparation on the development of autoimmune mediated type 1 diabetes in non-obese diabetic (NOD) mice. In this mouse model the diabetes is (predominantly) caused by Th1 cell-mediated destruction of insulin-producing β-cells in the islets of Langerhans of the pancreas. We showed that treatment of NOD mice with a commercial hCG preparation or a low molecular weight fraction thereof (NMPF) before the onset of clinical symptoms decreased or even prevented the increased blood glucose levels. Also the established inflammatory infiltrate in and around the islets of Langerhans was eliminated, and the development of diabetes was inhibited for prolonged time.

hCG consists of an α and a β chain, and occurs in a variety of forms in serum and urine of pregnant women, but only intact hCG stimulates the hCG receptor. Besides heterodimeric hCG, β-core fragment and nicked hCG are two other major forms of hCG that are found in pregnancy serum and urine samples. The nicked hCG and β-core fragment have peptide bond cleavages in between residues 44-54 of loop 2 of β-hCG and completely miss loop 2 (residues 41-54), respectively.

Several investigators have studied the biological effects of heterodimeric hCG and its variants on gonadal tissues and/or their role in immune modulation. So far, however, the possible biological activity of fragments liberated from the breakdown of hCG into the different variants was ignored. We postulated that the loop 2 region, consisting of residues 41-54 with amino acid sequence MTRVLQGVLPALPQ, which is absent in β-core, can also be nicked *in vivo* and can liberate smaller fragments (3-7 amino acids long oligopeptides) with biological activity.

We have tested the biological activities of the designed oligopeptides in various animal models for acute inflammation such as inflammation induced abortion, shock induced by septic (*E.coli*) or aseptic (LPS; hemorrhage and resuscitation) inflammation, renal failure by ischemia-reperfusion injury and *Listeria* infection, and we have evaluated the effect of several of the peptides in a tumor transplantation model in mice. In these animal models various oligopeptides designed from loop-2 of β-hCG had therapeutic effects (MTR, MTRV, LQGV, VLPALP, VLPALPQ). These therapeutic effects were confirmed in a nonhuman primate model for septic shock (combination treatment of LQGV, VLPALP and AQGV, an alanine-analogue of LQGV). We selected AQGV as the lead compound for clinical development. In collaboration with EBI Anti Sepsis we performed and successfully completed a phase 1 study with AQGV for various clinical indications including SIRS/Sepsis, renal failure and radiation sickness. Subsequently we confirmed the therapeutic effect of AQGV in human volunteers challenged with LPS. EBI Anti Sepsis BV has announced a Phase 2 study to test the safety and efficacy of AQGV in reducing kidney damage in patients undergoing on-pomp cardiovascular surgery. Our studies suggest that the oligopeptides from loop-2 of β-hCG may have an important function in the regulation of inflammation and in the proliferation and differentiation of cells. Various cell signaling pathways, including Notch, Wnt, Hedgdhog and NF-kB pathways, are candidates for further mechanistic studies.

Our experiments on Lewis Lung Carcinoma (LLC) in mice showed that LQGV can inhibit the growth of this tumor in a transplantation model. This anti-tumor activity was likely due to stimulation of the tumor vascularity in combination with disrupting the tumor associated vascular (TAV) integrity. Furthermore, AQGV has the ability to reduce gamma irradiation induced morbidity and mortality. In these experiments AQGV had profound effects on blood, spleen and femoral bone marrow cellularity, and on the regeneration of hemopoietic stem cells.

The cellular parameters affected by the designed oligopeptides were proinflammatory cytokines (TNF-α, IL-6, MIF), MHC-II expression on B-cells and macrophages, E-selectin expression, neutrophil infiltration in inflammatory sites, proliferation, regeneration and organ damage repair. In the employed models the affected inflammatory parameters varied. Apparently, individual oligopeptides may inhibit a particular spectrum of inflammatory responses. The common pathway of immunity that is affected by the designed hCG-related oligopeptides is the innate immune system. This especially appeared from the studies on the effect of LQGV, VLPALP and AQGV on *Listeria monocytogenes* infection in mice.

Our work supports the concept that tissue and organ specific proteins are degraded by peptidases, resulting in a large group of (small) peptides that represent the tissue or organ specific peptide pool or peptidome. This peptide pool may represent an important class of bioregulators resulting from the sequel genome, transcriptome, proteome, peptidome.

We also designed a series of antiproliferative oligopeptides based on the primary structure of several other human proteins than hCG (i.e. C-reactive protein, β-catenin, p53) and tested these in a plant model. Many of the designed peptides appeared capable to inhibit the pCDG and DR5::GUS genes involved in cellular proliferation in this model system. The fact that these peptides, that are part of human proteins, can inhibit the proliferation of plant cells suggests that these oligopeptides are part of a conserved regulatory system in biology.

We hypothesize that many endogenous (and possibly exogenous) proteins are broken down *in vivo* yielding small peptides with regulatory properties. In our view these normally occurring regulatory peptides play a crucial role in the fine-tuning of physiological processes and in pathophysiological processes. This likely represents a novel level of regulation that will find its way into biological and biomedical research in the coming years. Should this be true, this will allow the development of many potential pharmaceutical molecules.

SAMENVATTING

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Zwangerschap is een unieke fysiologische gebeurtenis. Het ongeboren kind is, immunologisch gezien, vreemd aan de moeder omdat het de helft van de erfelijke eigenschappen van de vader heeft. Uit immunologisch gezichtspunt zijn er overeenkomsten tussen het ongeboren kind en een lichaamsvreemd transplantaat. Zonder voldoende immunosuppressieve therapie wordt een vreemd transplantaat afgestoten, maar het ongeboren kind ontwikkelt zich ongestoord in de baarmoeder. Interessant is, dat veel vrouwen die lijden aan een autoimmuunziekte (bijvoorbeeld reumatoïde arthritis, multipele sclerose, en de ziekte van Crohn), daar tijdens hun zwangerschap minder last van hebben. Het lijkt erop dat de natuurlijke suppressie van hun immuunreactie tegen het ongeboren kind als neveneffect heeft dat ook hun autoimmuunziekte wordt onderdrukt. Er is echter geen algemene immunosuppressie van het afweersysteem van een zwangere, want zwangere vrouwen krijgen in het algemeen niet vaker een infectieziekte dan niet-zwangere vrouwen. Klaarblijkelijk wordt tijdens de zwangerschap het afweersysteem zo gereguleerd dat het ongeboren kind immunologisch wordt geaccepteerd.

Wij zijn in het onderzoek voor dit proefschrift uitgegaan van de hypothese dat het humaan chorion gonadotropine (hCG) hormoon (‘zwangerschapshormoon’) verantwoordelijk is voor de immunosuppressie tijdens de zwangerschap. hCG is een van de eerste hormonen die worden geproduceerd bij zwangerschap. Wij hebben het effect getest van een commercieel beschikbaar hCG preparaat op de ontwikkeling van autoimmuun gemedieerde type 1 diabetes in non-obese diabetische (NOD) muizen. In dit muizenmodel wordt de diabetes veroorzaakt door (vooral) Th1 cel gemedieerde destructie van insulineproducerende β-cellen in de eilandjes van Langerhans in de pancreas. Wij hebben aangetoond dat door behandeling van NOD muizen met een commercieel hCG preparaat, of een fractie daarvan met een laag molecuulgewicht (NMPF) vóór het begin van de klinische symptomen, de toename in bloedglucosespiegel verminderd of zelfs voorkomen werd. Ook werd het ontstekingsinfiltraat in en rond de eilandjes van Langerhans tenietgedaan en werd de ontwikkeling van diabetes voor langere tijd geremd.

hCG bestaat uit een α en een β keten, en komt in een variëteit van vormen voor in serum en urine van zwangere vrouwen, maar alleen intact hCG stimuleert de hCG receptor. Naast heterodimeer hCG zijn β-core fragment en ‘nicked’ hCG twee andere belangrijke vormen van hCG die voorkomen in serum en urine van zwangeren. Het ‘nicked’ hCG en β-core fragment hebben breuken in de peptideketen tussen de aminozuurresiduen 44-54 van ‘loop 2’ van β-hCG, respectievelijk missen volledig ‘loop 2’ (residuen 41-54).

Verschillende onderzoekers hebben de biologische effecten van heterodimeer hCG en zijn varianten bestudeerd op de geslachtsorganen en hun rol in immuunmodulatie. Tot dusver werd echter de mogelijke biologische activiteit van de bij de afbraak van hCG vrijgekomen fragmenten genegeerd. Centraal in dit proefschrift staat onze veronderstelling dat het 'loop 2' gebied, bestaande uit de residuen 41-54, met de aminozuurvolgorde MTRVLQGVLPALPQ, dat afwezig is in β-core, *in vivo* door bepaalde enzymen geknipt kan worden, waardoor kleinere fragmenten (oligopeptiden van 3-7 aminozuren) met biologische activiteit vrijkomen.

Wij hebben de biologische activiteit van zulke synthetisch geproduceerde oligopeptiden getest in verschillende diermodellen voor acute ontsteking, zoals abortus geïnduceerd door ontsteking, shock geïnduceerd door septische (*E.coli*) of aseptische (LPS; bloeding en reperfusie) ontsteking, nierfalen door schade bij ischemie-reperfusie en *Listeria* infectie, en wij hebben de invloed van enkele peptiden getest op de groei van een bepaalde tumor in een muizenmodel. In deze diermodellen hadden verschillende oligopeptiden die gemaakt waren van 'loop-2' van β-hCG (o.a. MTR, MTRV, LQGV, VLPALP, VLPALPQ) een therapeutisch effect. Deze therapeutische effecten werden bevestigd in een apenmodel voor septische shock (combinatiebehandeling met LQGV, VLPALP en AQGV; de laatste is een alanine-analoog van LQGV). Wij hebben AQGV geselecteerd als belangrijkste verbinding voor verdere klinische ontwikkeling. In samenwerking met de firma EBI Anti Sepsis BV hebben wij een Fase 1 studie met vrijwilligers, die AQGV kregen toegediend, succesvol afgerond. Vervolgens hebben wij in vrijwilligers het therapeutische effect van AQGV na toediening van LPS kunnen bevestigen. Deze studies zijn van belang in verband met de mogelijke klinische toepassing van AQGV voor de behandeling van SIRS/Sepsis, nierfalen en stralingsziekte. EBI Anti Sepsis BV heeft een Fase 2 studie aangekondigd om de veiligheid en effectiviteit van AQGV te testen om nierschade te reduceren bij patiënten die een cardiovasculaire operatie ondergaan. Onze studies suggereren dat de oligopeptiden van 'loop-2' van β-hCG een belangrijke functie kunnen hebben bij de regulatie van ontsteking, en bij proliferatie en differentiatie van cellen. Wat dit betreft zijn verschillende cel signaleringsroutes, inclusief Notch, Wnt, Hedgdhog en NF-kB, kandidaten voor mechanistische studies.

Onze experimenten met Lewis Long Carcinoom (LLC) in muizen toonden aan dat LQGV de groei van deze tumor in een transplantatiemodel kan remmen. Deze anti-tumor activiteit werd vermoedelijk veroorzaakt door stimulatie van de bloedvatontwikkeling in de tumor in combinatie met verstoring van de tumorgeassocieerde vasculaire (TAV) integriteit. Voorts kan AQGV de morbiditeit en mortaliteit reduceren die geïnduceerd wordt door radioactieve straling. In deze experimenten bleek AQGV het herstel van de bloed-, milt- en beenmergcellulariteit, en de regeneratie van hemopoetische stamcellen te bevorderen.

De cellulaire parameters, die beïnvloed werden door de door ons ontworpen oligopeptiden, waren de productie van ontstekingsbevorderende cytokinen (TNF-α, IL-6, MIF), MHC-II expressie op B-cellen en macrofagen, E-selectine expressie, infiltratie van neutrofiele granulocyten bij ontstekingen, proliferatie, regeneratie en herstel van orgaanschade. In de geteste modellen bleken de betreffende ontstekingsparameters, die werden beïnvloed door de verschillende oligopeptiden, deels te verschillen. Blijkbaar kan elk van de door ons ontworpen oligopeptiden een bepaald spectrum van ontstekingsreacties onderdrukken. De gemeenschappelijke route, die wordt beïnvloed door deze hCG-gerelateerde oligopeptiden in de door ons gebruikte diermodellen voor ontsteking, is het aangeboren ('innate') immuunsysteem. Dit bleek vooral uit de studies naar het effect van LQGV, VLPALP en AQGV bij *Listeria monocytogenes* infectie bij muizen.

Ons onderzoek sluit aan bij het concept dat weefsel- en orgaanspecifieke eiwitten *in vivo* worden afgebroken door peptidasen, resulterend in een grote groep (kleine) peptiden die een weefsel- of orgaanspecifieke verzameling van peptiden (peptidoom) vormen. Deze groep peptiden zou een belangrijke klasse van bioregulatoren kunnen vertegenwoordigen als onderdeel van de sequentie genoom – transcriptoom – proteoom – peptidoom.

Wij hebben tevens een serie antiproliferatieve oligopeptiden ontworpen, gebaseerd op de primaire structuur van verschillende andere humane eiwitten dan hCG (namelijk C-reactive protein, β-catenin, p53), en hebben deze getest in een plantenmodel. Veel van de ontworpen peptiden bleken in staat om de pCDG en DR5::GUS genen voor proliferatie in dit modelsysteem te remmen. Het feit dat deze peptiden, die onderdeel zijn van menselijke eiwitten, de proliferatie van plantencellen kunnen remmen, suggereert dat deze peptiden deel zijn van een geconserveerd regulatiesysteem in de natuur.

Wij veronderstellen dat veel endogene (en wellicht ook exogene) eiwitten *in vivo* worden afgebroken tot kleine peptiden met regulerende eigenschappen. In onze visie spelen deze normaal voorkomende regulatoire peptiden een cruciale rol in de fijnregulatie van fysiologische processen en in pathofysiologische processen. Zij vertegenwoordigen vermoedelijk een nieuw regulatiesysteem dat van belang zal blijken in toekomstig biologisch en biomedisch onderzoek. Als dit waar blijkt te zijn, zal dit de ontwikkeling van een groot aantal potentiële geneesmiddelen mogelijk maken.

ABBREVIATIONS

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α	alpha
AC	adenylate cyclase
ACTH	adrenocorticotropic hormone
AHA	hemolytic anemia
ALT	alanine aminotransferase
APC	antigen presenting cells
Arg	Arginine
Asn	asparagine
AST	aspartate aminotransferase
ATD	autoimmune thyroid disease
ATP	autoimmune thrombocytopenia
β	beta
BUN	blood urea nitrogen
CAM	chorioallantoic membrane
cAMP	cyclic adenosine monophostphate
CREAT	creatinine
CREBP	cAMP response element protein
CRH	corticotropin releasing hormone
CTP	C-terminal peptide
Cys	cysteine
E.coli	*Escherichia coli*
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
FSH	follicle-stimulating hormone
GH	growth hormone
GM-CSF	granulocyte macrophage cdolony stimulating factor
GnRH	gonadotropin releasing hormone
GRH	gonadotropin-releasing hormone
GTT	glucose tolerance test
hCG	human chorionic gonadotropin
His	histidine
HKLM	heat-killed *Listeria monocytogenes*
HLA	human leukocyte antigens
hPL	human placental lactogen
HPLC	high performance liquid chromatography
ICAM	intracellular adhesion molecule
ICU	intensive care units

IL	interleukin
INF-γ	interferon-gamma
L. monocytogenes	*Listeria monocytogenes*
LH	luteinizing hormone
LIF	leukemia inhibitory factor
LLC	lewis lung carcinoma
LLO	listeriolysin O
LPS	lipopolysaccharide
LQG	leucine-glutamine-glycine
LQGV	leucine-glutamine-glycine-valine
LRR	leucine-rich repeat
Lys	Lysine
MCP	monocyte chemoattractant protein
MHC	major histocompatibility complex
MIF	macrophage migration inhibitory factor
MODS	multiple organ dysfunction syndrome
mRNA	messenger ribonucleic acid
MTR	methionine-threonine-arginine
MTRV	methionine-threonine-arginine-valine
NCR	N-terminal cysteine-rich
NKT	natural killer T cell
NMPF	natural (immuno)modulatory pregnancy factor
NOD	non-obese diabetic
PGE2	prostaglandin E2
Phe	Phenylalanine
pI	isoelectric point
PKA	protein kinase A
PKC	protein kinase C
RA	rheumatoid arthritis
RER	rough endoplasmatic reticulum
SCID	severe combined immunodeficiency
Ser	Serine
SIRS	systemic inflammatory response syndrome
SLE	systemic lupus erythematosus
Th	T helper
Thr	Threonine
TLR	Toll-like receptor
TM	Transmembrane
TNF-α	tumor necrosis factor-alpha

Try	Tryptophan
TSH	thyroid-stimulating hormone
uNK	uterine natural killer cell
VLPALP	valine-leucine-proline-alanine-leucine-proline
VLPALPQ	valine-leucine-proline-alanine-leucine-prolineglutamine

APPENDIX I

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(54) **OLIGOPEPTIDE ACETATE AND FORMULATIONS THEREOF**

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Continuation-in-part of application No. 11/037,972, filed on Jan. 18, 2005, which is a continuation of application No. 09/821,380, filed on Mar. 29, 2001, now Pat. No. 6,844,315, which is a continuation-in-part of application No. 09/716,777, filed on Nov. 20, 2000, now Pat. No. 6,921,751, which is a continuation of application No. PCT/NL99/00313, filed on May 20, 1999.

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(57) **ABSTRACT**

The invention relates to acetates of the tetrapeptides AQGV (SEQ ID NO:2) and LQGV (SEQ ID NO:3), pharmaceutical compositions comprising the acetates of the tetrapeptides, and methods of treating using the acetates of the tetrapeptides or pharmaceutical compositions to treat acute inflammatory conditions including sepsis.

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FIG. 1

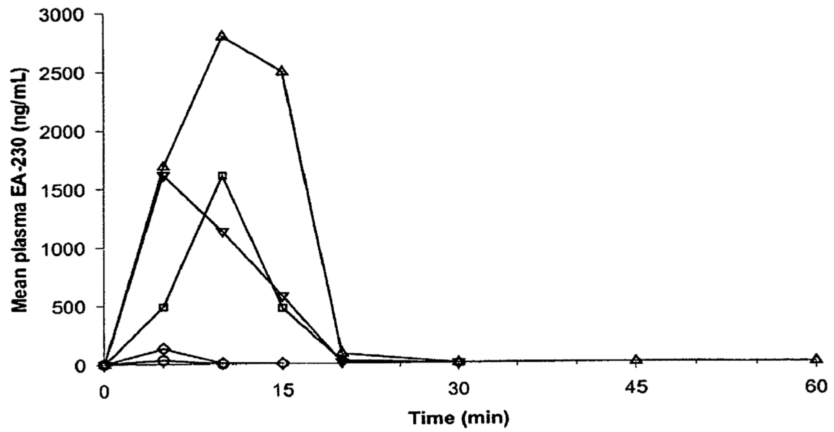

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FIG. 2A

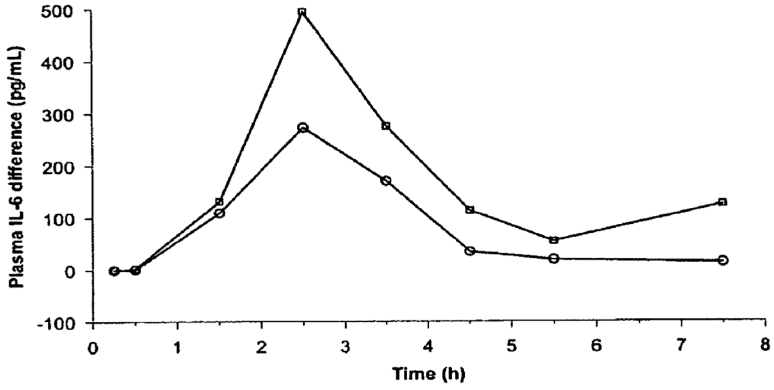


FIG. 2B

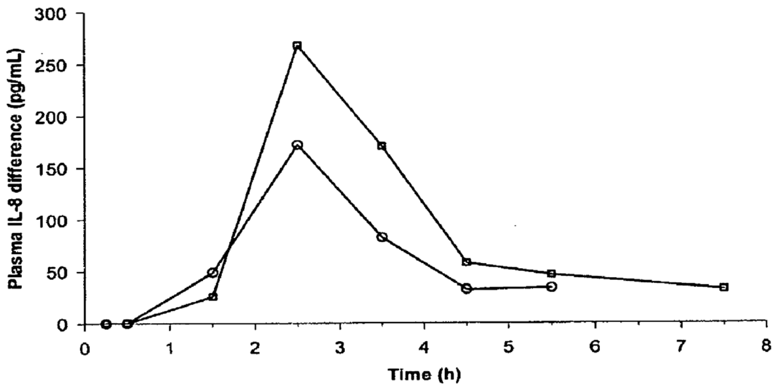


FIG. 3A

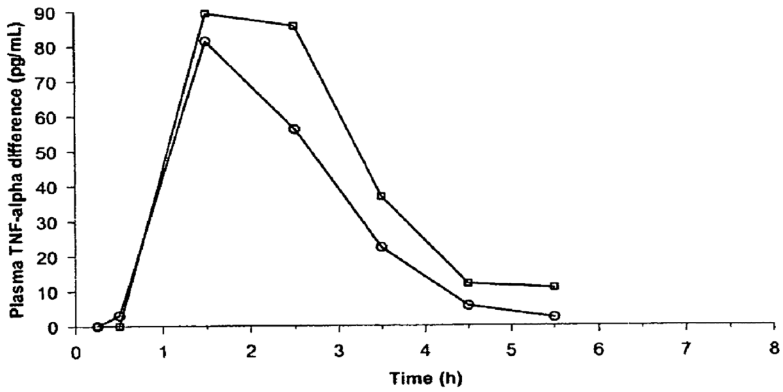
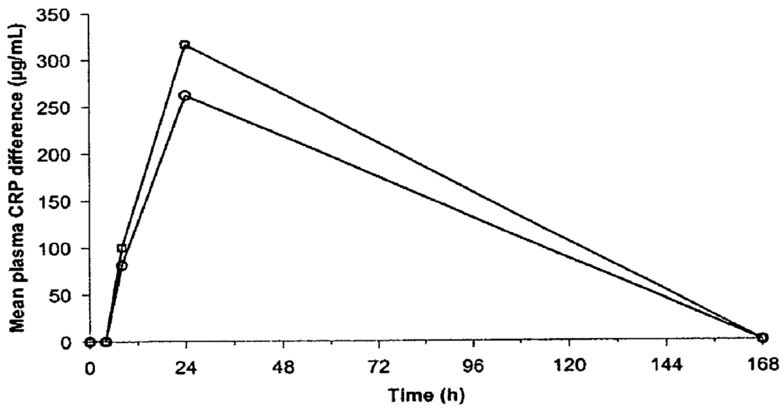


FIG. 3B



OLIGOPEPTIDE ACETATE AND FORMULATIONS THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation-in-part of co-pending U.S. patent application Ser. No. 10/409,668, filed Apr. 8, 2003, and a continuation-in-part of co-pending U.S. patent application Ser. No. 11/037,972, filed Jan. 18, 2005, which is a continuation of U.S. patent application Ser. No. 09/821,380, filed Mar. 29, 2001, now U.S. Pat. No. 6,844,315 B2, which is a continuation-in-part of earlier U.S. patent application Ser. No. 09/716,777, filed Nov. 20, 2000, now U.S. Pat. No. 6,921,751 B1, which is a continuation of co-pending International Application No. PCT/NL99/00313, filed May 20, 1999, designating the United States of America, which itself claims priority from EP 98201695.8, filed on May 20, 1998, and EP 98202706.2, filed on Aug. 12, 1998, the contents of the entirety of all of which are incorporated herein by this reference.

TECHNICAL FIELD

[0002] The invention relates generally to biotechnology and medicine, and, more particularly, to anti-inflammatory activity found in short (tri- to hepta-meric) peptides derived from human chorionic gonadotropin particularly AQGV (SEQ ID NO:2 of the incorporated herein SEQUENCE LISTING) and LQGV (SEQ ID NO:3), as pharmaceutical compounds.

BACKGROUND

[0003] Human chorionic gonadotropin ("hCG") is a heterodimeric placental glycoprotein hormone required in pregnancy. In the urine of human pregnancy and in commercial hCG preparations, it occurs in a variety of forms, including breakdown products. Several investigators have studied the effects of heterodimeric hCG and its variants on the immune system because of their putative role in preventing the rejection of the fetal allograft during pregnancy. Several reports have suggested modulation of the immune system by intact hormone, but such effects of breakdown products have not been reported.

[0004] Khan et al., *Hum Immunol.* 2002 January; 63(1):1-7, reported inhibition of septic shock in mice by a 6-mer oligopeptide (VLPALP(SEQ ID NO:6)) derived from the beta-chain of human chorionic gonadotropin hormone. A single treatment with this hexapeptide after high dose lipopolysaccharide ("LPS") injection inhibited septic shock in mice. Benner and Khan (*Scand J Immunol.* 2005 July; 62 Suppl 1:62-6) studied the possible immunological activity of the in vivo liberated peptide fragments originating from nicking of the sequence MTRVLQGVLPALPQVVC (residues 41-57) of loop 2 of the beta-subunit of hCG (SEQ ID NO:7). It is there reported that several of 3-7 amino acid-long peptides taken from loop 2 of the beta-subunit—and alanine-replacement peptides derived of some—displayed significant anti-inflammatory activity as measured by the inhibition of septic shock syndrome in mice. Selection was based on the known preferential cleavage sites of the sequence MTRVLQGVLPALPQVVC (residues 41-57) of loop 2 of the beta-subunit of hCG (SEQ ID NO:7).

[0005] (Cole et al., *J Clin Endocr Metab* 1993; 76:704-710; Alfthan H, Stenman U H. *Mol Cell Endocrinol* 1996; 125:107-120; Kardana A, et al., *Endocrinology* 1991; 129:1541-1550; Cole et al., *Endocrinology* 1991; 129:1559-1567; Birken S, Maydelman Y, Gawinowicz M A. Methods 2000; 21:3-14) The peptides LQGV (SEQ ID NO:3) and AQGV (SEQ ID NO:2) were selected for synthesis, of which the results are presented here.

BRIEF SUMMARY OF THE INVENTION

[0006] Khan et al., *Hum Immunol.* 2002 January; 63(1):1-7, reported inhibition of septic shock in mice by a 6-mer oligopeptide (VLPALP (SEQ ID NO:6)) derived from the beta-chain of human chorionic gonadotropin hormone. A single treatment with this hexapeptide after high dose LPS injection inhibited septic shock in mice. Here, we show that several other short (from trimeric peptides up) peptides derived from the beta chain of hCG, and modifications of some of said peptides obtained by alanine substitution of single amino acids, have similar anti-inflammatory activity. Furthermore, we provide our rational for selecting two of these (AQGV (SEQ ID NO:2) and LQGV (SEQ ID NO:3)) as therapeutic compounds for treatment of conditions such as human acute inflammatory conditions (e.g., sepsis).

[0007] In certain embodiments, the invention provides an acetate of tetrapeptide, wherein the tetrapeptide is AQGV (SEQ ID NO:2) and/or LQGV (SEQ ID NO:3). In certain embodiments, the acetate of a tetrapeptide is an acetate salt of the tetrapeptide. In a further embodiment the acetate of a tetrapeptide is an acetate ester of the tetrapeptide.

