IMMUNOREGULATORY AND ANTITUMOR EFFECTS OF INTERFERON-γ

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IMMUNOREGULERENDE EN ANTITUMOR EFFECTEN VAN INTERFERON-γ

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De axioma's die men door redenering vindt kunnen nooit voldoende zijn om nieuwe dingen te ontdekken, want de subtiliteit van de natuur is vele malen groter dan de subtiliteit van de redenering

Francis Bacon

Aan mijn ouders
Aan Margriet

CONTENTS

Part A

GENERAL INTRODUCTION

Chapter 1

THE IMMUNE SYSTEM

1.1 Introduction

Vertebrates possess an immune system that discriminates between "self" and "non-self". Reactions mediated by the immune system may lead to elimination of foreign organisms, such as viruses, bacteria and parasites.

Cells of the immune system produce cytokines, proteins that function as mediators between various cells of an organism. Due to recent progress in molecular biology a great number of cytokines has been identified and produced in large quantities by recombinant DNA techniques. One of the first cytokines that became available for research by these techniques was interferon-γ (IFN-γ). Based upon an impressive amount of in vitro experiments pleiotropic immunoregulatory and antiproliferative activities of IFN-γ were found. Today, knowledge of the function of IFN-γ in physiologic and pathologic conditions still mainly depends on extrapolation of in vitro findings.

From a clinical point of view it is of great interest to analyse the effects of IFN-γ in vivo. Being released by activated T cells at the centre of the immune response, this cytokine may play a pivotal role in immunoregulation. Thus, manipulation of its production might offer new perspectives for clinical entities in which suppression or stimulation of the immune system is essential, such as in organ transplantation and cancer immunotherapy.

For example, rejection of a donor organ by the recipient is known to be an immunological process (1), initiated by T lymphocytes. These cells recognize antigenic components on the cell surface of "non-self" structures and initiate, by release of cytokines, including IFN-γ, reactions that eventually lead to rejection of foreign cells. At present, T cell activation can adequately be prevented by cyclosporine, a potent immunosuppressive drug. However, cyclosporine surpresses T cell dependent immune functions non-specifically and thereby also reduces other host defense mechanisms necessary to offer resistance to invading micro-organisms. Further analysis of the function of cytokines released by activated T cells, such as interferon-γ, may lead to a more selective manipulation of the immune system.

Whereas organ transplantation might benefit from selective immunosuppression, selective immunostimulation might be to the advantage of cancer immunotherapy. During the last decades several theories have been postulated on cancer and the immune system. In the 1950's Foley demonstrated in mice that, after removal of a primary tumor, growth of reimplanted tumor tissue could be inhibited by allogenic-

like immune reactions (2). It was found that cancer cells could express different proteins leading to altered antigenic cell surface components. These altered antigens, called tumor associated antigens (TAA) could induce allogeneic immune reactions. Various types of TAA were identified, dependent upon the tumor-inducing factor, *i.e.* chemical, physical or viral.

In 1909 Ehrlich proposed the concept of an immune surveillance theory to explain how tumor growth could be prevented by the defense mechanisms of an organism (3). During the 1970s Burnett further developed this theory (4). He assumed that de novo tumor cells arose daily and were more or less antigenic to their host. Cancer cells would be recognized as "non-self" by T lymphocytes, be killed and eliminated via immunologic reactions. However, gradually it became clear that de novo tumor cells did not arise frequently (5) and that spontaneous tumors most often lack detectable surface antigens (6). Experimental studies demonstrated that the incidence of spontaneous tumors in immunodeficient mice was not higher than in normal mice (7) and that tumors which metastasized in normal mice failed to do so in immunocompromised mice (8). In clinical studies immunosuppressive therapy was found to enhance the risk of skin tumors and lymphomas, but not of other tumors of rapidly dividing tissues, as would be expected according to the immune surveillance theory (9,10).

Hence, the theory was adapted to newer insights and the concept of natural resistance was developed. In this concept the importance of natural killer (NK) cells, known to be able to kill cancer cells without antigenic stimulation, was stressed. In the 1980's interest in cancer and immune reactions revived with the discovery of cytokines. Cytokines were demonstrated to kill cancer cells directly and, in addition, to stimulate immune functions, including NK activity. Interferons were one of the first groups of cytokines discovered and three different classes were identified, IFN-α, IFN-β and IFN-γ. Of these, IFN-γ was shown to have the strongest immunomodulatory and antiproliferative potency *in vitro*.

The importance of these findings for immunoregulation and immunotherapy in vivo still has to be established. The aim of the experiments presented in this thesis was to determine the effect of IFN-γ on immune functions and on cancer growth in vivo, using the rat model.

As an introduction to the experiments a brief description of the main immune reactions is presented first.

1.2 Cells mediating immune responses

Immunological reactions can be divided into a humoral and a cellular part. B lymphocytes are of main importance for humoral responses, whereas T lymphocytes, macrophages and natural killer cells are mainly involved in cellular reactions. Commonly accepted functions of these cells are described.

B lymphocytes

Humoral immunological reactions are provided by B cells that differentiate into antibody producing plasma cells in response to antigenic stimulation. In 1982 Grey *et al* (11) demonstrated that B cells act as antigen presenting cells (APC), processing antigen for specific T cells recognizing the presented antigen. It was shown that B cells can capture antigen present in the corporal fluid at very low concentrations with specific receptors (surface immunoglobulins) without the help of other APC, such as macrophages (12). The interaction between B and T cells provides the B cell with helper signals for proliferation, differentiation and specific antibody production. Thus, amplification of the humoral immune response occurs after B-T cell contact.

Antibodies secreted by B cells bind specifically to inducing antigens, forming immune complexes. Subsequently, antigen-coated cells are damaged via complement dependent cellular cytotoxicity (CDCC) and antibody dependent cellular cytotoxicity (ADCC). In CDCC, the complement system, consisting of a series of protein components acting in sequence, is activated via binding to an immune complex. Lysis of the cell to which the immune complex-complement system is attached, occurs through membrane destruction. In ADCC an effector cell mediates cytolysis after binding to an immune complex on a foreign cell via its receptor for the constant part (F_c) of immunoglobulin (Ig) molecules.

T lymphocytes

The population of T lymphocytes can be divided according to functional assays in helper T (T_h), suppressor T (T_s) and cytotoxic T lymphocytes (CTL). As determined by these assays, activated helper T cells are essential for the activation of other immune mechanisms via release of several soluble, non-specific molecules (lymphokines). T_s-cells are capable to inhibit certain immune responses and CTL exert cytotoxic activity against cells with specific antigens.

With the introduction of T cell cloning and the availability of monoclonal antibodies directed against molecules expressed on the cell membrane, T cells were redefined phenotypically according to the presence of specific cluster differentiation (CD) antigens. All T cells appear to have a common determinant, CD3, being an invariant component of the T cell receptor (TCR) complex. A subdivision into those expressing differentiation antigen CD4 and CD8 was made. Although functional and

phenotypical identification may not be considered to be identical, CD4 T cells are commonly referred to as T_h-cells and CD8 T cells as T_s/CTL.

T cells recognize antigen presented by APC in complex with cell membrane molecules coded by genes of the major histocompatibility complex (MHC) (13). CD4 T cells mainly recognize antigen in association with MHC class II molecules, whereas CD8 T cells are restricted to MHC class I molecules (14). The accessory molecules CD4 and CD8 are thought to be responsible for the MHC restriction via a preferential binding site on certain parts of MHC molecules (15). In addition to the antigen-MHC class II complex the accessory molecules form a positive signal for the TCR during T cell activation.

Studying the functional aspects of cloned murine CD4 T cells, Killar *et al.* (16) found a distinction into two major functional cell types. One providing help to B cells to produce specific antibody directed against extracellular pathogens (helper CD4 T cell or T_h2) and the other mediating killing and delayed type hypersensitivity required for immunity against intracellular pathogens (inflammatory CD4 T cells or T_h1). Mosmann and Coffman (17) demonstrated that the distinct functions of the two types of CD4 T cell lines correlated with the production of a distinct panel of lymphokines. For example, T_h1 cells produce interleukin-2 (IL-2), lymphotoxin (LT) and interferon-gamma (IFN-γ), whereas T_h2 cells produce among others IL-4.

As in mice, subsets of CD4 T cells have been identified in rat and man. To date, no subdivision based upon differentiation antigens has been demonstrated for CD8 T cells. T cells that exert suppressive activity are identified in subsets expressing CD4 as well as CD8; cytotoxic T lymphocytes able to kill virus-infected cells and virally induced tumor cells express the accessory molecule CD8.

Natural killer cells

The existence of a third category of lymphocytes different from either T or B cells is acknowledged (18). These cells are called natural killer (NK) cells, referring to the fact that they do not require prior sensitisation in vivo, but are already cytotoxic in vitro to a variety of cell lines. NK cells can be defined as CD3 and TCR negative large granular lymphocytes. In contrast to CTL, NK cells are not MHC class I restricted. In fact, there seems to be an inverse relationship between the MHC class I expression of cells and susceptibility to NK-cytotoxicity. Recently, Yeoman and Robins (19) demonstrated that treatment of rat tumor cell lines with IFN-γ increased the expression of MHC class I molecules and reduced the sensitivity to NK-cytolysis. Interestingly, these treated cells were killed more effectively by CTL than untreated cells, suggesting a complementary function. In addition to NK cells, a minor population of CD3 T cells can be discerned that do not require MHC class I expression on cells either to exert cytotoxicity; these T cells express NK-like activity.
NK cells may serve as a rapidly acting first line of defense mechanism involved in the

early destruction of malignant cells after entrance into the circulation. Studies on the survival of transplanted radiolabeled tumor cells in vivo revealed that 24-48 hours after inoculation twice as many cells of a NK-resistant tumor cell line were alive as compared to those of a NK-sensitive tumor cell line. The growth rate and doubling time of both tumor lines were similar (20). Notably, NK-mediated resistance against tumor growth in vivo can only be observed when a small number of tumor cells is inoculated. Studies in beige mice, that have an inherited low NK activity, showed an enhanced incidence in experimental and spontaneous metastasis of NK-sensitive B 16 melanoma cells (21). In rat and mice depletion or inactivation of NK cells in vivo with antibodies enhances the survival of radiolabeled circulating tumor cells and the number of pulmonary and extra-pulmonary metastases of several tumors (22). These data strongly suggest that NK cells are of importance to prevent the take or seeding of circulating tumor cells.

Cell lysis can occur directly via cell-cell contact or indirectly, *e.g.* via the release of tumor necrosis factor (TNF). The NK activity can be influenced by several lymphokines. Using cloned NK cell lines responsiveness was demonstrated to IL-3 for maintenance of growth and to IL-2 and IL-4 for activation of cytotoxicity. In vivo it has been shown that considerable synergies can be obtained in mouse peritoneal NK activity by local injections of IL-2 and IFN-γ.

Macrophages

Monocytes/macrophages are bone-marrow derived cells that can exert a variety of functions. They can act as APC, produce IL-1 and trigger T lymphocytes. In response, the activated T cells produce a number of lymphokines that regulate monocyte activity. Macrophage chemotactic factor causes migration of monocytes to those sites where lymphokine production takes place, migration inhibiting factor (MIF) arrests monocytes at these locations and macrophage activating factor (MAF) induces lysis of intracellular organisms. MIF as well as MAF-like functions can be exerted by IFN-γ (23). IL-2, stimulating all three populations of lymphocytes, may influence the activity of monocytes as well. Malkovsky *et al.* (24) demonstrated that certain human monocytes can display an enhanced cytotoxic activity in response to IL-2, but not to IFN-γ.

Activated macrophages have an enhanced expression of certain cell membrane proteins, such as MHC class II molecules and F_c-receptors. They express an increased phagocytosis, non-specific antimicrobial activity and tumor cytotoxicity. The release of cytokines, such as tumor necrosis factor, is enhanced, which may contribute to the killing of tumor cells.

1.3 Determinants of immune responses

In the 1980s detailed information became available on the mechanisms by which immune cells are able to recognize foreign antigens. While B cells recognize antigen directly via immunoglobulin molecules, T cells can only recognize antigen in association with "self" molecules coded by genes of the MHC. These molecules play a pivotal role in antigen activation of T cells. They can be divided in class I and class II molecules according to their structure, function and tissue distribution. MHC class I molecules are expressed on most, if not all nucleated cells and function as receptors for peptide fragments, such as degradation products of viral and endogenously synthesized proteins. Antigen-specific CD8 T cells can recognize these MHC class I-peptide complexes and are triggered by this complex to execute their effector function, *i.e.* lysis of the target cell.

MHC class II molecules are normally expressed on a limited number of cells, including B cells, macrophages, dendritic and Langerhans cells (25). Antigen in association with MHC class II molecules on APC is mainly recognized by CD4 T cells leading to release of lymphokines regulating antigen-specific immune reactions such as expansion of CTL clones and enhancement of B cell responses. In the artificial situation of organ transplantation a donor MHC-peptide complex can induce alloreactive responses, thereby making complex formation of the donor peptide with the recipients MHC-molecules not a prerequisite for immune activation.

Although MHC-antigens are of major importance for the outcome of an allograft, other antigens may have an effect as well. In the mouse system another set of determinants has been demonstrated that can function as strong stimuli for proliferative responses of T-cells. They are called minor lymphocyte stimulating (Mls) antigens and defined by their ability to induce T cell responses in mixed lymphocyte reactions of MHC-identical strains (26). Furthermore, tissue specific antigens have been identified for skin, heart, liver and pancreas (27), as well as endothelial cells (28). These antigens are of minor importance for the rejection of an allograft compared to the MHC-antigens, except when presensitization has occurred.

1.4 Cell signaling and immune regulation

Activation, proliferation and differentiation of cells mediating immune reactions is controlled by a complex system of molecular mechanisms. Originally, these molecules were called interleukins, referring to their function as intermediates in the cooperation between leukocytes. However, since the first interleukins have been discovered a variety of (glyco)proteins have been identified that are produced by and regulate the growth and immune function of other cells than lymphocytes. Thus, the

term "interleukin" has become broadened in its use to encompass these other molecules. Strictly speaking the word interleukin is a misnomer to define the entire group of immunoregulating proteins and is at present replaced by the term cytokines.

During the 1980s an impressive number of cytokines have been discovered and made available for study by recombinant DNA techniques, such as interleukins, colony stimulating factors (CSF), tumour necrosis factors (TNF), and interferons (Table 1.1) (29).

Table 1.1 Main activity of various cytokines

Name	main activity
IL-1	proinflammatory protein
	activation of T lymphocytes
IL-2	growth factor for activated T cells
IL-3	multipotential hematopoietic cell
	growth factor
IL-4	B cell growth factor
	induction of IgE production
IL-5	activation, growth and differentiation
	factor for B cells
	differentiation of eosinophils
IL-6	proinflammatory protein
	stimulation of B cells
	weak antiviral activity
	growth factor for hybridoma/plasmocytoma
IL-7	T and B cell differentiation
TNF-α, -ß	proinflammatory proteins
	cytotoxic activity
	catabolic activity
GM-CSF	growth and stimulation factor for
G-CSF	hematopoietic cells (granulocytes (G)
M-CSF	and macrophages (M))
IFN-α, -ß	antiviral activity
	antiproliferative activity
	immunomodulation
IFN-γ	immunomodulation
	antiproliferative activity
	antiviral activity

In vitro studies, mainly performed with mouse and human cell lines, have shown that many of these cytokines have pleiotropic effects, such as IFN-γ, and more or less interchangeable biological activities, *e.g.* IFN-γ as well as IL-2 may enhance monocyte activity. Multiple cytokines may be required for the development of an immune cell, such as the B lymphocyte, where a series of factors, including IL-1, IL-2, IL-4, IL-5,

IL-6 and IFN-γ, is invoked to control activation and differentiation (30). For example, the production of different Ig is induced by distinct cytokines: in mice IL-4 induces IgG_1 and IgE production, IL-5 IgA, and IFN-γ IgG2.

These data clearly demonstrate the complexity of immunoregulation by the cytokine system. And although cell line studies have produced an overwhelming amount of detailed information many aspects of immunoregulation are still poorly understood.

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Chapter 2

INTERFERON-γ

2.1 Introduction

In 1957 Isaacs and Lindenmann (1) discovered a protein, designated interferon, that could confer resistance upon cells against a variety of viruses. In addition to its antiviral activity (2), IFN was found to inhibit cell growth (3) and influence many activities of cells belonging to the immune system (4-6). During the last decade multiple biologic effects of IFN were demonstrated (Table 2.1) (7). Most recently, a protein secreted by the early embryo and involved in maternal recognition of pregnancy was shown to be an IFN (8,9).

Table 2.1.Biological activities of IFN

antiviral
antitumor
antimicrobial
MHC antigen expression
cell differentiation
cell growth inhibition
enhancement of ADCC
enhancement of NK-activity
macrophage activation
modulation of B cell response
inhibition of T suppressor cells
enhancement of T cell cytotoxicity

Today, interferons are defined as proteins synthesized by vertebrate cells after exposure to a variety of stimuli and able to exert a broad spectrum of biological activities. Three functionally related, but antigenically distinct groups of proteins, designated IFN-α, -ß, and -γ (10) are identified in all mammalian species investigated.