[0008] An additional aspect of the invention provides a pharmaceutical composition comprising acetate of a tetrapeptide, wherein the tetrapeptide is SEQ ID NO:2 and/or SEQ ID NO:3 and a pharmaceutically acceptable carrier, adjuvant, or excipient.

[0009] A further aspect of the invention provides a method of treating an acute inflammatory condition in a subject comprising administering the acetate of a tetrapeptide SEQ ID NO:3 and/or SEQ ID NO:2 to a subject having an acute inflammatory condition in an amount efficacious to reduce the inflammation in the subject as may be determined by a decrease in serum IL-6 levels in the subject. An additional aspect of the invention provides a method of treating an acute inflammatory condition in a subject comprising administering to the subject a pharmaceutical composition of the invention in an amount efficacious to reduce the inflammation in the subject as may be determined by a decrease in serum IL-6 levels in the subject. In certain embodiments, the acute inflammatory condition is sepsis.

BRIEF DESCRIPTION OF THE FIGURES

[0010] FIG. **1**. is a graph depicting mean EA-230 concentrations in the plasma of subjects who received various dosages of EA-230 at various times after administration. Circles indicate an initial dose of 1 mg/kg; diamonds indicate an initial dose of 3 mg/kg; squares indicate an initial dose of 10 mg/kg; upright triangles indicate an initial dose of 30 mg/kg; and inverted triangles indicate a dosage of LPS followed by initial dose of 10 mg/kg EA-230.

[0011] FIGS. **2**A and **2**B. are graphs depicting average changes in the concentration of IL-6 and IL-8 in the plasma of subjects respectively. All subjects were treated with 4 ng/kg LPS 30 minutes prior to administration of a placebo (squares) or 10 mg/kg EA-230 (circles).

[0012] FIGS. 3A and 3B. are graphs depicting average changes in the concentration of TNF-α and CRP in the plasma of subjects respectively. All subjects were treated with 4 ng/kg LPS 30 minutes prior to administration of a placebo (squares) or 10 mg/kg EA-230 (circles).

DETAILED DESCRIPTION OF EXAMPLE EMBODIMENTS THE INVENTION

[0013] As used herein, a "purified, synthetic or isolated" peptide is one that has been purified from a natural or biotechnological source, or, more preferably, is synthesized as described herein.

[0014] As used herein, the phrases "treatment of an acute inflammatory condition," and "management of an acute inflammatory condition" are used interchangeably and do not necessarily imply the realization of a complete cure of the condition. These phrases include reduction of the symptoms of the underlying disease and/or reduction of one or more of the underlying cellular, physiological, or biochemical indicators, causes, risks or mechanisms associated with an acute inflammatory condition, including reduction of such symptoms or underlying indicators, causes, risks or mechanisms to below detectable levels. "Reduced," as used in this context, means a reduction in characteristic indicators, causes or mechanisms of the disease state or reduction in a risk of an associated disease state relative to the untreated state of the disease, including, but not limited to cellular, physiological, or biochemical indicators or risks of the diseased state or associated with the disease state.

[0015] As used herein, an "effective amount" means an amount of a composition administered to a subject that is effective to improve, prevent, reduce the symptoms of, or treat the disease condition in the subject.

[0016] As used herein, an "acute inflammatory condition" refers to a disease (or diseases) commonly associated with a one time inflammatory event. Examples of acute inflammatory conditions include, but are not limited to, sepsis, septic shock, anaphylactic shock, and hyper-acute transplant rejection.

[0017] "Composition," as used herein, refers to chemical compounds that contain or consist of the oligopeptide. The oligopeptide may be isolated before inclusion within the composition. The oligopeptide most preferably consists of three (3) to six (6) amino acids. Examples of an oligopeptide include, but are not limited to, SEQ ID NO:2 and SEQ ID NO:3.

[0018] For instance, a preferred compound could, in certain embodiments be: NT A Q G V CT wherein NT at the N-terminus is selected from the group of H—, CH3-, an acyl group, or a general protective group; and CT at the C-terminus is selected from the group of small (e.g., 1 to 5 amino acids) peptides, —OH, $—OR^1$, $—NH_2$, $—NHR^1$, $—NR^1R^2$, or $—N(CH_2)_{1-6}NR^1R^2$, wherein R^1 and R^2, when present, are independently selected from H, alkyl, aryl, (ar)alkyl, and wherein R^1 and R^2 can be cyclically bonded to one another.

[0019] "Alkyl" as used herein, is preferably a saturated branched or unbranched hydrocarbon having one to six carbon atoms, for example, methyl, ethyl, and isopentyl.

[0020] "Aryl" as used herein, is an aromatic hydrocarbon group, preferably having 6 to 10 carbon atoms, such as phenyl or naphthyl.

[0021] "(Ar)alkyl" as used herein, is an arene group (having both aliphatic and aromatic portions), preferably having 7 to 13 carbon atoms such as benzyl, ethylbenzyl, n-propylbenzyl, and isobutylbenzyl.

[0022] "Oligopeptide" as used herein, are peptides having from 3 to 12 amino acids joined together by peptide bonds. Equivalent to oligopeptides are compounds having the same or equivalent side chains as the particular amino acids used in an oligopeptide, and arranged sequentially in the same order as the peptides, but joined together by non-peptide bonds, e.g., by isosteric linkages such as the keto isostere, hydroxy isostere, diketo isostere, or the keto-difluoromethylene isostere.

[0023] "Composition" also includes, for example, an acceptable salt, counter ion, or ester of the oligopeptide or a labeled oligopeptide. As used herein, "acceptable salt" refers to salts that retain the desired activity of the oligopeptide or equivalent compound, but preferably do not detrimentally affect the activity of the oligopeptide or other component of a system in which uses the oligopeptide. Examples of such salts (or counter ions) are acid addition salts formed with inorganic acids, for example, hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid, and the like. Salts may also be formed with organic acids such as, for example, acetic acid, trifluoroacetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, pamoic acid, alginic acid, polyglutamic acid, and the like. Salts may be formed with polyvalent metal cations such as zinc, calcium, bismuth, barium, magnesium, aluminum, copper, cobalt, nickel and the like or with an organic cation formed from N,N'-dibenzylethylenediamine or ethylenediamine, or combinations thereof (e.g., a zinc tannate salt). Non-limiting examples of compositions according the invention include acetate salts or esters of AQGV (SEQ ID NO:2) and/or LQGV (SEQ ID NO:3).

[0024] Such a composition may be administered to the subject parenterally or orally. Such a composition may consist essentially of oligopeptide and PBS. It is preferred that the oligopeptide is of synthetic origin. Suitable treatment for example entails administering the oligopeptide in the composition to the patient intravenously in an amount of from about 0.1 to about 35 mg/kg body mass of the subject. It may be useful that the pharmaceutical composition consists essentially of from one to three different oligopeptides.

[0025] The thus developed chemical entity can be administered and introduced in-vivo systemically, topically, or locally. The peptide, or its modification or derivative, can be administered as the entity as such or as a pharmaceutically acceptable acid- or base addition salt, formed by reaction with an inorganic acid (such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid); or with an organic acid (such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid); or by reaction with an inorganic base (such as sodium hydroxide, ammonium hydroxide, potassium hydroxide); or with an organic base (such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines). A selected peptide and any of the derived entities may also be conjugated to sugars, lipids,

other polypeptides, nucleic acids and PNA; and function in-situ as a conjugate or be released locally after reaching a targeted tissue or organ.

[0026] A "substitution" with regard to the various amino acids generally relate to substituting a group such as alkoxy, halogen, hydroxy, nitro, or lower alkyl onto an aromatic ring for hydrogen that would usually be present. Substitutions can also be made on the alkyl chain connecting the aromatic portion to the peptide backbone, with, for instance lower alkyl groups substituting for hydrogen. Still further substitutions can be made at the alpha position of an amino acid, also using an alkyl group.

[0027] Preferred substitutions involve the use of fluorine or chlorine as a halogen, and methoxy as an alkoxy group. With regard to alkyl and lower alkyl, generally alkyl groups having fewer (1 to 3) carbon atoms are preferred.

[0028] The compounds according to the general formula may be prepared in a manner conventional for such compounds. To that end, suitably N alpha protected (and side-chain protected if reactive side-chains are present) amino acid derivatives or peptides are activated and coupled to suitably carboxyl protected amino acid or peptide derivatives either in solution or on a solid support. Protection of the alpha-amino functions generally takes place by urethane functions such as the acid-labile tertiary-butyloxycarbonyl group ("Boc"), benzyloxycarbonyl ("Z") group and substituted analogs or the base-labile 9-fluoremyl-methyloxycarbonyl ("Fmoc") group. The Z group can also be removed by catalytic hydrogenation. Other suitable protecting groups include the Nps, Bmv, Bpoc, Aloc, MSC, etc. A good overview of amino protecting groups is given in *The peptides, Analysis, Synthesis, Biology*, Vol. 3 E. Gross and J. Meienhofer, eds. (Academic Press, New York, 1981). Protection of carboxyl groups can take place by ester formation, for example, base-labile esters like methyl or ethyl, acid labile esters like tert. butyl or, substituted, benzyl esters or hydrogenolytically. Protection of side-chain functions like those of lysine and glutamic or aspartic acid can take place using the aforementioned groups. Protection of thiol, and although not always required, of guanidino, alcohol and imidazole groups can take place using a variety of reagents such as those described in *The Peptides, Analysis, Synthesis, Biology, id.* or in *Pure and Applied Chemistry,* 59(3), 331-344 (1987). Activation of the carboxyl group of the suitably protected amino acids or peptides can take place by the azide, mixed anhydride, active ester, or carbodiimide method especially with the addition of catalytic and racemization-suppressing compounds like 1-N-N-hydroxybenzotriazole, N-hydroxysuccinimide, 3-hydroxy-4-oxo-3,4-dihydro-1,2,3,-benzotriazine, N-hydroxy-5 norbornene-2,3-dicar-boxyimide. Also the anhydrides of phosphorus based acids can be used. See, e.g., *The Peptides, Analysis, Synthesis, Biology*, supra and *Pure and Applied Chemistry,* 59(3), 331-344 (1987).

[0029] It is also possible to prepare the compounds by the solid phase method of Merrifield. Different solid supports and different strategies are known see, e.g., Barany and Merrifield in *The Peptides, Analysis, Synthesis, Biology*, Vol. 2, E. Gross and J. Meienhofer, eds. (Acad. Press, New York, 1980), Kneib-Cordonier and Mullen *Int. J. Peptide Protein Res.,* 30, 705-739 (1987) and Fields and Noble *Int. J. Peptide Protein Res.,* 35, 161-214 (1990). The synthesis of compounds in which a peptide bond is replaced by an isostere, can, in general, be performed using the previously described protecting groups and activation procedures. Procedures to synthesize the modified isosteres are described in the literature e.g., for the —CH_2—NH— isostere and for the —CO—CH_2— isostere.

[0030] Removal of the protecting groups, and, in the case of solid phase peptide synthesis, the cleavage from the solid support, can take place in different ways, depending on the nature of those protecting groups and the type of linker to the solid support. Usually deprotection takes place under acidic conditions and in the presence of scavengers. See, e.g., volumes 3, 5 and 9 of the series on *The Peptides Analysis, Synthesis, Biology*, supra.

[0031] Another possibility is the application of enzymes in synthesis of such compounds; for reviews see, e.g., H. D. Jakubke in *The Peptides, Analysis, Synthesis, Biology*, Vol. 9, S. Udenfriend and J. Meienhofer, eds. (Acad. Press, New York, 1987).

[0032] Although possibly not desirable from an economic point of view, oligopeptides according to the invention could also be made according to recombinant DNA methods. Such methods involve the preparation of the desired oligopeptide thereof by means of expressing recombinant polynucleotide sequence that codes for one or more of the oligopeptides in question in a suitable microorganism as host. Generally the process involves introducing into a cloning vehicle (e.g., a plasmid, phage DNA, or other DNA sequence able to replicate in a host cell) a DNA sequence coding for the particular oligopeptide or oligopeptides, introducing the cloning vehicle into a suitable eucaryotic or prokaryotic host cell, and culturing the host cell thus transformed. When a eucaryotic host cell is used, the compound may include a glycoprotein portion.

[0033] As used herein, a "functional analogue" or "derivative" of a peptide includes an amino acid sequence, or other sequence monomers, which has been altered such that the functional properties of the sequence are essentially the same in kind, not necessarily in amount. An analogue or derivative can be provided in many ways, for instance, through "conservative amino acid substitution." Also peptidomimetic compounds can be designed that functionally or structurally resemble the original peptide taken as the starting point but that are for example composed of non-naturally occurring amino acids or polyamides. With a "conservative amino acid substitution," one amino acid residue is substituted with another residue with generally similar properties (size, hydrophobicity), such that the overall functioning is likely not to be seriously affected. However, it is often much more desirable to improve a specific function. A derivative can also be provided by systematically improving at least one desired property of an amino acid sequence. This can, for instance, be done by an Ala-scan and/or replacement net mapping method. With these methods, many different peptides are generated, based on an original amino acid sequence but each containing a substitution of at least one amino acid residue. The amino acid residue may either be replaced by alanine (Ala-scan) or by any other amino acid residue (replacement net mapping). This way, many positional variants of the original amino acid sequence are synthesized. Every positional variant is screened for a spe-

cific activity. The generated data are used to design improved peptide derivatives of a certain amino acid sequence.

[0034] A derivative or analogue can also be, for instance, generated by substitution of an L-amino acid residue with a D-amino acid residue. This substitution, leading to a peptide that does not naturally occur in nature, can improve a property of an amino acid sequence. It is, for example, useful to provide a peptide sequence of known activity of all D-amino acids in retro inversion format, thereby allowing for retained activity and increased half-life values. By generating many positional variants of an original amino acid sequence and screening for a specific activity, improved peptide derivatives comprising such D-amino acids can be designed with further improved characteristics.

[0035] A person skilled in the art is well able to generate analogous compounds of an amino acid sequence. This can, for instance, be done through screening of a peptide library. Such an analogue has essentially the same functional properties of the sequence in kind, not necessarily in amount. Also, peptides or analogues can be circularized, for example, by providing them with (terminal) cysteines, dimerized or multimerized, for example, by linkage to lysine or cysteine or other compounds with side-chains that allow linkage or multimerization, brought in tandem- or repeat-configuration, conjugated or otherwise linked to carriers known in the art, if only by a labile link that allows dissociation. Synthetic versions of these oligopeptides as described above, and functional analogues or derivatives or breakdown products, are herein provided to be used in methods to the treatment of radiation injury and subsequent disease.

[0036] The term "pharmaceutical composition", as used herein, includes both the active composition alone or a composition containing the composition of the invention together with a pharmaceutically acceptable carrier, diluent or excipient. Acceptable diluents of an oligopeptide as described herein in the detailed description are for example physiological salt solutions or phosphate buffered salt solutions. In certain embodiments, an oligopeptide or composition is administered in an effective concentration to an animal or human systemically, for example, by intravenous, intramuscular or intraperitoneal administration. Another way of administration comprises perfusion of organs or tissue, be it in vivo or ex vivo, with a perfusion fluid comprising an oligopeptide or composition according to the invention. The administration may be done as a single dose, as a discontinuous sequence of various doses, or continuously for a period of time sufficient to permit substantial modulation of gene expression. In the case of a continuous administration, the duration of the administration may vary depending upon a number of factors that would readily be appreciated by those skilled in the art.

[0037] The administration dose of the active molecule may be varied over a fairly broad range. The concentrations of an active molecule that can be administered would be limited by efficacy at the lower end and the solubility of the compound at the upper end. The optimal dose or doses for a particular patient should and can be determined by the physician or medical specialist involved, taking into consideration well-known relevant factors such as the condition, weight and age of the patient, etc.

[0038] Oligopeptides according to the invention, such as, for example, acetate salts or esters of SEQ ID NO:2 and/or SEQ ID NO:3, are generally used in pharmaceutical compositions containing the active ingredient with a carrier, vehicle, diluent and/or excipient in an amount of about 0.1 to 99 wt % and preferably about 25-85 wt %. Pharmaceutical compositions may be formulated using carriers, diluents and/or excipients known in the art, for example, see *Remington's Pharmaceutical Sciences*, Remington, J. P., Easton, Pa.: Mack Pub. Co., 1990. The compounds may be administered in any desired form, including, for example, parenterally, orally, injection, transdermally or by suppository using known methods. intraperitoneal delivery is a preferred means of administration.

[0039] The active molecule may be administered directly in a suitable vehicle, such as, for example, phosphate-buffered saline ("PBS") or solutions in alcohol or DMSO. Pursuant to preferred embodiments of the invention, however, the active molecule is administered through a single dose delivery using a drug-delivery system. A suitable drug-delivery system would be pharmacologically inactive or at least tolerable. It should preferably not be immunogenic nor cause inflammatory reactions, and should permit release of the active molecule so as to maintain effective levels thereof over the desired time period. Alternatives are known in the art as suitable for purposes of sustained release and are contemplated as within the scope of the invention. Suitable delivery vehicles include, but are not limited to, the following: microcapsules or microspheres; liposomes and other lipid-based release systems; viscous instillates; absorbable and/or biodegradable mechanical barriers and implants; and polymeric delivery materials, such as polyethylene oxide/polypropylene oxide block copolymers, polyesters, cross-linked polyvinyl alcohols, polyanhydrides, polymethacrylate and polymethacrylamide hydrogels, anionic carbohydrate polymers, etc. Useful delivery systems are well known in the art.

[0040] One formulation to achieve the active molecule release comprises injectable microcapsules or microspheres made from a biodegradable polymer, such as poly(dl-lactide), poly(dl-lactide-co-glycolide), polycaprolactone, polyglycolide, polylactic acid-co-glycolide, poly(hydroxybutyric acid), polyesters or polyacetals. Injectable systems comprising microcapsules or microspheres having a diameter of about 50 to about 500 micrometers offer advantages over other delivery systems. For example, they generally use less active molecules and may be administered by paramedical personnel. Moreover, such systems are inherently flexible in the design of the duration and rate of separate drug release by selection of microcapsule or microsphere size, drug loading and dosage administered. Further, they can be successfully sterilized by gamma irradiation.

[0041] The design, preparation, and use of microcapsules and microspheres are well within the reach of persons skilled in the art and detailed information concerning these points is available in the literature. Biodegradable polymers (such as lactide, glycolide and caprolactone polymers) may also be used in formulations other than microcapsules and microspheres; e.g., pre-made films and spray-on films of these polymers containing the active molecule would be suitable for use in accordance with the invention. Fibers or filaments comprising the active molecule are also contemplated as within the scope of the invention.

[0042] Another highly suitable formulation for a single-dose delivery of the active molecule in accordance with the

invention involves liposomes. The encapsulation of an active molecule in liposomes or multilamellar vesicles is a well-known technique for targeted drug delivery and prolonged drug residence. The preparation and use of drug-loaded liposomes is well within the reach of persons skilled in the art and well documented in the literature.

[0043] Yet another suitable approach for single-dose delivery of an active molecule in accordance with the invention involves the use of viscous installates. In this technique, high molecular weight carriers are used in admixture with the active molecule, giving rise to a structure that produces a solution with high viscosity. Suitable high molecular weight carriers include, but are not limited to, the following: dextrans and cyclodextrans; hydrogels; (cross-linked) viscous materials, including (cross-linked) viscoelastics; carboxymethylcellulose; hyaluronic acid; and chondroitin sulfate. The preparation and use of drug-loaded viscous instillates is well known to persons skilled in the art.

[0044] Pursuant to yet another approach, the active molecule may be administered in combination with absorbable mechanical barriers such as oxidized regenerated cellulose. The active molecule may be covalently or non-covalently (e.g., jonically) bound to such a barrier, or it may simply be dispersed therein.

[0045] Either fluid or solid unit dosage forms can be readily prepared for oral administration. For example, acetate salts or esters of SEQ ID NO:2 and/or SEQ ID NO:3 can be admixed with conventional ingredients such as dicalcium phosphate, magnesium aluminum silicate, magnesium stearate, calcium sulfate, starch, talc, lactose, acacia, methyl cellulose and functionally similar materials as pharmaceutical excipients or carriers. A sustained release formulation may optionally be used where appropriate or desirable. Capsules may be formulated by mixing, for example, acetate salts or esters of SEQ ID NO:2 and/or SEQ ID NO:3, with an inert pharmaceutical diluent and inserting this mixture into a hard gelatin capsule having the appropriate size. If soft capsules are desired, then a slurry of the compound with an acceptable vegetable, light petroleum or other inert oil can be encapsulated in a gelatin capsule or similar capsules.

[0046] Suspensions, syrups and elixirs may be used for oral administration of fluid unit dosage forms. A fluid preparation including oil may be used for oil soluble forms. A vegetable oil such as corn oil, peanut oil or sunflower oil, for example, together with flavoring agents, sweeteners and any preservatives produces an acceptable fluid preparation. A surfactant may be added to water to form a syrup for fluid unit dosages. Hydro-alcoholic pharmaceutical preparations may be used having an acceptable sweetener (such as sugar, saccharin, or a biological sweetener, preferably a low carbohydrate sweetener, such as manitol or sorbitol) and a flavoring agent in the form of an elixir.

[0047] Pharmaceutical compositions for parenteral and suppository administration can also be obtained using techniques standard in the art. In an exemplary embodiment, acetate salt or ester of SEQ ID NO:2 and/or SEQ ID NO:3 is administered as a pharmaceutical agent suitable for oral administration. In another exemplary embodiment, acetate salts or esters of SEQ ID NO:2 and/or SEQ ID NO:3 may be injected using an appropriate vehicle such as saline.

[0048] The pharmaceutical carriers acceptable for the purposes of this invention include carriers that do not adversely affect the drug, the host, or the material comprising the drug delivery device. Suitable pharmaceutical carriers include sterile water, saline, dextrose, dextrose in water or saline condensation products of castor oil and ethylene oxide (combining about 30 to 35 moles of ethylene oxide per mole of castor oil), liquid acid, lower alkanols, oils such as corn oil, peanut oil, sesame oil and the like, with emulsifiers such as mono- or diglyceride of a fatty acid; or a phosphatide, e.g., lecithin, and the like; glycols, polyalkylene glycols, aqueous media in the presence of a suspending agent, for example, sodium carboxymethyl cellulose, sodium alginate, poly(vinylpyrrolidone), and the like, alone, or with suitable dispensing agents such as lecithin, polyoxyethylene stearate, and the like. The carrier may also contain adjuvants such as preserving agents, stabilizing agents, wetting agents, emulsifying agents and the like together with penetration enhancers and an oligopeptide of the invention such as acetate salts or esters of SEQ ID NO:2 and/or SEQ ID NO:3.

[0049] The effective dose for mammals may vary due to such factors as age, body mass, activity level or condition of the subject being treated. For example, an effective dosage of acetate salts or esters of SEQ ID NO:2 and/or SEQ ID NO:3 is from about 0.1 to about 35 milligrams per kilogram when administered parenterally. Dosages may be significantly higher for administration in oral or other external form.

[0050] In an exemplary embodiment, the method includes administering an effective amount of an oligopeptide of the invention, such as acetate salts or esters of SEQ ID NO:2 and/or SEQ ID NO:3, or an effective amount of a pharmaceutical composition containing an oligopeptide of the invention, such as acetate salts or esters of SEQ ID NO:2 and/or SEQ ID NO:3, to a subject, such as a mammal (e.g., a human), thought to be in need of such treatment. For example, a subject that may benefit from the method is a subject believed to be suffering from an acute inflammation disorder such as sepsis or septic shock.

[0051] The invention is further explained with the aid of the following illustrative examples.

EXAMPLES

Peptide Synthesis

[0052] Peptides mentioned here were prepared commercially by solid-phase synthesis using the fluorenylmethoxycarbonyl (Fmoc)/tert-butyl-based methodology with 2-chlorotrityl chloride resin as the solid support. The side-chain of glutamine was protected with a trityl function. The peptides were synthesized manually. Each coupling consisted of the following steps: (i) removal of the alpha-amino Fmoc-protection by piperidine in dimethylformamide (DMF), (ii) coupling of the Fmoc amino acid (3 eq) with diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt) in DMF/N-methylformamide (NMP) and (iii) capping of the remaining amino functions with acetic anhydride/diisopropylethylamine (DIEA) in DMF/NMP. Upon completion of the synthesis, the peptide resin was treated with a mixture of trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (TIS) 95:2.5:2.5. After 30 minutes TIS was added until decolorization. The solution was evaporated in vacuo and the peptide precipitated with diethyl ether. The crude peptides were dissolved in water (50-100 mg/ml) and purified by reverse-phase high-performance liquid chromatography (RP-

HPLC). HPLC conditions were: column: Vydac TP21810C18 (10×250 mm); elution system: gradient system of 0.1% TFA in water v/v (A) and 0.1% TFA in acetonitrile (ACN) v/v (B); flow rate 6 ml/min; absorbance was detected from 190-370 nm. There were different gradient systems used. For example for peptides LQG and LQGV (SEQ ID NO:3): 10 minutes 100% A followed by linear gradient 0-10% B in 50 minutes. For example for peptides VLPALP (SEQ ID NO:6) and VLPALPQ (SEQ ID NO:5): 5 minutes 5% B followed by linear gradient 1% B/minute. The collected fractions were concentrated to about 5 ml by rotation film evaporation under reduced pressure at 40° C. The remaining TFA was exchanged against acetate by eluting two times over a column with anion exchange resin (Merck II) in acetate form. The elute was concentrated and lyophilized in 28 hours. Peptides later were prepared for use by dissolving them in PBS.

NO Experiment

[0053] Cell culture. The RAW 264.7 murine macrophage cell line, obtained from American Type Culture Collection (Manassas, Va., USA), were cultured at 37° C. in 5% CO_2 using DMEM containing 10% fetal calf serum (FCS), 50 U/ml penicillin, 50 μg/ml streptomycin, 0.2 M Na-pyruvate, 2 mM glutamine and 50 μM 2-mercaptoethanol (Bio Whittaker, Europe). The medium was changed every 2 days.

[0054] Nitrite measurements. Nitrite production was measured in the RAW 264.7 macrophage supernatants. The cells (7.5×10^5/ml) were cultured in 48-well plates in 500 ml of culture medium. The cells were stimulated with LPS (10 microg/ml) and/or peptide (1 pg/ml, 1 ng/ml, 1 μg/ml) for 24 hours, then the culture media were collected. Nitrite was measured by adding 100 microl of Griess reagent (Sigma) to 100 microl samples of culture medium. The OD_{540} was measured using a microplate reader, and the nitrite concentration was calculated by comparison with the OD_{540} produced using standard solutions of sodium nitrite in the culture medium.

Results NO Experiment

[0055] NO production is a central mediator of the vascular and inflammatory response. Our results show that macrophages (RAW 264.7) stimulated with LPS produce large amount of NO. However, those cells co-stimulated with SEQ ID NO:3 or SEQ ID NO:2 even in a very low dose (1 pg/ml) inhibited the production of NO.

Example I

[0056] SEQ ID NO:2 was tested and compared with PBS (control) in a double blind animal study for the peptide's relative ability to aid recovery in a mouse renal ischemia reperfusion test. In this test, the mice were anesthetized, and one kidney from each mouse was removed. The other kidney was tied off for 25 minutes, and the serum urea levels were allowed to increase. Both before and after tying off, the peptide was administered to thirty (30) different mice (5 mg oligopeptide/kg body mass intravenously), after which, the mortality of the mice was determined for each oligopeptide as well as was the BUN concentration at two hours, 24 hours and 72 hours. The results are shown in Table 1 below.