On basis of its genetic origin, production and pleiotropic effects IFN-γ can be considered as a distinct entity when compared with other IFN. With the availability of recombinant IFN-γ (11-15) an impressive amount of research has been performed *in vitro* and *in vivo* to elucidate its physiologic role and potential therapeutic use.

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2.2 Molecular biology

2.2.1 *Induction and expression*

IFN-α and -ß are structurally related (16) and their genes located on the same chromosome (9 in man) (17), indicating their origin from a common ancestral gene (18). IFN-γ has a very limited structural homology to IFN-α and -ß; its genetic code is located on a different chromosome (12 in man) (19-21). There is evidence for only one IFN-γ gene in all species analysed. The gene codes for single polypeptides with an overall length of 146, 137 and 136 amino acids for human, rat and mouse IFN-γ, respectively. In man, the protein has a molecular weight of 17.147 daltons (22,23). In all three species the IFN-γ molecule contains two potential glycosylation sites. Dependent upon the carbohydrate groups attached at these sites IFN-γ can be secreted as a glycoprotein with a different molecular weight, *i.e.* in man 20.000 or 25.000 dalton (24). The presence of carbohydrate moieties on IFN-γ affects its biochemical, but not its functional properties. Glycosidase treatment of IFN-γ does not reduce its antiviral activity, affect the interaction with a specific antibody or change its target cell specificity (25).

IFN-γ is produced by activated T cells and probably by NK cells (26,27). Its production can be induced by many and diverse substances such as mitogens, antigens, allogeneic molecules coded for by the MHC complex (28) and antibodies directed against lymphocyte surface antigens (29).

The interaction between an IFN-γ inducer and a sensitive T cell leads to derepression of the specific genes, transcription of the cDNA sequence, *de novo* synthesis of mRNA and protein synthesis. In primary and secondary anamnestic immune responses, different kinetics of production can be distinguished, varying from days to hours, respectively (30,31).

Different T cell subpopulations, phenotypically defined as helper cells and suppressor/cytotoxic cells, may produce IFN-γ, depending on the type of inducing stimulus (32,33). Notably, these cells can also produce IFN-α and -ß, if stimulated by viral antigens, demonstrating an independent regulation of the distinct IFN-genes (34,35).

The production of IFN-γ is mediated by other cytokines released by cells of the immune system. Interleukin 2 (IL-2), mainly secreted by helper T cells (36) and interleukin-1 (IL-1), secreted by antigen-presenting cells, can enhance IFN-γ release directly as well as indirectly. IL-1 induces the expression of IL-2 receptors (IL-2R) in T cells that recognize the appropriate antigen (37). Subsequently, IL-2 promotes the proliferation of IL-2R positive cells and enhances the secretion of IFN-γ (38). The intracellular pathway induced by IL-2 to regulate the production of IFN-γ acts independently from the mechanism by which IL-2 enhances cellular proliferation (39).

Although IL-2 is significantly involved in the production of IFN-γ, its requirement is not an absolute one (40,41).

2.2.2 *Receptor interaction*

IFN-molecules interact with cells by binding to specific membrane receptors (42). All human cells tested so far carry a receptor for IFN-γ characterized by high affinity and specificity to the ligand. The observed species-specificity normally associated with IFN-γ seems to be dependent on the interaction of the molecule with the cell membrane-receptor. Sanceau *et al.* (43) demonstrated that murine cells transfected with a cDNA encoding for a human IFN-γ protein, accumulated the protein inside the cell, did not secrete it, and yet developed an antiviral state. In addition, human and murine IFN-γ proteins encapsulated within liposomes can induce activation of human as well as murine macrophages after phagocytosis (44).

Two molecular forms of the human IFN-γ receptor have been identified (45). The two receptor types differ in molecular weight, stability to acid and regulation of receptor expression. It is suggested that receptor variability may contribute to the different activities of IFN-γ on different cell types. The transmembranous signal produced by ligand binding leads to intracellular signals, mediated by and requiring the presence of other factors (46). Interaction of IFN-γ with its receptor as such may not be sufficient to initiate cell activation, as demonstrated by Hamilton *et al.* (47) who investigated the receptor mechanism of macrophages derived from A/J mice. The functional and biochemical reactions of these macrophages are deficient in response to IFN-γ when compared with macrophages of normal mice. It could be demonstrated that this was not due to different expression of the IFN-γ receptor or to altered affinity of the receptor for its ligand. Using another stimulus (ionophore combined with phorbolmyristic acetate), functional competence, comparable to the IFN-γ induced in normal macrophages, could be achieved.

Binding of IFN-γ to its receptor is followed by receptor-mediated endocytosis and ligand degradation (48). It is not clear whether IFN necessarily needs to be internalized to exert its biologic effect (49). Experiments with anti-receptor antibodies suggest that activation of the receptor may be sufficient to initiate transmembranous and intracellular signalling. However, if IFN-γ is manipulated into a cell via liposomes, thus bypassing the receptor, biologic effects can be induced too, indicating the existence of different mechanisms.

2.2.3 *Biochemical actions*

Receptor-bound IFN-γ initiates a series of metabolic modifications leading to the expression of a large series of genes, *de novo* synthesis of RNA and synthesis of polypeptides. Like other types of IFN, IFN-γ induces the production of at least 12 distinct proteins (50). A protein kinase (51), a nuclease (52) and a mRNA methylation pathway (53) have been identified. Viral protein synthesis is inhibited, probably by inhibition of viral RNA translation (54).

All types of IFN can down-regulate protein synthesis and it has been suggested by Clemens (55) that such an effect on oncogene expression might be an important pathway by which IFN exerts antitumour activity. In agreement with this theory, Jonak and Knight (56) observed concurrent inhibition of cell growth and oncogene expression by IFN-ß added to a cell line with abnormal oncogene expression. However, Chang *et al.* (57) found that IFN-γ treatment of a human carcinoma cell line resulted in growth inhibition and enhanced oncogene expression. Thus, IFN-γ can also exert anti-proliferative effects while oncogene expression is increased.

2.3 Biological effects

2.3.1 *Antiviral action*

In contrast to other interferons, IFN-γ is not primarily an antiviral agent. Within hours after viral infection, IFN-α and -ß are released by cells at the portal of viral entry (*e.g.* epithelium of the upper respiratory tract) and rapidly (58) inhibit virus replication by the induction of enzymes that interfere with the translation of viral RNA (59).

In physiologic conditions IFN-γ is produced much later during the course of a viral infection than IFN-α and -ß. Viral antigens that have penetrated into the circulation will be mitogenic to lymphocytes and induce IFN-γ production. Antiviral activity of IFN-γ has been demonstrated *in vitro* (60) and *in vivo* (61). IFN-γ has been demonstrated to potentiate the antiviral activities of IFN α/ß (62). Rubin and Gupta (60) compared IFN α/ß and IFN-γ for relative antiviral activities against different viruses using human fibroblast cell lines. Replication of vesicular stomatitis and encephalomyocarditis virus was found to be inhibited more strongly by IFN α/ß, whereas reovirus and vaccinia virus displayed more sensitivity to IFN-γ treatment. Thus, viruses differ in their sensitivity to IFN-α/ß or -γ. During a viral infection the synergy between distinct types of IFN will be most effective. IFN-α/ß will act as a first line of defense, secondarily amplified by antiviral activities of IFN-γ.

2.3.2 *Immunomodulatory action*

Action on B lymphocytes

IFN-γ is one of the lymphokines released by T cells upon T-B cell and T cell-macrophage interaction. Its role in B cell maturation is not well established. In 1984 Sidman *et al.* (63) described IFN-γ as being one of a family of B cell maturation factors. It was suggested that IFN-γ was functioning as an obligatory early (64) and late-acting (65) B cell differentiation factor.

Recent studies indicate that IFN-γ in fact inhibits the proliferation of resting B cells that can be stimulated by B cell stimulatory factor 1 (BSF-1), also called interleukin-4 (66). The identification of two subsets of helper T cells (T_h1 and T_h2) can explain these seemingly contradicting results. T_h1 mainly secretes IL-2 and IFN-γ, whereas T_h2 mainly produces BSF-1. Reynolds *et al.* (67) nicely demonstrated, using cloned murine cell lines, that the supernatant of T_h1 cells is able to induce proliferation of B cells when monoclonal antibodies directed against IFN-γ are added, whereas the activity of B cell stimulating T_h2 supernatant can be reversed by addition of IFN-γ. Thus, it was shown that both helper T subsets produce B cell growth and maturation inducing factors and that IFN-γ, produced by Th1 cells, is a potent inhibitor of B cell activation, especially for the IgG_1 and IgE response.

However, the relationship between IFN-γ and B cells is more complex. IFN-γ also plays a role in phenotypic changes of B cells and in the differentiation into Ig-secreting plasma cells. Recently, Snapper and Paul (68) demonstrated that IFN-γ stimulates the expression of Ig of the IgG_{2a} isotype and inhibits the production of others. By contrast, IL-4 markedly inhibits IgG_2a expression, but promotes the expression of IgG_1 and IgE, which in turn is depressed by IFN-γ. Thus, IFN-γ and IL-4 act as antagonists in the regulation of Ig-isotype responses.

Action on T lymphocytes

IFN-γ is one of the lymphokines involved in the generation of CTL from resting precursors (69-72). Bach *et al.* (73) investigated the relationship between various lymphokines and the stage of differentiation of T cells. Using monoclonal antibodies directed against the CD2 surface molecule present on T cells, a proliferative response in CD4 as well as CD8 T cells was induced without the induction of cytotoxicity. Subsequent addition of IFN-γ or IL-2 to these activated cells led to the acquisition of cytotoxicity by CD8 T cells, but not CD4 T cells. In contrast to IL-2, IFN-γ did not have the capacity to stimulate further proliferation of activated cells.

Action on NK-cells

During the last decade much attention has been given to the NK-IFN system. Mice with absent or low NK-activity have an increased incidence of experimental and spontaneous metastasis of NK-sensitive B16 melanoma cells (74). Bloom (75) stressed the immunomodulatory effect of IFN on NK-activity. Herberman (76) suggested that deficiencies of the NK-IFN system could play an important role in immune surveillance against tumours. Different findings were reported on the NK-IFN system in patients with advanced cancer disease. Catalona *et al.* (77) demonstrated a significant enhancement of NK-activity by IFN-γ, and Weigent *et al.* (78) demonstrated that IFN-γ had a 50-fold greater ability to enhance NK-activity in human lymphocytes than IFN α/ß. Enhancement of NK-activity in response to IL-2 was suggested to be an IFN-γ mediated effect (79). Analyzing lymphoid cytotoxicity in mice, Braakman *et al.* (80) demonstrated that at least three phenotypical distinct subsets of NK-cells could be identified. Addition of IL-2 and/or IFN-γ resulted in enhancement of NK-activity in all subsets. In one subset, however, the absence of an IFN-γ receptor could be demonstrated and NK-activity of cells belonging to this category could only be enhanced by IL-2. Furthermore, the IFN-γ induced enhancement of NK-activity of the other subsets could be completely inhibited by antibodies directed against IFN-γ. These antibodies did not interfere with IL-2 cytotoxicity. Thus, this study indicates that distinct lymphoid subsets have a variable expression of lymphokine-receptors and enhancement of NK-activity by IL-2 is only partially mediated by IFN-γ. Shalaby *et al.* (81) demonstrated that the NK-activity of peritoneal derived cells of mice treated with IFN-γ or IL-2 alone exhibited significantly enhanced cytolytic activities. Treatment of mice with both lymphokines in sequence resulted in a synergistic enhancement of NK-activity if IFN-γ was administered 24 hours before IL-2. The time-dependent relationship between both lymphokines may be explained by the induction of IL-2 receptors by IFN-γ.

In 1978 Trinchieri and Santoli (82) reported that IFN could protect tumor cells from NK-mediated cytolysis. Recently, the results of this contradicting report were clarified by the work of Yeoman and Robbins (83). They demonstrated that IFN-γ treated target cells become less sensitive to NK-cytotoxicity, if the expression of MHC class I molecules is concurrently enhanced.

Action on macrophages

IFN-γ has the ability to induce differentiation of myelomonocytic cells (84) and to exert pleiotropic effects on mature macrophages (85,86). It is well established that IFN-γ induces enhanced MHC class II and F_c-receptor expression, inhibition of bac-

terial growth, phagocytosis, tumor cytotoxicity and cytokine release, such as TNF.

Celada *et al.* (87) found that the receptor for IFN-γ on monocytes differs from that on non-hematopoietic cells. Both receptors behave as hormone receptors, *i.e.* binding of the ligand is followed by receptor-mediated endocytosis and ligand degradation with release of degradation products (45). However, when tested by excess of the specific ligand, receptor down-regulation can be observed in the case of monocytes, but not in the case of non-hematopoietic cells (88).

In 1983 Schultz and Kleinschmidt (89) demonstrated that MAF is identical to IFN-γ. Bancroft *et al.* (90) discovered the existence of a T cell independent mechanism of macrophage activation in mice having no detectable B or T cell functions due to an inherited severe combined immunodeficiency mutation. With monoclonal antibodies against IFN-γ, this lymphokine was identified as macrophage activating factor, leading to enhanced expression of MHC class II expresssion and inhibition of bacterial growth. Leu and Herriott (91) demonstrated that IFN-γ induces specific antibody-dependent and non-specific killing of bacteria and tumor cells. In the murine model, proteins distinct from IFN-γ have been identified that are able to execute MAF-like activity. Meltzer *et al.* (92) found that a murine cloned T cell line treated with phorbol myristate released a factor that activated macrophages for non-specific tumor cytotoxicity. This factor was physicochemically and biologically different from IFN-γ or one of its breakdown products.

In the human system similar observations have been made. Malkovsky *et al.* (93) demonstrated that certain human monocytes can display an increased cytotoxic activity as direct and rapid response to IL-2, but not to IFN-γ. Futhermore, IL-2 may enhance the production of TNF-α and -ß by human monocytes in a similar way as IFN-γ (94). Although activation of macrophages seems to be one of the major functions of IFN-γ, other cytokines may exert MAF-like activity as well.

IFN-γ is thought to play a pivotal role in the induction of antigen processing and signalling by APC. It is well established that IFN-γ rapidly enhances the expression of MHC class II molecules on the cell membrane of macrophages. The relevance of this mechanism was demonstrated by Gonwa *et al.* (95), who showed that the antigen-presenting capacity of human monocytes correlates with the expression of MHC class II molecules. In addition, IFN-γ is known to enhance the release of IL-1 (96), a lymphokine acting as accessory signal in T cell activation. These observations strongly favor the concept that IFN-γ is involved in macrophage activation.

Action on the expression of cell membrane molecules

IFN-γ has the capacity to induce the expression of cell membrane molecules, including IgG receptors (97), tumor associated antigens (98) and molecules encoded

by MHC genes (99). Following treatment with IFN-γ, an increased expression of MHC class I molecules has been detected on a wide variety of cells. In a similar way class II expression may be enhanced by IFN-γ. For MHC class II antigens a qualitative change can be observed when cells that are normally class II negative are induced by IFN-γ to express class II molecules *de novo*.

IFN-γ has a strong effect on the expression of MHC class II molecules of a large number of cell types (100, 101) among which endothelial cells. Since antigen recognition by cytotoxic and helper T cells is restricted to class I and II molecules, respectively, the qualitative and quantitative variation in MHC antigen expression may have important immunomodulatory effects. Janeway *et al.* (102) demonstrated that lymphocyte reactions are enhanced when the number of MHC molecules expressed on allogeneic cells is increased. Thus, the release of IFN-γ by activated T cells during ongoing alloreactive responses may lead to enhanced expression of MHC molecules on target cells and thereby to amplification of these responses.

IFN-γ and autoimmune disease

Among many other humoral factors, such as prostaglandins, leukotrienes and corticoids, IFN play a modulatory role in the pathogenesis of inflammation (103). Billiau *et al.* (104) reported that IFN-γ may act as a positive factor triggering or promoting the inflammatory response. Studying the Schwartzman reaction in mice, it was found that the local hemorrhagic and necrotic reaction, normally observed if bacterial endotoxin is given intravenously 24 hours after an intradermal injection of toxin, was completely absent in mice treated systemically with monoclonal antibodies directed against murine IFN-γ. Antibodies against IFN-α/ß had no effect. This finding suggests that IFN-γ may function as an up-regulator of the primary phase of an inflammatory response.