[0057] Under inhalation anesthesia, the left kidney with its artery and vein was isolated and occluded for 25 minutes using a microvascular clamp. During surgery animals were placed on a heating path to maintain body temperature at 37° C. Five minutes before placing the clamp, and 5 minutes before releasing the clamp, 5 mg/kg of peptide, dissolved in 0.1 mL of sterile saline, was administered intravenously. After reperfusion of the left kidney the right kidney was removed. Kidney function was assessed by measuring blood urea nitrogen before clamping, and at 2, 24, and 72 hours after reperfusion.

Results - Table 1 (mortality at 72 hours post-reperfusion).

PBS	(AQGV) (SEQ ID NO:2)
6/10	0/10
*P < (vs PBS)	0.01

*2 × 2 Chi-square test. df = 1

[0058] As can be seen, mice administered the oligopeptide AQGV (SEQ ID NO:2) did much better in terms of both survival (a significant reduction in mortality versus the PBS control group) and reduced BUN concentration than the control group.

Example II

[0059] SEQ ID NO:3 was tested for its capacity to reduce BUN levels in the mice test as described above. The results are shown in Table 2.

TABLE 2

BUN after 25 mm renal ischemia tested in mice with peptides A-J

Peptide		t = 0 hr	2 hr	24 hr	72 hr	C-term: N-term:	CARBOXYL FREE
AQGV (SEQ ID NO:2)	Mean	9.713333	16.62	26.36	22.31	NMPF-46	AQGV (SEQ ID NO:2)
	Sd	1.882722	2.185203	20.62105	15.96444		
	N	30	10	20	10		
LQGV	mean	7.518182	17.53333	56.08333	73.17778	NMPF-4	LQGV

TABLE 2-continued

BUN after 25 mm renal ischemia tested in mice with peptides A-J						
Peptide		t = 0 hr	2 hr	24 hr	72 hr	C-term: CARBOXYL N-term: FREE
(SEQ ID NO:3)						(SEQ ID NO:3)
	SD	1.537356	2.956913	14.53573	23.3083	
	N	22	3	18	9	
PBS control	mean	8.172414	15.0875	56.81	82.075	
	SD	1.549169	2.215167	22.4659	34.82713	
	N	29	8	15	4	

[0060] At 24 hour post-reperfusion statistical analyses revealed P-values of:

p = 0.0008 NMPF-46 AQGV (SEQ ID NO:2)

p = 0.9301 NMPF-4 LQGV (SEQ ID NO:3)

[0061] At 24 hour post-reperfusion statistical analyses revealed P-values of:

p = 0.0001 NMPF-46 AQGV (SEQ ID NO:2)

p = 0.8328 NMPF-4 LQGV (SEQ ID NO:3)

P values were calculated by Mann Whitney U-test (SPSS for Windows).

Example III

[0062] To determine dose-response relationships SEQ ID NO:2, was tested in a dose-response manner in the mice test as described above. Peptides were tested at 0.3, 1, 3, 10 and 30 mg/kg dosages given as described in Example I. Serum urea levels are presented in Table 3. Statistical significance of changes in serum urea levels is presented in Table 4. Mortality data is presented in Table 5.

TABLE 3

Urea Levels in dose-response experiment		
	24 h	72 h
PBS	57.8	85.4
Peptide D (AQG) 0.3 mg/kg	38.4	30.4
Peptide D (AQG) 1.0 mg/kg	48.4	38.4
Peptide D (AQG) 3.0 mg/kg	39.3	40.3
Peptide D (AQG) 10.0 mg/kg	46.8	25.8
Peptide D (AQG) 30.0 mg/kg	52.8	58.9
Peptide B (AQGV (SEQ 1ID NO:2)) 0.3 mg/kg	62.4	86.7
Peptide B (AQGV (SEQ ID NO:2)) 1.0 mg/kg	50.0	52.6
Peptide B (AQGV (SEQ ID NO:2)) 3.0 mg/kg	37.4	19.6
Peptide B (AQGV (SEQ ID NO:2)) 10.0mg/kg	41.2	37.1
Peptide B (AQGV (SEQ ID NO:2)) 30.0 mg/kg	47.8	38.0
standard error	24 h	72 h
PBS	7.1	14.7
Peptide D (AQG) 0.3 mg/kg	8.6	3.5
Peptide D (AQG) 1.0 mg/kg	7.2	10.2
Peptide D (AQG) 3.0 mg/kg	3.5	10.7
Peptide D (AQG) 10.0 mg/kg	8.0	3.4
Peptide D (AQG) 30.0 mg/kg	9.5	12.9
Peptide B (AQGV (SEQ ID NO:2)) 0.3 mg/kg	10.8	14.1
Peptide B (AQGV (SEQ ID NO:2)) 1.0 mg/kg	11.7	14.3
Peptide B (AQGV (SEQ ID NO:2)) 3.0 mg/kg	7.6	2.6
Peptide B (AQGV (SEQ ID NO:2)) 10.0 mg/kg	8.5	6.9
Peptide B (AQGV (SEQ ID NO:2)) 30.0 mg/kg	5.8	7.8

[0063]

TABLE 4

statistical significance /p values (Mann Whitney U-Test) of serum urea levels in dose-response experiment 72 hours post-clamping. PBS control compared to peptide administered groups. (See, FIG. 3).	
	72 h
PBS	NA
AQG 0.3 mg/kg	0.001
AQG 1.0 mg/kg	0.009
AQG 3.0 mg/kg	0.02
AQG 10.0 mg/kg	0.000
AQG 30.0 mg/kg	0.23
AQGV (SEQ ID NO:2) 0.3 mg/kg	0.88
AQGV (SEQ ID NO:2) 1.0 mg/kg	0.054
AQGV (SEQ ID NO:2) 3.0 mg/kg	0.000
AQGV (SEQ ID NO:2) 10.0 mg/kg	0.001
AQGV (SEQ ID NO:2) 30.0 mg/kg	0.003

[0064]

TABLE 5

Mortality in dose-response experiment

	24 h	72 h
PBS	0-9	4-8
AQGV (SEQ ID NO:2) 0.3 mg/kg	0-9	2-10
AQGV (SEQ ID NO:2) 1.0 mg/kg	0-10	1-8
AQGV (SEQ ID NO:2) 3.0 mg/kg	1-10	0-10
AQGV (SEQ ID NO:2) 10.0 mg/kg	0-10	0-8
AQGV (SEQ ID NO:2) 30.0 mg/kg	0-8	3-10

Example IV

[0065] Septic shock experiments were set up to determine which peptide was best suited to battle acute inflammation.

[0066] Mice used in sepsis or septic shock experiments: Female BALB/c mice of 8-12 weeks of age were used for all experiments. The animals were bred in our facility under specific pathogen-free conditions according to the protocols described in the Report of European Laboratory Animal Science Associations (FELASA) Working group on Animal Health (Laboratory Animals 28: 1-24, 1994).

[0067] Injection protocols: For the endotoxin model, BALB/c mice were injected i.p. with 150-300 μg LPS (*E. coli* 026:B6; Difco Lab., Detroit, Mich., USA). Control groups were treated with PBS i.p. only. To test the effect of peptides, they were dissolved in PBS and injected i.p. at predetermined points in time after LPS treatment.

[0068] Mice were scored for sickness severity using the following measurement scheme:

[0069] 0 No abnormalities.

[0070] 1 Percolated fur, but no detectable behavior differences compared to normal mice.

[0071] 2 Percolated fur, huddle reflex, responds to stimuli (such as tap on cage), just as active during handling as healthy mouse.

[0072] 3 Slower response to tap on cage, passive or docile when handled, but still curious when alone in a new setting.

[0073] 4 Lack of curiosity, little or no response to stimuli, quite immobile.

[0074] 5 Labored breathing, inability or slow to self-right after being rolled onto back (moribund, sacrificed).

[0075] D Dead

[0076] A first set of septic shock experiments were set up to determine if the peptide SEQ ID NO:3 was capable of inhibiting LPS-induced septic shock in mice by treating mice with a single dose of peptide at 2 hours after LPS treatment. Peptides were used at 5 mg/kg bodyweight. ALB/c mice were injected i.p. with escalating doses LPS (*E. coli* 026:B6; Difco Lab., Detroit, Mich., USA), predetermined to be leading to 80-100% mortality in 24 72 hours. Control groups were treated with PBS i.p. only and showed no mortality. Results are presented in Table 6.

[0077] A second set of septic shock experiments were set up to determine if the peptides SEQ ID NO:3 and SEQ ID NO:2 were able to inhibit high dose LPS-induced septic shock in mice by treating mice with a double dose of peptide at 2 and 24 hours after LPS treatment. At each treatment, peptides were used at 5 mg/kg bodyweight. BALB/c mice were injected i.p. with high doses LPS (*E. coli* 026:B6; Difco Lab., Detroit, Mich., USA), predetermined to be leading to 80-100% mortality in 24-72 hours. Control groups were treated with PBS i.p. only and showed no mortality. Results are presented in Table 7.

[0078] A further set of septic shock experiments were set up to determine if the peptides SEQ ID NO:3 and SEQ ID NO:2 were most suited to be used early and/or late after or throughout the development of shock. For determining the percent of endotoxin shock survival after late or early treatment with peptide, BALB/c mice were injected i.p. with 300 μg LPS (*E. coli* 026:B6; Difco Lab.), predetermined to be leading to 100% mortality in 48 hours without peptide treatment. Control groups were treated with PBS i.p. only and showed no mortality. Results are presented in Table 8.

[0079] A comparative trial was set up to compare peptide MTR and SEQ ID NO:2. The comparative trial comprised 6 groups of 6 animals; two groups (1A and 1B) receiving placebo (PBS), one group (2) peptide MTR (source Pepscan, Lelystad, NL), one group (3) receiving peptide MTR (source Ansynth), one group (4) receiving peptide SEQ ID NO:2 (source Pepscan) and one group peptide SEQ ID NO:2 (source Ansynth). Peptide/placebo in these groups was administered 2 hours after LPS. LPS (source) was used at 10-11 mg/kg. Sickness scores were done at 0, 2, 22 26 42 and 48 hours after LPS injection. Results are presented in Table 9.

Results

[0080] To test the effect of peptide early in the development of development of shock, mice were treated at 2 hours or at 24 after treatment with varying doses of LPS by i.p. injection with test peptide at 5 mg/kg bodyweight. All LPS doses resulted in 100% mortality at 48-72 hours in the non-peptide treated mice. The results are shown in Table 6. SEQ ID NO:3 showed a marked protective effect against LPS-induced sepsis.

TABLE 6

Peptide tested	LPS dose	n	single dose administration 5 mg/kg — effect at t = 24 hr 0	1	2	3	4	5	D	effect at t = 48 hr 0	1	2	3	4	5	D
LQGV	7*	6		2	4						6					
(SEQ ID	7**	6		6							6					
NO:3)	8	6			5	1					5	1				
	8	6		3	3					4	2					
	10	6			6						6					
	10	6		2	2	1	1				4	1				1

[0081]

TABLE 7

P	LPS dose	n	Peptide administered twice (t = 2 hr and t = 24 hr) 5 mg/kg at high LPS dose — effect at t = 24 hr 0	1	2	3	4	5	D	effect at t = 48 hr 0	1	2	3	4	5	D
LQGV (SEQ ID NO:3)	10.5	5						5								5
	11	6					2	4						2		4
AQGV (SEQ ID NO:2)	10.5	6		2	3						5					
	11	4		2	4						6					

[0082] To evaluate the effect of peptide treatment at an early or late point in time of development of shock, mice were treated at 2 hours or at 24 after LPS injection by i.p. injection with test peptide at 5 mg/kg bodyweight. The mice were followed for 84 hours instead of for 48 hours in the earlier experiments. Table 8 depicts the results. SEQ ID NO:2 showed 100% survival and no remaining clinical signs of shock at 84 hours after LPS-treatment when given both early or late in the development of shock.

TABLE 8

Percent of mice surviving LPS-induced sepsis after treatment with a single injection of test peptide (at 5 mg/kg body weight) at 2 or 24 hours after induction of sepsis by treatment with LPS.

	% SURVIVAL IN TIME (HOURS) 0	14	24	48	84
TREATMENT 2 HOURS AFTER LPS TREATMENT					
PBS	100	100	100	0	0
LQGV (SEQ ID NO:3)	100	100	100	100	100
AQGV (SEQ ID NO:2)	100	100	100	100	100
TREATMENT 24 HOURS AFTER LPS TREATMENT					
PBS	100	100	100	0	0
LQGV SEQ ID NO:3	100	100	100	0	0
AQGV SEQ ID NO:2	100	100	100	100	100

[0083]

TABLE 9

comparative trial MTR and AQGV, each from two sources		Sickness scores						
	No.	0 hrs	2 hrs	22 hrs	26 hrs	42 hrs	48 hrs	Survival %
Group 1A	1	0	2	4	4	5	dead	
PBS	2	0	2	3	3	5	dead	
	3	0	2	5	dead			
	4	0	2	3	3	3	2	
	5	0	2	4	4	dead		
	6	0	2	5	5	dead		
Group 1B	1	0	2	4	4	4	dead	
PBS	2	0	2	5	dead			
	3	0	2	3	3	2	1	
	4	0	2	4	dead			
	5	0	2	4	4	5	dead	
	6	0	2	5	5	dead		17%
Group 2	1	0	2	dead				
#970	2	0	2	3	3	5	dead	
MTR	3	0	2	2	2	2	2	
	4	0	2	2	2	2	2	
	5	0	2	2	2	4	dead	
	6	0	2	4	5	dead		33%
Group 3	1	0	2	2	1	1	1	
#Ansynth 12	2	0	2	2	2	2	1	
MTR	3	0	2	4	5	dead		
	4	0	2	3	4	4	dead	
	5	0	2	2	3	5	dead	
	6	0	2	2	2	2	2	50%
Group 4	1	0	2	2	2	2	2	
#971	2	0	2	3	3	2	1	
AQGV	3	0	2	2	2	2	2	
(SEQ ID	4	0	2	2	2	2	2	
NO:2)	5	0	2	4	5	dead		
	6	0	2	2	3	2	1	83%
Group 5	1	0	2	3	3	2	1	
#Ansynth 46	2	0	2	3	3	3	1	
AQGV	3	0	2	2	2	1	1	
(SEQ ID	4	0	2	4	4	5	dead	
NO:2)	5	0	2	2	2	1	1	
	6	0	2	2	2	1	1	83%

LPS = 10-11 mg/kg
#970 = 5 mg/kg
#971 = 5 mg/kg
#Ansynth 12 LPS treatment
#Ansynth 46 compound treatment

Example V

Human Phase Trials

Materials

[0084] SEQ ID NO:2) was Used in Human Trials Identified as EA-230

[0085] The active compound (H-Ala-Gln-Gly-Val-OH.xAcOH) was manufactured by Diosynth, Oss, NL, whereas filling and finishing of the final product was performed by Octoplus, Leiden, NL. The injection fluid was supplied in 20 mL vials containing 11 mL of 40 mg/mL EA-230. The final product was a clear to slightly opalescent injection fluid. Batch No 05C21701-01A. An analyses report of the producer is included as Appendix 1.

[0086] Phosphate buffered saline (PBS) was used as placebo.

[0087] Lipopolysaccharide (LPS) was used in human trials

[0088] LPS is a USP reference standard derived from *E. coli* (EC6, Catalog No. 1235503), and was obtained from the following supplier:

[0089] U.S. Pharmacopoeia

[0090] 12601 Twinbrook Parkway

[0091] Rockville, Md. 20852 (USA)

[0092] LPS was supplied in vials containing 1000 ng from a single batch.

Methodology:

The study consisted of two parts:

[0093] Part 1: A Phase I, double blind, randomized, single dose, placebo controlled, dose escalation study with 4 groups of 8 subjects (Groups 1-4). In each group 6 subjects were randomized to active medication and 2 to placebo. The first group received a single intravenous injection of EA-230 or placebo. After all subjects had completed Day 7 assessments, the safety review board decided on continuation of the study until the maximum tolerated dose had been assessed or the highest dose. Placebo subjects were pooled for analysis.

[0094] Part 2: A Phase I, double blind, randomized, single dose, placebo controlled study. The second part consisted of 1 group of 12 subjects (Group 5). All twelve subjects received LPS (4 ng/kg *E. coli* EC6) as a 2-minute injection, 30 minutes prior to EA-230 administration. Eight subjects received 10 mg/kg EA-230 (the most effective dose, as established by a biological assay (whole blood cell proliferation) during Part 1 of the study), and 4 subjects received placebo. Cytokines profiles were assessed to establish the effect of EA-230 on inflammatory parameters.

[0095] Number of Subjects:

[0096] 44 planned, 44 randomized, 44 treated, 44 completed.

[0097] 32 (4 groups of 8) in the first part and 12 (1 group) in the second part of the study.

Diagnosis and Main Criteria for Inclusion:

[0098] Healthy Caucasian male subjects, aged 18-50 years inclusive, with a BMI within 18-29 kg/m^2 inclusive.

Test Product, Dose and Mode of Administration, Batch Number:

[0099] Group 1: EA-230 1 mg/kg body weight, intravenous 2-min infusion, batch No 05C21701-01A.

[0100] Group 2: EA-230 3 mg/kg body weight, intravenous 2-min infusion, batch No 05C21701-01A.

[0101] Group 3: EA-230 10 mg/kg body weight, intravenous 15-min infusion, batch No 05C21701-01A.

[0102] Group 4: EA-230 30 mg/kg body weight, intravenous 15-min infusion, batch No 05C21701-01A.

[0103] Group 5: LPS, followed by EA-230 10 mg/kg, intravenous 15-min infusion, batch No 05C21701-01A

Duration of Treatment:

[0104] Per subject, an hospitalization from the evening before administration until 24 hours post-dose and a follow up visit 7 days after hospitalization. The screening visit occurred within 21 days before study drug administration.

Reference therapy, dose and mode of administration:

[0105] Placebo: Phosphate buffered saline (PBS)

[0106] Group 1 to 4: PBS, intravenous 2- or 15-min infusion.

[0107] Group 5: LPS, followed by PBS, intravenous 15-min infusion.

Criteria for Evaluation:

[0108] Pharmacokinetics (part 1 and part 2):

[0109] AUC, clearance, volume of distribution and elimination half-life.

[0110] Safety (part 1 and part 2):

[0111] Adverse events (AEs), vital signs, ECG, hematology, clinical chemistry, and urinalysis.

[0112] Whole blood cell proliferation (part 1 and part 2)

[0113] Pharmacodynamics (part 2):

[0114] Measurement of body temperature, vital signs, and the following cytokines: TNFα, IL-6, IL-8, IL-10, CRP and granulocyte response following stimulation with LPS. Additional cytokines may be measured after completion of the present study on stored plasma samples.

Statistical Methods:

[0115] Pharmacokinetics (Part 1 and Part 2):

[0116] Plasma concentration time profiles of EA-230 were analyzed. Pharmacokinetic parameters AUC, clearance, volume of distribution and elimination half-life were obtained for EA-230.

[0117] Safety (Part 1 and Part 2):

[0118] Incidence of treatment emergent AEs and treatment emergent treatment related AEs were summarized. Hematological, biochemical, urinalysis parameters, vital signs and their changes from baseline were summarized over time using n, mean, standard deviation, median and range.

Results

Summary of Results:

Pharmacokinetics:

[0119] PK samples were collected pre-dose and from 5 minutes to 4 hours after the start of study drug administration.

[0120] Mean EA-230 concentration in plasma is depicted in FIG. 1.

[0121] Inter-subject variability in EA-230 concentration in plasma and derived PK parameters was high. The drug was rapidly eliminated: average concentrations were below LOQ by 15 minutes after the 1-mg/kg dose to 2 h after dosing after the 30-mg/kg dose. No first-order terminal elimination phase could be observed in the profiles. C_{max} and AUC_{last} values are summarized in the table below.

Study part	Treatment	C_{max} (μg/mL)	AUC_{last} (μg · min/mL)
1	1 mg/kg (N = 6)	0.0325±	0.150 ± 0.112
	3 mg/kg (N = 6)	0.132 ± 0.0764	0.706 ± 0.390
	10 mg/kg (N = 6)	1.60 ± 1.13	13.0 ± 9.11
	30 mg/kg (N = 6)	3.22 ± 1.08	35.7 ± 12.9
2	LPS + 10 mg/kg	1.71 ± 1.33	18.2 ± 15.7

Values are arithmetic means ± SD.

[0122] 90% CIs of the mean C_{max} and AUC_{last} treatment ratios calculated using ANOVA model were wide, making estimates little reliable. Therefore, no conclusion on dose-linearity of EA-230 PK can be drawn.

Pharmacodynamics:

[0123] PD analysis was performed on data from Part 2 only. Samples for cytokines (IL-6, IL-8, IL-10, and TNF a) and granulocytes (WBC, basophils, eosinophils, lymphocytes, monocytes, and neutrophils) were taken up to 7.5 h after dosing. Samples for CRP measurement were taken up to 168 h after dosing.

[0124] Cytokines, WBC, neutrophils, and CRP showed an increase consecutive to LPS challenge. Other granulocytes differentials showed a decrease. Most cytokines and monocytes were back to baseline values by 7.5 h after dosing. Average concentration changes of IL-6, IL-8, TNF-α, and CRP were generally larger in the placebo subjects than in the active subjects, as depicted in FIGS. **2**A, **2**B, **3**A, and **3**B respectively.

[0125] Other PD variables profiles were similar in placebo and active subjects. No significant difference between placebo and active subjects in PD variables changes was observed at any time, except in 1 test out of the 84 performed.

Safety (Adverse Events):

[0126] Thirteen treatment-emergent AEs ("TEAEs") in 8 subjects occurred during the first part of the study and 28 in 12 subjects during the second part. All TEAEs were mild, except two moderate events in 1 placebo subject having been challenged with LPS. TEAEs deemed treatment-related by the investigator were few and included mostly mild nervous system disorders. No difference in AE occurrence was observed between placebo and active subjects. No increase in the number or intensity of AEs with the dose of EA-230 was observed during the escalating dose part. AEs occurred more frequently during Part 2 because of the LPS challenge. No SAE occurred during the study.

[0127] Twenty-four hours after study drug administration, 30 subjects (68%) presented at least 1 laboratory abnormality. The most frequently recorded laboratory abnormalities were a low total bilirubin level, a low cholesterol level, and a high protein level. Low total bilirubin level and low cholesterol level were more encountered in active than in placebo subjects. This difference was already observed at screening visit. None of the abnormalities were deemed clinically significant by the Investigator.

[0128] All 44 subjects had weak positive or positive level of urine urobilinogen 24 h after study drug administration.

The same abnormality was already observed at screening visit for each subject. Five subjects had weak positive or positive level of occult blood in urine 24 h after treatment. Three subjects had urine pH value above normal range and two subjects had rare squamous epithelial cells in urine.

[0129] Twenty-four hours after study drug administration, 6 subjects (14%) presented vital signs abnormalities (3 low HR, 2 high DBP, and 1 low SBP). At follow-up, 5 subjects (1%) presented vital signs abnormalities (3 low SBP, 1 high DBP, and 1 high HR). These abnormal parameters were not considered clinically significant by the Investigator.

[0130] Subjects of Group 5 showed an increase of oral body temperature starting approximately 2 h after study drug administration and peaking around 4 h. Treatment with EA-230 seemed to significantly reduce the increase in body temperature (+0.6° C. in active subjects vs +1.6° C. in placebo subjects), 4.5 h after LPS challenge (p-value=0.018).

[0131] LPS challenge also caused a decrease in blood pressure which was less marked in active than in placebo subjects and an increase in HR. No abnormality was observed in physical examinations performed 24 h after study drug administration and at follow-up.

[0132] No abnormal QTc was observed during the study. None of the occasional abnormal ECG parameters were considered clinically significant by the investigator.

[0133] EA 230 was rapidly eliminated from plasma. No conclusion on dose-linearity of EA 230 PK can be drawn because of large inter-subject variations in PK parameters.

[0134] Average concentration changes of IL-6, IL-8, TNF-α, and CRP were generally larger in the placebo subjects than in the active subjects after LPS challenge, but these differences were not significant.

[0135] Administration of a single dose of EA-230 was safe and well tolerated. No difference in AE number, abnormal laboratory parameters, vital signs, and ECG abnormalities were observed during the escalating dose part (from 1 mg/kg to 30 mg/kg).

[0136] The number of AEs, vital signs abnormalities and abnormal laboratory parameters were higher in Part 2 than in Part 1, because of the LPS challenge. EA-230 tended to reduce changes in blood pressure and body temperature in LPS-challenged subjects.

[0137] In conclusion, 44 healthy male subjects were included in the study, and all completed the study. The study was designed in two parts (Part 1 and Part 2).

[0138] Part 1 was a dose escalating study with 4 groups of 8 subjects (Group 1-4). In each group 6 subjects were randomized to active medication and 2 to placebo.

[0139] Group 1 received a single intravenous injection of EA-230 (1 mg/kg) or placebo.

[0140] Group 2 received a single intravenous injection of EA-230 (3 mg/kg) or placebo.

[0141] Group 3 received a single intravenous injection of EA-230 (10 mg/kg) or placebo.

[0142] Group 4 received a single intravenous injection of EA-230 (30 mg/kg) or placebo.

[0143] Part 2 consisted of 1 group of 12 subjects (Group 5) receiving LPS (4 ng/kg *E. coli* EC6) as a 2-minutes injection, 30 minutes prior to EA-230 administration. Eight subjects received EA-230 at the most effective dose (10 mg/kg), as established during Part 1, and 4 subjects received placebo.

Pharmacokinetics

[0144] PK samples were collected pre-dose and from 5 minutes to 4 h after the start of study drug administration.

[0145] Theoretically, t_{max} should have been the end of infusion time. However, it was observed 5 minutes before the end of infusion in subjects treated with 10 mg/kg EA-230 alone and in 2 subjects treated with 30 mg/kg EA-230, and 10 minutes before the end of infusion in most subjects treated with 10 mg/kg EA-230 with LPS challenge. The investigator did not report any infusion problem for these subjects. Therefore, no obvious explanation can be provided for this peculiarity.

[0146] Inter-subject variability in EA-230 concentration in plasma and derived PK parameters was high. The drug was rapidly eliminated: average concentrations were below LOQ by 15 minutes after the 1-mg/kg dose to 2 h after dosing after the 30-mg/kg dose. No first-order terminal elimination phase could be observed in the profiles. 90% CIs of the mean C_{max} and AUC_{last} treatment ratios were wide, making estimates little reliable. Therefore, no conclusion on dose-linearity of EA-230 PK can be drawn.

Pharmacodynamics

[0147] PD analysis was performed on data from Part 2 only. Samples for cytokines (IL-6, IL-8, IL-10, and TNF-α) and granulocytes (WBC, basophils, eosinophils, lymphocytes, monocytes, and neutrophils) were taken up to 7.5 h after dosing. Samples for CRP measurement were taken up to 168 h after dosing.

[0148] Cytokines, WBC, neutrophils, and CRP showed an increase consecutive to LPS challenge. Other granulocytes differentials showed a decrease. Most cytokines and monocytes were back to baseline values by 7.5 h after dosing. Average concentration changes of IL-6, IL-8, TNF-α; and CRP were generally larger in the placebo subjects than in the active subjects. Other PD variables profiles were similar in placebo and active subjects. No significant difference between placebo and active subjects in PD variables changes was observed at any time, except in one test out of the 84 performed.

Safety

[0149] Thirteen treatment-emergent AEs (TEAEs) in 8 subjects occurred during the first part of the study and 28 in 12 subjects during the second part. All TEAEs were mild, except two moderate events in 1 placebo subject having been challenged with LPS. TEAEs deemed treatment-related by the investigator were scarce and included mostly mild nervous system disorders. No difference in AE occurrence was observed between placebo and active subjects. No increase in the number or intensity of AEs with the dose of EA-230 was observed during the escalating dose part. AEs occurred more frequently during Part 2 because of the LPS challenge. No SAE occurred during the study.

[0150] Twenty-four hours after study drug administration, 30 subjects (68%) presented at least 1 laboratory abnormal-

ity. The most frequently recorded laboratory abnormalities were a low total bilirubin level, a low cholesterol level, and a high protein level. Low total bilirubin level and low cholesterol level were more encountered in active than in placebo subjects. This difference was already observed at screening visit. None of the abnormalities were deemed clinically significant by the Investigator.

[0151] All 44 subjects had weak positive or positive level of urine urobilinogen 24 h after study drug administration. The same abnormality was already observed at screening visit for each subject. Five subjects had weak positive or positive level of occult blood in urine 24 h after treatment. Three subjects had urine pH value above normal range and two subjects had rare squamous epithelial cells in urine.

[0152] Twenty-four hours after study drug administration, 6 subjects (14%) presented vital signs abnormalities (3 low HR, 2 high DBP, and 1 low SBP). At follow-up, 5 subjects (11%) presented vital signs abnormalities (3 low SBP, 1 high DBP, and 1 high HR). These abnormal parameters were not considered clinically significant by the Investigator.

[0153] Subjects of Group 5 showed an increase of oral body temperature starting approximately 2 hours after study drug administration and peaking around 4 h. Treatment with EA-230 seemed to significantly reduce the increase in body temperature (+0.6° C. in active subjects vs +1.6° C. in placebo subjects), 4.5 h after LPS challenge (p-value= 0.018).

[0154] LPS challenge also caused a decrease in blood pressure which was less marked in active than in placebo subjects and an increase in HR. No abnormality was observed in physical examinations performed 24 h after study drug administration and at follow-up.

[0155] No abnormal QTc was observed during the study. None of the occasional abnormal ECG parameters were considered clinically significant by the Investigator.

[0156] In conclusion, administration of a single dose of EA-230 was safe and well tolerated. No difference in AE number, abnormal laboratory parameters, vital signs, and ECG abnormalities were observed between the different EA-230 doses (from 1 mg/kg to 30 mg/kg) during the escalating dose part.

[0157] The number of AEs, vital signs abnormalities and abnormal laboratory parameters were higher in Part 2 than in Part 1, due to the LPS challenge. EA-230 tended to reduce changes in blood pressure and body temperature in LPS-challenged subjects.

[0158] While this invention has been described in certain embodiments, the invention can be further modified within the spirit and scope of this disclosure. This application is therefore intended to cover any variations, uses, or adaptations of the invention using its general principles. Further, this application is intended to cover such departures from the present disclosure as come within known or customary practice in the art to which this invention pertains and which fall within the limits of the appended claims.

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What is claimed is:

1. An acetate of a tetrapeptide, wherein the terapeptide is AQGV (SEQ ID NO:2) or LQGV (SEQ ID NO:3).

2. The acetate of a tetrapeptide of claim 1, wherein the acetate of a tetrapeptide is an acetate salt or ester of the tetrapeptide.

3. A composition comprising the acetate of a tetrapeptide of claim 1 together with a pharmaceutically acceptable excipient.

4. A method of treating an acute inflammatory condition in a subject, said method comprising:

administering the acetate of a tetrapeptide of claim 1 to a subject having an acute inflammatory condition in an amount efficacious to reduce the inflammation in the subject as may be determined by a decrease in serum IL-6 levels in the subject.

* * * * *

APPENDIX II

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(54) Title: USE OF PEPTIDES FOR THE CONTROL OF RADIATION INJURY

(57) Abstract: The invention relates to the field of drug development against acute radiation injury caused by exposure to high-energy electromagnetic waves (X-rays, gamma rays) or particles (alpha particles, beta particles, neutrons). To date, there is no effective drug to ameliorate radiation injury after accidental exposure to ionizing irradiation. The invention provides a method of treating radiation injury of a subject in need thereof comprising administering to the subject a peptide, or functional analogue or derivative thereof, of smaller than 30 amino acids. Furthermore, the invention provides use of a peptide, or functional analogue or derivative thereof, of smaller than 30 amino acids for the production of a pharmaceutical composition for the treatment of a subject suffering from or believed to be suffering from radiation injury. In particular, the invention provides anti-radiation peptides having a dose reduction factor (DRF) against acute gamma irradiation of at least 1.10, said DRF determinable by testing which dose of radiation results in 50% mortality at 30 days (LD50/30) after whole body radiation (WBI) in a test group of mice treated with said peptide at 72 hours after WBI and, testing which dose of radiation results in 50% mortality at 30 days (LD50/30) after whole body radiation (WBI) in a control group of mice treated only with the vehicle of said peptide at 72 hours after WBI and wherein the DRF is calculated by dividing the LD5O/3O of the peptide-treated animals by the LD50/30 of the vehicle-treated animals.

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USE OF PEPTIDES FOR THE CONTROL OF RADIATION INJURY

5 TECHNICAL FIELD

[0001] The invention relates to the field of drug development against acute radiation injury caused by exposure to high-energy electromagnetic waves (X-rays, gamma rays) or particles (alpha particles, beta particles, neutrons). To date, there is no effective drug to ameliorate radiation injury after accidental exposure to ionizing irradiation.

10

BACKGROUND

[0002] Radiation injury is damage to tissues caused by exposure to radiation. Herein, radiation refers to ionizing radiation caused by high-energy electromagnetic waves (X-rays, gamma rays) or particles (alpha particles, beta particles, neutrons). Such radiation is emitted by 15 radioactive substances (radioisotopes), such as uranium, radon, and plutonium. Such radiation is also produced by man-made sources, such as x-ray and radiation therapy machines. Radiation dose is measured in several different units, but all relate to the amount of energy deposited. The units include the roentgen (R), the gray (Gy), and the sievert (Sv). The sievert and gray are similar, except the sievert takes into account the biologic effects of different types of radiation. 20 The two main types of radiation exposure are irradiation and contamination. Many radiation accidents expose a person to both.

[0003] Irradiation is exposure to radiation waves that pass directly through the body from outside the body. Irradiation can make a person sick immediately (acute radiation illness). Additionally, irradiation, particularly in high doses, can damage a person's genetic material 25 (DNA), causing chronic (delayed) disorders, such as cancer and birth defects. However, irradiation does not make the person or his tissues radioactive. Contamination is contact with and retention of radioactive material, typically in the form of a dust or liquid. The radioactive material may stay on the skin, where it can fall or be rubbed off, contaminating other people and objects. The material also may be absorbed by the body through the lungs, digestive tract, or 30 breaks in the skin. The absorbed material is transported to various sites in the body, such as the bone marrow, where it continues to release radiation. This internalized radiation does not cause

acute radiation illness such as internal bleeding only but may produce chronic disorders such as cancer as well.

[0004] People are constantly exposed to low levels of natural radiation (background radiation). Radiation comes from outer space (cosmic radiation), although much of it is blocked
5 by the earth's atmosphere. Exposure to cosmic radiation is greater for people living or working at high radioactive elements, particularly radon gas, also are present in many rocks and minerals. These elements end up in a variety of substances, including food and construction materials. In addition, people are exposed to radiation from man-made sources, including the environmental radiation that results from nuclear weapons testing and radiation from various medical tests and
10 treatments. The average person receives a total of about three to four mSv (1 mSv = $^1/_{1000}$ Sv) per year from natural radiation and man-made sources. People who work with radioactive materials and with x-ray sources are at risk of exposure to higher levels of radiation. People who are receiving radiation treatments for cancer may receive very high levels of radiation. Nuclear weapons release massive amounts of radiation. These weapons have not been used against
15 people since 1945. However, a number of nations now possess nuclear weapons, and several terrorist groups have also tried to obtain them, raising the possibility that these weapons could once again be used.

[0005] The damaging effects of radiation depend on several factors, including the amount (dose) and duration of exposure. A single, rapid dose of radiation to the entire body can
20 be fatal, but the same total dose given over a period of weeks or months may have much less effect. For a given dose, genetic damage is more likely with rapid exposure. The effects of radiation also depend on how much of the body is exposed. For example, more than 6 Gy generally causes death when the radiation is distributed over the entire body; however, when concentrated in a small area, as in radiation therapy for cancer, three or four times this amount
25 can be given without serious harm for the subject as a whole. The distribution of radiation is also important, because certain parts of the body are more sensitive to radiation. Organs and tissues in which cells are multiplying quickly, such as the intestines and bone marrow, are harmed more easily by radiation than those in which cells multiply more slowly, such as muscles and tendons. The genetic material of sperm and egg cells can be damaged by radiation. During

radiation therapy for cancer, therefore, every attempt is made to shield the more vulnerable parts of the body from radiation so that high doses can be delivered primarily to the cancer. Radiation exposure produces two types of injury: acute (immediate) and chronic (delayed). Acute radiation injury triggers inflammation through vascular endothelial damage leading to leaking vessels. A vascular response and a cellular response follow. Ionizing radiation depresses immunity and damages intestinal epithelium, both of which promote microbial translocation from the intestines.

[0006] Radiation therapy for cancer mainly produces symptoms in the part of the body that receives radiation. For example, in radiation therapy for rectal cancer, abdominal cramping and diarrhea are common because of the effects of radiation on the small intestine.

[0007] The search for non-toxic radioprotective agents which can protect normal tissue against radiation damage began soon after World War II. Extensive radiobiological research yielded numerous agents which, when given before radiation exposure, protected animals (primarily rodents) against radiation injuries [Prasad KN. Handbook of radiobiology. 2nd edn. Boca Raton, FL: CRC Press, 1995]. From these studies it became clear that agents, which scavenge free radicals and/or cause hypoxia, may be of radioprotective value. Unfortunately most of these compounds at radioprotective doses were found to be toxic to humans. With the decreased risk of nuclear confrontation experienced during the evolution of the cold war and later, the interest in the study of radioprotective agents markedly decreased. Due to rapid growth of X-ray-based diagnostic equipments and increased use of radiological procedures in the early diagnosis of disease, concerns are being raised about increased frequency somatic and heritable mutations that can enhance the risk of gene-linked diseases in present and future generations. Therefore, it has become imperative that normal tissues be protected against potential radiation damage no matter how small that damage might be.

[0008] Commonly, radioprotective agents are defined as compounds that are administered before exposure to ionizing radiation to reduce its damaging effects, including radiation-induced lethality [H.B. Stone et al., Models for evaluating agents intended for the prophylaxis, mitigation and treatment of radiation injuries. Report of an NCI Workshop, December 3-4, 2003, Radiat Res 162: 711-728.]. They have applications in radiological terrorism, military scenarios, clinical oncology, space travel, radiation site cleanup [R.H.

Johnson, Dealing with the terror of nuclear terrorism, Health Phys 87: S3-7., F.A.J. Mettler, G.L. Voelz, Major radiation exposure—what to expect and how to respond, N Engl J Med 346: 1554-1561, 2001] C.K. Nair, D.K. Parida, T. Nomura, Radioprotectors in radiotherapy, J Radiat Res (Tokyo) 42:21-37, J.K. Waselenko, T.J. MacVittie, W.F. Blakely, N. Pesik, A.L. Wiley, W.E. Dickerson, H.Tsu, D.L. Confer, C.N. Coleman, T. Seed, P. Lowry, J.O. Armitage, N. Dainiak, Medical management of the acute radiation syndrome: Recommendations of the Strategic National Stockpile Radiation Working Group, Ann Intern Med 140: 1037-1051.]. Recently, the U.S. Office of Science and Technology Policy and the Homeland Security Council have made the development of new radioprotectors a top research priority. Although synthetic radioprotectors such as the aminothiols have yielded the highest protective factors, typically they are more toxic than naturally occurring protectors. In general, the best radioprotective agents also have been reported to result in the highest behavioral toxicity.

[0009] In a military radiation scenario, the effective mitigation of radiation induced health consequences and performance-degrading effects can reduce the casualty load at medical treatment facilities, sustain a more effective operational force after a radiation exposure event, allow commanders to conduct operations in radiation field environments with reduced risk of decremented performance due to acute tissue injury, and reduce the negative psychological impact on personnel tasked to operate in contaminated environments. The ideal radioprotectant would be nontoxic, would not degrade performance, and would be effective after a single administration, particularly when expedited entry is required into an area with potential external radiation hazards.

[0010] In a paper (Landauer et al., NATO RTG-099 2005) presented at the NATO Human Factors and Medicine Panel Research Task Group 099 "Radiation Bioeffects and Countermeasures" meeting, held in Bethesda, Maryland, USA, June 21-23, 2005, and published in AFRRI CD 05-2, genisteine was forwarded as giving prevention of gamma radiation-induced mortality in mice, having a "Dose reduction Factor" (DRF) at the best dose (200 mg/kg; which resulted in the highest survival rate when administered to mice 24 hr before irradiation) of 1.16. When given at 1 hour prior to whole body irradiation (WBI), no radioprotection was observed. Other studies describing the radiation protection activity of a drug code-named ON-01210 that were presented at the 51st Radiation Research Society (April, 2004), show that this particular

drug ON-01210 (like other drugs that are currently under investigation for radiation-exposure) is protective only if it is given pre-radiation exposure. This particular drug has a sulfhydryl component (4-carboxystyrl-4-chlorobenzylsulfone) that works as an antioxidant, scavenging the free radicals that are generated as the radiation damages the cells.

5 **[0011]** Also, as stated in the annual report to the Congress of the US Department of Defense (March 2005; http://medchembio.amedd.army.mil/docs/CBDP_Report_To_Congress.pdf), currently, there are no commercially-available non-toxic pharmaceutical agents or diagnostic capabilities suitable for use in military operational environments. An aminothiol compound, amifostine, is FDA

10 approved for use in patients receiving chemotherapy or radiation therapy, but its performance-degrading toxic side effects prohibit its use in a fit fighting force, and its intravenous route of administration requires that medical professionals be available.. Other pharmacologic agents, such as hematopoietic cytokines for treating bone marrow injury, may be used off-label on a case-by-case basis by an individual physician, but regulatory restrictions for such use make it

15 impractical for treating large numbers of casualties during military operations. Antibiotics are commonly used to treat the infectious sequelae of radiological injuries, but they must be appropriately selected to effectively treat exogenous and endogenous systemic infections while only little affecting beneficial intestinal anaerobic bacteria. In addressing the issue of currently limited medical countermeasure alternatives, a novel compound, 5-androstenediol (5-AED;

20 Whitnall et al., Experimental Biology and Medicine 226:625-627 (2001)), has been under study at the Armed Forces Radiobiology Research Institute (AFRRI). Again, the compound showed good efficacy as a radioprotectant when administered prior to irradiation challenge in a mouse model. Improvements in survival were observed when AED was administered by sc injection between 24 h before and 2 h after gamma-irradiation of mice. A dose reduction factor of 1.3 was

25 calculated from probit survival curves for the administration prior to WBI. Protection was observed in both male and female mice, with and without subsequent inoculation with lethal doses of *Klebsiella pneumoniae.* No protection was observed with a number of other steroids: dehydroepiandrosterone (DHEA), 5-androstene-3B,7B, 17B-triol (AET), androstenedione, or estradiol. However, expanded studies in a nonhuman primate (NHP) model during the past year

30 in preparation for the IND application proved 5-AED is far less effective than in the mouse

model when administered as a radioprotectant but yielded good efficacy in the NHP model when administered therapeutically in serial doses shortly following irradiation.

[0012] *Acute Radiation Illness.* Acute radiation illness generally occurs in people whose entire body has been exposed to radiation. Acute radiation illness progresses through several stages, beginning with early symptoms (prodrome) and followed by a symptom-free period (latent stage). Various syndromes (patterns of symptoms) follow, depending on the amount of radiation the person received. The greater the amount of radiation, the more severe the symptoms and the quicker the progression from the early symptoms to the actual syndrome. The symptoms and time course are consistent from person to person for a given amount of radiation exposure. Doctors can predict a person's radiation exposure from the timing and nature of the symptoms. Doctors divide acute radiation syndromes into three groups based on the main organ system affected, although there is overlap among these groups.

[0013] The hematopoietic syndrome is caused by the effects of radiation on the bone marrow, spleen, and lymph nodes—the primary sites of blood cell production (hematopoiesis). Loss of appetite (anorexia), lethargy, nausea, and vomiting begin 2 to 12 hours after exposure to 2 Gy or more of radiation. These symptoms resolve within 24 to 36 hours after exposure, and the person feels well for a week or more. During this symptom-free period, the blood-producing cells in the bone marrow, spleen, and lymph nodes begin to waste away and are not replaced, leading to a severe shortage of white blood cells, followed by a shortage of platelets and then red blood cells. The shortage of white blood cells can lead to severe infections. The shortage of platelets may cause uncontrolled bleeding. The shortage of red blood cells (anemia) causes fatigue, weakness, paleness, and difficulty breathing during physical exertion. After four to five weeks, if the person survives, blood cells begin to be produced once more, but the person feels weak and tired for months.

[0014] The gastrointestinal syndrome is due to the effects of radiation on the cells lining the digestive tract. Severe nausea, vomiting, and diarrhea begin 2 to 12 hours after exposure to 4 Gy or more of radiation. The symptoms may lead to severe dehydration, but they resolve after two days. During the next four or five days, the person feels well, but the cells lining the digestive tract, which normally act as a protective barrier, die and are shed. After this

time, severe diarrhea—often bloody—returns, once more resulting in dehydration. Bacteria from the digestive tract invade the body, producing severe infections. People who have received this much radiation also likely develop the hematopoietic syndrome, which results in bleeding and infection and increases their risk of death.

5 **[0015]** The cerebrovascular (brain) syndrome occurs when the total dose of radiation exceeds 20 to 30 Gy. A person rapidly develops confusion, nausea, vomiting, bloody diarrhea, and shock. Within hours, blood pressure falls, accompanied by seizures and coma. The cerebrovascular syndrome is considered always fatal.

[0016] *Chronic Effects of Radiation.* Chronic effects of radiation result from damage 10 to the genetic material in dividing cells. These alterations may cause abnormalities of cell growth, such as cancer. In severely irradiated animals, damage to reproductive cells has been shown to lead to defective offspring (birth defects). However, little deformities resulting from irradiation have been observed in the offspring of survivors of the nuclear blasts in Japan. It may be that radiation exposure below a certain (unknown) level does not alter genetic material 15 enough to cause birth defects.

[0017] Irradiation injury is suspected when a person becomes ill after receiving radiation therapy or being exposed to radiation in an accident. No specific tests are available to diagnose the condition, although certain tests may be used to detect infection, low blood count, or organ malfunction. To determine the severity of radiation exposure, doctors measure the 20 number of lymphocytes (a type of white blood cell) in the blood. The lower the lymphocyte count 48 hours after exposure, the worse the radiation exposure.

[0018] Radioactive contamination, unlike irradiation, can be determined by surveying a person's body with a Geiger counter, a device that detects radiation. Swabs from the nose, throat, and any wounds also are checked for radioactivity.

25 **[0019]** The outcome of radiation injury depends on the dose, dose rate (how quickly the exposure has occurred), and distribution over the body as well as on the person's underlying state of health. In general, most people who have received more than 6 Gy of WBI die of gastrointestinal syndrome. Because doctors are unlikely to know the exact amount of radiation a person has received, they usually judge outcome by the person's symptoms. The 30 cerebrovascular syndrome is fatal within hours to a few days. The gastrointestinal syndrome

generally is fatal within three to ten days, although some people survive for a few weeks. Many people who receive proper medical care survive the hematopoietic syndrome, depending on their total amount of radiation; those who do not survive typically die after 8 to 50 days.

[0020] Irradiation has no current emergency treatment, but doctors closely monitor the person for the development of the various syndromes and treat the symptoms as they arise. Also, and unfortunately, very few medical products exist to counter the variety of acute and long-term toxicities that can result from nuclear or radiological attacks. Contamination requires immediate removal of the radioactive material to prevent it from being taken up by the body. Skin contaminated by radioactive materials should be scrubbed immediately with large amounts of soap and water or with a solution designed for this purpose, when available. Small puncture wounds should be cleaned vigorously to remove all radioactive particles, even though scrubbing may cause pain. Contaminated hair is clipped off, not shaved—shaving may abrade the skin and allow contamination to enter the body. Scrubbing continues until the Geiger counter shows that the radioactivity is gone. If a person has recently swallowed radioactive material, vomiting is induced. Some radioactive materials have specific antidotes that can prevent absorption of swallowed material. Most such antidotes are given only to people exposed to significant radioactive contamination, such as from a major reactor accident or nuclear explosion. Potassium iodide prevents the thyroid gland from absorbing radioactive iodine and lowers the risk of thyroid cancer. Other drugs, such as diethylene triamine pentaacetic acid (DTPA), ethylenediamine tetraacetic acid (EDTA), and penicillamine, can be given intravenously to remove certain radioactive elements after they have been absorbed.

[0021] When contamination is not suspected, nausea and vomiting can be reduced by taking drugs to prevent vomiting (antiemetics); such drugs are routinely given to people undergoing radiation therapy. Dehydration is treated with fluids given intravenously.

[0022] People with the gastrointestinal or hematopoietic syndrome are kept isolated so that they do not contact infectious microorganisms. Blood transfusions and injections of growth factors (such as erythropoietin and colony-stimulating factor) that stimulate blood cell production are given to decrease bleeding and increase blood counts. If the bone marrow is severely damaged, these growth factors are ineffective, and sometimes bone marrow transplantation is performed, although the success rate is low.

[0023] People with the gastrointestinal syndrome require anti-emetics, fluids given intravenously, and sedatives. Some people may be able to eat a bland diet. Antibiotics, such as neomycin, are given to kill bacteria in the intestine that may invade the body. Antibiotics as well as antifungal and antiviral drugs are given intravenously when necessary. Treatment for the
5 cerebrovascular syndrome is geared toward providing comfort by relieving pain, anxiety, and breathing difficulties. Drugs are given to control seizures.

[0024] People with chronic effects of radiation or disorders caused by radiation therapy receive treatment directed at their symptoms. Sores or ulcers can be removed or repaired surgically and can be helped to heal with the use of high-pressure (hyperbaric) oxygen therapy.
10 Radiation-induced leukemia is treated with chemotherapy. Blood cells can be replaced through transfusions. No treatment can reverse sterility, but low levels of sex hormones as a result of abnormal ovarian and testicular functioning can be treated with replacement hormones. Investigators are currently exploring ways to prevent or reduce radiation-induced normal tissue injury using cytokines, growth factors, and various other therapies. Amifostine or pilocarpine-
15 HCl have been shown to decrease the severity of dry mouth (xerostomia) in people with head and neck cancer treated with radiation therapy.

[0025] Clinical and experimental studies of the acute and late effects of radiation on cells have enhanced our knowledge of radiotherapy and have led to the optimization of radiation treatment schedules and to more precise modes of radiation delivery. However, as both normal
20 and cancerous tissues have similar response to radiation exposure, radiation-induced injury on normal tissues may present either during, or after the completion of, the radiotherapy treatment. Studies on both NSAIDs and prostaglandins have indeed shown some evidence of radioprotection. Both have the potential to increase the survival of cells but by entirely different mechanisms. Studies of cell kinetics reveal that cells in the mitotic (M) and late G2 phases of
25 the cell cycle are generally most sensitive to radiation compared with cells in the early S and G1/G0 phases. Furthermore, radiation leads to a mitotic delay in the cell cycle. Thus, chemical agents that either limit the proportion of cells in the M and G2 phases of the cell cycle or enhance rapid cell growth could in principle be exploited for their potential use as radioprotectors to normal tissue during irradiation. NSAIDs have been shown to exert
30 anti-cancer effects by causing cell-cycle arrest, shifting cells towards a quiescence state (G0/G1).

The same mechanism of action was observed in radioprotection of normal tissues. An increase in arachidonic acid concentrations after exposure to NSAIDs also leads to the production of an apoptosis-inducer ceramide. NSAIDs also elevate the level of superoxide dismutase in cells. Activation of heat shock proteins by NSAIDs increases cell survival by alteration of cytokine
5 expression. A role for NSAIDs with respect to inhibition of cellular proliferation possibly by an anti-angiogenesis mechanism has also been suggested. Several *in vivo* studies have provided evidence suggesting that NSAIDs may protect normal tissues from radiation injury. Prostaglandins do not regulate the cell cycle, but they do have a variety of effects on cell growth and differentiation. PGE2 mediates angiogenesis, increasing the supply of oxygen and nutrients,
10 essential for cellular survival and growth. Accordingly, PGE2 at sufficiently high plasma concentrations may enhance cellular survival by inhibiting pro-inflammatory cytokines such as TNF-a and IL-1ß. Thus, PGE2 acts as a modulator, rather than a mediator, of inflammation. Prospective studies have suggested the potential use of misoprostol, a PGE1 analogue, before irradiation, in prevention of radiation-induced side effects. The current understanding of the
15 pharmacology of NSAIDs and prostaglandins shows some potential to minimize the adverse effects of radiation on normal tissue when used preventively.

[0026] In addition to transiently inhibiting cell cycle progression and sterilizing those cells capable of proliferation, irradiation disturbs the homeostasis affected by endogenous mediators of intercellular communication (humoral component of tissue response to radiation).
20 Changes in the mediator levels may modulate radiation effects either by assisting a return to normality (e.g., through a rise in H-type cell lineage-specific growth factors) or by aggravating the damage. The latter mode is illustrated with reports on changes in eicosanoid levels after irradiation and on results of empirical treatment of radiation injuries with anti-inflammatory drugs. Prodromal, acute and chronic effects of radiation are accompanied by excessive
25 production of eicosanoids (prostaglandins, prostacycline, thromboxanes and leukotrienes). These endogenous mediators of inflammatory reactions may be responsible for the vasodilatation, vasoconstriction, increased microvascular permeability, thrombosis and chemotaxis observed after radiation exposure. Glucocorticoids inhibit eicosanoid synthesis primarily by interfering with phospholipase A2 whilst non-steroidal anti-inflammatory drugs
30 prevent prostaglandin/thromboxane synthesis by inhibiting cyclooxygenase. When administered

after irradiation on empirical grounds, drugs belonging to both groups tend to attenuate a range of prodromal, acute and chronic effects of radiation in man and animals.