In addition to its role in the local inflammatory response to exogenous agents, IFN-γ may be involved in the reaction to endogenous stimuli as well. It is well established that the first step in the induction of immune responses, whether humoral or cell mediated, requires the interaction between antigen-presenting cells, expressing MHC class II molecules, and MHC-restricted T cells. As mentioned above, the induction of MHC class II expression seems to be one of the major functions of IFN-γ. It has been found that in certain autoimmune diseases, such as thyroiditis (105), insulin-dependent diabetes (106) and rheumatoid arthritis (107), MHC class II molecules are expressed by endocrine epithelial cells that normally do not express them. These class II molecules appear to have a normal protein structure. Weetman and Rees (108) reported MHC class II antigen induction on thyrocytes by IFN-γ, while Londei *et al.* (109) demonstrated that human thyroid cells expressing class II antigens may act as

APC activating T cells. In a recent study CD4 T cell mediated killing of antigen-presenting cells was described (110). Once activated by APC expressing the appropriate class II molecule and antigen, CD4 T cell clones autonomously killed their target APC. Furthermore, normal macrophages treated with IFN-γ to increase class II expression, were killed too. The authors suggested that target tissues in autoimmune diseases, such as thyroiditis, act as APC for infiltrating autoreactive immune cells, which in turn produce cytokines, leading to enhanced expression of class II molecules, thus maintaining ongoing autoimmune responses.

The initiating event leading to this self-destructing mechanism may be caused by viral infection. Studying the pathogenesis of beta-cell destruction in insulin-dependent diabetes Campbell *et al.* (111) showed that viruses may increase MHC antigen expression independent of and before the action of cytokines such as IFN-γ and TNF.

It should be noted that stimulation by IFN-γ alone may not be sufficient to induce class II expression in all cell types, *i.e.* islet beta cells. In these cells TNF or lymphotoxin (LT) are required in addition to IFN-γ to induce class II expression (112). Since activated T cell clones may produce TNF and LT as well as IFN-γ (113), it can be speculated that these cytokines are released at the same time during an immune response and act synergistically upon the target cell, a process which may have important consequences for cell destruction in autoimmune diseases.

2.3.3 *Antitumor action*

Several mechanisms may be involved in the antitumor activities of IFN-γ. Depending upon the tumor cell line tested, IFN-γ may have direct antiproliferative effects on cells mediated through specific membrane receptor binding. A relationship between the number of cell membrane receptors and the efficacy of IFN-γ has been demonstrated by Uecer *et al.* (114). IFN-γ has been shown to have a stronger antitumor efficacy on a number of human and mouse tumors than either IFN-α or -ß (115,116). IFN-γ may exert a direct cytolytic effect on certain tumor cell lines, as shown with murine and human preparations. Tyring *et al.* (117) investigated the cytolytic activity of murine IFN-γ on 11 murine tumor cell lines and observed a 20-fold difference between the most sensitive and most resistant cell type. Synergistic effects of IFN-γ and IFN-α or -ß were demonstrated and sublytic concentrations of IFN-γ combined with another IFN did lead to cytolysis. Enhancement of cytotoxicity was also observed when sublytic concentrations of IFN-γ were combined with spleen cells or peripheral blood mononuclear cells (118). This effect might be caused by reinforcement of the cytolytic activity of IFN-γ on target cells and/or activation of cellular cytotoxicity of added mononuclear cells.

Using murine lymphoma cells, Suzuki *et al.* (119) observed IFN-γ induced cell-mediated cytotoxicity in the absence of a direct effect of IFN-γ on the tumor cells. The lymphoma cells used are characterized by growth inhibition in peritoneal cell cultures prepared from mice harboring the tumor cells in their peritoneal cavity in a dormant state. Treatment of these cultures with antibodies to IFN-γ and TNF receptors (but not with antibody to IL-2 receptors) eliminated the restraint on tumor cell growth, permitting proliferation. The lymphoma cells appeared to be resistant to direct treatment with IFN-γ and/or TNF. Treatment of peritoneal cultures from tumor-dormant mice with IFN-γ resulted in a marked inhibition of tumor cell growth. Since no soluble tumor-cytotoxic factors could be detected in the cell cultures, these findings suggest that the IFN-γ induced cytotoxicity was cell-mediated.

Several *in vivo* studies demonstrated that *in vitro* insensitivity to IFN-γ does not exclude effectiveness of *in vivo* treatment. Belardelli *et al.* (120) injected IFN-resistant and IFN-susceptible leukemia cells into mice treated with IFN. Treatment was active against both cell types, suggesting that host defense mechanisms were involved in the antitumor action of IFN. Similar findings were reported by our group (121) using a rat colon carcinoma cell line insensitive to *in vitro* treatment by IFN-γ. It was found that animals treated with IFN-γ developed a significantly lower number of liver metastases after injection of tumor cells into the portal vein. These findings strongly suggest that IFN-γ may have antitumor effects through activation of effector cells, such as T cells, NK-cells and macrophages, of which is known that they can destroy tumor cells *in vitro*.

Down-regulation of oncogene expression has been speculated to be another mechanism by which IFN-γ may exert antiproliferative efficacy. Einert *et al.* (122) described a relationship between reduction of oncogene expression (c-myc) by IFN-γ and arrest in the G_0/G_1 cell cycle, indicating inhibition of proliferative activity. The reduction of oncogene expression could be reinforced by addition of TNF.

It should be noted that these findings may depent on the cell line used. Other investigators have reported growth inhibition of a carcinoma cell line concurrently with an enhancement of oncogene expression (123). Whatever the mechanism involved, as yet the effect of IFN-γ on oncogene expression is not clear.

2.4 Clinical studies

The results of the first phase I trials investigating toxicity of IFN-γ in patients were reported in 1983, utilizing partially purified, non-recombinant IFN-γ preparations. Adverse effects reported included flu-like symptoms such as fever, chills, fatigue and anorexia, nausea and diarrhea, all reversible on cessation of the drug. An *in vivo* half-life of 30-60 min was found (124), based on serum antiviral activity. To obtain higher

serum concentrations during an extended period of time without serum peak levels continuous infusion instead of bolus injections were designed. Gutterman *et al.* (125) observed that low dosages of IFN-γ not leading to measurable serum levels, did cause clinical toxicity, such as chills and fever. This finding suggests that low doses of IFN-γ may be sufficient to induce physiological effects.

Reports on phase I studies using recombinant IFN-γ products described clinical toxicities identical to those observed in studies in which partially purified IFN-γ preparations were used. Thus, the adverse effects are indeed caused by IFN-γ itself and not by contaminating products. Toxic effects appear to be independent of the route of administration. Using high dosages of IFN-γ, dose-related toxicities were reported, including leukopenia, transaminase elevations, proteinuria, respiratory distress, uncontrollable pyrexia and hypotension (126,127).

Kleinerman *et al.* (128) examined the antitumor properties of blood monocytes obtained from cancer patients undergoing recombinant IFN-γ treatment. Remarkably, monocytes of patients treated with low doses of IFN-γ showed an enhanced cytotoxicity, whereas those of patients treated with high doses showed a reduced one. Studying biological activities of IFN-γ in patients with melanomas, Maluish *et al.*(129) observed immunomodulatory effects of IFN-γ treatment in early stage malignancies. Macrophage activation, enhancement of F_c receptor expression on mononuclear cells, increased NK cell cytoxicity and enhancement of MHC class II antigen expression were demonstrated after IFN-γ treatment.

Recently, the first results of phase II trials using rIFN-γ have been published. Responses have been observed in patients with distinct pathological entities. Garnick *et al.* (130) reported antitumor activity in patients with advanced metastatic renal cell carcinoma, giving recombinant IFN-γ at a daily dose of 10^3-$3x10^3$ microgram/m^2 for 7 days, which was repeated every 2 to 3 weeks. Of 41 patients evaluable for response, one patient demonstrated a complete response 6 months in duration, while three other patients showed a partial response. The responders were patients with pulmonary, lymphatic or hepatic metastases. In this study osseous and primary renal lesions were refractory to treatment. Most recently, Sedman *et al.* (131) reported that IFN-γ can augment the *in vitro* cytotoxic activity of IL-2 activated killer cells of patients with gastrointestinal cancer. Whether this finding may have therapeutic consequences, remains to be determined.

A number of studies have reported positive effects of IFN-γ treatment in other diseases, such as rheumatoid arthritis (132,133) and relapsing ovarian carcinoma (134). Murphy *et al.* (135) investigated the incidence of bacterial infections in patients with the aquired immunodeficiency syndrome (AIDS) treated by IL-2 or IFN-γ. A marked disparity was noted in favor of IFN-γ treated patients, who suffered less frequently of bacterial infections.

Today however, no convincing results have been published about diseases that can be successfully treated by single IFN-γ therapy. Considering the complexity of cellular and cytokine interactions within the immune system, it must be realized that the administration of only one of its components, such as IFN-γ, may not be sufficient to achieve therapeutic effects. At present studies are being performed to investigate the antitumor effect of IFN-γ in combination with other cytokines or treatment modalities.

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Chapter 3

OBJECTIVES OF INVESTIGATION

In the following chapters studies are described in which various immunological and antitumor effects of rRIFN-γ are investigated *in vivo*. The aims of the studies are, respectively:

- To assess the effect of rRIFN-γ on immune functions, including NK cell activity, phagocytosis and mitogen-induced blastogenesis (Chapter 4).

- To assess the effect of rRIFN-γ on MHC class II antigen expression in different organs of various rat strains (Chapter 5).

- To study cardiac allograft survival in recipients treated with rRIFN-γ (Chapter 6).

- To study the effect of donor pretreatment with rRIFN-γ on MHC class II antigen expression on dendritic-like cells (Chapter 7) and endothelial cells (Chapter 8) in heart tissue, and the effect of donor pretreatment on cardiac allograft rejection by unmodified recipients.

- To study the influence of the recipient's immune status on the rejection of skin allografts derived from rRIFN-γ pretreated donors (Chapter 9).

- To determine the antitumor capacity of rRIFN-γ in a rat colon adenocarcinoma model (Chapter 10).

- To determine the toxicity and antitumor capacity of rTNF in combination with rRIFN-γ (Chapter 11).

Part B

ORIGINAL STUDIES

Chapter 4

IMMUNOMODULATION BY rRIFN-γ IN VIVO

Abstract

Recently, recombinant rat IFN-γ (rRIFN-γ) became available, enabling extensive studies on its physiologic role in rats. In the experiments described here a LEW rat model was used to determine the efficacy of rRIFN-γ on immune functions *in vivo*. LEW rats were treated with rRIFN-γ by continuous intravenous infusion at a dosage of 1.5 x 10^5 U/kg/hr for 2 consecutive days. Twelve hours after cessation of rRIFN-γ administration, immune functions, including NK cell activity, phagocytosis and mitogen-induced blastogenesis, were assessed. All experimental animals displayed a marked reduction in the number of peripheral blood and bone marrow cells when compared with controls ($p < 0.005$). Assessment of immune functions revealed a significant enhancement of NK cell activity ($p < 0.001$), phagocytosis ($p < 0.05$) and mitogen-induced blastogenesis ($p < 0.05$). These findings indicate that rRIFN-γ, when given in high dosages, has a stimulatory effect on various immune functions, which substantiates its important immunological role *in vivo*.

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Introduction

IFN-γ is a lymphokine predominantly produced by activated T lymphocytes. A multitude of biologic activities exerted by IFN-γ on cells of the immune system is well documented. It has been demonstrated that IFN-γ is involved in proliferation and differentiaton of helper (CD4) and cytotoxic (CD8) T lymphocytes and the acquisition of cytotoxicity by CD8 T lymphocytes (1,2). IFN-γ has been shown to enhance the activity of NK cells (3-5), monocytic cytotoxicity (6,7) and macrophage activity (8,9). In addition, it is well documented that IFN-γ can exert pleiotropic effects on cells not belonging to the immune system. A direct cytotoxic effect on certain tumor cell lines has been reported (10,11). Furthermore, IFN-γ may induce the expression of surface antigens on a diversity of cell types, including tumor cells (12,13), endothelial cells (14,15) and macrophages (16,17).

The relevance of these distinct *in vitro* findings for the physiologic efficacy of IFN-γ *in vivo* remains to be determined. Because of its species specificity human IFN-γ cannot be tested appropriately in rats or mice. Recent advances in molecular biology have resulted in recombinant DNA-derived rat IFN-γ (18). By the introduction of this recombinant lymphokine, the rat system with its cancer-, infection- and transplantation models, has become accessible to study the biologic activities of IFN-γ *in vivo*. These studies may contribute considerably to our understanding of IFN-γ and offer new perspectives towards the clinical application of human IFN-γ. In the present study we demonstrate the *in vivo* efficacy of rRIFN-γ on various immune functions , including NK cell activity, mitogen-induced blastogenesis and phagocytic activity of peritoneal exudate cells.

Materials and methods

Animals Male rats of the inbred LEW ($RT1^l$) strain were used. The animals were 12-14 weeks of age, weighed 200-220 g and were bred under specific pathogen free conditions.

IFN-γ Recombinant DNA-derived rat IFN-γ (rRIFN-γ) was prepared from cultures of a transformed chinese hamster ovary cell line carrying the relevant gene encoding for RIFN-γ. Details about the methods of production and purification have been described elsewhere (18). The rRIFN-γ preparation used contained $2.5x10^6$ laboratory reference units per mg protein and had a purity of more than 99%. The product was diluted in PBS at pH 7.8 and administered i.v. with a flow of 1.0 ml/hr.

Bone marrow and blood cell countings One ml of heparinized blood was obtained from the tail vein to determine hemoglobin, red blood cell (RBC), white blood cell (WBC) and platelet (Plt) count.

Resection of both femoral bones was performed, each femur opened at the mid-shaft and the bone marrow obtained by flushing of the medulla with a fixed amount (2ml) of Hanks balanced salt solution (HBSS). Cells were counted using a Coulter counter.

NK cytotoxicity assay Spleen lymphocytes were isolated by means of a Ficoll Isopaque density gradient, washed, resuspended in RPMI containing 10% fetal calf serum (FCS) and incubated for one hour at 37°C in tissue culture flasks. Viability was assesed by means of trypan blue exclusion and was always more than 90%. Natural killer cytotoxicity was measured in round-bottem microtiter plates (Nunc, Denmark) in a 4-hr assay using ^{51}Cr labeled YAC target cells (19,20). Lymphocyte effector cells were added, in triplicate, at various concentrations according to the ratio of 25:1, 50:1, 100:1, and 200:1 respectively. To harvest cells, plates were centrifuged for one minute at 150 g and the supernatants were removed using the method described by Hirschberg (21). The release was determined by counting radioactivity in a gamma counter (LKB Wallace Ultragamma II). The maximum release was calculated by adding 10% cetavlon (ICI, UK) to an aliquot of target cells. Spontaneous release was defined as the ^{51}Cr released from target cells incubated with medium alone. This value was usually 6-10% of the maximum. The percentage specific lysis was calculated according to the formula described by Reynolds (22).

Mitogen stimulation assay Lymphocyte response to mitogen was tested using the phytohaemagglutination (PHA) and Concanavalin A (Con A) stimulation assay as described by Kruisbeek (19). Spleen lymphocytes were adjusted to a concentration of 7.5 x 10^6 cells per ml in RPMI 1640 medium enriched with 10% FCS. Cultures of 0.2 ml supplemented with 1 μg PHA (Wellcome, UK) or 1 μg Con A were maintained at 37°C in a 5% CO_2 humidified incubator for three days. Six hours before cells were harvested, 0.8 μCi of methyl-3-H-thymidine (^{3}H-Tdr, specific activity 2CI/mmol; Amersham, UK) was added to each culture. Harvesting was performed using an automatic system (microtiter-automash, Dynatech, Holland) collecting cells on fiber glass filters. After drying, the filters were placed in scintillation vials and 3 ml scintillation fluid was added. The uptake of ^{3}H-Tdr (cpm) was determined with a liquid scintillation counter. Activity was expressed as stimulation index (SI) which was calculated by dividing the TdR uptake in stimulated cells by the uptake in non-stimulated cells.

Phagocytic activity Peritoneal exudate cells (PEC) were obtained by injecting 25 ml of HBSS into the abdominal cavity, followed by gentle massage of the abdomen and aspiration of the peritoneal lavage. Viability of cells was checked by means of trypan blue exclusion. Formalinization and phagocytosis of sheep red blood cells (SRBC) was performed as described earlier (23,24). Briefly, 10^6 PECs resuspended in 2.5 ml RPMI supplemented with 10% FCS were plated in 35 mm plastic Petri dishes (Costar, USA) containing 24 mm square glass cover slips. The dishes were placed in a 5% CO_2 humidified incubator at 37°C for 2 hours. Subsequently, the dishes were washed twice to remove nonadherent cells and incubated for one hour with 2.5 ml RPMI supplemented

with 10% FCS and containing 10^8 formaldehyde-treated SRBC. After this incubation period, the cover slips were rinsed with PBS and immediately fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for 10 min at 4°C. The cover slips were stained with hematoxylin and phagocytosis was quantified by counting the number of SRBC ingested per cell. The percentage of macrophages which contained one or more SRBC was determined (%phagocytosis) and by multiplying this percentage with the mean number of ingested SRBC a phagocytic index was calculated.