[0027] U.S. Patent 5,380,668 to Herron (Jan. 10, 1995), the contents of the entirety of which are incorporated by this reference, discloses, among other things, various compounds
5 having the antigenic binding activity of hCG. The oligopeptides disclosed therein are disclosed generally for use in diagnostic methods. Various patents and patent applications to Gallo et al. (*e.g.*, U.S. Patent 5,677,275 (corresponding to WO 96/04008 A1), U.S. Patent 5,877,148 (also corresponding to WO 96/04008 A1), WO 97/49721 A1, U.S. Patent 6,319,504 (corresponding to WO 97/49373), U.S. Patent Application 2003/0049273 A1 (also corresponding to WO
10 97/49373), U.S. Patent 5,968,513 (corresponding to WO 97/49418), U.S. Patent 5,997,871 (corresponding to WO 97/49432), U.S. Patent 6,620,416, U.S. Patent 6,596,688, WO 01/11048 A2, WO 01/10907 A2., and U.S. Patent 6,583,109) relate to various oligopeptides and their use in, among other things, "inhibiting HIV infection," "treating or preventing HIV infection," "treating or preventing cancer," "treating or preventing a condition characterized by loss of body
15 cell mass," "treating or preventing a condition associated with pathological angiogenesis," "treating or preventing hematopoietic deficiency," "*ex vivo* gene therapy," "expanding blood cells *in vitro*," and/or "providing blood cells to a subject." As described in PCT International Publication No. WO 03/029292 A2 (published April 10, 2003), PCT International Publication No. WO 01/72831 A2 (published October 4, 2001), and U.S. Patent Application Publications
20 20020064501 A1 (published May 30, 2002), 20030119720 A1 (published June 26, 2003), 20030113733 A1 (published June 19, 2003), and 20030166556 A1 (published September 4, 2003), U.S. Patent Application No. 11/249,541, filed on October 13, 2005, International Application No. PCT/EP2005/003707, filed on April 8, 2005, U.S. Patent Application No. 10/821,256, filed on April 8, 2004, U.S. Patent Application No. 10/262,522, filed on September
25 30, 2002, International Application No. PCT/NL01/00259 (International Publication No. WO 01/72831 A2) filed March 3, 2001, U.S. Patent 6,844,315 and U.S. Patent 6,921,751, the contents of all of which are incorporated by this reference, compositions containing some of the oligopeptides described herein have immunoregulatory activity useful in, for example, the treatment of sepsis and other disease states and conditions.

[0028] The current invention relates to the body's innate way of modulating important physiological processes and builds on insights reported in PCT International Publications WO99/59617 and WO01/72831 and PCT International Application PCT/NL02/00639, the contents of the entirety of all of which are incorporated herein by this reference. These applications describe small gene-regulatory peptides that are present in pregnant women and are derived from proteolytic breakdown of placental gonadotropins, such as hCG. These breakdown products are often only about 2 to 6 amino acids long and were shown to have unsurpassed immunological activity that is exerted by regulating expression of genes encoding inflammatory mediators such as cytokines. Surprisingly, it was found that breakdown of hCG provides a cascade of peptides that helps maintain a pregnant woman's immunological homeostasis. These peptides balance the immune system to assure that the mother stays immunologically sound while her fetus does not get prematurely rejected during pregnancy, but instead is safely carried until its time of birth.

[0029] Furthermore, the current invention relates to US application 10/821,240 which provides methods for screening and identifying further small gene-regulatory peptides and using the results from such screens, for example, with peptides derived from a reference peptide. For example, peptides to be analyzed were derived from C-Reactive Protein (CRP) (*e.g.*, human CRP), such peptides include, LTSL, FVLS, NMWD, LCFL, MWDF, FSYA, FWVD, AFTV, and WDFV; peptides derived from Beta-catenin (*e.g.*, human CTNB), such as GLLG, TAPS, VCQV, CLWT, VHQL, GALH, LGTL, TLVQ, QLLG, YAIT, LCEL, GLIR, APSL, ITTL, QALG, HPPS, GVLC, LCPA, LFYA, NIMR, NLIN, LHPP, LTEL, SPIE, VGGI, QLLY, LNTI, LWTL, LYSP, YAMT, LHNL, TVLR, and LFYA; peptides derived from beta-hCG (*e.g.*, human CG), such as GLLLLLLLS, MGGTWA, TWAS, TLAVE, RVLQ, VCNYRDV, FESI, RLPG, PRGV, NPVVS, YAVALS, LTCDDP, EMFQ, PVVS, VSYA, GVLP, FQGL, and AVAL; peptides derived from Bruton's tyrosine kinase (*e.g.*, human BTK), such as LSNI, YVFS, LYGV, YVVC, FIVR, NILD, TIMY, LESI, FLLT, VFSP, FILE, TFLK, FWID, MWEI, QLLE, PCFW, VHKL, LYGV, LESI, LSNI, YVFS, IYSL, and NILD; and peptides derived from matrix metalloproteinase-2 (*e.g.*, human MM02), such as FKGA, FFGL,GIAQ, LGCL, YWIY, AWNA, ARGA, PFRF, APSP, CLLS, GLPQ, TFWP, AYYL, FWPE, CLLG, FLWC, RIIG, WSDV,

PIIK, GLPP, RALC, LNTF, LSHA, ATFW, PSPI, AHEF, WRTV, FVLK, VQYL, KFFG, FPFR, IYSA, and FDGI, and others

[0030] DISCLOSURE OF THE INVENTION

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The invention relates to the field of developing drugs against acute radiation injury caused by exposure to high-energy electromagnetic waves (x-rays/photons and/or natural gamma rays) and/or other high energy ionising particles (alpha particles, beta particles, neutrons, protons, pi-mesons). To date, there is no effective drug to ameliorate 10 radiation injury after accidental exposure to ionizing irradiation, or after damage to healthy tissues during therapeutic radiation or radio-mimetic agents; nor is there an effective prophylactic drug to prevent or minimize such injuries when administered before the event (for example, to first responders). The present inventors surprisingly observed that relatively small, non-toxic peptides can be effective as drugs against radiation damage. Importantly, the 15 anti-radiation peptides of the invention are not only useful as prophylactic agents but can also protect when administered several hours after exposure to radiation. This makes them extremely suitable for use in a military radiation scenario e.g. when dealing with the terror of nuclear terrorism. Accordingly, the invention provides a method of preventing or treating radiation injury of a subject in need thereof comprising administering to the subject a 20 peptide, or functional analogue thereof, of smaller than 30 amino acids. Preferably, said peptide or functional analogue thereof is administered to said subject post radiation, i.e. following exposure of said subject to a source of radiation. Furthermore, the invention provides use of a peptide, or functional analogue thereof, of smaller than 30 amino acids for the production of a pharmaceutical composition for the treatment of a subject suffering from 25 or believed to be suffering from radiation injury. In particular, the invention provides anti-radiation peptides having a dose reduction factor (DRF) against acute whole body irradiation of at least 1.10, said DRF determinable by testing which dose of whole body irradiation (WBI) results in 50% mortality at 30 days (LD50/30) in a test group of experimental rodents (e.g. mice) treated with said peptide immediately or up to 72 hours after WBI, versus the 30 dose of WBI that results in 50% mortality at 30 days (LD50/30) in an untreated control

group, and wherein the DRF is calculated by dividing the LD50/30 radiation dose of the peptide- treated animals by the LD50/30 radiation dose of the vehicle-treated animals.

[0031] The invention provides a method for treating a subject suffering from or believed to be suffering from radiation injury, said method comprising providing the subject with a pharmaceutical composition comprising an anti-radiation peptide of smaller than 30 amino acids. Current radioprotection agents are of a non-peptide nature or comprise large proteins such as cytokines. The present invention discloses that peptides, such as MTRVLQGVLPALPQVVC, that are smaller than 30 amino acids are useful for protection against and treatment of radiation injury. For the first time it is shown that a peptide drug is capable of reducing damaging effects of radiation when it is administered after exposure to radiation has taken place. For example, the anti-radiation peptide consists of up to 29, up to 28, up to 27, up to 26, up to 25, up to 24, up to 23, up to 22, up to 21, up to 20, up to 19, up to 18, up to 17, up to 16 or up to 15 amino acids.

[0032] It is however preferred that said peptide is smaller than 15 amino acids. For example, the anti-radiation peptide preferably consists of up to 14, up to 13, up to 12, up to 11, up to 10, up to 9 or up to 8 amino acids. Some examples of useful peptides are LPGCPRGVNPVVS, DINGFLPAL and QPLAPLVG. However, when peptides are used for self-medication, for example as is provided herein with an autoinjector, from a safety viewpoint it is preferred that said peptide is smaller than 7 amino acids.Such a peptide will generally not bind to the MHC receptors, thereby decreasing the risk of the development of autoimmunity initiated by an immune response against administered peptide.

[0033] This size of smaller than 7 amino acids (aa) is also particularly preferred because it was determined (when comparing peptides derived from the human proteome with those derived from pathogen proteomes, in particular of viruses or bacteria (Burroughs et al., Immunogenetics, 2004, 56: 311-320)) that with a peptide size of 7 aa only 3% overlap between self or non-self is found. For peptides of 6 aa, that overlap in human self with pathogen non-self was determined to be 30%, for peptides of 5 aa, 90%, and for 4 aa long (and smaller) peptides, 100% overlap between the peptides present in the human proteome and the peptides present in the proteome of pathogens was determined. Based on these data, it is now herein recognized that when the self-non-self difference is not present, risk of adverse immune reactions, such as

anaphylactic shock, is greatly diminished, which is a distinct advantage when non-medically trained persons administer any drug to themselves or to others.

[0034] From the viewpoint of preventing adverse reactions such as anaphylactic shock it is thus preferred that the peptide consists of 2 to 6 amino acids, more preferably consists of 3 to 5 amino acids, and most preferably consists of 3 or 4 amino acids. From the viewpoint of activity, based on a general insight that activity is broader with increasing peptide size, if only to withstand full proteolysis longer whereby metabolic fragments of 3 aa still have activity, it is herein preferred that said peptide consists of 4 amino acids. Above and below described compositions are preferably used for the treatment of acute radiation injury.

[0035] The use of peptides to protect against radiation injury has been proposed in the art. The Japanese patent applications JP09157291 and JP09157292 describe specific 6-mer and 9-mer peptide sequences having an *in vitro* inhibiting effect on activated oxygen, scavenging actions of activated oxygen free radicals and antioxidizing activity. It is speculated that the peptides are useful *in vivo* to suppress the adverse effects of various types of events known to be associated with active oxygen formation, including radiation damage. No *in vivo* radiation experiments were conducted.

[0036] JP09176187 teaches a histidine-containing 6-mer peptide analog having active oxygen-scavenging activity. Peritoneal administration of a peptide at 660 mg/kg of body weight 20 minutes prior to irradiation increased the survival rate of mice from 10% in the control group to 70% in the treated group. No *in vivo* post-radiation experiments were conducted.

[0037] WO2006/032269 describes a homogenate of blood cells from which components having a molecular weight of more than 3 kDa have been removed. The homogenate is reported to be suitable for improving the cellular immune response in a subject. Among a long list of diverse immunological diseases and pathological conditions, it is proposed that the homogenate can be administered to a patient in a prophylactic fashion in a treatment using chemotherapeutical agents and/or radiation in order to optimize the patient's general condition. It is noted that the study does not involve any radiation experiments. Furthermore, although the homogenate likely comprises among others a mixture of proteins, the identity of the active component(s) is not clear at all and they may well be of non-proteinaceous nature. At any rate, no distinct peptides have been isolated or identified therein.

[0038] EP 0572688 discloses a specific peptide comprising 14 amino acid residues which at 20 mg/kg body weight was found to confer protection in mice against whole body radiation. The effect was only observed when the compound was applied 1 hour before radiation. However, no difference relative to control data was observed when peptide was administered 1 hour after exposure to irradiation.

[0039] These prior art disclosures are in marked contrast to the present invention; the anti-radiation peptides as disclosed herein can provide protection even if administered several hours after whole body irradiation.

[0040] Subjects having received sub-lethal radiation doses will already benefit from the anti-inflammatory properties of some of the small peptides identified herein, but surprisingly most benefit will come from the anti-gastrointestinal syndrome activity of the small peptides, notably of the 3- and 4-mer peptides at dosages of above 1 mg/kg, preferably of above 5 mg/kg, more preferably of above 10 mg/kg bodyweight. Considering the low immunogenic nature of the small peptides (i.e. those of 3 to 4 aa), dosing to up to 100 mg/kg with small peptides, and in some cases when need for treatment is determined to be acute considering the condition of the subject in need of treatment, of up to 200 mg /kg, 500 mg/kg or even 1 g/kg will be possible. Consequently, the treatment of subjects who have radiation injury that comprises damage of the lining of the intestinal tract of said subject, the so-called gastrointestinal syndrome, has now been made possible; the peptides allowing the epithelial lining to slowly recover.

[0041] For a better activity of peptides under high radiation dosage it is preferred to select a peptide for inclusion in a pharmaceutical composition of the invention or for inclusion in an autoinjector according to the invention that has a dose reduction factor (DRF) against acute gamma irradiation of at least 1.10, said DRF determinable by testing which dose of radiation results in 50% mortality at 30 days (LD50/30) after whole body radiation (WBI) in a test group of mice treated with said peptide at 72 hours after WBI and, testing which dose of radiation results in 50% mortality at 30 days (LD50/30) after whole body radiation (WBI) in a control group of mice treated only with the vehicle of said peptide at 72 hours after WBI and, wherein the DRF is calculated by dividing the LD50/30 of the peptide-treated animals by the LD50/30 of the vehicle-treated animals.

[0042] It is even more preferred to use a peptide having a dose reduction factor (DRF) of at least 1.20, more preferably of at least 1.25, especially when said radiation injury is irradiation injury. Such peptides as identified herein are also called anti-radiation peptide. The invention provides a method and pharmaceutical composition for the treatment of irradiation injury irrespective whether the radiation is emitted by radioactive substances (radioisotopes), such as uranium, radon, and plutonium, or is produced by man-made sources, such as x-ray and radiation therapy machines.

[0043] The invention also provides use of a peptide of smaller than 30 amino acids for the production of a pharmaceutical composition for the treatment a subject suffering from or believed to be suffering from radiation injury. As above, it is preferred that said peptide be smaller than 15 amino acids, and, for self-medication or for administration by laymen, it is even more preferred that said peptide be smaller than 7 amino acids. Several useful 3-mer peptides for use in the production of a pharmaceutical composition for treatment of radiation injury are identified herein as VVC, LAG, and AQG.

[0044] Similarly, several useful 4-mer peptides for treatment of radiation injury are LQGV, QVVC, MTRV, AQGV, LAGV, LQAV, PGCP, VGQL, RVLQ, EMFQ, AVAL, FVLS, NMWD, LCFL, FSYA, FWVD, AFTV, LGTL, QLLG, YAIT, APSL, ITTL, QALG, GVLC, NLIN, SPIE, LNTI, LHNL, CPVQ, EVVR, MTEV, EALE, EPPE, LGTL, VGGI, RLPG, LQGA, and LCFL, useful 5-mer peptides for treatment of radiation injury are TLAVE, VEGNL, and LNEAL, useful 6-mer peptides for treatment of radiation injury are VLPALP, MGGTWA, LTCDDP, useful 7-mer peptides for treatment of radiation injury are VLPAPLQ, VCNYRDV, and CPRGVNP, a useful 8-mer peptide for treatment of radiation injury is QPLAPLVG and a useful 9-mer peptide for treatment of radiation injury is DINGFLPAL.

[0045] Other peptides, especially 3 or 4-mer peptides, can be found by testing for anti-cell cycle activity in proliferation assays, for example by using the plant growth assay as provided herein. Use of a peptide for the production of a pharmaceutical composition for the treatment a subject suffering from or believed to be suffering from radiation injury wherein said peptide consists of 2 to 6 amino acids is herein particularly provided. Again, from the viewpoint of preventing adverse reactions such as anaphylactic shock it is thus preferred that the pharmaceutical composition is made with a peptide that consists of 2 to 6 amino acids, more

preferably consists of 3 to 5 amino acids, and most preferably consists of 3 or 4 amino acids. If only from the viewpoint of activity, based on a general insight that activity is broader with increasing peptide size, if only to withstand full proteolysis (after administration) longer whereby metabolic fragments of 3 aa still have activity, it is herein preferred that said peptide consists of 4 amino acids.

[0046] Furthermore, it is particularly useful that subjects in need of treatment for radiation injury can now be treated via a mere subcutaneous or intra-muscular injection, thereby allowing self-treatment with an autoinjector or treatment by non-trained or non-medical personnel, thereby greatly facilitating the organisation of help in emergency scenarios where thousands of people may need to be treated. If only intravenous or similarly risky intraperitoneal injections had been found to be useful, subjects in need of treatment would be harder to help, when compared now to the situation that simple administration tools such as autoinjectors are provided herein.

[0047] In particular, the invention also provides use of a peptide smaller than 30 amino acids for the production of a pharmaceutical composition for the treatment of radiation injury wherein said pharmaceutical composition is contained in an autoinjector. An autoinjector is a medical device designed to deliver a single dose of a particular (typically life-saving) drug, sometimes also described as a pre-filled syringe for self-injection or injection by non-medical personnel or laymen. As used herein, the term "autoinjector" does not refer to an injector for the automated application of a biological (e.g. peptide) sample in an analytical system, such as a chromatography apparatus, as is described for example in Husek et al. (J. of Chromatography B: Biomedical Sciences & Applications, Elsevier, Amsterda, Vol. 767, no.1, (2002) pg. 169-174).

[0048] By design, autoinjectors are easy to use and are intended for self-administration by patients or administration by laymen to patients. The site of injection typically is into the thigh or the buttocks, wherein said treatment comprises subcutaneous or intramuscular injection with said peptide. Because autoinjectors may be designed to automatically and reliably deliver a desired dose of medicament, they facilitate quick, convenient, and accurate delivery of medicaments. In particular, autoinjectors are well suited for use by subjects who must self-administer therapeutic substances or by healthcare workers who must inject multiple subjects over a relatively short period of time, for instance in an emergency situation. Moreover,

autoinjectors incorporating a needled injection mechanism may be designed so that the needle is hidden from view before, during, and even after an injection operation, thereby reducing or eliminating any anxiety associated with the act of penetrating a visible needle into the subject's tissue. Though their precise specifications vary widely, needled autoinjectors generally include a
5 body or housing, a needled syringe or similar device, and one or more drive mechanisms for inserting a needle into the tissue of the subject and delivering a desired dose of liquid medicament through the inserted needle. The drive mechanisms included in state of the art needled autoinjectors generally include a source of energy capable of powering the drive mechanism. This energy source may be, for example, mechanical (i.e., spring-loaded),
10 pneumatic, electromechanical, or chemical, as described in U.S. Pat. Nos. 6,149,626, 6,099,504, 5,957,897, 5,695,472, 5,665,071, 5,567160, 5,527,287, 5,354,286, 5,300,030, 5,102,393, 5,092,843, 4,894,054, 4,678,461, and 3,797,489, the contents of each such patent being incorporated herein by reference. International Publications numbered WO 01/17593, WO 98/00188, WO 95/29720, WO 95/31235, and WO 94/13342 also describe various injectors
15 including different drive mechanisms. Most autoinjectors are (optionally spring-loaded) syringes. An autoinjector of the present invention, in particular the body or housing thereof which is in direct contact with the peptide, is preferably made of a material which has a minimal affinity for peptides. This can reduce unwanted adhesion or sticking of peptides to the autoinjector to a minimum. A very suitable material is polypropylene, in particular essentially pure
20 polypropylene.

[0049] Autoinjectors were initially designed to overcome the hesitation associated with self-administration of needle-based drugs. Examples of such autoinjectors are Epipen® or the recently introduced Twinject®, which is often prescribed to persons who are at risk for anaphylaxis. Another example of an autoinjector is the Rebiject® for interferon beta used to treat
25 Multiple Sclerosis. Autoinjectors are often used in the military to protect personnel from chemical warfare agents. In the United States Military, an autoinjector is part of every Biological or Chemical Weapons Response kit. It is issued to every soldier in the event they may face Biological or Chemical Weapons. The needle automatically injects the person once you activate it, piercing any clothes (even multiple layers) one may be wearing. An autoinjector herein not
30 only comprises above described injection devices, that usually is spring-driven, whereby the skin

penetration and/or the injection of the drug takes place automatically, but also comprises pre-filled syringes, or autoinjector cartridges and the like.

[0050] The invention provides such an autoinjector useful for the treatment of (ir)radiation injury irrespective of whether the radiation is emitted by radioactive substances (radioisotopes), such as uranium, radon, and plutonium, or is produced by man-made sources, such as x-ray and radiation therapy machines. The invention also provides an autoinjector comprising a pharmaceutical composition consisting of a peptide of smaller than 30 amino acids (herein also called anti-radiation peptide) and a suitable excipient. Suitable excipients are known in the art, see for example the Handbook of Pharmaceutical Manufacturing Formulations (edited by Sarfaraz K Niazi; ISBN: 0849317460 and incorporated herein by reference).

[0051] Suitable excipients for example are composed of water, propylene glycol, ethyl alcohol, sodium benzoate and benzoic acid as buffers, and benzyl alcohol as preservative; or of mannitol, human serum albumin, sodium acetate, acetic acid, sodium hydroxide, and water for injections. Other exemplary compositions for parenteral administration via an autoinjector include injectable solutions or suspensions which may contain, for example, suitable non-toxic, parenterally acceptable diluents or solvents, such as mannitol, 1,3-butanediol, water, Ringer's solution, an isotonic sodium chloride solution, or other suitable dispersing or wetting and suspending agents, including synthetic mono- or diglycerides, and fatty acids, including oleic acid.

[0052] In one embodiment, an autoinjector comprises as an active ingredient an anti-radiation peptide (or functional analog thereof) that is capable of reducing adverse effects of radiation in a subject when administered after the subject has been exposed to radiation. Preferably, said peptide can confer at least a partial protection against radiation damage if administered at least 30 minutes, more preferably at least one hour, most preferably at least several hours or even several days (e.g. 3 days) post irradiation. This type of autoinjector is also referred to as an "emergency-autoinjector", reflecting its applicability in unexpected emergency situations.

[0053] In one embodiment, the invention provides an autoinjector containing a sterile solution packaged within a syringe-like device that delivers its entire 5 mL contents automatically upon activation. Each mL contains 100 mg, preferably 200 mg, anti-radiation

peptide compounded with an excipient, such as an excipient comprising propylene glycol, ethyl alcohol, sodium benzoate and benzoic acid as buffers, and benzyl alcohol as preservative. In a preferred embodiment, the autoinjector for the treatment of radiation injury carries an anti-radiation peptide smaller than 15 amino acids, more preferably smaller than 7 amino acids.

5 **[0054]** Preferred are autoinjectors for the treatment of acute radiation injury carrying a peptide of 3 to 4 aa in length, preferably a peptide which has a dose reduction factor (DRF) against acute gamma irradiation of at least 1.10, said DRF determinable by testing which dose of radiation results in 50% mortality at 30 days (LD50/30) after whole body radiation (WBI) in a test group of mice treated with said peptide at 72 hours after WBI and, testing which dose of
10 radiation results in 50% mortality at 30 days (LD50/30) after whole body radiation (WBI) in a control group of mice treated only with the vehicle of said peptide at 72 hours after WBI and, wherein the DRF is calculated by dividing the LD50/30 of the peptide-treated animals by the LD50/30 of the control animals.

[0055] Even more preferred is an autoinjector carrying a peptide which has a dose
15 reduction factor (DRF) of at least 1.20, more preferably of at least 1.25. Suitable peptides for inclusion in an autoinjector are also those that have anti cell-cycle activity in plants, as determined herein. Very suitable peptides for use in an autoinjector of the invention are VVC, LAG, AQG, LQGV, QVVC, MTRV, AQGV, LAGV, LQAV, PGCP, VGQL, RVLQ, EMFQ, AVAL, FVLS, NMWD, LCFL, FSYA, FWVD, AFTV, LGTL, QLLG, YAIT, APSL, ITTL,
20 QALG, GVLC, NLIN, SPIE, LNTI, LHNL, CPVQ, EVVR, MTEV, EALE, EPPE, LGTL, VGGI, RLPG, LQGA, LCFL, TLAVE, VEGNL, or LNEAL.

[0056] The invention also provides a pharmaceutical composition for the treatment of a subject suffering from or believed to be suffering from radiation injury, said pharmaceutical composition comprising: a pharmacologically effective amount of anti-radiation peptide, or a
25 functional analogue thereof, or pharmaceutical composition as identified herein together with a pharmaceutically acceptable diluent. The invention herewith provides a method of treating or preventing radiation injury in a subject in need thereof or in potential need thereof, said method comprising: administering to the subject a pharmaceutical composition comprising: means for treating or preventing radiation injury, and a pharmaceutically acceptable excipient, wherein said

means comprise an anti-radiation peptide or pharmaceutical composition as identified herein, in particular wherein said radiation injury comprises irradiation injury.

[0057] In one embodiment, the invention provides a method of treating radiation injury of a subject in need thereof comprising administering to the subject a composition comprising an
5 oligopeptide obtainable or derivable from the peptide MTRVLQGVLPALPQVVC or from the peptide LPGCPRGVNPVVS. It is preferred that the oligopeptide is selected from the group consisting of MTR, MTRV, LQG, LQGV, VLPALP, VLPALPQ, QVVC, VVC, AQG, AQGV, LAG, LAGV and any combination thereof. In another embodiment, it is preferred that the oligopeptide is selected from the group consisting of LPGC, CPRGVNP and PGCP. Such
10 oligopeptides are particularly useful when said radiation injury comprises irradiation injury. The invention also provides a pharmaceutical composition for the treatment of radiation injury comprising an oligopeptide obtainable or derivable from the peptide MTRVLQGVLPALPQVVC or from the peptide LPGCPRGVNPVVS, such as an oligopeptide is selected from the group of MTR, MTRV, LQG, LQGV, VLPALP, VLPALPQ, QVVC, VVC,
15 AQG, AQGV, LAG, LAGV, LPGC, CPRGVNP and PGCP, and combinations of any thereof and use of an such (oligo)peptide(s) for the production of a pharmaceutical composition for the treatment of radiation injury.

[0058] Earlier we reported inhibition of septic shock in mice by a 6-mer oligopeptide (VLPALP) derived from the beta-chain of human chorionic gonadotropin hormone. Also, we
20 showed that several other short (from trimeric peptides up) peptides derived from loop 2 of the beta chain of hCG (residues 41-57), and modifications of some of said peptides obtained by alanine substitution of single amino acids, have similar anti-inflammatory activity. Furthermore, we provide our rational for selecting several of these for continuing development towards a therapeutic compound for treatment of acute inflammatory conditions after accidental exposure
25 to ionizing irradiation.

[0059] Human chorionic gonadotropin (hCG) is a heterodimeric placental glycoprotein hormone required in pregnancy. In human pregnancy urine and in commercial hCG preparations it occurs in a variety of forms, including breakdown products. Several investigators have studied the effects of heterodimeric hCG and its variants on the immune system because of their putative
30 role in preventing the rejection of the fetal allograft during pregnancy. Several reports have

suggested modulation of the immune system by intact hormone, but such effects of breakdown products have not been reported. Earlier we (Khan et al., *Hum. Immunol.* 2002 Jan; 63(1):1-7), reported inhibition of septic shock in mice by a 6-mer oligopeptide (VLPALP) derived from the beta-chain of human chorionic gonadotropin hormone. A single treatment with this hexapeptide after high dose lipopolysaccharide (LPS) injection inhibited septic shock in mice. Benner and Khan (*Scand. J. Immunol.* 2005 Jul; 62 Suppl 1:62-6) studied the possible immunological activity of the *in vivo* liberated peptide fragments originating from nicking of the sequence MTRVLQGVLPALPQVVC (residues 41-57) of loop 2 of the beta-subunit of hCG. Here it is reported that several of 3 – 7 amino acid-long peptides taken from loop 2 of the beta-subunit --and alanine-replacement peptides derived of some-- displayed significant anti-inflammatory activity as measured by the inhibition of septic shock syndrome in mice and are beyond that considered useful for treatment of radiation injury, in particular of radiation injury comprising gastrointestinal syndrome and for the production of a pharmaceutical composition for the treatment of radiation injury, in particular of radiation injury comprising gastrointestinal syndrome.

The invention also provides a pharmaceutical composition having anti-cell cycle activity. The cell cycle is an ordered set of events, culminating in cell growth and division into two daughter cells. The stages of the cell cycle are G1-S-G2-M. The G1 stage stands for "GAP 1". The S stage stands for "Synthesis". This is the stage when DNA replication occurs. The G2 stage stands for "GAP 2". The M stage stands for "mitosis", and is when nuclear (chromosomes separate) and cytoplasmic (cytokinesis) division occur. The term ''anti-cell cycle activity'' as used herein is meant to indicate that the peptide is capable of altering cell cycle dynamics. For example, it comprises altering, i.e. increasing or reducing, the frequency of cell division. In one embodiment, it refers to an anti-proliferative activity.

[0060] Provided is a pharmaceutical composition having anti-cell cycle activity comprising PGCP, a pharmaceutical composition having anti-cell cycle activity comprising VGQL, a pharmaceutical composition having anti-cell cycle activity comprising RVLQ, a pharmaceutical composition having anti-cell cycle activity comprising EMFQ, a pharmaceutical composition having anti-cell cycle activity comprising AVAL, a pharmaceutical composition having anti-cell cycle activity comprising FVLS, a pharmaceutical composition having anti-cell

cycle activity comprising NMWD, a pharmaceutical composition having anti-cell cycle activity comprising LCFL, a pharmaceutical composition having anti-cell cycle activity comprising FSYA, a pharmaceutical composition having anti-cell cycle activity comprising FWVD, a pharmaceutical composition having anti-cell cycle activity comprising AFTV, a pharmaceutical
5 composition having anti-cell cycle activity comprising LGTL, a pharmaceutical composition having anti-cell cycle activity comprising QLLG, a pharmaceutical composition having anti-cell cycle activity comprising YAIT, a pharmaceutical composition having anti-cell cycle activity comprising APSL, a pharmaceutical composition having anti-cell cycle activity comprising ITTL, a pharmaceutical composition having anti-cell cycle activity comprising QALG, a
10 pharmaceutical composition having anti-cell cycle activity comprising GVLC, a pharmaceutical composition having anti-cell cycle activity comprising NLIN, a pharmaceutical composition having anti-cell cycle activity comprising SPIE, a pharmaceutical composition having anti-cell cycle activity comprising LNTI, a pharmaceutical composition having anti-cell cycle activity comprising LHNL, a pharmaceutical composition having anti-cell cycle activity comprising
15 CPVQ, a pharmaceutical composition having anti-cell cycle activity comprising EVVR, a pharmaceutical composition having anti-cell cycle activity comprising MTEV, a pharmaceutical composition having anti-cell cycle activity comprising EALE, a pharmaceutical composition having anti-cell cycle activity comprising EPPE, a pharmaceutical composition having anti-cell cycle activity comprising LGTL, a pharmaceutical composition having anti-cell cycle activity
20 comprising VGGI, a pharmaceutical composition having anti-cell cycle activity comprising RLPG, a pharmaceutical composition having anti-cell cycle activity comprising LQGA, a pharmaceutical composition having anti-cell cycle activity comprising LCFL, a pharmaceutical composition having anti-cell cycle activity comprising TLAVE, a pharmaceutical composition having anti-cell cycle activity comprising VEGNL, a pharmaceutical composition having anti-
25 cell cycle activity comprising LNEAL, a pharmaceutical composition having anti-cell cycle activity comprising MGGTWA, a pharmaceutical composition having anti-cell cycle activity comprising LTCDDP, a pharmaceutical composition having anti-cell cycle activity comprising VCNYRDV, a pharmaceutical composition having anti-cell cycle activity comprising CPRGVNP, and a pharmaceutical composition having anti-cell cycle activity comprising
30 DINGFLPAL.

[0061] Legends

[0062] Figure 1 Whole Body Irradiation of mice treated with AQGV (peptide EA-230).

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[0063] "WBI" stands for Whole Body Irradiation. Radiation damage protection *in vivo* was assessed after WBI (6.5 to 9.8Gy, Philips MG 30 at 81cGy/min) on anesthetized C57Bl/6 mice, and survival differences were measured by Kaplan-Meirer analysis. All groups of mice got the first injection with peptide or vehicle (control animals) 3 hours after the WBI. The group that
10 got a placebo injection experienced 80% mortality, as predicted in this model. The dose of radiation of 8.6 Gray(= 8.6 Gy) is known, in this species, to cause about an 80% mortality, so it is called the LD80 (lethal dose for 80%). The deaths starting around Day 10 - which is typical for what happens in WBI to animals or humans: By around Day 10, the gut lining is so damaged and leaky from the radiation that bacteria get into the circulation and cause gastrointestinal
15 syndrome, and the bone marrow is so damaged that there aren't enough white blood cells to fight the infection ("Bone Marrow Syndrome"), and death ensues. The group with "x" as the symbol got the first injection IV, and the second injection subcutaneously (SC) 3 hours after that first injection. 100% of these animals survived. What the graph does not show is that they didn't show *any* signs of sickness at all. To an outside observer, they would look like perfectly normal mice.
20 The group with the triangle symbol got its first injection of the peptide via the SC route. Then it got additional SC injections every 48 hours, for a total of 3 doses (in addition to the first dose) - meaning, on Day 3, Day 5, and Day 7. Note that only one of these animals died. The group with the square symbol was identical in procedures to the group with the triangle symbol, except that the 48 hour SC injections continued on until a total of 6 doses had been injected (in addition to
25 the first dose). So the dosing continued on until Day 13. Note that this prolonging of the treatment was associated with complete protection (no mortality whatsoever in this group). The animals in this group showed no signs of sickness. What we can conclude from this data is that when the animals got two doses of peptide in the first day (with the first one being IV), there was complete protection against a highly-lethal dose of WBI. When the animals got lower-level

treatment (SC only), if the treatment was prolonged until the 2nd week, here again there was complete protection.

[0064] Figure 2 Second set of radioprotection experiments with peptide AQGV

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[0065] Escalating doses of Whole Body Irradiation (WBI), with one single exposure given to any particular cohort, and the exposure dose going progressively higher, for each subsequent cohort. A single dose of peptide EA-230 (AQGV) was administered subcutaneously, but with treatment delayed until three days (72 hr) after the WBI. The test is called the Dose
10 Reduction Factor ("DRF"), which is defined as ratio between the LD50 of the treated group to the LD50 of the control group. The LD50 refers to that dose which is lethal to 50% of the animals tested. An acceptable DRF value is 1.20. To pass the test, at Day 30 after WBI a candidate drug must have an LD50 at a radiation dose that is at least 20% higher (an increase by a factor of 1.20) than the LD50 dose for the control animals. If e.g. the LD50 for the control
15 animals is 8.2 Gy, then, the LD50 for a candidate drug would have to be at least 20% higher, which in this case would mean a dose of 8.2 x 1.20 = 10.4 Gy.

[0066] Figure 3 An example of the effect of oligopeptides in *Arabidopsis thaliana* cell-cycle analysis. Compounds NAK 4 (LQGV) and NAK 9 (VVC) show a clear effect on the tested
20 markers. For the cell cycle marker (pCDG), a clear effect was observed, for both time points, in the roots. In the transition zone and cotyledons the effect was observed in a time- and/or dose-dependent manner. In the case of the auxin responsive marker (DR5::GUS) the same was observed as with the cell cycle marker. NAK 26 (DINGFLPAL) shows less consistent and time-dependent effect. An effect was only observed in time in the roots. No effect was observed in the
25 transition zone and cotyledons.

[0067] Figure 4. A test on representative oligopeptides for their effect on proliferation during rapid growth of murine monocytes induced by CD3 when avid cell division occurs. Mice (n=5) were treated i.p. PBS, Nak4 (LQGV), Nak47 (LAGV), Nak46 (AQGV) provided by
30 Ansynth BV,The Netherlands, or Nak46*(AQGV provided by Diosynth BV,The Netherlands).

Mice were treated with 0.5 mg/kg of 5 mg/kg peptide for one hour after which spleens were isolated and spleen cell suspensions were made. Spleen cell suspensions were pooled per group and cultured *in vitro* (in three-fold) in the presence of PBS or of anti-CD3 antibody and proliferation was tested at 0, 12, 24 and 48 hours after culture.

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[0068] DETAILED DESCRIPTION OF THE INVENTION

[0069] As used herein, a "purified, synthetic or isolated" peptide is one that has been purified from a natural or biotechnological source, or, more preferably, is synthesized as

10 described herein.

[0070] "Composition," as used herein, refers to chemical compounds that contain or consist of the oligopeptide. The oligopeptide is preferably isolated before inclusion within the composition. The oligopeptide preferably consists of two (2) to six (6) amino acids, most preferably of three (3) to four (4) amino acids.

15 **[0071]** For instance, a preferred compound could, in one embodiment be: NT A Q G V CT wherein NT at the N-terminus is selected from the group of H--, CH3--, an acyl group, or a general protective group; and CT at the C-terminus is selected from the group of small (e.g. 1 to 5 amino acids) peptides, --OH, $--OR^1$, $--NH_2$, $--NHR^1$, $--NR^1 R^2$, or $--N(CH_2)_{1\text{-}6} NR^1 R^2$, wherein R^1 and R^2, when present, are independently selected from H, alkyl, aryl, (ar)alkyl, and wherein

20 R^1 and R^2 can be cyclically bonded to one another.

[0072] "Alkyl" as used herein, is preferably a saturated branched or unbranched hydrocarbon having one to six carbon atoms, for example, methyl, ethyl, and isopentyl.

[0073] "Aryl" as used herein, is an aromatic hydrocarbon group, preferably having 6 to 10 carbon atoms, such as phenyl or naphthyl.

25 **[0074]** "(Ar)alkyl" as used herein, is an arene group (having both aliphatic and aromatic portions), preferably having 7 to 13 carbon atoms such as benzyl, ethylbenzyl, n-propylbenzyl, and isobutylbenzyl.

[0075] "Oligopeptide" as used herein, are peptides having from 2 to 12 amino acids joined together by peptide bonds. Equivalent to oligopeptides are compounds having the same

30 or equivalent side chains as the particular amino acids used in an oligopeptide, and arranged

sequentially in the same order as the peptides, but joined together by non-peptide bonds, *e.g.*, by isosteric linkages such as the keto isostere, hydroxy isostere, diketo isostere, or the keto-difluoromethylene isostere.

[0076] "Composition" also includes, for example, an acceptable salt of the oligopeptide
5 or a labeled oligopeptide. As used herein, "acceptable salt" refers to salts that retain the desired activity of the oligopeptide or equivalent compound, but preferably do not detrimentally affect the activity of the oligopeptide or other component of a system in which uses the oligopeptide. Examples of such salts are acid addition salts formed with inorganic acids, for example, hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid, and the like.
10 Salts may also be formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, pamoic acid, alginic acid, polyglutamic acid, and the like. Salts may be formed with polyvalent metal cations such as zinc, calcium, bismuth, barium, magnesium, aluminum, copper, cobalt, nickel and the like or with an organic cation formed from
15 N,N'-dibenzylethylenediamine or ethylenediamine, or combinations thereof (*e.g.*, a zinc tannate salt).

[0077] Such a pharmaceutical composition may be administered to the subject parenterally or orally. Such a pharmaceutical composition may consist essentially of oligopeptide and PBS. It is preferred that the oligopeptide is of synthetic origin. Suitable
20 treatment for example entails administering the oligopeptide in the pharmaceutical composition to the patient intravenously in an amount of from about 0.1 to about 35 mg/kg body mass of the subject. It may be useful that the pharmaceutical composition consists essentially of from one to three different oligopeptides.

[0078] The thus developed chemical entity can be administered and introduced *in vivo*
25 systemically, topically, or locally. The peptide, or its modification, can be administered as the entity as such or as a pharmaceutically acceptable acid- or base addition salt, formed by reaction with an inorganic acid (such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid); or with an organic acid (such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid,
30 succinic acid, maleic acid, and fumaric acid); or by reaction with an inorganic base (such as

sodium hydroxide, ammonium hydroxide, potassium hydroxide); or with an organic base (such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines). A selected peptide and any of the derived entities may also be conjugated to sugars, lipids, other polypeptides, nucleic acids and PNA; and function in-situ as a conjugate or be released locally after reaching a targeted tissue or organ.

[0079] A "substitution" with regard to the various amino acids generally relate to substituting a group such as alkoxy, halogen, hydroxy, nitro, or lower alkyl onto an aromatic ring for hydrogen that would usually be present. Substitutions can also be made on the alkyl chain connecting the aromatic portion to the peptide backbone, with, for instance lower alkyl groups substituting for hydrogen. Still further substitutions can be made at the alpha position of an amino acid, also using an alkyl group.

[0080] Preferred substitutions involve the use of fluorine or chlorine as a halogen, and methoxy as an alkoxy group. With regard to alkyl and lower alkyl, generally alkyl groups having fewer (1 to 3) carbon atoms are preferred.

[0081] The compounds according to the general formula may be prepared in a manner conventional for such compounds. To that end, suitably N alpha protected (and side-chain protected if reactive side-chains are present) amino acid analogs or peptides are activated and coupled to suitably carboxyl protected amino acid or peptide derivatives either in solution or on a solid support. Protection of the alpha-amino functions generally takes place by urethane functions such as the acid-labile tertiary-butyloxycarbonyl group ("Boc"), benzyloxycarbonyl ("Z") group and substituted analogs or the base-labile 9-fluoremyl-methyloxycarbonyl ("Fmoc") group. The Z group can also be removed by catalytic hydrogenation. Other suitable protecting groups include the Nps, Bmv, Bpoc, Aloc, MSC, etc. A good overview of amino protecting groups is given in *The peptides, Analysis, Synthesis, Biology*, Vol. 3, E. Gross and J. Meienhofer, eds. (Academic Press, New York, 1981). Protection of carboxyl groups can take place by ester formation, for example, base-labile esters like methyl or ethyl, acid labile esters like tert. butyl or, substituted, benzyl esters or hydrogenolytically. Protection of side-chain functions like those of lysine and glutamic or aspartic acid can take place using the aforementioned groups. Protection of thiol, and although not always required, of guanidino, alcohol and imidazole groups can take place using a variety of reagents such as those described in *The Peptides, Analysis,*

Synthesis, Biology, id. or in *Pure and Applied Chemistry*, 59(3), 331-344 (1987). Activation of the carboxyl group of the suitably protected amino acids or peptides can take place by the azide, mixed anhydride, active ester, or carbodiimide method especially with the addition of catalytic and racemization-suppressing compounds like 1-N-N-hydroxybenzotriazole,
5 N-hydroxysuccinimide, 3-hydroxy-4-oxo-3,4-dihydro-1,2,3,-benzotriazine, N-hydroxy-5 norbornene-2,3-dicar-boxyimide. Also the anhydrides of phosphorus based acids can be used. *See, e.g., The Peptides, Analysis, Synthesis, Biology, supra* and *Pure and Applied Chemistry*, 59(3), 331-344 (1987).

[0082] It is also possible to prepare the compounds by the solid phase method of
10 Merrifield. Different solid supports and different strategies are known *see, e.g.* Barany and Merrifield in *The Peptides,* Analysis, *Synthesis, Biology*, Vol. 2, E. Gross and J. Meienhofer, eds. (Acad. Press, New York, 1980); Kneib-Cordonier and Mullen, *Int. J. Peptide Protein Res.*, 30, 705-739 (1987); and Fields and Noble, *Int. J. Peptide Protein Res.*, 35, 161-214 (1990). The synthesis of compounds in which a peptide bond is replaced by an isostere, can, in general, be
15 performed using the previously described protecting groups and activation procedures. Procedures to synthesize the modified isosteres are described in the literature, e.g., for the --CH_2--NH-- isostere and for the --CO--CH_2 -- isostere.

[0083] Removal of the protecting groups, and, in the case of solid phase peptide synthesis, the cleavage from the solid support, can take place in different ways, depending on the
20 nature of those protecting groups and the type of linker to the solid support. Usually deprotection takes place under acidic conditions and in the presence of scavengers. *See, e.g.* volumes 3, 5 and 9 of the series on *The Peptides Analysis, Synthesis, Biology, supra.*

[0084] Another possibility is the application of enzymes in synthesis of such compounds; for reviews see, *e.g.*, H.D. Jakubke in *The Peptides, Analysis, Synthesis, Biology*,
25 Vol. 9, S. Udenfriend and J. Meienhofer, eds. (Acad. Press, New York, 1987).

[0085] Although possibly not desirable from an economic point of view, oligopeptides according to the invention could also be made according to recombinant DNA methods. Such methods involve the preparation of the desired oligopeptide thereof by means of expressing recombinant polynucleotide sequence that codes for one or more of the oligopeptides in question
30 in a suitable microorganism as host. Generally the process involves introducing into a cloning

vehicle (*e.g.*, a plasmid, phage DNA, or other DNA sequence able to replicate in a host cell) a DNA sequence coding for the particular oligopeptide or oligopeptides, introducing the cloning vehicle into a suitable eukaryotic or prokaryotic host cell, and culturing the host cell thus transformed. When a eukaryotic host cell is used, the compound may include a glycoprotein
5 portion.
As used herein, a "functional analogue" of a peptide includes an amino acid sequence, or other sequence monomers, which has been altered such that the functional properties of the sequence are essentially the same in kind, not necessarily in amount.

[0086] The functionality of a peptide or a functional analogue thereof can be
10 determined using an *in vivo* and/or *in vitro* testing. In vitro testing is preferred. In one embodiment, a functional peptide analogue is subjected to comparative testing using a reference or control peptide, for instance a peptide analog consisting solely of L-amino acids. A suitable test comprises determining the capability of the candidate peptide to affect cell cycle dynamics. For example, the effect on cell cycle progression may be determined using a plant model system,
15 e.g. the *Arabidopsis* system exemplified herein below, or using cultured (mammalian) cells. In a further aspect it involves determining the ability of the candidate peptide to inhibit apoptosis, for example by inducing a (temporary) G2-M cell cycle arrest.

[0087] An analogue can be provided in many ways, for instance, through "conservative amino acid substitution." Also, peptidomimetic compounds can be designed that
20 functionally or structurally resemble the original peptide taken as the starting point but that are for example composed of non-naturally occurring amino acids or polyamides. With "conservative amino acid substitution," one amino acid residue is substituted with another residue with generally similar properties (size, hydrophobicity), such that the overall functioning is likely not to be seriously affected. However, it is often much more desirable to improve a
25 specific function. An analogue can also be provided by systematically improving at least one desired property of an amino acid sequence. This can, for instance, be done by an Ala-scan and/or replacement net mapping method. With these methods, many different peptides are generated, based on an original amino acid sequence but each containing a substitution of at least one amino acid residue. The amino acid residue may either be replaced by alanine (Ala-scan) or
30 by any other amino acid residue (replacement net mapping). This way, many positional variants

of the original amino acid sequence are synthesized. Every positional variant is screened for a specific activity. The generated data are used to design improved peptide derivatives of a certain amino acid sequence.

An analogue can also be, for instance, generated by substitution of an L-amino acid residue with
5 a D-amino acid residue. This substitution, leading to a peptide that does not naturally occur in nature, can improve a property of an amino acid sequence. It is, for example, useful to provide a peptide sequence of known activity of all D-amino acids in retro inversion format, thereby allowing for retained activity and increased half-life values. By generating many positional variants of an original amino acid sequence and screening for a specific activity, improved
10 peptide derivatives comprising such D-amino acids can be designed with further improved characteristics. It has been shown in the art that peptides that are protected by D-amino acids at either one or both termini were found to be more stable than those consisting of L-amino acids only. Other types of modifications include those known in the art of peptide drug development to have beneficial effects for use of the peptide in a pharmaceutical composition. These effects may
15 include improved efficacy, altered pharmacokinetics, increasing stability resulting in a longer shelf-life and less stringent cold chain handling requirements.

[0088] In one embodiment of the invention, an anti-radiation peptide comprises a sequence of amino acids joined together in a chain by peptide bonds between their amino and carboxylate groups, wherein at least one amino acid is a D-amino acid. For example, the anti-
20 radiation peptide is selected from the group consisting of VVC, LAG, AQG, LQGV, QVVC, MTRV, AQGV, LAGV, LQAV, PGCP, VGQL, RVLQ, EMFQ, AVAL, FVLS, NMWD, LCFL, FSYA, FWVD, AFTV, LGTL, QLLG, YAIT, APSL, ITTL, QALG, GVLC, NLIN, SPIE, LNTI, LHNL, CPVQ, EVVR, MTEV, EALE, EPPE, LGTL, VGGI, RLPG, LQGA, LCFL, TLAVE, VEGNL, LNEAL, VLPALP, MGGTWA, LTCDDP, VLPAPLQ, VCNYRDV, CPRGVNP,
25 QPLAPLVG and DINGFLPAL, wherein at least one of the amino acid residues indicated by the standard one-letter code is a D -amino acid.

[0089] A person skilled in the art is well able to generate analogous compounds of an amino acid sequence. This can, for instance, be done through screening of a peptide library. Such an analogue has essentially the same functional properties of the sequence in kind, not
30 necessarily in amount. Also, peptides or analogues can be circularized, for example, by

providing them with (terminal) cysteines, dimerized or multimerized, for example, by linkage to lysine or cysteine or other compounds with side-chains that allow linkage or multimerization, brought in tandem- or repeat-configuration, conjugated or otherwise linked to carriers known in the art, if only by a labile link that allows dissociation. Synthetic versions of these oligopeptides as described above, and functional analogues or breakdown products, are herein provided to be used in methods to the treatment of radiation injury and subsequent disease.

[0090] As used herein, a "functional analogue" of a peptide is preferably smaller than the peptide from which it is derived and thus rather made by deletions and/or substitutions than by additions in size. Also, as used herein, a "functional analogue" of a peptide, does not refer to a larger protein or peptide merely containing an amino acid sequence identified as an anti-radiation peptide that is flanked by more amino acids at one or both sides.

[0091] The term "pharmaceutical composition" as used herein is intended to cover both the active composition of the invention alone or a composition containing the composition of the invention together with a pharmaceutically acceptable carrier, diluent or excipient. Of course, a pharmaceutical composition may comprise a mixture of at least two anti-radiation peptides or analogs as disclosed herein. Acceptable diluents of an oligopeptide as described herein in the detailed description are, for example, physiological salt solutions or phosphate buffered salt solutions. In one embodiment, an oligopeptide or composition is administered in an effective concentration to an animal or human systemically, for example, by intravenous, intra-muscular or intraperitoneal administration. Another way of administration comprises perfusion of organs or tissue, be it *in vivo* or *ex vivo*, with a perfusion fluid comprising an oligopeptide or composition according to the invention. The administration may be done as a single dose, as a discontinuous sequence of various doses, or continuously for a period of time sufficient to permit substantial modulation of gene expression. In the case of a continuous administration, the duration of the administration may vary depending upon a number of factors that would readily be appreciated by those skilled in the art.

[0092] The administration dose of an active molecule may be varied over a fairly broad range. The concentrations of an active molecule that can be administered are typically limited by efficacy at the lower end and the solubility of the compound at the upper end. The optimal dose or doses for a particular patient should and can be determined by the physician or medical

specialist involved, taking into consideration well-known relevant factors such as the condition, weight and age of the patient, etc.

[0093] The active molecule may be administered directly in a suitable vehicle, such as, for example, phosphate-buffered saline ("PBS") or solutions in alcohol or DMSO. Pursuant to preferred embodiments of the present invention, however, the active molecule is administered through a single dose delivery using a drug-delivery system. A suitable drug-delivery system would be pharmacologically inactive or at least tolerable. It should preferably not be immunogenic nor cause inflammatory reactions, and should permit release of the active molecule so as to maintain effective levels thereof over the desired time period. Alternatives are known in the art as suitable for purposes of sustained release and are contemplated as within the scope of the present invention. Suitable delivery vehicles include, but are not limited to, the following: microcapsules or microspheres; liposomes and other lipid-based release systems; viscous instillates; absorbable and/or biodegradable mechanical barriers and implants; and polymeric delivery materials, such as polyethylene oxide/polypropylene oxide block copolymers, polyesters, cross-linked polyvinyl alcohols, polyanhydrides, polymethacrylate and polymethacrylamide hydrogels, anionic carbohydrate polymers, etc. Useful delivery systems are well known in the art.

[0094] One formulation to achieve the active molecule release comprises injectable microcapsules or microspheres made from a biodegradable polymer, such as poly(dl-lactide), poly(dl-lactide-co-glycolide), polycaprolactone, polyglycolide, polylactic acid-co-glycolide, poly(hydroxybutyric acid), polyesters or polyacetals. Injectable systems comprising microcapsules or microspheres having a diameter of about 50 to about 500 micrometers offer advantages over other delivery systems. For example, they generally use less active molecules and may be administered by paramedical personnel. Moreover, such systems are inherently flexible in the design of the duration and rate of separate drug release by selection of microcapsule or microsphere size, drug loading and dosage administered. Further, they can be successfully sterilized by gamma irradiation.

[0095] The design, preparation, and use of microcapsules and microspheres are well within the reach of persons skilled in the art and detailed information concerning these points is available in the literature. Biodegradable polymers (such as lactide, glycolide and caprolactone

polymers) may also be used in formulations other than microcapsules and microspheres; *e.g.*, pre-made films and spray-on films of these polymers containing the active molecule would be suitable for use in accordance with the present invention. Fibers or filaments comprising the active molecule are also contemplated as within the scope of the present invention.

5 **[0096]** Another highly suitable formulation for a single-dose delivery of the active molecule in accordance with the present invention involves liposomes. The encapsulation of an active molecule in liposomes or multilamellar vesicles is a well-known technique for targeted drug delivery and prolonged drug residence. The preparation and use of drug-loaded liposomes is well within the reach of persons skilled in the art and well documented in the literature.

10 **[0097]** Yet another suitable approach for single-dose delivery of an active molecule in accordance with the present invention involves the use of viscous installates. In this technique, high molecular weight carriers are used in admixture with active molecule(s), giving rise to a structure that produces a solution with high viscosity. Suitable high molecular weight carriers include, but are not limited to, the following: dextrans and cyclodextrans; hydrogels;
15 (cross-linked) viscous materials, including (cross-linked) viscoelastics; carboxymethylcellulose; hyaluronic acid; and chondroitin sulfate. The preparation and use of drug-loaded viscous instillates is well known to persons skilled in the art.

[0098] Pursuant to yet another approach, active molecule(s) may be administered in combination with absorbable mechanical barriers such as oxidized regenerated cellulose. The
20 active molecule may be covalently or non-covalently (*e.g.*, ionically) bound to such a barrier, or it may simply be dispersed therein.

[0099] The invention is further explained with the aid of the following illustrative examples.

25 EXAMPLES

Peptide selection

[00100] Selection was based on the known preferential cleavage sites of the sequence MTRVLQGVLPALPQVVC (residues 41-57) of loop 2 of the beta-subunit of hCG (Cole et al.,
30 *J. Clin. Endocr. Metab.* 1993; 76:704-710; H. Alfthan, U.H. Stenman, *Mol. Cell. Endocrinol.*

1996; 125:107-120; A. Kardana, et al., *Endocrinology* 1991; 129:1541-1550; Cole et al., *Endocrinology* 1991; 129:1559-1567; S. Birken, Y. Maydelman, M.A. Gawinowicz, *Methods* 2000; 21:3-14), and on aa sequences taken from C-Reactive Protein (CRP) (Beta-catenin, *e.g.*, human CTNB), Bruton's tyrosine kinase (*e.g.*, human BTK), matrix metalloproteinase-2 and p-
5 53.