Experimental design During a period of 48 consecutive hours rRIFN-γ was administered to LEW rats by continuous intravenous infusion at a dosage of 1.5×10^5 U/kg/hr. Continuous infusion was achieved by means of a catheter manipulated into the jugular vein and connected to an external compression pump. Rats were housed in metabolic cages and allowed to drink, eat and move freely during the long-term infusion. Since IFN-γ induced effects are known to be executed relatively slowly, an interval of 12 hours was introduced between cessation of rRIFN-γ treatment and assesment of immune functions. Controls were treated in a similar way, but received PBS adjusted at pH 7.8 instead of rRIFN-γ. Three series of identical experiments were performed. In the first series the experimental and control group consisted of 4 animals each; in the second and third series of 3 animals. Abdominal lavage, splenectomy and resection of the femoral bones was performed during one operation using ether anaesthesia.

Statistics Data are presented as mean ± SEM unless otherwise stated. The results were analyzed using Student's *t* test. A probabilty value smaller than 5% was taken as level of significance.

Results

Continuous infusion of rRIFN-γ produced weight loss (8-12%) and weakness of all experimental animals. Marked changes in the number of peripheral blood and bone marrow cells were found (Table 4.1). When compared with controls, rRIFN-γ treated rats displayed an impressive anemia, leukopenia and thrombocytopenia, accompanied by a reduction in the number of bone marrow cells. The number of spleen cells was equal in both groups.

Assessment of NK cell response (Fig. 4.1) demonstrated that rRIFN-γ treatment did lead to a significant enhancement of NK activity ($p < 0.001$). The increase of NK activity in the first series of experiments shown in Fig. 4.1 is representative for the results obtained in the second and third series. Enhancement of NK activity was found in all rRIFN-γ treated rats.

The response of spleen cells from rRIFN-γ treated rats to mitogen stimulation is shown in Table 4.2. A significant increase ($p < 0.05$) of the stimulation index of the experimental group was observed and found to be more than twice as large as the control value.

Table 4.1. Effect of rRIFN-γ on Hb and the number peripheral blood-, bone marrow- and spleen cells.

		rRIFN-γ	Controls	p
Hb	(mmol/L)	4.4 ± 1.3	8.0 ± 0.6	<0.005
RBC	(x 10^{12}/L)	3.0 ± 0.3	5.9 ± 0.2	<0.005
WBC	(x 10^{9}/L)	3.8 ± 1.3	9.7 ± 1.6	<0.005
Plt	(x 10^{12}/L)	75.6 ± 10.7	300.0 ± 68.5	<0.005
BM	(x 10^{9}/L)	4.5 ± 0.4	10.4 ± 0.7	<0.005
Spleen	(x 10^{9}/L)	11.1 ± 1.5	9.4 ± 2.5	

rRIFN-γ was given by continuous i.v. infusion for 2 days (n=4). Controls were given PBS (n=4).
Hb: hemoglobin; RBC: red blood cells; WBC: white blood cells; Plt: platelets; BM: bone marrow.

Table 4.2. Effect of rRIFN-γ on mitogen-induced blastogenesis of spleen cells

Mitogen	rRIFN-γ	PBS	p
Con A	126.0 ± 27.5	44.4 ± 10.0	<0.05
PHA	57.7 ± 12.1	19.0 ± 4.0	<0.05

rRIFN-γ was given by continuous i.v. infusion for two days; controls were given PBS.
Each group consisted of 3 animals. Data represent stimulation index ± SEM.

The effect of rRIFN-γ treatment on the phagocytic capacity of peritoneal exudate cells is shown in Table 4.3. Although phagocytosis by PEC of some rRIFN-γ treated rats was quite impressive, there was no significant difference between the number of SRBC ingested per cell in the experimental and control group. In the second and third series of experiments one out of three rRIFN-γ treated rats was found to have PEC that ingested more than twice the number of SRBC per PEC when compared with controls. The results found in other experimental animals, however, were not different from the controls. In the second and third series of experiments, the percentage of cells involved in phagocytosis appeared to be significantly higher in rRIFN-γ treated rats. All PECs from these animals were found to execute phagocytic activity. The phagocytic index of peritoneal cells obtained from rRIFN-γ treated animals was significantly enhanced in the second and third series of experiments.

Table 4.3. Effect of rRIFN-γ on phagocytosis of SRBC by peritoneal exudate cells (PEC).

	PEC/ml	%phagocytosis	SRBC/cell	PI	p
rRIFN-γ	3.0 ± 1.0	92.5 ± 7.5	9.8 ± 0.7	906	<0.05
PBS	2.3 ± 0.9	84.7 ± 2.3	7.1 ± 1.1	601	
rRIFN-γ	nd	100.0 ± 0.0	22.2 ± 7.8	2220	<0.001
PBS	nd	78.7 ± 1.3	8.9 ± 0.3	700	
rRIFN-γ	4.3 ± 2.3	100.0 ± 0.0	18.4 ± 6.7	1840	<0.001
PBS	3.9 ± 0.5	68.0 ± 3.2	10.0 ± 1.8	680	

rRIFN-γ was given by continuous infusion for 2 days; controls were given PBS. PEC/ml: number of peritoneal exudate cells/ml harvested after i.p. injection of 25 ml HBSS. % phagocytosis: % of PEC involved in phagocytosis ± SD; SRBC/cell: mean ± SD of SRBC ingested per PEC. PI: phagocytic index, number of ingested SRBC per 100 PEC. The experiments were performed in triplicate; each group consisted of 3-4 animals.

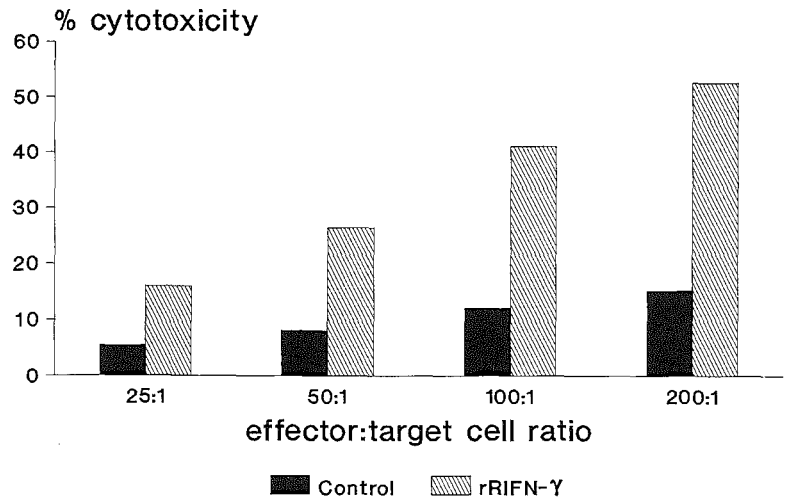

Figure 4.1. Enhancement of NK cell activity of spleen lymphocytes after rRIFN-γ treatment. rRIFN-γ was given by continuous i.v. infusion at a dosage of 1.5×10^5 U/kg/hr for two consecutive days. Controls received PBS. The percentage of specific lysis is shown as determined in a 4hr ^{51}Cr release assay. A significant difference in NK cell activity is found between rRIFN-γ and PBS treated animals at all indicated effector to target cell ratios ($p<0.001$).

Discussion

The results presented in this study demonstrate conclusively that rRIFN-γ has immunomodulatory properties *in vivo*. Our findings in the rat model are in agreement with those of mouse and human studies investigating the effects of recombinant mouse and human IFN-γ, respectively (1-9). However, most of these studies were performed *in vitro* and few experimental models have been presented so far that are representative for the biologic activities of IFN-γ *in vivo (25)*.

All animals that were treated with rRIFN-γ displayed a marked reduction in body weight, up to 12% in 2 days, and were in a bad condition. It is well known from human studies that treatment with IFN-γ may induce flu-like symptoms, including fever, shivering and gastro-intestinal distress leading to nausea and loss of appetite. These toxic side-effects may well explain the observed changes in the experimental animals.

When compared with treatment schedules used in clinical studies (26), it must be noted that a very high dosage of rRIFN-γ was given. This dosage was administered by continuous infusion to obtain high serum levels of rRIFN-γ, up to 200 U/ml (P.H. van der Meide, personal communication). Earlier, we demonstrated that rRIFN-γ given by single i.v. injections was rapidly removed from the circulation and induced no changes in NK cell activity, phagocytosis and mitogen-induced blastogenesis (27). In physiologic conditions, no serum levels of IFN-γ can be detected systemically, not even with very sensitive biologic assays. However, locally at the intercellular level, high concentrations of IFN-γ may be present, since lymphocytes will release IFN-γ directly into their pericellular environment. Most recently, it has been reported that T cells may even direct their lymphokine secretory apparatus towards the site of the cell part that is stimulated (28,29). Thus, very high concentrations of IFN-γ may be produced exactly at the location needed. By cell binding, dilution into plasma and renal filtration, IFN-γ will be cleared and the serum concentration will be very low. Considering the toxicity of exogenous administered IFN-γ, the rapid physiologic clearance may be directed to protect the organism against adverse effects of its own products.

Examination of peripheral blood cells showed a marked pancytopenia in all rRIFN-γ treated rats. Similar hematologic changes have been described in clinical phase I studies investigating the toxicity of recombinant human IFN-γ (30,31). Direct cytolysis may well be responsible for this effect as blood cells were continuously exposed to high levels of IFN-γ (10,11). Reduction of the number of bone marrow cells may be caused by the same mechanism. Recently, Steiniger reported a significant increase of myelo-monocytic cells in the peripheral blood after IFN-γ treatment (32). Although some increase of younger cells in differential white blood cell counts was noted, we did not observe an increase of myelo-monocytic cells.

In this study, it is demonstrated for the first time that rRIFN-γ may induce a significant enhancement of NK cell activity *in vivo*. This finding offers new perspectives to study the

relevance of the NK-IFN system in immune surveillance against tumors (33). It has been suggested that absence of NK cell activity is associated with increased incidence of spontaneous and experimental metastasis of several tumors (34,35). With the availability of the model used in the current experiments, new studies can be designed to determine the effect of enhanced NK cell activity on the process of carcinogenesis and metastasis.

Weigent (36) reported that IFN-γ had a 50-fold greater ability to enhance NK-activity in human lymphocytes than IFN-α/ß. Earlier, we found a stimulating effect of IFN-α on NK cell activity in the rat model. However, a comparison with the potency of rRIFN-γ can not be made, since partially purified IFN-α preparations were used.

IFN-γ is known to exert pleiotropic effects on mature macrophages (8,37). Activation of macrophages is thought to be one of the major physiologic functions of IFN-γ. In the present study, rRIFN-γ treatment was found to increase phagocytosis by peritoneal exudate cells significantly. It can be assumed that IFN-γ, released during infectious diseases, stimulates phagocytic cells to enhanced activity, leading to the removal of foreign material. Several experimental studies have shown that IFN-γ has the capacity to alter the course of microbial infections (38,39). Hershman found that IFN-γ treatment in a murine burn wound model reduced mortality after bacterial challenge (39). Since severe burn wounds may cause depression of immune functions (40), IFN-γ therapy may be of importance in these cases to activate the immune system.

rRIFN-γ treatment was found to enhance mitogen-induced blastogenesis of T cells. This finding suggests that IFN-γ is involved in the induction of nonspecific enhancement of host T cell defense mechanisms. Gromo and co-workers demonstrated that the addition of IFN-γ to activated T cells led to the acquisition of cytotoxicity by CD8 T cells *in vitro* (2). As demonstrated in this study, rRIFN-γ enhanced the activity of NK cells, macrophages and T lymphocytes, all of which are able to destroy tumor cells, at least *in vitro* (41,42). Recently, we reported on the efficacy of rRIFN-γ on a weakly immunogenic adenocarcinoma of the colon in a liver metastasis model (43). While *in vitro* no direct antiproliferative effect of rRIFN-γ was found, a reduction of the number of liver metastases was observed in rRIFN-γ treated rats. This finding suggests that indirect mechanisms, such as rRIFN-γ induced immune responses, are responsible for the antitumor effect. The results presented in the current study are in agreement with this hypothesis.

In the last decade much experimental and clinical research has been focused on the impact of anaesthesia, surgery and blood transfusions on the immune system. Depression of immune functions by these procedures have been reported (44-47). Considering the immunostimulating effects of rRIFN-γ presented in this study, IFN-γ treatment may offer new perspectives to modulate immune functions perioperatively.

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Chapter 5

INDUCTION OF MHC CLASS II ANTIGENS BY rRIFN-γ IN VIVO

Abstract

In this chapter the expression of MHC class II antigens is studied in different organs of various rat strains after treatment with rRIFN-γ. The recombinant lymphokine was administered intravenously by continuous infusion during 36 hours. MHC class II antigens were demonstrated with a monoclonal antibody (OX-6) and immuno-peroxidase staining. All organs studied showed increased expression of MHC class II antigens after treatment with rRIFN-γ. Differences in the pattern of expression were found on vascular endothelial cells. MHC class II antigen expression was induced on endothelial cells of larger vessels in different organs of LEW rats, but not on endothelial cells of BN and WAG rats. Changes in the pattern of MHC class II expression in different organs are described in detail.

Introduction

Cell membrane molecules encoded by genes of the MHC play an important role in immune responses. T cells recognize antigen presented in complex with MHC molecules on the cell membrane of antigen-presenting cells (1). Allogeneic MHC molecules have been demonstrated to be highly immunogenic in transplantation responses.

The expression of MHC antigens on the cell membrane is regulated by cytokines. MHC antigen expression is increased or induced *de novo* by IFN-γ (2,3), a cytokine predominantly produced by activated T lymphocytes (4,5). It is well established that IFN-γ has pleiotropic effects on cells involved in immune reactions (6). Based upon *in vitro* studies, the capacity of IFN-γ to induce the expression of MHC class II antigens is thought to be one of its main functions. In autoimmune and alloimmune reactions *de novo* induction of MHC class II antigens can be observed on cells that normally do not express class II antigens. It is speculated that cells expressing *de novo* MHC class II antigens may function as APC and reinforce ongoing immune responses. However, the relevance of *de novo* induction of MHC class II antigens *in vivo* remains to be determined.

With the production of recombinant IFN-γ for the rat species, a new model has become available to study the importance of MHC class II antigen expression *in vivo*. The present study was initiated to investigate the effect of recombinant rat IFN-γ on MHC class II antigen expression in different organs of various rat strains.

Materials and methods

Animals Male inbred BN ($RT1^n$) rats, WAG ($RT1^u$) rats and LEW ($RT1^l$) rats were used. The animals were bred under specific pathogen-free conditions, weighed 200-250 g, and were 10-12 weeks old.

rRIFN-γ Recombinant DNA-derived rat interferon-gamma (rRIFN-γ) was prepared from cultures of a transformed chinese hamster ovary cell line carrying the relevant gene encoding for rat IFN-γ. Details about the methods of production and purification have been described elsewhere (7). The rRIFN-γ preparation used, contained 2.5 x 10^6 laboratory reference units per mg protein. The product was diluted in PBS at pH 7.8 and administered at a flow of 1.0 ml/hr.

Immunoperoxidase staining After removal, organs were embedded in Tissue Tek (Miles Laboratories, Naperville, USA), snap-frozen in liquid nitrogen, and cut in 5 μm sections using a cryostat microtome (Bright Instrument Comp, Huntingdon, England) at -20 °C. Frozen sections were mounted on to glass slides, allowed to air dry for six hr, fixed in acetone for 10 min and washed 3 times in PBS. Subsequently,

the sections were exposed for 45 min at room temperature in a humidified atmosphere to OX-6 (Flow Laboratories, England), a monoclonal antibody directed against MHC class II antigens. Peroxidase-conjugated rabbit anti-mouse immunoglobulin (Nordic, The Netherlands) was used as secondary step. Diaminobenzidine was used as chromogen. After each step the sections were washed 3 times in PBS. Finally, they were mounted in malinol.

Luconyl staining Luconyl blue (No 708, BASF, FRG) is a dye consisting of macromolecules unable to pass the vascular endothelial lining. When injected intravenously, Luconyl blue remains in the vascular system and therefore can be used to identify vessels of any caliber in histological sections. Luconyl blue was kindly provided by Dr. Reinhold (TNO, Rijswijk, The Netherlands). The dye was diluted 4:6 in PBS and injected i.v. at a dose of 1.5 ml/rat.

Experimental design rRIFN-γ was given to LEW (n=4), BN (n=3), and WAG (n=3) rats at a dosage that induces class II antigen expression on endothelial cells of LEW rats, as was demonstrated in a pilot study. rRIFN-γ was administered by continuous intravenous infusion at dosages of 1.5 x 10^5 U/kg/hr during a period of 36 consecutive hr. Intravenous infusion was achieved by means of a catheter introduced into the jugular vein. The catheter was connected to an external compression pump (Perfusor, Braun, FRG). Rats were housed in special cages provided with a system to protect the catheter, but allowing free movement and intake of water and food. Of each strain two animals were used as controls; they were treated with PBS at the same pH (7.8) and flow-rate (1ml/hr) as the experimental animals. Twelve hours after cessation of rRIFN-γ treatment, the animals were killed and their organs removed for immunohistological staining. One animal of each experimental group was injected with Luconyl blue, 5 min before sacrifice, to enable identification of small vascular structures, such as capillaries. Heart, kidney, liver, pancreas, spleen, colon, and skin were examined for MHC class II antigen expression.

Results

Analysis of MHC class II antigen expression after rRIFN-γ treatment showed an increase of class II antigen positive cells in different organs of all strains (Table 5.1). Induction of MHC class II antigen expression was mainly observed on non-parenchymal interstitial cells. The pattern of MHC class II antigen expression on cells of heart, kidney, liver and pancreas of LEW rats after rRIFN-γ treatment is summarized in Table 5.2. Compared with BN and WAG rats, a different pattern of MHC class II antigen expression was found on endothelium of large vessels of LEW rats. Normally, vascular endothelial cells of BN, LEW, and WAG rats do not express MHC class II antigens. After treatment with rRIFN-γ, MHC class II antigen expression was found

on vascular endothelial cells of different organs of LEW rats. The same dosage of rRIFN-γ did not induce *de novo* induction of MHC class II antigen expression on vascular endothelium of BN and WAG rats.

Table 5.1. Induction of MHC class II antigen expression after rRIFN-γ treatment on interstitial and endothelial cells of LEW, WAG and BN rats.

	control	rRIFN-γ
Interstitial cells[a]	+	++
Endothelium of large vessels	–	+[b]
Endothelium of capillaries	–	–

a) interstitial cells having a dendritic-like appearance
b) in LEW but not in BN or WAG rats; rRIFN-γ was administered at a dose of 1.5 x 10^5 U/kg/hr during a period of 36 hours

Except for the difference in expression of MHC class II antigens on vascular endothelium between LEW rats and BN/WAG rats, the pattern of induction in different organs and strains showed great resemblance. An increase in the number of MHC class II positive dendritic-like cells was a major finding in all organs of different strains.

In hearts of rRIFN-γ treated animals an increase in the number of MHC class II positive interstitial cells was observed. Class II positive interstitial cells were equally distributed among myocardial cells that remained class II negative. *De novo* induction of class II antigen expression was observed on endothelium of large vessels and endocardium of LEW rats, but not on endothelium of BN or WAG rats. Endothelial cells of capillaries did not express class II antigens in any strain.

In kidneys of control rats class II antigen expression was found on interstitial cells, mainly located in the cortical part and in glomeruli, and on epithelial cells of proximal convoluted tubules (Fig. 5.1). MHC class II antigen expression was not observed on any other renal structure. Epithelial cells of the proximal convoluted tubules of BN rats showed stronger class II antigen expression than those of WAG and LEW rats. In BN rats, the change in MHC class II antigen expression could even be seen macroscopically in the cortex. On microscopic examination, an increase in the number of class II positive cells was found in the cortical interstitium and in glomeruli. rRIFN-γ induced class II antigen expression on the parietal layer of Bowman's capsule surrounding the glomerular structures. A strong class II antigen induction was found on proximal convoluted tubular cells; the distal convoluted cells only stained faintly. Within the medullary zone weak class II expression was found on Henle's loop

and, more strongly, on collecting tubules. Vascular endothelial cells of arteries expressed MHC class II antigens, whereas cells of capillaries and veins did not.

Table 5.2. MHC class II antigen expression after rRIFN-γ treatment of LEW rats.

Organ	Cell type	Control	rRIFN-γ
Heart	myocytes	–	–
	endocardium	–	+
Kidney	glomeruli		
	- Bowman's capsule	–	+
	- interstitial cells	+	++
	prox. convoluted tubules	+	++
	prox. straight tubules	–	–
	Henle's loop	–	±
	dist. straight tubule	–	±
	dist. convoluted tubules	–	±
	collecting tubules	–	±
Liver	hepatocytes	–	–
	bile ducts	–	±
	Kuppfer cells	–	+
Pancreas	exocrine acinar cells	–	±
	endocrine islet cells	–	–
	epithelial duct cells	–	+

rRIFN-γ was administered at a dose of 1.5 x 10^5 U/kg/hr during a period of 36 hours

In a normal liver (BN, LEW or WAG) interstitial dendritic-like cells are the only structures expressing class II antigens. The distribution of these cells is mainly restricted to the portal triads. After rRIFN-γ treatment an increase in the number of these cells was observed in all strains (Fig. 5.2). In addition, class II antigen expression was found on epithelial bile duct cells and on endothelium of arteries of LEW rats. MHC class II expression was not found on endothelial cells of central or portal veins. Hepatocytes did not express class II antigens either.

In control animals the only class II positive structures in pancreas are dendritic-like interstitial cells. rRIFN-γ increased the number of class II positive interstitial cells markedly in all rat strains. A strong induction of class II antigen expression was found on acinar cells, epithelial pancreatic duct cells and vascular endothelial cells of large arteries and veins of LEW rats (Fig. 5.3). In none of the strains class II antigen expression was found on pancreatic islet cells or on capillary cells.

In spleens of control animals class II positive cells are mainly present in the marginal zone, in follicles and in the red pulp. After treatment with rRIFN-γ an increase in the number of class II positive cells was found in the red pulp. In addition, induction of class II expression was found on the vascular endothelium of the central arteries of LEW rats.

In the colon of control animals dendritic-like class II positive cells can be found in the *lamina propria.* Enterocytes normally do not express class II antigens. After treatment with rRIFN-γ a strong increase in the number of dendritic-like class II positive cells was observed in the lamina propria. In contrast to liver and heart parenchymal cells, colon enterocytes strongly expressed class II antigens after rRIFN-γ treatment in all rat strains. As observed in all other organs, vascular endothelium of large arteries were induced to express class II antigens in LEW rats, but not in WAG or BN rats.

In skin biopsies of normal rats class II antigen expression is found on dendritic-like cells that are mainly located in the dermal layer and, rarely, in the basal layer of the epidermis. After treatment with rRIFN-γ the number of class II positive cells was strongly increased in the dermal layer as well as in the basal layer of the epidermis. Again, the vascular endothelium of arteries expressed class II antigens in LEW rats, but not WAG or BN rats.

The toxicity of rRIFN-γ treatment differed markedly between various strains. BN rats treated with rRIFN-γ remained in a fairly good condition. In contrast, the condition of WAG and LEW rats treated with rRIFN-γ deteriorated and most of these animals became severly cachectic. A weight loss of 20% was noted for experimental animals compared with 5% for control animals. One LEW rat died during rRIFN-γ treatment at the second day. At autopsy pleural and peritoneal exudate was found. Microscopic examination of HE sections showed multiple small infarctions of liver, spleen and kidneys. Pleural exudate cultures showed bacterial growth.

Discussion

In this study it is clearly demonstrated that continuous infusion of rRIFN-γ induces *de novo* class II antigen expression in different rat organs. This finding confirms earlier reports by Steiniger *et al.* (8). The pattern of class II antigen expression was found to be highly reproducible. Interestingly, cell types of common origin but located in different organs, such as epithelial or endothelial cells, reacted with the same pattern of expression. Epithelial cells, such as bile duct cells, keratinocytes and enterocytes, reacted most strongly to rRIFN-γ treatment. The induction on vascular endothelial cells of large vessels was only observed in one strain and appeared to be less intense,

although consistently present. In none of the strains studied cell types, such as capillaries and parenchymal cells of heart, liver and endocrine islet cells did show any response to rRIFN-γ treatment.

As mentioned above, the pattern of class II induction varied between different strains with respect to class II induction on endothelial cells of large vessels. Using the same dose of rRIFN-γ, *de novo* induction of class II antigen induction was observed on vascular endothelium of organs of LEW rats, but not of BN or WAG rats. We did not determine serum levels of rRIFN-γ to investigate the pharmacodynamics of rRIFN-γ. It might be argued that the process of elimination of rRIFN-γ by clearance or inactivation differs between distinct rat strains. Thus, if LEW rats have a lower clearance rate of rRIFN-γ than the other rat strains, LEW rats might have had higher serum concentrations of the lymphokine. To support this hypothesis it can be argued that rRIFN-γ induced only minor toxic effects in BN rats, while it induced markedly more toxicity in LEW rats. However, WAG rats suffered from rRIFN-γ toxicity to the same degree as LEW rats, but had no class II induction on the vascular endothelium. In addition, the changes in the number of class II positive dendritic-like cells strongly suggest that the serum concentrations of rRIFN-γ achieved in BN and WAG rats were sufficient to induce class II antigen expression. More likely, it can be assumed that there is a genetic difference in the potency of endothelial cells of distinct strains to express class II antigens. In addition, the affinity of receptors for IFN-γ may vary between cell types (9) and in some cells it has been demonstrated that a combination of mediators may be necessary to induce class II expression (10).

It is well established that the outcome of transplantation experiments varies with rat strain combinations. Graft immunogenicity is determined significantly by MHC incompatibility and the degree of mismatch will affect the immune response of the host. It will be of interest to investigate whether different patterns of class II antigen expression on dendritic-like interstitial cells and/or vascular endothelial cells of a graft influence its immunogenicity and thereby graft rejection. Thus, differences in the outcome of transplantation experiments could be explained.

In this study it has been demonstrated that rRIFN-γ may induce different patterns of class II antigen expression in distinct rat strains. If rRIFN-γ treatment is used to induce class II antigen expression prior to transplantation experiments, attractive models become available to investigate the importance of class II antigen expression on different structures to the process of graft rejection.

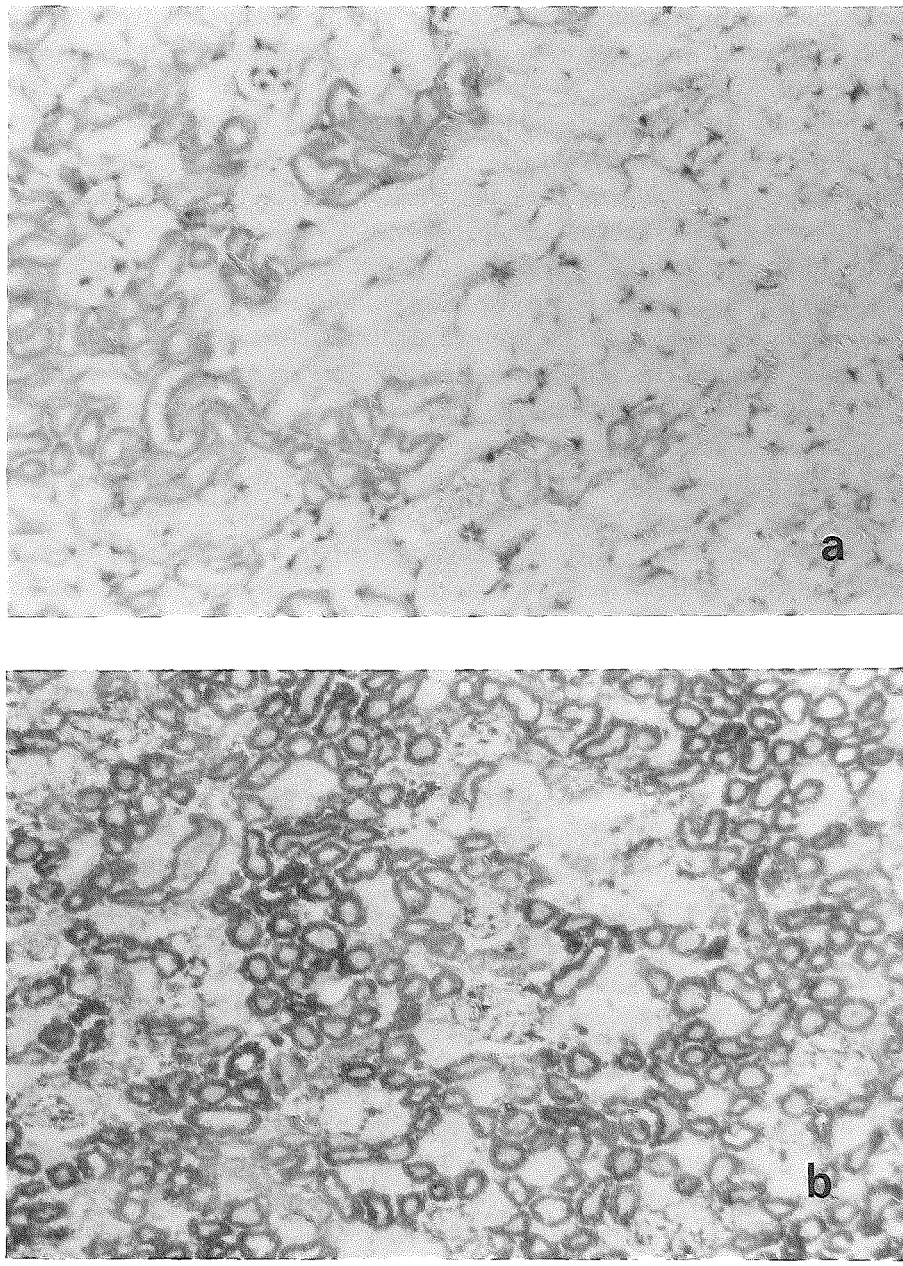

Figure 5.1. Photomicrograph showing MHC class II antigen expression on renal structures of PBS (a) and rRIFN-γ (b) treated LEW rats after immunoperoxidase staining using monoclonal antibody OX-6. x 200.

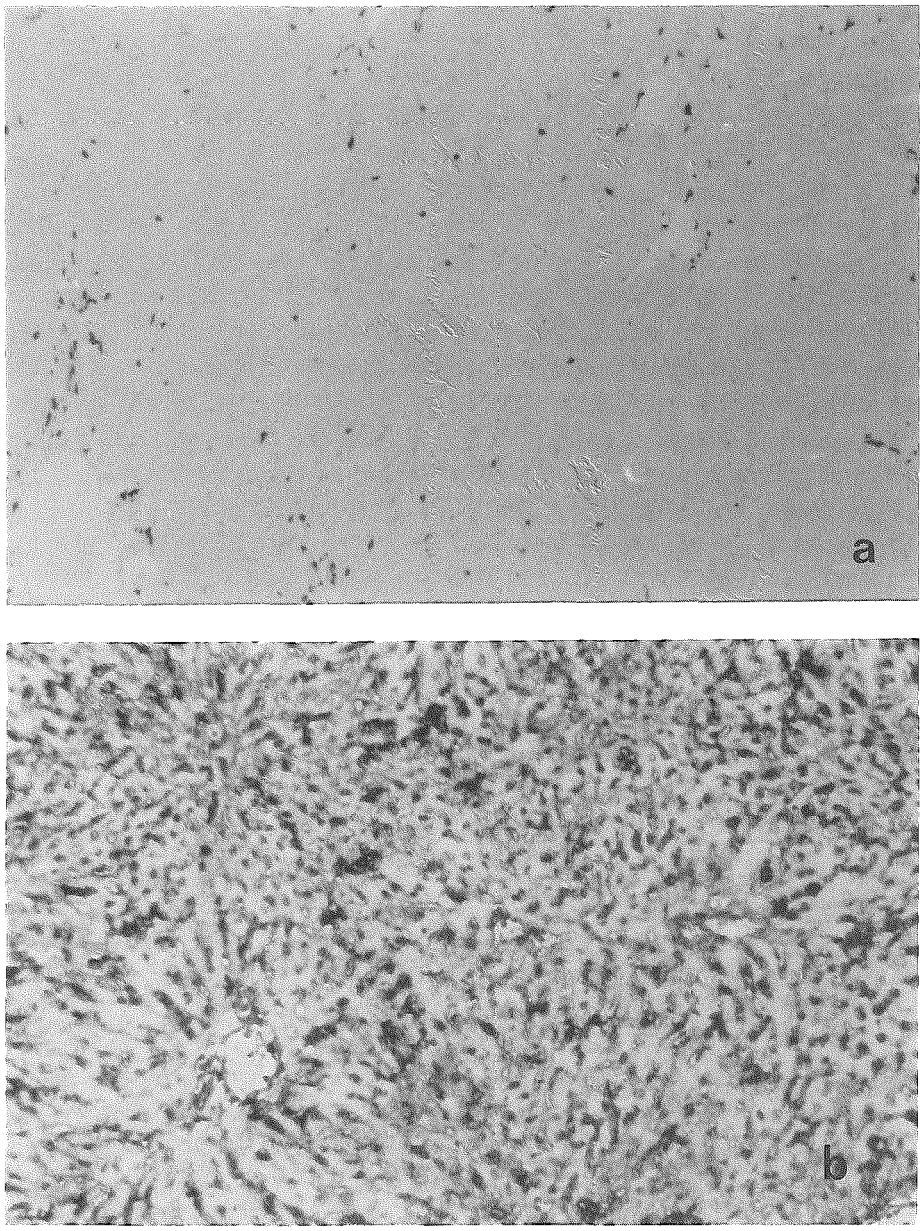

Figure 5.2. Photomicrograph showing MHC class II antigen expression on hepatic structures of PBS (a) and rRIFN-γ (b) treated LEW rats after immunoperoxidase staining using monoclonal antibody OX-6. x 90.