Peptide synthesis

[00101] Peptides mentioned here were prepared commercially by a proprietary process (Diosynth BV) or by solid-phase synthesis (Ansynth BV) using the fluorenylmethoxycarbonyl
10 (Fmoc)/tert-butyl-based methodology with 2-chlorotrityl chloride resin as the solid support. The side-chain of glutamine was protected with a trityl function. The peptides were synthesized manually. Each coupling consisted of the following steps: (i) removal of the alpha-amino Fmoc-protection by piperidine in dimethylformamide (DMF), (ii) coupling of the Fmoc amino acid (3 eq) with diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt) in
15 DMF/N-methylformamide (NMP) and (iii) capping of the remaining amino functions with acetic anhydride/diisopropylethylamine (DIEA) in DMF/NMP. Upon completion of the synthesis, the peptide resin was treated with a mixture of trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (TIS) 95:2.5:2.5. After 30 minutes, TIS was added until decolorization. The solution was evaporated *in vacuo* and the peptide precipitated with diethyl ether. The crude peptides were
20 dissolved in water (50-100 mg/ml) and purified by reverse-phase high-performance liquid chromatography (RP-HPLC). HPLC conditions were: column: Vydac TP21810C18 (10 x 250 mm); elution system: gradient system of 0.1% TFA in water v/v (A) and 0.1% TFA in acetonitrile (ACN) v/v (B); flow rate 6 ml/minute; absorbance was detected from 190-370 nm. There were different gradient systems used. For example for peptides LQG and LQGV:
25 10 minutes 100% A followed by linear gradient 0-10% B in 50 minutes. For example for peptides VLPALP and VLPALPQ: 5 minutes 5% B followed by linear gradient 1% B/minute. The collected fractions were concentrated to about 5 ml by rotation film evaporation under reduced pressure at 40°C. The remaining TFA was exchanged against acetate by eluting two times over a column with anion exchange resin (Merck II) in acetate form. The elute was

concentrated and lyophilized in 28 hours. Peptides later were prepared for use by dissolving them in PBS.

Example 1 and Example 2

5 **[00102]** In the first experiment, 12-week-old female BALB/c mice were treated intraperitoneally with a single injection of either PBS (n=9) or with a peptide (LGQV, VLPALP, LPGCPRGVNPVVS, MTRVLQGVLPALPQVVC; n=8, 10 mg/kg). One and a half hours after the treatment mice were exposed whole body to a single dose of 10 Gy ^{137}Cs-γ-irradiation. In the second experiment 12-week-old female BALB/c mice were first exposed whole body to a single
10 dose of 10 Gy ^{137}Cs-γ-irradiation and then 1.5 hours after the irradiation treated intraperitoneally with a single injection of either PBS (n=9) or with a peptide (n=8 or 9, 10 mg/kg). During the experiment mortality and clinical signs (e.g., watery eyes indicating conjunctivitis and weight loss) were observed at different time points. As can be seen from Table 2, all peptides tested had good effect on reducing conjunctivitis in the treated mice, however, no effects were seen on
15 mortality, leading us to select a peptide most suited to combat acute inflammation for testing at a later phase with repeated doses and lowered irradiation.

Appendix II

Table 1. Effects of Intraperitoneal treatment with peptide (10 mg/kg) in 12-wks old female BALB/c mice 1.5 hours before whole body exposure to (10 Gy) γ-irradiation.

Mortality

	Days after irradiation					
	0	3	4	5	6	7
PBS	0/9	0/9	0/9	0/9	5/9	9/9
LQGV	0/8	0/8	0/8	0/8	7/8	8/8
VLPALP	0/8	0/8	0/8	0/8	7/8	8/8
LPGCPRGVNPVVS	0/8	0/8	0/8	0/8	4/8	8/8
MTRVLQGVLPALPQVVC	0/8	0/8	0/8	0/8	7/8	8/8

Watery eyes

	Days after irradiation	
	0	4
PBS	0/9	0/9
LQGV	0/8	0/8
VLPALP	0/8	0/8
LPGCPRGVNPVVS	0/8	1/8
MTRVLQGVLPALPQVVC	0/8	1/8

Weight loss

	Days after irradiation	
	0	4
PBS	0/9	9/9
LQGV	0/8	8/8
VLPALP	0/8	8/8
LPGCPRGVNPVVS	0/8	8/8
MTRVLQGVLPALPQVVC	0/8	1/8

Table 2. Effects of Intraperitoneal treatment with peptide (10 mg/kg) in 12-wks old female BALB/c mice 1.5 hours after whole body exposure to (10 Gy) γ-irradiation.

Mortality

	Days after irradiation					
	0	3	4	5	6	7
PBS	0/9	0/9	0/9	0/9	6/9	9/9
LQGV	0/9	0/9	0/9	1/9	6/9	9/9
VLPALP	0/9	0/9	0/9	0/9	3/9	9/9
LPGCPRGVNPVVS	0/8	0/8	0/8	0/8	6/8	8/8
MTRVLQGVLPALPQVVC	0/9	0/9	0/9	0/9	5/9	9/9

Watery eyes

	Days after irradiation	
	0	4
PBS	0/9	6/9
LQGV	0/9	3/9
VLPALP	0/9	0/9
LPGCPRGVNPVVS	0/8	0/8
MTRVLQGVLPALPQVVC	0/9	0/9

Weight loss

	Days after irradiation	
	0	4
PBS	0/9	9/9
LQGV	0/9	9/9
VLPALP	0/9	9/9
LPGCPRGVNPVVS	0/8	8/8
MTRVLQGVLPALPQVVC	0/9	9/9

5 Example 3

[00103] Six oligopeptides (*i.e.*, A: LAGV , B: AQGV , C: LAG, D: AQG, E: MTR, and F: MTRV) were tested and compared with PBS (control) in a double blind animal study for each peptide's relative ability to aid recovery in a mouse renal ischemia reperfusion test. In this test, the mice were anesthetized, and one kidney from each mouse was removed. The other kidney 10 was tied off for 25 minutes, and the serum urea levels were allowed to increase. Both before and after tying off, each of the separate peptides was administered to thirty (30) different mice (5 mg

oligopeptide / kg body mass intravenously), after which, the mortality of the mice was determined for each oligopeptide as well as was the BUN concentration at two hours, 24 hours and 72 hours. The results are shown in Table 3 below (excluding the results of peptide A (LAGV (SEQ ID NO:4)) obtained in example 3).

5 **[00104]** Under inhalation anesthesia, the left kidney with its artery and vein was isolated and occluded for 25 minutes using a microvascular clamp. During surgery animals were placed on a heating path to maintain body temperature at 37°C. Five minutes before placing the clamp, and 5 minutes before releasing the clamp, 5 mg/kg of peptide, dissolved in 0.1 mL of sterile saline, was administered intravenously. After reperfusion of the left kidney the right kidney was 10 removed. Kidney function was assessed by measuring blood urea nitrogen before clamping, and at 2, 24, and 72 hours after reperfusion.

[00105] Results - Table 3 (mortality at 72 hours post-reperfusion).

PBS	A (LAGV)	B (AQGV)	C (LAG)	D (AQG)	E (MTR)	F (MTRV)
6/10	6/10	0/10	4/10	4/10	4/10	2/10
*P< (vs PBS)	NS	0.01	0.01	0.01	0.01	0.01

15 *2x2 Chi-square test. df=1

[00106] Peptide A (SEQ ID NO:4) was the first peptide administered in the renal ischemia reperfusion test. The personnel who performed the experiments went through a learning curve while working with peptide A. During administration of the peptide in the 20 inferior caval vein, some animals experienced moderate blood loss from the site of injection, whereas others did not. Inadvertently the animals were returned to the stable without drinking water present in their cages the first night after surgery. Also, by mistake, the animals that were intended to be sacrificed at 72 hours were killed 48 hours after reperfusion. None of these or other problems were encountered during the experiments with peptides B-F.

25 **[00107]** As can be seen, mice administered the oligopeptides MTRV and especially AQGV did much better in terms of both survival (a significant reduction in mortality versus the

PBS control group) and reduced BUN concentration than the control group (PBS) or the group administered the other oligopeptides, with more mice surviving and the serum urea levels being much lower than in the other groups. However, the oligopeptides LAG, AQG, and MTR, in this experiment having no reducing effect on BUN concentration, each caused a significant reduction of mortality compared to the PBS control, where MTR did significantly raise BUN levels in the tested mice at 72 hours.

Example 4

[00108] One oligopeptide (A) was retested for its capacity to reduce BUN levels in the mice test for the reasons as described above. The results are shown in Table 4 below. As can be seen, mice administered the oligopeptide LAGV now did much better in terms of both survival (a significant reduction in mortality versus the PBS control group) and reduced BUN concentration than the control group (PBS).

Example 5

[00109] Four additional oligopeptides (G (VLPALPQ), H (VLPALP), I (LQGV) and J (LQG)) were tested for their capacity to reduce BUN levels in the mice test as described above. The results are shown in Table 4 below. As can be seen, mice administered the oligopeptide LQG did show reduced BUN concentration early in the experiment (at 24 hours post-reperfusion) and mice administered VLPALPQ did much better in terms of reduced BUN concentration late in the experiment (at 72 hours post-reperfusion) than the control group (PBS) or the group administered the other oligopeptides, with more mice surviving and the serum urea levels being much lower than in the other groups.

TABLE 4. BUN after 25 min renal ischemia tested in mice with peptides A-J

Peptide		t=0 hr	2 hr	24 hr	72 hr	C-term: N-term:	CARBOXYL FREE
A	Mean	8.166667	14.03333	38.86364	32.8875	NMPF-47	LAGV
	sd	1.774658	1.011599	14.54711	14.31228		
	N	18	3	11	8		
B	Mean	9.713333	16.62	26.36	22.31	NMPF-46	AQGV
	sd	1.882722	2.185203	20.62105	15.96444		
	N	30	10	20	10		
C	Mean	10.15185	18.13333	59.24375	74.4	NMPF-44	LAG
	SD	1.789794	1.88326	16.19662	33.12546		
	N	29	6	16	6		
D	Mean	9.303846	17.7	66.75625	91.18333	NMPF-43	AQG
	SD	1.502127	1.561135	24.50445	51.22154		
	N	26	8	16	6		
E	mean	8.403846	17.13	66.23333	104.0167	NMPF-12	MTR
	SD	1.739076	1.625526	17.55069	48.97193		
	N	26	10	6	6		
F	mean	7.462963	15.08571	34.57368	39.8375	NMPF-11	MTRV
	SD	1.338526	1.422941	15.18083	21.45973		
	N	30	7	18	8		
G	mean	8.256667	13.58	37.79375	37.6375	NMPF-7	VLPALPQ
	SD	1.304021	1.927462	18.33007	29.32872		
	N	30	7	18	8		
H	mean	8.423333	16.24	62.4	47.05	NMPF-6	VLPALP
	SD	1.255521	1.370482	13.33867	20.92728		
	N	30	10	9	7		

I	mean	7.518182	17.53333	56.08333	73.17778	NMPF-4	LQGV
	SD	1.537356	2.956913	14.53573	23.3083		
	N	22	3	18	9		
J	mean	7.82069	16.75	26.74	83.95714	NMPF-3	LQG
	SD	1.330515	1.44123	15.51796	40.32129		
	N	29	8	9	8		
PBS	mean	8.172414	15.0875	56.81	82.075		
control	SD	1.549169	2.215167	22.4659	34.82713		
	N	29	8	15	4		

At 2 hour post-reperfusion statistical analyses revealed P-values of:

A p=0.0491 NMPF-47 LAGV
- B p=0.0008 NMPF-46 AQGV
5 - C p=0.9248 NMPF-44 LAG
- D p=0.4043 NMPF-43 AQG
- E p=0.1848 NMPF-12 MTR
- F p=0.0106 NMPF-11 MTRV
- G p=0.1389 NMPF-7 VLPALPQ
10 - H p=0.5613 NMPF-6 VLPALP
- I p=0.9301 NMPF-4 LQGV
- J p=0.0030 NMPF-3 LQG

At 24 hour post-reperfusion statistical analyses revealed P-values of:

A p=0.0017 NMPF-47 LAGV
15 - B p<0.0001 NMPF-46 AQGV
- C p=0.8186 NMPF-44 LAG
- D p=0.2297 NMPF-43 AQG
- E p=0.0242 NMPF-12 MTR
- F p=0.0021 NMPF-11 MTRV
20 G p=0.0049 NMPF-7 VLPALPQ
H p=0.3297 NMPF-6 VLPALP

- I p=0.8328 NMPF-4 LQGV
- J p=0.9445 NMPF-3 LQG

P values were calculated by Mann Whitney U-test (SPSS for Windows).

5 Example 6

[00110] To determine dose-response relationships, two peptides (D (AQG, having a good effect on mortality on the mice tested in Example 3) and B (AQGV), also having superior effect on BUN of the mice tested in Example 3) were also tested in a dose-response manner in the mouse renal failure test as described above. Peptides were tested at 0.3, 1, 3, 10 and 30
10 mg/kg dosages given as described in Example 3. P values (calculated by Mann Whitney U-test (SPSS for Windows)) of serum urea levels of PBS compared to peptide D groups at 72 hours post-clamping were at 0.3 mg/kg 0.001, at 1 mg/kg 0.009, at 3 mg/kg 0.02, at 10 mg/kg 0.000, and at 30 mg/kg 0.23, for peptide B groups these P-values were 0.88, 0.054, 0.000, 0.001 and 0.003. As can be seen, peptide D (AQG) did reduce BUN levels surprisingly well at the lower
15 dosages tested, as compared with peptide B (AQGV), while the beneficial effect on mortality was also still notable at the lower dosages tested.

Table 5 Mortality in dose-response experiment	24h	72h
PBS	0-9	4-8
AQG 0.3 mg/kg	0-10	2-8
AQG 1.0 mg/kg	0-10	1-8
AQG 3.0 mg/kg	0-10	0-10
AQG 10.0 mg/kg	0-8	1-10
AQG 30.0 mg/kg	0-8	1-8
AQGV 0.3 mg/kg	0-9	2-10
AQGV 1.0 mg/kg	0-10	1-8
AQGV 3.0 mg/kg	1-10	0-10
AQGV 10.0 mg/kg	0-10	0-8
AQGV 30.0 mg/kg	0-8	3-10

Table 6 – Urea Levels in dose-response experiment		
	24h	72h
PBS	*57.8*	85.4
Peptide D (AQG) 0.3 mg/kg	38.4	30.4
Peptide D (AQG) 1.0 mg/kg	48.4	38.4
Peptide D (AQG) 3.0 mg/kg	39.3	40.3
Peptide D (AQG) 10.0 mg/kg	46.8	25.8
Peptide D (AQG) 30.0 mg/kg	52.8	58.9
Peptide B (AQGV 0.3 mg/kg	62.4	86.7
Peptide B (AQGV 1.0 mg/kg	50.0	52.6
Peptide B (AQGV 3.0 mg/kg	37.4	19.6
Peptide B (AQGV 10.0 mg/kg	41.2	37.1
Peptide B (AQGV 30.0 mg/kg	47.8	38.0

standard error	24h	72h
PBS	7.1	14.7
Peptide D (AQG) 0.3 mg/kg	8.6	3.5
Peptide D (AQG) 1.0 mg/kg	7.2	10.2
Peptide D (AQG) 3.0 mg/kg	3.5	10.7
Peptide D (AQG) 10.0 mg/kg	8.0	3.4
Peptide D (AQG) 30.0 mg/kg	9.5	12.9
Peptide B (AQGV) 0.3 mg/kg	10.8	14.1
Peptide B (AQGV) 1.0 mg/kg	11.7	14.3
Peptide B (AQGV) 3.0 mg/kg	7.6	2.6

Peptide B (AQGV) 10.0 mg/kg	8.5	6.9
Peptide B (AQGV) 30.0 mg/kg	5.8	7.8

Table 7 statistical significance / p values (Mann Whitney U-Test) of serum urea levels in dose-response experiment 72 hours post-clamping. PBS control compared to peptide administered groups.	72h
PBS	NA
AQG 0.3 mg/kg	0.001
AQG 1.0 mg/kg	0.009
AQG 3.0 mg/kg	0.02
AQG 10.0 mg/kg	0.000
AQG 30.0 mg/kg	0.23
AQGV 0.3 mg/kg	0.88
AQGV 1.0 mg/kg	0.054
AQGV 3.0 mg/kg	0.000
AQGV 10.0 mg/kg	0.001
AQGV 30.0 mg/kg	0.003

[00111] Septic shock experiments were set up to determine which peptide was best
5 suited to battle acute inflammation.

[00112] Mice used in sepsis or septic shock experiments: Female BALB/c mice of 8 to 12 weeks of age were used for all experiments. The animals were bred in our facility under specific pathogen-free conditions according to the protocols described in the Report of European Laboratory Animal Science Associations (FELASA) Working group on Animal Health
10 (*Laboratory Animals* 28: 1-24, 1994).

[00113] Injection protocols: For the endotoxin model, BALB/c mice were injected i.p. with 150-300 µg LPS (*E. coli* 026:B6; Difco Lab., Detroit, MI, USA). Control groups were

treated with PBS i.p. only. To test the effect of peptides, they were dissolved in PBS and injected i.p. at predetermined points in time after LPS treatment

[00114] Mice were scored for sickness severity using the following measurement scheme:

5 0 No abnormalities.

1 Percolated fur, but no detectable behavior differences compared to normal mice.

2 Percolated fur, huddle reflex, responds to stimuli (such as tap on cage), just as active during handling as healthy mouse.

3 Slower response to tap on cage, passive or docile when handled, but still curious
10 when alone in a new setting.

4 Lack of curiosity, little or no response to stimuli, quite immobile.

5 Labored breathing, inability or slow to self-right after being rolled onto back (moribund, sacrificed).

D Dead

15

[00115] A first set of septic shock experiments were set up to determine which of the peptides LQG, LQGV, VLPALP, VLPALPQ, MTR, MTRV, VVC or QVVC were capable of inhibiting lipopolysaccharide (LPS) induced septic shock in mice by treating mice with a single dose of peptide at two hours after LPS treatment. Peptides were used at 5 mg/kg bodyweight
20 BALB/c mice were injected i.p. with escalating doses LPS (E. coli 026:B6; Difco Lab., Detroit, MI, USA), predetermined to be leading to 80 - 100% mortality in 24-72 hours. Control groups were treated with PBS i.p. only and showed no mortality.

[00116] A second set of septic shock experiments were set up to determine which of the peptides LQG, LQGV, VLPALP, VLPALPQ, MTR, MTRV, VVC or AQG, AQGV, LAG and
25 LAGV were capable of inhibiting high dose LPS induced septic shock in mice by treating mice with a double dose of peptide at 2 and 24 hours after LPS treatment. At each treatment, peptides were used at 5 mg/kg bodyweight. BALB/c mice were injected i.p. with high doses LPS (E. coli 026:B6; Difco Lab., Detroit, MI, USA), predetermined to be leading to 80 - 100% mortality in 24 - 72 hours. Control groups were treated with PBS i.p. only and showed no mortality.

[00117] A further set of septic shock experiments were set up to determine which of the peptides LQG, LQGV, VLPALP, VLPALPQ, MTR, MTRV, VVC or AQGV under study were most suited to be used early and/or late after or throughout the development of shock. For determining the percent of endotoxin shock survival after late or early treatment with peptide,
5 BALB/c mice were injected i.p. with 300 μg LPS (*E. coli* 026:B6; Difco Lab., Detroit, MI, USA), predetermined to be leading to 100% mortality in 48 hours without peptide treatment. Control groups were treated with PBS i.p. only and showed no mortality.

[00118] A comparative trial was set up to compare peptide MTR and AQGV, each obtained from two commercial sources. The comparative trial comprised six groups of six
10 animals; two groups (1A and 1B) receiving placebo (PBS), one group (2) peptide MTR (source Pepscan), one group (3) receiving peptide MTR (source Ansynth), one group (4) receiving peptide AQGV (source Pepscan) and one group peptide AQGV (source Ansynth). Peptide/placebo in these groups was administered two hours after LPS. LPS (source) was used at 10-11 mg/kg. Sickness scores were done at 0, 2, 22 26 42 and 48 hours after LPS injection.
15

Results

Peptide selection

[00119] We selected for synthesis the peptides MTR, MTRV, LQG, LQGV, VLPALP and VLPALPQ, as well as QVVC and VVC. In a later phase of the study we also selected for
20 synthesis alanine-replacement-peptides variants derived from LQG and LQGV, whereby a single substitution of one amino acid with alanine was made; four (AQG, AQGV, LAG, and LAGV) of which the results are presented here.

Septic shock experiments

25 **[00120]** To test the effect of peptide early in the development of development of shock, mice were treated at two hours or at 24 hours after treatment with varying doses of LPS by i.p. injection with test peptide at 5 mg/kg bodyweight. All LPS doses resulted in 100% mortality at 48 - 72 hours in the non-peptide treated mice. The results are shown in Table 8. Of the seven peptides tested, peptide VLPALP and LQGV showed a marked protective effect against
30 LPS-induced sepsis.

Appendix II

Table 8			single dose administration 5 mg/kg														
			effect at t=24 hr							effect at t=48 hr						
Peptide tested	LPS dose	n	0	1	2	3	4	5	D	0	1	2	3	4	5	D
LQG	7*	6					3	3							2	4
	7**	6					3	2	1					1		5
	8	6			2	1	3						1	2	3	
	8	6					1	4	1							6
	10	6					2	4							2	4
	10	6				1	2	3						1	2	3
LQGV	7*	6		2	4						6					
	7**	6		6							6					
	8	6			5	1					5	1				
	8	6		3	3					4	2					
	10	6			6						6					
	10	6		2	2	1	1				4	1				1
VLPALP	7*	6		4	2						5	1				
	7**	6		5	1						5	1				
	8	6			5	1					3	2	1			
	8	6		3	3					2	3	1				
	8	6			2	4					5	1				
	8	6		2	4						5	1				
	9	5			3	1	1				1	2	1			1
	9	6		2	4						5	1				
	9	6			3	2	1				2	1	1		2	
	10	6			3		3				3		2		1	

Table 8			single dose administration 5 mg/kg														
			effect at t=24 hr							effect at t=48 hr							
Peptide tested	LPS dose	n	0	1	2	3	4	5	D	0	1	2	3	4	5	D
	10	6			4		2				2	2				2
VLPALPQ	7*	6				3	3							2	2	2
	7**	6						5	1							6
	8	6			1	3	2						1	3	2	
	8	6					1	3	2							6
	10	6					2	4							1	5
	10	6				1		2	3						1	5
VVC	7*	6				4	2					4			2	
	7**	6				2	1	2	1		1	1	1			3
	10	6			2	2	2				2	2			2	
MTRV	7*	6				3	3					4			2	
	7**	6				2	3	1			3		1			2
	10	6			2	1	2	1			2	1	1		1	1
	10	6			1	2	1	2				3				3
MTR	7*	6				3	3							2	3	1
	7**	6			1	3	1	1			3					3
	10	6				2	3		1		2				1	3

Table 9			
peptide administered twice (t=2 hr and t=24 hr) 5 mg/kg at high LPS dose			

			effect at t=24 hr							effect at t=48 hr						
Peptide tested	LPS dose	n	0	1	2	3	4	5	D	0	1	2	3	4	5	D
LQG	10.5	5						5								5
	11	6					6									6
LQGV	10.5	5						5								5
	11	6					2	4						2		4
VLPALP	10.5	5						5								5
	11	6					2	4						1		6
VLPALPQ	10.5	5			1	2		2				3				2
	11	6						6				4				2
	11	6						6				2		3	1	
VVC	10.5	5			5						2	3				
	11	6			6						2	4				
MTRV	10.5	5			1	1	3				2	3				
	11	6			4		2				6					
MTR	10.5	5		4	1						5					
	11	6		6							6					
AQG	10.5	5		2	1	2					2	3				
	11	6		4		2						6				
LAG	10.5	5						5								6
	11	6						6								6
AQGV	10.5	6		2	3						5					
	11	4		2	4						6					
LAGV	10.5	5				1		4					1			4
	11	6				1	1	3	1				2			4

[00121] To evaluate the effect of peptide treatment at an early or late point in time of development of shock, mice were treated at two hours or at 24 after LPS injection by i.p. injection with test peptide at 5 mg/kg bodyweight. The mice were followed for 84 hours instead of for 48 hours in the earlier experiments. The results are shown in Table 10. Of all tested
5 peptides, only AQGV showed 100% survival and no remaining clinical signs of shock at 84 hours after LPS-treatment when given both early or late in the development of shock.

[00122] Table 10

Percent of mice surviving LPS-induced sepsis after treatment with a single injection of test peptide(at 5 mg/kg body weight) at 2 or 24 hours after induction of sepsis by treatment with LPS.

TREATMENT 2 HOURS AFTER LPS TREATMENT

	% SURVIVAL IN TIME (HRS)				
	0	14	24	48	84
PBS	100	100	100	0	0
LQG	100	100	100	0	0
LQGV	100	100	100	100	100
VLPALP	100	100	100	100	100
VLPALPQ	100	100	100	0	0
MTR	100	100	100	100	50
MTRV	100	100	100	50	30
VVC	100	100	100	80	50
AQGV	100	100	100	100	100

* 5 mg/kg BW

TREATMENT 24 HOURS AFTER LPS TREATMENT

	% SURVIVAL IN TIME (HRS)				
	0	14	24	48	84
PBS	100	100	100	0	0
LQG	100	100	100	0	0
LQGV	100	100	100	0	0
VLPALP	100	100	100	0	0
VLPALPQ	100	100	100	100	100
MTR	100	100	100	100	100
MTRV	100	100	100	100	100
VVC	100	100	100	100	100
AQGV	100	100	100	100	100

n=10

[00123] Comparative trial MTR and AQGV, each derived from two sources, all at 5mg/kg.

Table 11

LPS= 10-11 mg/kg
#970= 5 mg/kg LPS treatment compound treatment
#971= 5 mg/kg
#Ansynth 12
#Ansynth 46

Sickness scores

	No.	0hrs	2hrs	22hrs	26hrs	42hrs	48hrs	Survival %
Group 1A	1	0	2	4	4	5	dead	
PBS	2	0	2	3	3	5	dead	
	3	0	2	5	dead			
	4	0	2	3	3	3	2	
	5	0	2	4	4	dead		
	6	0	2	5	5	dead		
Group 1B	1	0	2	4	4	4	dead	
PBS	2	0	2	5	dead			
	3	0	2	3	3	2	1	
	4	0	2	4	dead			
	5	0	2	4	4	5	dead	
	6	0	2	5	5	dead		17%
Group 2	1	0	2	Dead				
#970	2	0	2	3	3	5	dead	

Table 11

LPS= 10-11 mg/kg
#970= 5 mg/kg
#971= 5 mg/kg
#Ansynth 12
#Ansynth 46

	No.	0hrs	2hrs	22hrs	26hrs	42hrs	48hrs	Survival %
		LPS treatment	compound treatment	Sickness scores				
Diosynth BV MTR	3	0	2	2	2	2	2	
	4	0	2	2	2	2	2	
	5	0	2	2	2	4	dead	
	6	0	2	4	5	dead		33%
Group 3	1	0	2	2	1	1	1	
#Ansynth 12	2	0	2	2	2	2	1	
MTR	3	0	2	4	5	dead		
	4	0	2	3	4	4	dead	
	5	0	2	2	3	5	dead	
	6	0	2	2	2	2	2	50%
Group 4	1	0	2	2	2	2	2	
#971	2	0	2	3	3	2	1	
Diosynth BV AQGV	3	0	2	2	2	2	2	
	4	0	2	2	2	2	2	

Table 11

LPS= 10-11 mg/kg
#970= 5 mg/kg LPS treatment compound treatment
#971= 5 mg/kg
#Ansynth 12
#Ansynth 46

Sickness scores

	No.	0hrs	2hrs	22hrs	26hrs	42hrs	48hrs	Survival %
	5	0	2	4	5	dead		
	6	0	2	2	3	2	1	83%
Group 5	1	0	2	3	3	2	1	
#Ansynth 46	2	0	2	3	3	3	1	
AQGV	3	0	2	2	2	1	1	
	4	0	2	4	4	5	dead	
	5	0	2	2	2	1	1	
	6	0	2	2	2	1	1	83%

[00124] Several reports have suggested modulation of the immune system by intact hCG, but such effects of breakdown products have not been reported in the scientific literature. Benner and Khan (*Scand. J. Immunol.* 2005 Jul; 62 Suppl 1:62-6) studied the possible immunological activity of the *in vivo* liberated peptide fragments originating from nicking of the sequence MTRVLQGVLPALPQVVC (residues 41-57) of loop 2 of the beta-subunit of hCG, in lieu of the fact that peptides as small as three to seven amino acids generally are not supposed to have significant biological activity.