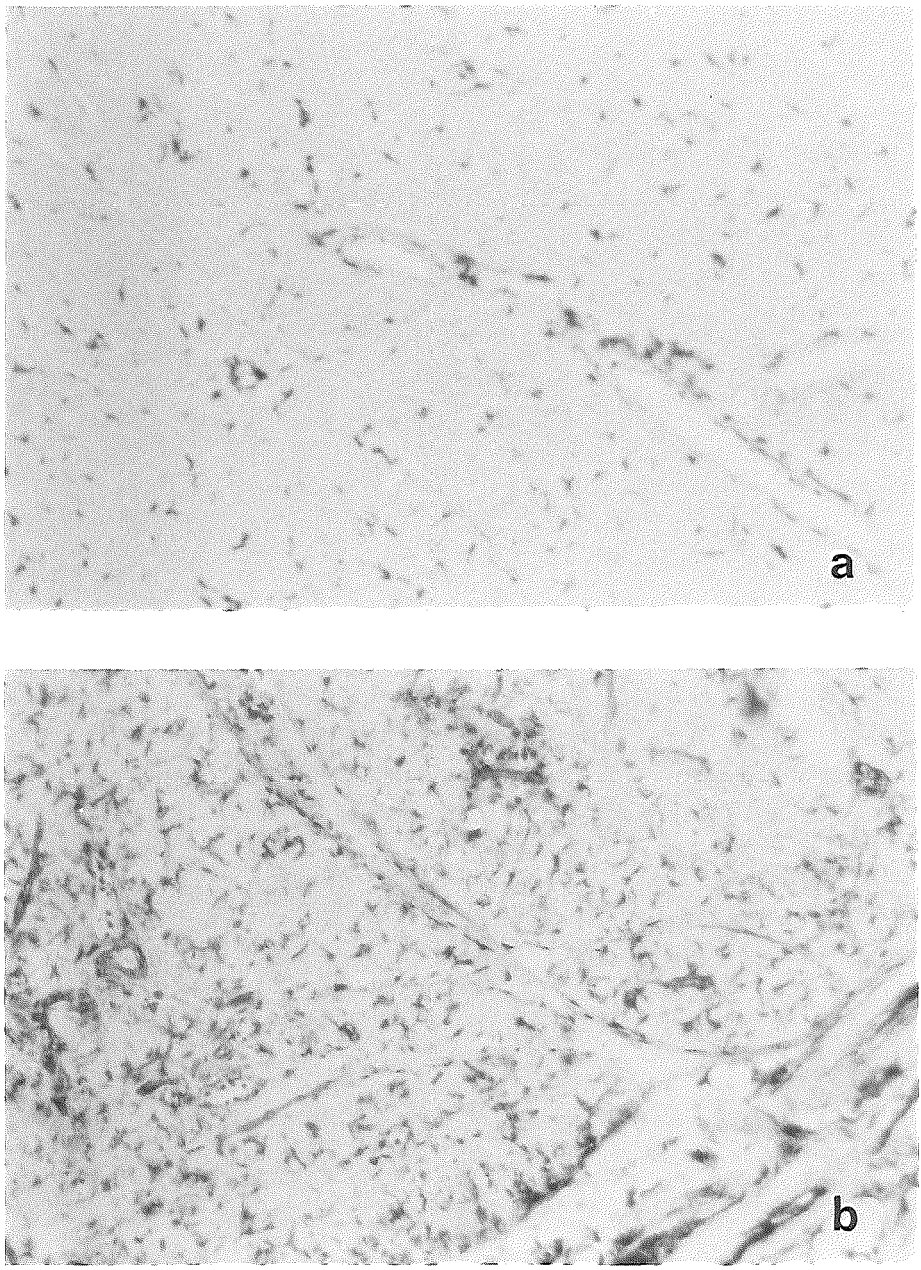

Figure 5.3. Photomicrograph showing MHC class II antigen expression on pancreatic structures of PBS (a) and rRIFN-γ (b) treated LEW rats after immunoperoxidase staining using monoclonal antibody OX-6. x 120.

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Chapter 6

TREATMENT WITH rRIFN-γ HAS NO EFFECT ON CARDIAC ALLOGRAFT REJECTION

Abstract

The effect of rRIFN-γ on allograft rejection was studied in BN ($RT1^n$) to WAG ($RT1^u$) and BN to LEW ($RT1^l$) rat heart transplantation models. Recipients were treated with rRIFN-γ by intraperitoneal or intravenous injection, either by bolus or continuous infusion. Treatment was started immediately after transplantation and continued during a period ranging from 4 to 10 days; dosages varied from 2.5×10^2 U/kg/day to 3×10^6 U/kg/day. Controls received PBS.

Treatment with rRIFN-γ had no effect on allograft survival, irrespective of the route of administration, the dosage used and the duration of treatment. Higher dosages of rRIFN-γ were found to be toxic with a mortality rate up to 75%. LEW rats were found to be more sensitive than WAG rats.

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Introduction

IFN-γ has the capacity to modulate several aspects of the immune response (1,2). It has been demonstrated that IFN-γ is involved in the activation of macrophages (3,4), and natural killer cells (4,5), differentiation of T-cells and acquisition of cytotoxicity by CD8 T-cells (6,7) as well as differentiation of B-cells into immunoglobulin-secreting plasma cells (8). In addition, IFN-γ induces enhanced expression of cell membrane molecules, including antigens encoded by genes of the MHC (1,2). As demonstrated *in vitro*, all of these mechanisms may be involved in immune responses that eventually lead to the rejection of an allograft (9,10). Thus, IFN-γ, released by activated T cells upon allogeneic stimulation, may play a pivotal immunoregulatory role in the rejection process.

Immunomodulating effects of rIFN-γ within the rat model have been demonstrated previously (11). In the present experiments the effect of rRIFN-γ on the process of graft rejection was studied in a rat heart transplantation model.

Materials and methods

Animals Male inbred BN rats ($RT1^n$), WAG rats ($RT1^u$) and LEW rats ($RT1^l$) were used. The animals were bred under specific pathogen free conditions, weighed 200-250 g, and were 10-12 weeks old.

IFN-γ Recombinant DNA-derived rat interferon-gamma (rRIFN-γ) was prepared from cultures of a transformed chinese hamster ovary cell line carrying the relevant gene encoding for rat IFN-γ. Details about the methods of production and purification have been described elsewhere (12). The IFN-γ preparation used, contained 2.5 x 10^6 laboratory reference units per mg protein. Dilutions were made in PBS at pH 7.8.

Heart transplantation Heterotopic heart transplantations were performed as described previously (13). Cessation of heart beat was taken as moment of graft rejection.

Experimental design After cardiac transplantation, rRIFN-γ treatment of recipients was started. Different routes of rRIFN-γ administration were selected, including i.p. and i.v. injection, either by bolus or continuous infusion. Group I and IX were designed to establish the moment of graft rejection in the unmodified BN to WAG and BN to LEW combination, respectively. In addition, the following experiments were performed: 1) daily treatment of WAG recipients with rRIFN-γ by i.v. bolus injection at a dosage of 1.5 x 10^4 U/kg (group III) and 2.5 x 10^5 U/kg (group IV) during 7 consecutive days; 2) treatment of WAG recipients with rRIFN-γ, 3 times daily i.p. at a dosage of 10^6 U/kg, during 4 (group V) or 10 consecutive days (group VII); 3) treatment of LEW recipients with rRIFN-γ, once daily i.p. at a dosage of 2.5

x 10^5 U/kg during 4 consecutive days (group XI) or 3 times daily i.p. at a dosage of 10^6 U/kg during 4 days (group XII) 3) treatment of LEW recipients with rRIFN-γ by means of continuous i.v. infusion at a dosage of 10^4 U/kg/hr (group XIV) or 5 x 10^4 U/kg/hr (group XV) during 4 days. Controls (group II, VI, VIII, X, and XIII) were treated with PBS adjusted at pH 7.8. Continuous i.v. infusion was achieved by a catheter introduced into the jugular vein and connected to an external compression pump (Perfusor^R, Braun, Melsungen, FRG).

Statistics Wilcoxon rank-sum test was used to determine differences in survival times of cardiac grafts. P-values smaller than 0.05 were considered statistically significant.

Results

Toxicity of rRIFN-γ treatment

In the first series of experiments, in which rRIFN-γ was injected i.v. daily in low dosages, no signs of toxicity were found (Table 6.1). In the other series, in which higher dosages of rRIFN-γ were administered, either by i.p. or continuous i.v. injection, treatment was associated with high morbidity and mortality. In these experiments, rRIFN-γ treated animals were in a bad condition and showed reduction of body weight. LEW rats appeared to be more sensitive to rRIFN-γ toxicity than WAG rats. All WAG rats treated i.p. with rRIFN-γ at a dosage of 3 x 10^6 U/kg/day during 4 days survived (Table 6.2, group VI), whereas prolongation of rRIFN-γ treatment to a period of 10 days resulted in a mortality of 37% (Table 6.2, group VIII). In the BN-LEW combination i.p. treatment with rRIFN-γ at dosages of 3 x 10^6 U/kg/day during 4 days led to a mortality of 45% (Table 6.3, group XIII). When the dosage of rRIFN-γ was reduced twelvefold to 2.5 x 10^5 U/kg (Table 6.3, group XII) all LEW recipients survived.

Administration of rRIFN-γ by continuous i.v. infusion did not reduce morbidity or mortality, although serum peak levels were avoided by this technique. On the contrary, 43% of LEW recipients treated with 10^4 U/kg/hr and 75% of those treated with 5 x 10^4 U/kg/hr died (Table 6.4). Pleural and peritoneal exudates were the main findings at autopsy.

Effect on graft survival

In the unmodified BN-WAG combination, grafts were rejected in 8-9 days (Table 1.1, group I). Treatment with rRIFN-γ led to shortening as well as prolongation of graft survival, but none of these changes was found to be of significance when compared with controls. Treatment of WAG recipients with rRIFN-γ at a dosage of 3 x 10^6 U/kg/day did lead to allograft survival times ranging from 6-13 days compared to

Table 6.1. Survival of BN hearts transplanted to WAG recipients treated i.v. with rRIFN-γ.

group	n	treatment	days	survival time
I	9	none	-	8 (8x), 9
II	6	PBS	7	8,8,8,8,9,9
III	7	rRIFN-γ $1.5x10^4$U/kg	7	8,8,8,8,8,9,9
IV	7	rRIFN-γ $2.5x10^5$U/kg	7	8,8,8,9,9,9,10

WAG rats were treated i.v. with rRIFN-γ for 7 consecutive days; treatment was started at the day of transplantation.

Table 6.2. Survival of BN hearts transplanted to WAG recipients treated i.p. with rRIFN-γ.

group	n	treatment	days	survival time
V	11	rRIFN-γ	4	6,6,6,7,7,8,9,11,11,11,13
VI	6	PBS	4	7,7,8,8,9,9
VII	8	rRIFN-γ	10	4,9,12,15,16 [7,10,10]
VIII	6	PBS	10	9,9,10,10,11,12

WAG rats were treated i.p. with rRIFN-γ at a dosage of 3 x 10^6 U/kg/day; treatment was started at the day of transplantation. [ ] recipients died with functioning graft

controls showing a range from 7-9 days (Table 6.2. group VI vs. VII; $p>0.05$). Extension of rRIFN-γ treatment to 10 days resulted in graft survival times from 4-16 days (group VIII), not significantly different from survival times of PBS treated controls (9-12 days, group IX; $p>0.05$).

Treatment of LEW recipients with rRIFN-γ, either i.p. (Table 6.3) or i.v. by continuous infusion (Table 6.4), had no significant effect on graft survival. A dose-response effect was found for rRIFN-γ toxicity (group XV vs XVI). Interestingly, it was observed that the method of drug administration, *i.e.* continuous infusion, had a small though significant effect on graft survival. Recipients treated with PBS by continuous infusion showed graft survival times that were prolonged compared to those of recipients treated with PBS i.p. (group XIV: 11.2 ± 1.5 days *vs* group XI: 7.4 ± 0.6, $p<0.035$).

Table 6.3. Survival of BN cardiac grafts in LEW recipients treated i.p. with rRIFN-γ.

group	n	treatment	days	survival time
IX	10	none	-	6(5x), 7(5x)
X	14	PBS	4	7(10x),8(3x),9
XI	8	rRIFN-γ $2.5x10^5$U/kg/day	4	7,7,8,8,9,9,14,15
XII	11	rRIFN-γ $3.0x10^6$U/kg/day	4	6,7,7,11,11,13 [4,4,5,6,7]

rRIFN-γ treatment was started at the day of transplantation.
[ ] recipients died with functioning graft

Table 6.4. Survival of BN cardiac grafts in LEW recipients treated i.v. with rRIFN-γ.

group	n	treatment	days	survival time
XIII	6	PBS	4	9,10,11,12,12,13
XIV	7	rRIFN-γ $1x10^4$U/kg/hr	4	5,7,12,19 [2,8,11]
XV	8	rRIFN-γ $5x10^4$U/kg/hr	4	11,13 [1,2,2,2,8,8]

rRIFN-γ was given by continuous infusion starting immediately after transplantation.
[ ] recipients died with functioning graft

Discussion

In the current study it was demonstrated that systemic treatment of graft recipients with rRIFN-γ has no effect on the outcome of the rejection process. Although various dosages, routes of administration and treatment periods were tested in different strain combinations, none of the treatment schedules revealed a significant effect of rRIFN-γ treatment on graft rejection.

Most recently, Paineau reported on the effects of rRIFN-γ on allograft rejection (14). He studied the effect of low dosages of rRIFN-γ (10^5 U/kg/day) in combination with blood transfusions. No effect on the survival of heart allografts was found if rRIFN-γ treatment was given alone. However, when recipients were transfused with donor strain blood, leading to a slight but significant prolongation of graft survival, rRIFN-γ treatment resulted in further prolongation. The author concluded that IFN-γ might have an immunosuppressive rather than immunostimulatory effect. If anything, the results from Paineau's and our study suggest that IFN-γ alone does not have the potency to activate the immune system. This concept may be supported by data reported by Billiau (15). This investigator found that IFN-γ alone is not able to increase the inflammatory process in mice, whereas antibodies directed against IFN-γ are able to inhibit it, indicating the involvement IFN-γ in inflammation. However, the effect of anti-IFN-γ treatment may depend on the experimental model used. In rat and monkey transplantation studies treatment with anti-IFN-γ serum and monoclonal antibodies, respectively, did not lead to changes in allograft survival (16,17). The findings of these studies and the present one indicate that positive or negative interference with the actions of IFN-γ alone is ineffective to manipulate allograft rejection.

High dosages of rRIFN-γ led to serious toxicity and even mortality with marked differences between various rat strains. Administration of rRIFN-γ by continuous intravenous infusion to avoid serum peak levels did not reduce morbidity and mortality. The use of high dosages of rRIFN-γ can therefore be excluded as a means to manipulate the process of rejection.

It was found that prolonged handling of graft recipients as such prolonged graft survival, irrespective of the drug administered. This finding stresses the importance of appropriate controls if minor effects of an experimental treatment are anticipated.

In conclusion, systemic treatment of allografted rats with rRIFN-γ in low and moderate dosages has no effect on graft survival; high dosages of rRIFN-γ are associated with severe toxic side-effects impeding possible immunoregulation by rRIFN-γ at these concentrations.

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Chapter 7

INCREASE OF MHC CLASS II POSITIVE CELLS IN CARDIAC ALLOGRAFTS BY rRIFN-γ HAS NO IMPACT ON GRAFT SURVIVAL

Abstract

The present study was initiated to investigate the efficacy of donor pretreatment with IFN-γ to induce MHC class II expression in heart tissue, and to investigate whether this pretreatment would influence heart allograft survival. BN rats were used as donors and RT-1 incompatible LEW rats as recipients. During a period of 3 consecutive days prior to transplantation, rRIFN-γ was administered to BN rats via continuous i.v. infusion at dosages of 10^3, 10^4 and $5x10^4$ U/hr. Control rats received PBS; each group consisted of 9 rats. Analysis of rRIFN-γ induced MHC class II expression by immunoperoxidase staining revealed a significant, fourfold increase of the number of dendritic-like cells, irrespective of the rRIFN-γ dose given (controls: 15 ± 4 vs. highest dose group: 57 ± 9 cells/mm^2; $p < 0.005$). Endothelial cells of arteries and venules remained MHC class II negative. Grafting of hearts of rRIFN-γ perfused donors resulted in the same survival time as compared to controls. These results indicate that upgrading of MHC class II antigen expression on dendritic-like cells is not likely to be of importance for the process of rejection.

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Introduction

Cell membrane molecules coded by genes of the MHC play an essential role in the interaction between cells of the immune system and a transplanted organ (1). MHC class I molecules are expressed on most, if not all nucleated cells, whereas class II molecules have a more restricted tissue distribution (2). MHC molecules of allografted tissue have the capacity to induce strong immune responses by activating T cells of the recipient. MHC class II molecules appear to be stronger transplantation antigens than class I molecules (3). Class II antigens are recognized by T lymphocytes having the surface marker CD4, referred to as T helper cells (4).

Activated T cells release IFN-γ, a lymphokine that has the capacity to induce the expression of MHC molecules (5). IFN-γ has been demonstrated to have a preferential effect on the expression of class II antigens in a large number of cells (6,7). MHC class II antigen expression may be enhanced in cells that constitutively express these molecules, or be induced *de novo* in cells that normally do not express them.

It has been demonstrated that the degree of T cell activation *in vitro* is positively correlated with the amount of MHC class II molecules on the stimulator cells (8). Grafts undergoing rejection are known to display an increased amount of class II antigens. These antigens can activate T cells that, subsequently, release IFN-γ leading to enhanced class II expression and reinforcement of T cell activation. By this mechanism a vicious circle is maintained that, without interference, eventually will lead to rejection. Thus, enhancement of MHC class II antigen expression on cells of a donor organ prior to transplantation might result in a stronger immune response after allografting and early rejection of the transplant.

The present study was initiated to test this hypothesis. Before cardiac allografts were transplanted, donor animals were treated with IFN-γ to enhance the expression of MHC class II antigens.

Materials and methods

Animals Male inbred BN ($RT1^u$) rats were used as organ donors and Lewis ($RT1^l$) rats as recipients. The animals were bred under specific pathogen-free conditions, were 10-12 weeks old, and weighed 200-250 g.

IFN-γ Recombinant DNA-derived rat IFN-γ (rRIFN-γ) was a gift from Dr. P.H. van der Meide (ITRI-TNO, Rijswijk, The Netherlands). The protein was prepared from cultures of a transformed chinese hamster ovary cell line carrying the relevant gene encoding for rat IFN-γ. Details about the methods of production and purification have been described elsewhere (10). The rRIFN-γ preparation used contained

2.5 x 10^6 laboratory reference units/mg protein. IFN-γ was diluted in PBS at pH 7.8 and administered i.v. with a flow of 0.5 ml/hr.

Donor treatment During a period of 3 consecutive days, rRIFN-γ was given by continuous intravenous infusion at dosages of 10^3 U/kg/hr (group I), 10^4 U/kg/hr (group II) and 5 x 10^4 U/kg/hr (group III). Controls (group IV) received PBS. All groups consisted of 9 animals. Continuous infusion was achieved by means of a catheter introduced into the jugular vein and connected to an external compression pump (Perfusor M, Braun Melsungen Ag, FRG). Infusion was started 3 days before and continued until the day of transplantation. Of each group 3 animals were sacrificed for immunohistologic staining; 1 animal per group was injected with Luconyl blue (see below) to visualize blood vessels, 5 min before sacrifice.

Heart transplantation Heterotopic heart transplantations were performed as described previously (9). Cessation of heart beat was taken as moment of graft rejection.

Luconyl staining Luconyl blue (No 708, BASF, FRG) is a dye consisting of macromolecules unable to pass the vascular endothelial lining. When injected intravenously, Luconyl blue remains in the vascular system and therefore can be used to identify vessels of any caliber in histologic sections. Luconyl blue was kindly provided by Dr. Reinhold (TNO, Rijswijk, The Netherlands). The dye was diluted 4:6 in PBS and injected i.v. at a dose of 1.5 ml/rat.

Immunoperoxidase staining OX-6 monoclonal antibody (Flow Laboratories, UK) was used to demonstrate MHC class II antigen expression. After removal, the hearts were embedded in Tissue Tek (Miles Laboratories, Naperville, USA), snap-frozen in liquid nitrogen, and cut in 5 μm sections using a cryostat microtome (Bright Instrument Comp, Huntingdon, UK) at -20°C. Frozen sections were mounted on glass slides, allowed to air dry for 6 hr, fixed in acetone for 10 min, and washed 3 times in PBS. Subsequently, the sections were exposed for 45 min at room temperature in a moist chamber with OX-6 (1:100). Peroxidase-conjugated rabbit anti-mouse immunoglobulin (Nordic, The Netherlands) was used as secondary step. Diaminobenzidine was used as chromogen. After each step the sections were washed 3 times in PBS. Finally, they were mounted in malinol.

Counting of dendritic-like cells MHC class II positive dendritic-like cells were counted on immunoperoxidase and Luconyl-stained sections. The sections were studied using a high-power objective (magnification x200), with diameter of the area under view of 300 μm. Fifteen fields were counted per section.

Statistical analysis The Chi-square test was used to determine differences in survival times of cardiac grafts. Student's *t* test was used in the experiments analyzing the expression of class II antigens on dendritic-like cells after rRIFN-γ treatment. In all cases, differences were considered to be significant at $p<0.05$.

Results

Number of MHC class II positive cells

After 3 consecutive days of continuous i.v. infusion of rRIFN-γ, the number of MHC class II positive cells in heart, liver and kidney was counted. A significant increase in the number of cells exhibiting MHC class II expression was found in all organs examined (data for liver and kidney not shown). In heart tissue, a fourfold increase in the number of cells exhibiting class II expression was seen, from 15 ± 4 to 61 ± 12 (Table 7.1; $p<0.005$). The increase in number of class II positive cells in rats infused with the low dose of rRIFN-γ did not differ significantly from those receiving the high dosages, indicating the absence of a dose-response relationship in the dose-range used.

MHC class II positive dendritic-like cells were equally distributed among the myocardial cells and could clearly be distinguished from small vessels, such as capillaries that were identified by Luconyl blue staining. Cardiomyocytes did not express class II antigens after rRIFN-γ treatment. The endocardium and endothelial cells of large arteries and venules remained negative as well. Induction of class II positive endothelial structures was not observed in any of the examined organs.

Table 7.1. Number of dendritic-like cells in hearts after treatment with rRIFN-γ.

rRIFN-γ (U/hr)	0	10^3	10^4	5.10^4
number of positive cells /mm^2	15±4	51±7	61±12	57±9

3 BN rats were treated with rRIFN-γ by continuous infusion during 3 days. Data represent the mean ± SD of dendritic-like cells in heart. rRIFN-γ significantly increased the numbers of dendritic-like cells ($p<0.005$).

Graft survival

The survival times of grafts obtained from rRIFN-γ and PBS-treated donors were similar in all groups (Table 7.2). The mean survival times (±SD) were: 6.3 ± 0.5 days for the control group; 6.3 ± 0.5 days for group II; 6.2 ± 0.4 days for group III; and 6.5 ± 0.8 days for group IV.

Table 7.2. Effect of donor pretreatment with rRIFN-γ on cardiac allograft survival

group	treatment	survival(days)	MST (±SD)
I	PBS	6,6,6,6,7,7	6.3 ± 0.5
II	10^3 U/hr	6,6,6,6,7,7	6.3 ± 0.5
III	10^4 U/hr	6,6,6,6,6,7	6.2 ± 0.4
IV	5.10^4 U/hr	6,6,6,6,7,8	6.5 ± 0.8

BN donors were treated with rRIFN-γ by continuous infusion for 3 days. Controls were infused with PBS. Following infusion, BN hearts were transplanted immediately to untreated WAG recipients.

Discussion

In this study, it has been clearly demonstrated that rRIFN-γ is able to enhance the expression of MHC class II *in vivo*. The enhancement of class II expression could be observed in different organs, including the heart. This finding confirms earlier observations made by ourselves and others (11,12). Analysis of MHC class II expression on vascular and nonvascular cells was done. Distinction between both structures might be of importance, since it has been demonstrated that class II positive vascular endothelial cells not only may function as target but also as antigen presenting cells (13). As endothelial cells of the donor organ are the first allogeneic structures recognized by the host's immune system, induction of class II antigens on endothelial cells might be of utmost importance for initiation of rejection. In the current model, no vascular endothelial cells were shown to express class II antigens after rRIFN-γ treatment. MHC class II expression was found on non-parenchymal non-endothelial dendritic-like cells, localized between cardiomyocytes. It is likely that a major proportion of these cells is identical to the dendritic cells, originally described by Hart and Faber (14). We did try to make a distinction between dendritic cells and tissue macrophages. It is well established that dendritic cells as well as macrophages express class II antigens and are able to function as antigen-presenting cells. In the allogenic situation, both cell types have been demonstrated to induce CD4-T cell dependent responses (15-17). Thus, enhancement of MHC class II positive cells within a donor organ, being expressed either by dendritic cells or tissue macrophages, might induce

reinforced immune responses by the host in both cases. However, in the present study it was found that a quantitative variation in the number of class II positive cells in the donor organ did not have any effect on the moment of graft rejection.

This finding indicates that enhancement of class II antigens within the donor organ does not necessarily result in increased immunogenicity of the graft. It can be speculated that the amount of class II antigens normally present on dendritic or dendritic-like cells within a graft is sufficient to induce adequate T cell activation. Subsequently, these activated T cells may lead to endogenous IFN-γ production, overwhelming the effect of exogenous IFN-γ. It should further be noted that in the current study an unmodified recipient was used. Consequently, the period needed to develop T cells primed against donor antigens may well have been a critical factor in the model used. Transplantation of MHC class II enriched organs to sensitized or immunosuppressed recipients leads to quite different results, as is described in chapter 9.

Lechler has reported that the antigenicity of rodent allografts could be modulated by treatments leading to depletion of dendritic cells (17). He concluded that the APC of the graft provided the main signal to initiate allograft rejection. However, the role of class II antigens in a transplant appears to be complex and variable. In previous experiments, we only found a marginal effect of dendritic-like cell depletion on heart allograft survival (18). In addition, there is accumulating evidence that, depending on the donor-host combination used, different types of graft rejection, leading to varying patterns of class II antigen expression, may be operational (19).

We and others have found that the unmodified rejection of rat cardiac allografts leads to induction of class II antigens on endothelial cells of the graft (20,21). It has been well established that endothelial cells expressing class II antigens have the capacity to induce strong immune responses (13). IFN-γ has been demonstrated to induce the expression of class II antigens on endothelial cells *in vitro* and *in vivo* (11,12,22). This effect was not observed in the present study, which is probably due to the rat strain used (see chapter 5). Steiniger *et al.* were able to induce class II expression on endothelial cells of LEW rats by 3-day continuous infusion with IFN-γ, using dosages that were within the range as used in our experiments (23). It thus seems that there exist marked differences in sensitivity for IFN-γ between various rat strains. Unfortunately, in the experiments of Steiniger no transplantation experiments were included. Therefore, it remains to be investigated whether the presence of class II antigens on endothelial cells in a graft at the time of transplantation may have an effect on the process of rejection.

In conclusion, in this study we have demonstrated that treatment with IFN-γ in vivo may lead to a fourfold increase in the number of MHC class II antigen-expressing, dendritic-like cells in hearts. However, transplantation of such class II antigen-enriched hearts did not have an effect on graft rejection in the unmodified recipient.

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Chapter 8

DE NOVO INDUCTION OF DONOR MHC CLASS II ANTIGENS ON ENDOTHELIAL CELLS PRIOR TO TRANSPLANTATION HAS NO EFFECT ON ALLOGRAFT REJECTION

Abstract

In the present chapter we investigated the effect of MHC class II antigen expression on vascular endothelium on allograft rejection. LEW rats were treated with rRIFN-γ to induce class II antigen expression on vascular endothelium of the graft prior to cardiac transplantation to WAG recipients. Expression of MHC class II antigens was demonstrated by immunoperoxidase staining using the monoclonal antibody OX-6. Vascular structures were identified by means of immunofluorescence staining using antibodies directed against FVIII, an endothelial surface antigen, or direct staining of the vascular lumen by means of Luconyl perfusion. Treatment of LEW rats with rRIFN-γ induced the expression of MHC class II antigens on vascular endothelium of arteries and veins, but not on endothelium of capillaries. No expression was found on cardiomyocytes. The induction of MHC class II antigen expression on vascular endothelium prior to transplantation had no effect on graft survival. This suggests that *de novo* expression of MHC class II antigens on vascular endothelium of a graft does not imply increased immunogenicity and accelerated graft rejection.

This chapter has been submitted for publication

Introduction

It is well established that allograft immunogenicity is mainly dependent on the presence of MHC antigens (1,2). A quantitative relationship between MHC antigen expression and T cell mediated responses has been demonstrated in vitro (3,4). MHC class II antigens appear to be stronger transplantation antigens than class I molecules (5). They play a pivotal role in the initiation of graft rejection by activating helper T cells (6) that subsequently release immunostimulatory cytokines, such as IL-2, IL-4, TNF-α and IFN-γ (7,8). The expression of class II antigens is variable and dependent on immunologic stimuli. Immunosuppressive drugs such as corticosteroids (9) and cyclosporine (10) reduce MHC antigen expression. Alternatively, upregulation of MHC class II antigen expression on graft cells is observed during allograft rejection (11,12). MHC class II antigen expression may be intensified on cells that constitutively express these antigens and induced *de novo* on cells that are class II negative under non-stimulatory conditions. Enhancement of MHC class II antigen expression on donor cells is thought to amplify T cell activation and facilitate alloreactive immune responses. Most recently however, it was demonstrated that an increase in the number of interstitial MHC class II positive cells within a heart graft prior to transplantation did not effect acute graft rejection in the rodent model (13). It was speculated that endothelial cells, being the first graft cells encountered by the host immune system and induced to express class II antigens *de novo*, would be of more importance to facilitate acute graft rejection (14). Endothelial cells expressing class II antigens may function as APC (15,16) and activate allogeneic lymphocytes (17,18). Ferry *et al.* found that the extent of the immunogenic potential of highly purified rat heart endothelial cells was directly proportional to the expression of MHC class II antigens on the cell surface (19). These findings suggest that the immunogenicity of a vascularized graft would be significantly enhanced if endothelial cells are induced to express class II antigens, thereby facilitating graft rejection.

In the present study a rat model is used in which *de novo* expression of MHC class II antigens can be induced on vascular endothelial cells by rRIFN-γ treatment. Earlier, it has been reported that class II antigen reactive endothelial cells remain detectable until 4 days after rRIFN-γ withdrawal (20). In the current experiments the effect of increased MHC class II expression on vascular endothelial cells on acute rejection is studied.

Materials and methods

Animals MHC class I and II incompatible rat strains were used. Male LEW ($RT1^l$) rats were selected as organ donors and WAG ($RT1^u$) rats as recipients. The animals

were 10-12 weeks old, weighed 200-250 g, and were bred under specific pathogen free conditions.

Heart transplantation Heterotopic heart transplantations were performed as described previously (21). Briefly, an anastomosis was made between the aorta and pulmonary artery of the donor heart and the aorta and caval vein of the recipient, respectively. The transplanted hearts were checked by daily palpation. Rejection was diagnosed by cessation of heart beat and confirmed by histologic examination.

IFN-γ Recombinant DNA-derived rat IFN-γ (rRIFN-γ) was prepared from cultures of a transformed chinese hamster ovary cell line carrying the relevant gene encoding for rat IFN-γ. Details about the methods of production and purification have been described elsewhere (22). The rRIFN-γ preparation used contained 2.5 x 10^6 laboratory reference units per mg protein. The product was diluted in PBS at pH 7.8 and administered with a flow of 0.5 ml/hr.

Luconyl staining Luconyl blue (No 708, BASF, FRG) is a dye consisting of macromolecules unable to pass the vascular endothelial lining. When injected intravenously (i.v.), Luconyl blue remains within the vascular system and therefore can be used to identify vessels of any caliber in histological sections. Luconyl blue was kindly provided by Dr. Reinhold (ITRI-TNO, Rijswijk, The Netherlands). The dye was diluted 4:6 in PBS and injected i.v. at a dose of 1.5 ml/rat.

Immunoperoxidase staining OX-6 and F-17 monoclonal antibodies (Flow Laboratories, England) were used to demonstrate MHC class II antigen expression. OX-6 monoclonal antibodies react with class II antigens of both LEW and WAG rats, whereas F-17 antibodies only react with class II antigens of LEW rats. After removal, hearts were embedded in Tissue Tek (Miles Laboratories, Naperville, USA), snap-frozen in liquid nitrogen, and cut in 5 μm sections using a cryostat microtome (Bright Instrument Comp, Huntingdon, England) at -20°C. Frozen sections were mounted onto glass slides, allowed to air dry for 6 hr, fixed in acetone for 10 min and washed 3 times in PBS. The sections were exposed to OX-6 (1:100) for 45 min. at room temperature in a humidified atmosphere. Peroxidase-conjugated rabbit anti-mouse immunoglobulin (Nordic, The Netherlands) was used as secondary step. Diaminobenzidine was used as chromogen. After each step the sections were washed 3 times in PBS. Finally, they were mounted in manilol.

Indirect immunofluorescence A specific rabbit antiserum, binding to human factor VIII and crossreacting with rat factor VIII (RAHu/FVIII, Nordic, The Netherlands), was used to identify vascular structures. Sections were exposed to RAHu/FVIII (1:25), OX-6 (1:100), goat-anti-rabbit fluorescein-isothiocyanate (GAR/FITC ;1:25) and goat-anti-mouse tetramethylrhodamine isothiocyanate (GAM/TRITC; 1:25), respectively for 45 min. at room temperature in a humidified atmosphere. NaN_3 (0.02%) was added to all incubation steps, 10% normal BN serum to the first two and 2% to the latter two steps. After each step the sections were washed 3 times in PBS.

Finally, the sections were air dried and mounted in PBS/glycerol (1:20). The preparations were examined using a Zeiss Universal Microscope equipped with an epi-illuminator IIIRS and filters for FITC and TRITC fluorescence.