[00125] We have designed peptides that block completely LPS induced septic shock in mice, in several cases even when treatment with these peptides is started up to 24 hours after

LPS injection. These peptides are also able to inhibit the production of MIF. This finding provides the possibility of therapeutic use of these peptides for the treatment of patients suffering from radiation injury.

[00126] Example 7

[00127] This example shows the results of experiment with peptide AQGV on Whole Body Irradiation (WBI) of mice with 8.6 Gy whereby all groups of mice got the first injection 3 hours after the WBI. The group that got a placebo injection experienced 80% mortality, as predicted in this model. The dose of radiation given (8.6 Gray = 8.6 Gy) is known, in this species, to cause about an 80% mortality, so it is called the LD80 (lethal dose for 80%). The deaths starting around Day 10 - which is typical for what happens in WBI to animals or humans: By around Day 10, the gut lining is so damaged and leaky from the radiation that bacteria get into the circulation and cause sepsis because of Gastrointestinal (GI) Syndrome, and the bone marrow is so damaged that there aren't enough white blood cells to fight the infection ("Bone Marrow Syndrome"), and death ensues.

[00128] A first group of peptide treated mice group with "x" as the symbol (figure 1) got the first injection with AQGV IV, and the second injection subcutaneously (SC) 3 hours after that first injection. Surprisingly, 100% of these survived. Furthermore, the animals didn't show any signs of sickness at all. To an outside observer, they would look like perfectly normal mice, in particular, the peptide-treated mice did not show the GI syndrome.

[00129] A second group of mice got its first injection of the peptide via the SC route. Then it got additional SC injections every 48 hours, for a total of 3 doses (in addition to the first dose) - meaning, on Day 3, Day 5, and Day 7. Note that only one of these animals died, others were not showing any symptoms of the GI syndrome.

[00130] The third group of mice was identical in procedures to the group with the second group, except that the q48 hour SC injections continued on until a total of 6 doses had

been injected (in addition to the first dose). So the dosing continued on until Day 13. Note that this prolonging of the treatment was associated with complete protection (no mortality whatsoever in this group). The animals in this group showed no signs of sickness, again not showing any symptoms of the GI syndrome.

[00131] What we can conclude from this data is that when the animals got two doses of peptide in the first day (with the first one being IV), with AQGV there was complete protection against a highly-lethal dose of WBI, and in particular against the GI syndrome associated with that dose. When the animals got lower-level treatment (SC only), if the treatment was prolonged until the 2nd week, here again there was complete protection and in particular against the GI syndrome associated with the high dose used.

[00132] When comparing these results to published information on studies describing the radiation protection activity of a drug code-named ON-01210 that were presented at the 51st Radiation Research Society (April, 2004), it shows that this particular drug ON-01210 (like several other non-peptide drugs that are currently under investigation for radiation-exposure) is protective only if it is given pre-radiation exposure. That makes it not very useful for protection against dirty bombs. This particular drug has a sulfhydryl component (4-carboxystyrl-4-chlorobenzylsulfone) that works as an antioxidant, scavenging the free radicals that are generated as the radiation damages the cells. If it's not present in the body at the time of the radiation exposure, it has no effect whatsoever. In contrast, treating with a peptide according to the invention works after exposure.

[00133] Also, when reviewing treatment with other available drugs all the current data given on such drugs (i.e. on treatment with anabolic steroids) show the need for current non-peptide drugs to being given prior (i.e. 24 hours) to the WBI, later administration has no supportive effect whatsoever on protection against acute radiation injury.

[00134] Example 8, DRF studies.

[00135] In this example we report on escalating dose studies of Whole Body Irradiation (WBI), with one single exposure given to any particular cohort, and the exposure dose going progressively higher, for each subsequent cohort. A single dose of peptide AQGV (10mg/kg) was administered subcutaneously, but with treatment delayed until three days (72 hr) after the WBI. The test is called the Dose Reduction Factor ("DRF"), which is defined as ratio between the LD50 of the treated group to the LD50 of the control group. The LD50 refers to that dose which is lethal to 50% of the animals tested.

[00136] An acceptable DRF ratio is a factor of at least 1.10; but preferred is at least 1.20 or even at least 1.25. To pass the test for DRF 1.20, at Day 30 after WBI a candidate drug must have an LD50 at a radiation dose that is at least 20% higher (an increase by a factor of 1.20) than the LD50 dose for the control animals. If e.g. the LD50 for the control animals is 8.2 Gy, then, to pass this test, the LD50 for a candidate drug would have to be at least 20% higher, which in this case would mean a dose of 8.2 x 1.20 = 10.4 Gy.

[00137] Numbers of animals tested and results in DRF test can be found in Table 12.

Table 12

Dose (Gy)	"n"	The absolute number of animals alive at 30 days post WBI	Percent alive at day 30	Percent dead at day 30
7.4	50	45	90%	10%
8	100	60	60%	40%
8.6	120	24	20%	80%
9.2	30	0	0%	100%
8.6 + AQGV at day 3	20	20	100%	0%
9.2 + AQGV at day 3	10	10	100%	0%
9.8 + AQGV at day 3	10	10	100%	0%
10.4 + AQGV at day 3	10	4	40%	60%
11.0 + AQGV at day 3	10	0	0%	100%

[00138] It is important to discuss the rationale for the decision to delay treatment for 72 hours: In some scenarios of radioactive exposure (e.g., explosion of a nuclear fission device on a cargo ship, or a plane crashing into a nuclear reactor near metropolitan centers, etc.), the amount of destruction could be such that it could take several days to get all the victims to treatment centers. Therefore military scientists (interested in protecting the first responders) and civilian scientists (interested in treating the mass casualties) would naturally want to determine if a candidate drug can do anything to diminish the acute radiation toxicity (GI Syndrome, Bone Marrow Syndrome) that will otherwise be fulminant after so long a delay.

[00139] Results of DFR test with AQGV. The radiation dose that kills 50% of the controls turns out to be ~8.2 Gy. Peptide AQGV is so protective that one has to increase the radiation dose by 25% (a factor of 1.25), all the way to ~10.4 Gy, to kill 50% of the animals, and this is with treatment delayed for three (3) days. If the treatment had been given sooner, e.g. at 24 hr or 48 hr, it would have taken an even higher dose of radiation to kill 50% of the animals.

[00140] Example 9

To further study anti-cell cycle activity of various oligopeptides a proliferation experiment in Arabidopsis Thaliana seedlings was performed. The aim was to test a group of 140 oligopeptides of varying length for their effect on plant marker gene expression during rapid growth when avid cell division occurs. Both marker genes are related to the cell-cycle process, high marker activity represents high cell-cycle activity and no marker activity represents no cell-cycle activity and hence no proliferation. Examples of the effect of oligopeptides in Arabidopsis Thaliana cell-cycle analysis is given in figure 3.

Method

The peptides were re-suspended in 1x Phosphate Buffer Saline (PBS) pH8 to a final concentration of 5 mg/ml. The obtained solutions were then divided through 96-well round bottom plates (Corning Incorporated) at 40 microliters per well. Plates were stored before use at

–200C for four days. Seeds of *Arabidopsis thaliana* ecotype Ws-0 were surface sterilized in 2% commercial bleach (Glorix) for 10 minutes and washed five times with sterile MQ water. The seeds were then re-suspended with 0.1% agar and plated on MS20 plates supplemented with 80 mg/l Kanamycin.
The plates were placed at 40C for 2 nights, and then transferred to a climate room at 210°C and a 16/8 hrs photoperiod. After four days of growth, the seedlings were transferred to 96-well plates containing the peptide solutions (4 seedlings per well) and incubated for 4 and 8 hours.

For this experiment *Arabidopsis* homozygous seedlings harbouring two reporter genes fused to GUS were used. The first reporter gene used was a cell cycle marker, pCDG (Carmona et al., The Plant Journal, 1999, 20(4), 503-508), and the second an auxin responsive marker, DR5::GUS (Ulmasov et al., The Plant Cell, Vol. 9, 1963-1971). After incubation with the compounds, the seedlings were stained for GUS. The staining reaction was performed in 100 mM sodium phosphate buffer (pH 7.0) that contained 10 mM EDTA, 10% DMSO, 0.1% Triton X-100, 2 mM X-Gluc, 0.5 mM K3Fe(CN)6 and 0.5 mM K4Fe(CN)6 at 370C for 16 hours. To stop the GUS reaction and remove chlorophyl, the seedlings were subsequently treated for one hour with 96% ethanol and then stored in 70% ethanol. Stained seedlings were observed under a stereomicroscope and slides were made with seedlings showing an effect of the compound treatment. Seedlings were fixed and cleared in chloral hydrate solution for detailed microscopic observation and photography under a microscope equipped with DIC optics.

Results

Peptides were tested for an effect on marker gene expression on rapidly growing Arabidopsis young seedlings. This was monitored by changes on GUS distribution in different organs: root, root-hypocotyl transition zone and cotyledons.

From the 140 compounds tested a total of 43 showed a clear effect on the expression of both markers tested. Examples of significant changes caused by the tested compounds are shown in detail at the microscopic level for peptides LQGV, VVC and DINGFLPAL in Figure 3. Surprisingly, the effects were clearly related to the length of the various peptides tested. As can

be seen in Table 13 below, anti-cell cycle activity was over-represented in the short peptides, none of the peptides longer than 9 amino acids gave reduction of cell cycle activity. Of the peptides of 5 to 9 amino acids in length, about 22% showed reduction, but of the trimers and tetramers tested more than 50% showed reduction of cell cycle activity.

Table 13

Frequency distribution of peptides tested positive/peptide length as found in cell cycle test in Arabidopsis Thaliana. #AA = peptide length in amino acids; # = number tested; #+ = number found positive; %+ = percentage positive

#AA	**3**	**4**	**5**	**6**	7	**8**	**9**	**10**	**12**	**13**	**>14**
#	6	65	9	11	10	2	17	7	3	5	5
#+	3	38	3	3	3	1	1	0	0	0	0
%+	**50%**	**58%**	**33%**	**27%**	**30%**	**50%**	**6%**	**0%**	**0%**	**0%**	**0%**

Example 10

To further study anti-cell cycle activity of various oligopeptides an *in vitro* experiment in peripheral blood cells of mice stimulated with anti-CD3 was performed. The aim was to test representative oligopeptides for their effect on proliferation during rapid growth induced by CD3 when avid cell division occurs. Mice (n=5) were treated i.p. PBS, Nak4 (LQGV), Nak47 (LAGV), Nak46 (AQGV) provided by Ansynth BV, The Netherlands, or Nak46*(AQGV provided by Diosynth BV, The Netherlands). Mice were treated with 0.5 mg/kg or 5 mg/kg peptide for one hour after which spleens were isolated and spleen cell suspensions were made. Spleen cell suspensions were pooled per group en cultured in vitro (in three-fold) in the presence of PBS or of anti-CD3 antibody and proliferation was tested at 0, 12, 24 and 48 hours after culture. All tested peptides showed a reduction of proliferation (see figure 4).

Results from examples 9 and 10.

From cell-cycle studies in plants and in-vitro reduction of proliferation studies in peripheral blood cells, useful 3-mer peptides for treatment of radiation injury were identified, VVC, LAG, AQG. Similarly, useful 4-mer peptides for treatment of radiation injury are LQGV, QVVC, MTRV, AQGV, LAGV, LQAV, PGCP, VGQL, RVLQ, EMFQ, AVAL, FVLS, NMWD, LCFL, FSYA, FWVD, AFTV, LGTL, QLLG, YAIT, APSL, ITTL, QALG, GVLC, NLIN, SPIE, LNTI, LHNL, CPVQ, EVVR, MTEV, EALE, EPPE, LGTL, VGGI, RLPG, LQGA, LCFL, useful 5-mer peptides for treatment of radiation injury are TLAVE, VEGNL, LNEAL, useful 6-mer peptides for treatment of radiation injury are VLPALP, MGGTWA, LTCDDP, useful 7-mer peptides for treatment of radiation injury are VLPAPLQ, VCNYRDV, CPRGVNP, a useful 8-mer peptide for treatment of radiation injury is QPLAPLVG and a useful 9-mer peptide for treatment of radiation injury is DINGFLPAL.

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Claims

1. A method for treating a subject suffering from or believed to be suffering from radiation injury, said method comprising providing the subject with a peptide, or functional analogue or derivative thereof, of smaller than 30 amino acids.
2. A method according to claim 1 wherein said peptide is smaller than 15 amino acids.
3. A method according to claim 1 or 2 wherein said peptide is smaller than 7 amino acids.
4. A method according to anyone of claims 1 to 3 wherein said peptide consists of 2 to 6 amino acids.
5. A method according to anyone of claims 1 to 4 wherein said peptide consists of 3 to 5 amino acids.
6. A method according to anyone of claims 1 to 5 wherein said peptide consists of 4 amino acids.
7. A method according to anyone of claims 1 to 6 wherein said radiation injury comprises acute radiation injury.
8. A method according to anyone of claims 1 to 7 wherein said radiation injury comprises damage of the lining of the intestinal tract of said subject, the so-called gastrointestinal syndrome.
9. A method according to anyone of claims 1 to 8 wherein said peptide has a dose reduction factor (DRF) against acute gamma irradiation of at least 1.10, said DRF determinable by
   a. testing which dose of radiation results in 50% mortality at 30 days (LD50/30) after whole body radiation (WBI) in a test group of mice treated with said peptide at 72 hours after WBI and,
   b. testing which dose of radiation results in 50% mortality at 30 days (LD50/30) after whole body radiation (WBI) in a control group of mice treated only with the vehicle of said peptide at 72 hours after WBI and,
   c. wherein the DRF is calculated by dividing the LD50/30 of the peptide-treated animals by the LD50/30 of the vehicle-treated animals.

10. A method according to claim 9 wherein said peptide has a dose reduction factor (DRF) of at least 1.20, more preferably of at least 1.25.
11. A method according to anyone of claims 1 to 10 wherein said radiation injury is irradiation injury.
12. A method according to anyone of claims 1 to 11 wherein said treatment comprises subcutaneous or intramuscular injection with said peptide.
13. Use of a peptide, or functional analogue or derivative thereof, of smaller than 30 amino acids for the production of a pharmaceutical composition for the treatment of a subject suffering from or believed to be suffering from radiation injury.
14. Use according to claim 13 wherein said peptide is smaller than 15 amino acids.
15. Use according to claim 13 or 14 wherein said peptide is smaller than 7 amino acids.
16. Use according to anyone of claims 13 to 15 wherein said peptide consists of 2 to 6 amino acids.
17. Use according to anyone of claims 13 to 16 wherein said peptide consists of 3 to 5 amino acids.
18. Use according to anyone of claims 13 to 17 wherein said peptide consists of 4 amino acids.
19. Use according to anyone of claims 13 to 18 wherein said radiation injury comprises acute radiation injury.
20. Use according to anyone of claims 13 to 19 wherein said radiation injury comprises damage of the lining of the intestinal tract of said subject, the so-called gastrointestinal syndrome.
21. Use according to anyone of claims 13 to 20 wherein said peptide has a dose reduction factor (DRF) against acute gamma irradiation of at least 1.10, said DRF determinable by
    a. testing which dose of radiation results in 50% mortality at 30 days (LD50/30) after whole body radiation (WBI) in a test group of mice treated with said peptide at 72 hours after WBI and,

b. testing which dose of radiation results in 50% mortality at 30 days (LD50/30) after whole body radiation (WBI) in a control group of mice treated only with the vehicle of said peptide at 72 hours after WBI and,

c. wherein the DRF is calculated by dividing the LD50/30 of the peptide-treated animals by the LD50/30 of the vehicle-treated animals.

22. Use according to claim 21 wherein said peptide has a dose reduction factor (DRF) of at least 1.20, more preferably of at least 1.25.

23. Use according to anyone of claims 13 to 22 wherein said radiation injury is irradiation injury.

24. Use according to anyone of claims 13 to 23 wherein said treatment comprises subcutaneous or intramuscular injection with said peptide.

25. Use according to anyone of claims 13 to 24 wherein said pharmaceutical composition is contained in an autoinjector.

26. An autoinjector comprising a peptide, or functional analogue or derivative thereof, of smaller than 30 amino acids.

27. An autoinjector according to claim 26 wherein said peptide is smaller than 15 amino acids.

28. An autoinjector according to claim 26 or 27 wherein said peptide is smaller than 7 amino acids.

29. An autoinjector according to anyone of claims 26 to 28 wherein said peptide consists of 2 to 6 amino acids.

30. An autoinjector according to anyone of claims 26 to 29 wherein said peptide consists of 3 to 5 amino acids.

31. An autoinjector according to anyone of claims 26 to 30 wherein said peptide consists of 4 amino acids.

32. An autoinjector according to anyone of claims 26 to 31 wherein said radiation injury comprises acute radiation injury.

33. An autoinjector according to anyone of claims 26 to 32 wherein said radiation injury comprises damage of the lining of the intestinal tract of said subject, the so-called gastrointestinal syndrome.

34. An autoinjector according to anyone of claims 26 to 33 wherein said peptide has a dose reduction factor (DRF) against acute gamma irradiation of at least 1.10, said DRF determinable by
    a. testing which dose of radiation results in 50% mortality at 30 days (LD50/30) after whole body radiation (WBI) in a test group of mice treated with said peptide at 72 hours after WBI and,
    b. testing which dose of radiation results in 50% mortality at 30 days (LD50/30) after whole body radiation (WBI) in a control group of mice treated only with the vehicle of said peptide at 72 hours after WBI and,
    c. wherein the DRF is calculated by dividing the LD50/30 of the peptide-treated animals by the LD50/30 of the vehicle-treated animals.
35. An autoinjector according to claim 34 wherein said peptide has a dose reduction factor (DRF) of at least 1.20, more preferably of at least 1.25.
36. An autoinjector according to anyone of claims 26 to 35 wherein said radiation injury is irradiation injury.
37. An autoinjector according to anyone of claims 26 to 36 wherein said radiation is emitted by radioactive substances.

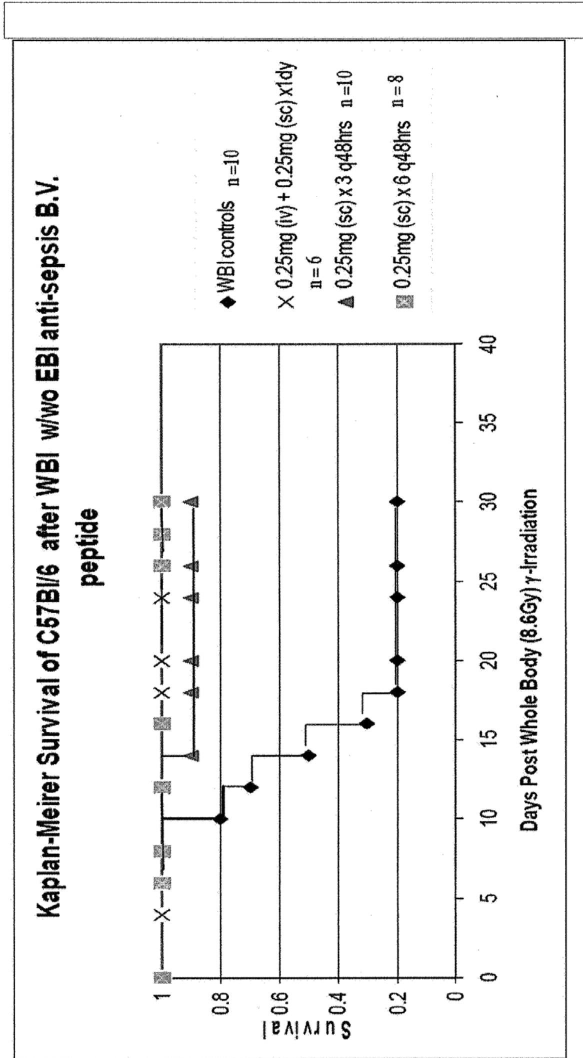

Fig. 1

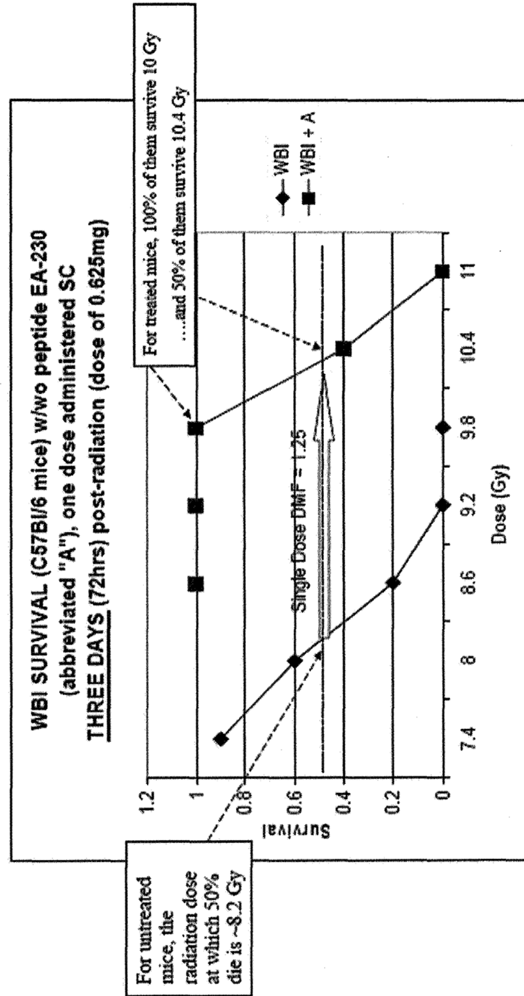

Fig. 2

WO 2007/102735 PCT/NL2007/050092

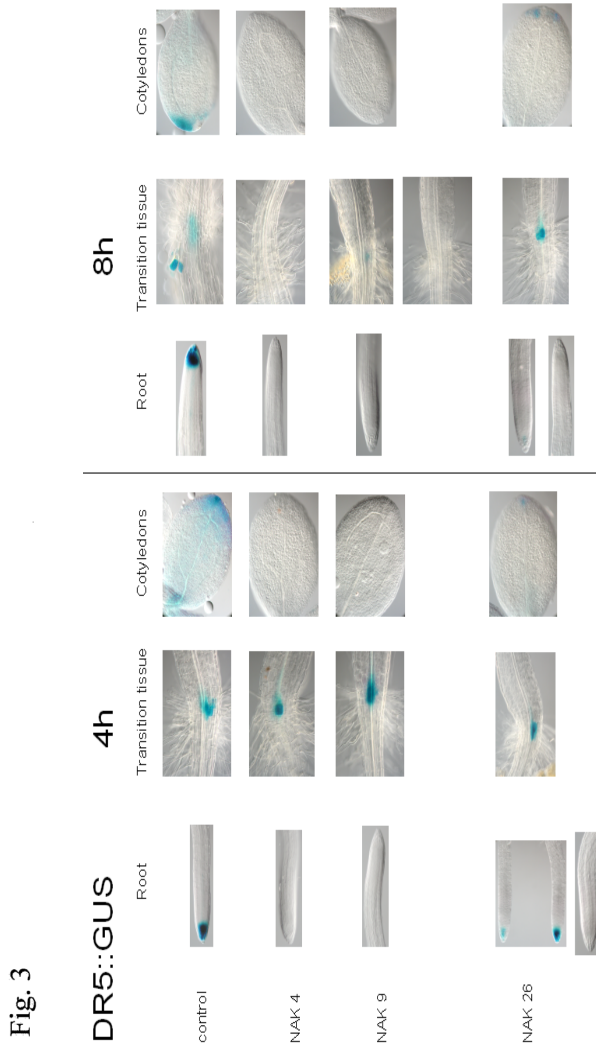

Fig. 3

Fig. 4

αCD3 proliferatie

(light shading) =P<0.05
(dark shading) =P0.08<0.06

T=12

groep	mg/kg I.p.	CCPM			AVG	SEM	p-value
PBS		413	766	591	590	101.9	
NAK-4	0.5	305	372	288	321.7	25.6	0.11
	5	449	616	492	519.0	50.1	0.58
NAK-46	0.5	154	465	444	354.3	100.4	0.17
	5	787	1760	1327	1291.3	281.5	0.12
NAK-47	0.5	148	273	378	266.3	66.5	0.07
	5	169	375	523	355.7	102.7	0.18
NAK-46*	0.5	299	60	105	154.7	73.3	0.03
	5	389	87	604	360.0	150.0	0.28

T=24

groep	mg/kg I.p.	CCPM			AVG	SEM	p-value
PBS		7114	9395	9287	8598.7	743.0	
NAK-4	0.5	2359	1904	2244	2169.0	136.6	0.01
	5	3017	3289	4143	3483.0	339.2	0.01
NAK-46	0.5	2552	4275	3367	3398.0	497.6	0.01
	5	4372	6860	8791	6674.3	1279.1	0.28
NAK-47	0.5	181	2586	3199	1988.7	921.0	0.01
	5	1554	2631	3518	2567.7	567.9	0.00
NAK-46*	0.5	1297	1306	162	921.7	379.9	0.00
	5	4527	72	2823	2474.0	1297.9	0.02

T=36

groep	mg/kg I.p.	CCPM			AVG	SEM	p-value
PBS		40051	56075	61125	52417.0	6352.7	
NAK-4	0.5	23826	23624	20520	22656.7	1070.0	0.04
	5	20352	25311	34935	26866.0	4281.1	0.04
NAK-46	0.5	22921	26487	39461	29623.0	5025.7	0.05
	5	33409	58790	43895	45364.7	7363.8	0.51
NAK-47	0.5	8888	20833	25977	18566.0	5061.9	0.02
	5	15397	22297	23183	20292.3	2461.1	0.02
NAK-46*	0.5	3751	2583	4191	3508.3	479.8	0.02
	5	29886	18485	33944	27438.3	4627.5	0.04

T=48

groep	mg/kg I.p.	CCPM			AVG	SEM	p-value
PBS		30814	45218	41992	39341.3	4364.3	
NAK-4	0.5	24325	21071	15146	20180.7	2687.0	0.03
	5	22413	24013	20795	22407.0	929.0	0.05
NAK-46	0.5	21410	29916	27099	26141.7	2501.8	0.07
	5	65312	58347	55436	59698.3	2930.0	0.02
NAK-47	0.5	10160	21079	20152	17130.3	3495.5	0.02
	5	11253	20791	17370	16471.3	2789.9	0.02
NAK-46*	0.5	3091	1174	2821	2362.0	599.1	0.01
	5	25844	16048	21713	21201.7	2839.5	0.03

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