Donor treatment In the first series of experiments six heart transplantations were performed using untreated LEW rats as donors and WAG rats as recipients (group I). In a second series of experiments LEW rats were treated with PBS (group II) or rRIFN-γ (group III) before heart transplantation was performed. PBS and rRIFN-γ (10^5 U/kg/hr) were given by continuous intravenous infusion during 2 days and continued until the day of transplantation. Group II and III consisted of 9 animals, 3 of which were killed for immunohistological staining. One animal of each group was injected with Luconyl blue, 5 min before sacrifice. Continuous infusion was achieved by means of a catheter introduced into the jugular vein and connected to an external compression pump (Perfusor, Braun, Melsungen, FRG). Following donor pretreatment hearts were transplanted to unmodified WAG recipients.

Statistical analysis Chi-square test was used to analyze differences in survival times of cardiac grafts. Differences with p-values smaller than 0.05 were considered to be significant.

Results

Patterns of MHC class II antigen expression

Interstitial dendritic-like cells were found the only sessile structures expressing MHC class II antigens in hearts of control LEW rats (Fig 8.1a). These interstitial cells were equally distributed among class II negative cardiomyocytes. Using immunoperoxidase and immunofluorescence techniques MHC class II and FVIII antigens could be detected. The localization of MHC class II antigen positive structures clearly differed from that of FVIII positive structures. Small vessels such as capillaries did react with FVIII but not with anti-class II antibodies. By means of Luconyl perfusion of the donor heart, vascular structures could be identified before immunohistologic sections were made. Concomitant immunoperoxidase staining using antibodies directed against class II antigens confirmed the results obtained by immunofluoresence techniques. A comparable vascular pattern was found and capillaries could easily be discriminated from MHC class II positive interstitial dendritic-like cells. Endothelial cells of large arteries and veins as well as endocardium did not express MHC class II antigens.

Rejected grafts were studied to compare the pattern of MHC class II expression induced by endogenous cytokines released during rejection with the one observed after exogenous rRIFN-γ treatment. Immunohistologic analysis of LEW cardiac allografts rejected by WAG rats (group I) did show a marked increase in the number of cells expressing class II antigens (Table 8.1). These cells were equally distributed

Table 8.1. Expression of MHC class II antigens in hearts of LEW rats.

cellular structure		control	rRIFN-γ	rejection
Interstitial cells		+	++	++
Endothelium	arteries	–	+	+
	veins	–	+	+
	capillaries	–	–	–
Endocardium		–	+	+
Cardiomyocytes		–	–	–

rRIFN-γ (10^5 U/kg/hr, 2 days) was administered by continuous infusion. Hearts of LEW rats were transplanted to untreated WAG rats to study the pattern of MHC class II expression induced by rejection.

among myocardial cells. Using LEW strain specific antibodies (F-17) the enhanced MHC class II antigen expression could be demonstrated not to be of donor origin, but instead host derived. Vascular endothelial cells of large arteries and veins were found to express class II antigens, whereas endothelial cells of capillaries remained class II negative. MHC class II antigen expression on vascular endothelium was demonstrated to be of donor origin by using strain specific antibodies (F-17) against LEW class II antigens. The phenotypic appearance of cardiomyocytes remained unchanged as far as the expression of class II antigens was concerned; expression of class II antigens could not be demonstrated intracellularly or on the cell surface of these parenchymal cells.

The pattern of class II antigen expression in hearts of LEW rats following rRIFN-γ treatment was comparable to that of rejected LEW cardiac allografts described above. A marked expression of class II antigens on vascular endothelial cells of larger vessels was seen (Fig. 8.1b). Capillaries were found to be negative for MHC class II antigens as studied by immunofluorescense and Luconyl staining techniques. In addition, cardiomyocytes of rRIFN-γ treated donors did not express class II antigens intracellularly or on their membranes either. Examination of HE sections by light microscopy did not show any signs of rRIFN-γ induced cellular damage of parenchymal or non-parenchymal structures.

Effect on graft survival

Graft survival of rRIFN-γ pretreated donor hearts of LEW rats transplanted to WAG recipients did not differ from that of controls pretreated with PBS (Table 8.2). Microscopic examination of rejected grafts revealed no differences in histology between transplants of experimental and control rats.

Table 8.2. Graft survival times of LEW hearts transplanted to WAG recipients.

group	treatment	survival(days)	Mean ± SD
I	none	9,9,9,19,21	13.4 ± 6.1
II	PBS	11,11,11,17,17,19	14.3 ± 3.7
III	rRIFN-γ	10,11,11,12,16,17	12.8 ± 2.9

Prior to heart transplantation LEW rats were treated with rRIFN-γ at a dosage of 10^5 U/kg/hr for 2 days.

Discussion

The results presented in this study demonstrate that rRIFN-γ treatment leads to *de novo* induction of MHC class II antigen expression on cardiac vascular endothelial cells comparable to that observed during acute cardiac allograft rejection. In contrast to reports by others (19,25) class II antigen expression was not found on capillary vascular endothelium or cardiomyocytes. Deleterious effects of rRIFN-γ treatment on heart parenchymal and non-parenchymal cells were not observed, confirming recent data indicating that MHC expression may be induced in the absence of cell injury (23).

Our results demonstrate that the presence of enhanced class II antigen expression on endothelial cells at the moment of transplantation does not facilitate acute graft rejection. This finding throws doubt upon the importance of the presence of class II positive vascular endothelium to graft immunogenicity *in vivo* as suggested by others (10,12-16). It may be explanatory that most of these studies were performed *in vitro* under well-defined conditions, whereas our results were obtained in an experimental model using unmodified recipients. More potent immune mechanisms might overrule immunostimulating activities of endothelial cells *in vivo*. To our knowledge there is only circumstantial evidence to support the hypothesis that class II antigen expression on vascular endothelial cells is of importance to graft immunogenicity *in vivo*. It is well recognized that induction of class II antigens and infiltration of leukocytes into an allograft are closely interrelated events and acute graft rejection almost never occurs without increased MHC class II expression (24). Moreover, Ruers *et al.* reported that intrarenal administration of corticosteroids to rat kidney allografts leads to reduced donor class II antigen expression and prolonged allograft survival (25). They found that the cellular infiltrate of treated and untreated grafts was comparable, whereas the expression of MHC class II antigens on renal target cells differed.

The absence of class II expression on renal target cells of treated rats was suggested to be responsible for the prolonged survival. However, others have reported contradicting results studying long-surviving rat kidney grafts (22,26). They described the presence of a cellular infiltrate concomitantly with an increased expression of class II antigens in non-rejected grafts. These data are in agreement with findings of ourselves (unpublished) and others (27) in the rat heart transplantation model showing that long surviving cardiac allografts may express class II antigens on vascular endothelium without being rejected.

There is no uniformity concerning the changes in the expression of class II antigens during acute rejection, whereas most studies report similar changes in the expression of class I antigens during rejection (28). This lack of uniformity concerning class II antigen expression may be explained by the use of different experimental models. Forbes *et al.* investigated the pattern of class II expression within rat cardiac allografts using different donor-recipient combinations (29). They found that in certain strain combinations acute graft rejection occured in the absence of donor class II antigen expression on vascular endothelium, whereas in other combinations prolonged graft survival was observed in the presence of class II antigen expression on arterial endothelium. In addition, Sachs *et al.* demonstrated that differences between mice strains for class II antigens may not in themselves constitute transplantation antigens capable of causing acute graft rejection, but that additional genetic differences might be needed to induce acute graft rejection (30).

It is well established that graft immunogenicity may be reduced by down-regulation of class II antigen expression on APC (24,31,32). However, as our results show, this concept does not imply that upregulation of these antigens on APC, *e.g.* on endothelial cells, enhances immunogenicity. These data are in agreement with our earlier findings indicating that an increase in the number of class II positive interstitial dendritic-like cells within a graft prior to transplantation has no influence on acute graft rejection (13).

It is likely that acute graft rejection is initiated when a number of donor APC exceeds a certain threshold. These APC can be represented by dendritic or endothelial cells (33). Once rejection is initiated a cascade of events will take place that is independent of the potency of the primary stimulation. However, upregulation of MHC class II antigen expression may affect acute graft rejection by recipients whose immune system has been modified. Thus, in rodents MHC class II enriched skin grafts have been shown to be rejected more rapidly by weakly immunosuppressed or sensitized recipients than unmodified recipients (34, see also Chapter 9). This finding indicates that there are certain conditions, defined by the recipient's immune system, in which an increase of MHC class II antigen expression on grafts affect the outcome of rejection.

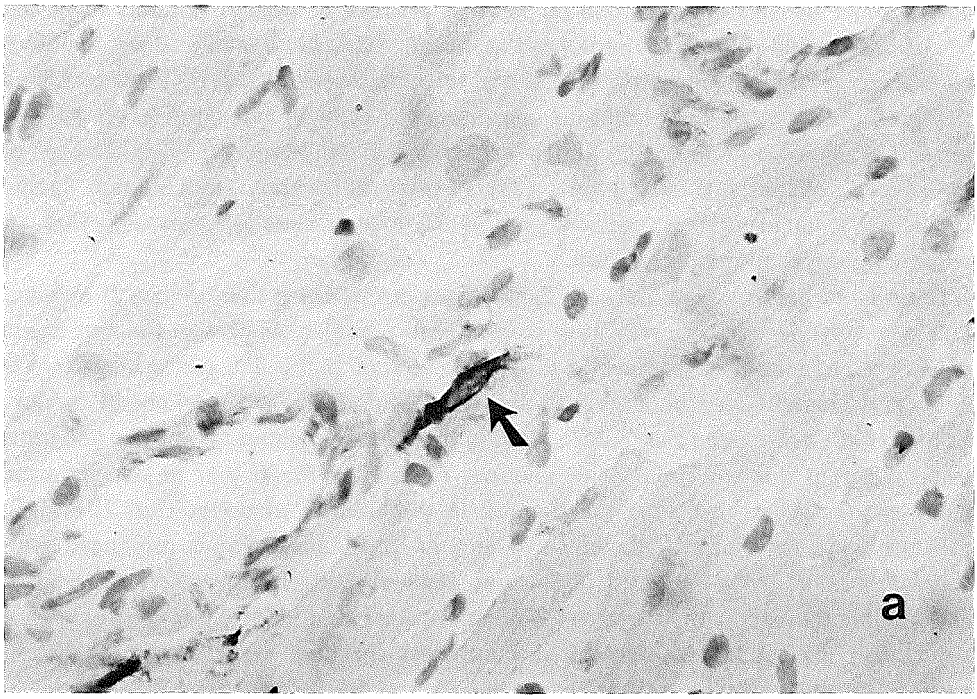
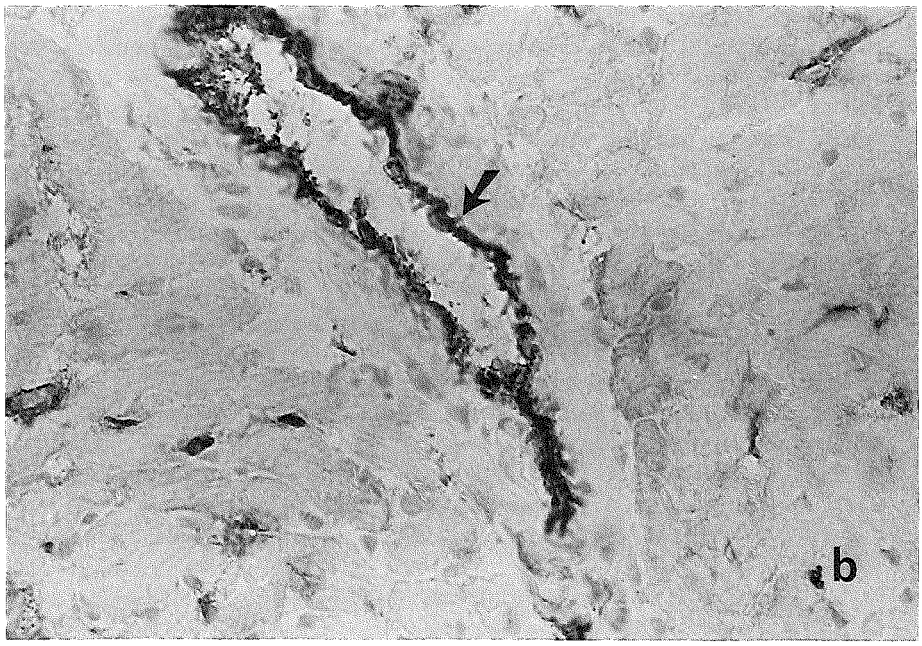


Figure 8.1. Expression of MHC class II antigens on cardiac structures of LEW rats. (a) Photomicrograph of a control heart showing MHC class II antigen expression on an interstitial cell (arrow), but not on endothelial cells or cardiomyocytes. x 600. (b; facing page) Photomicrograph of a vessel showing MHC class II antigen expression on endothelial cells (arrow) after rRIFN-γ treatment. x 490.



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Chapter 9

ACCELERATED REJECTION OF MHC CLASS II ANTIGEN ENRICHED GRAFTS BY IMMUNOMODIFIED RECIPIENTS

Abstract

Allogeneic MHC class II antigens play an important role in the initiation of T cell activation leading to graft rejection. In this chapter a rat model was used in which MHC class II expression was induced on epidermal skin cells of LEW rats prior to skin transplantation by continuous i.v. infusion of rRIFN-γ at a dosage of 1.5 x 10^5 U/kg/hr for 2 consecutive days. Induction of class II expression on epidermal skin cells was evaluated by immunoperoxidase staining using the monoclonal antibody OX-6. After pretreatment skin grafts were transplanted to unmodified, sensitized and immunosuppressed WAG rats. Graft survival in unmodified recipients was unchanged when compared to controls (MST 9.5 $\pm$ 0.14 vs 9.75 $\pm$ 0.14), whereas a shortened graft survival was found on sensitized (MST 5.3 $\pm$ 0.14 vs 6.1 $\pm$ 0.10; $p<0.01$) and CsA immunosuppressed recipients (MST 12.5 $\pm$ 0.42 vs 11.1 $\pm$ 0.23; $p<0.04$). It can be concluded that upregulation of MHC class II antigen expression does not affect unmodified immune responses, but may accelerate rejection if immune responses are modified.

This chapter was published in a modified form in Transplant Proc 22: 1941-1942, 1990.

Introduction

The initiating event in the induction of immune responses requires the interaction between antigen-presenting cells (APC) and T lymphocytes that recognize antigen in complex with molecules of the MHC expressed on cell membranes (1,2). It is well established, that MHC antigens of allografted cells have the capacity to induce strong immune responses (3). MHC class I molecules are mainly recognized by CD8 T cells and class II molecules by CD4 T cells (4,5).

IFN-γ appears to have a preferential effect on the expression of class II molecules and has been demonstrated to induce transcription of class II genes in cells that normally do not express these molecules (6,7). Upon exposure to IFN-γ, cells may display a qualitative and quantitative variation in class II expression. Marked changes in the expression of MHC class II antigens on allografted cells have been observed during the rejection of grafts, including skin (8,9). It is assumed that IFN-γ, released by activated CD4 T cells during the process of rejection, may induce this phenomenon *in vivo*. The immunologic relevance of this effect remains to be determined. *In vitro* it has been demonstrated that the degree of T cell activation is positively correlated with the concentration of MHC molecules on target cells (10). If the same mechanism is operative *in vivo* it follows that immune reactions will be facilitated and the process of graft rejection accelerated by enhanced MHC expression.

The present study was initiated to investigate the immunologic relevance of enhanced graft MHC class II expression at the moment of transplantation on the outcome of rejection. Using a rat model, donor pretreatment with rRIFN-γ was used to induce class II antigen expression on cells of a skin graft. The process of skin graft rejection was studied using unmodified, sensitized or weakly immunosuppressed recipients.

Materials and methods

Animals Male inbred Lewis rats ($RT1^l$) were used as skin donors and WAG rats ($RT1^u$) as recipients. The animals were 10-12 weeks old, weighed 200-250 g, and were bred under specific pathogen free conditions.

IFN-γ Recombinant DNA-derived rat IFN-γ (rRIFN-γ) was kindly provided by Dr. P.H. van der Meide (ITRI-TNO, Rijswijk, The Netherlands). The protein was prepared from cultures of a transformed chinese hamster ovary cell line carrying the relevant gene encoding for rat IFN-γ. Details about the methods of production and purification have been described elsewhere (11). The rRIFN-γ preparation used, contained 2.5 x 10^6 laboratory reference units per mg protein. The product was diluted in PBS at pH 7.8 and administered at a flow of 1.0 ml/hr.

Donor treatment During a period of 2 consecutive days rRIFN-γ was given by continuous intravenous infusion at a dosage of 1.5 x 10^5 U/kg/hr (group II, IV and VI). In chapter 5 we demonstrated that this treatment leads to MHC class II expression. Controls (group I, III and V) received PBS in the same volume and pH of 7.8. Groups consisted of at least 8 animals. Continuous infusion was achieved by means of a catheter introduced into the jugular vein and connected to an external compression pump (Dascon, Uden, The Netherlands). After rRIFN-γ infusion, a period of 12 hrs was waited before transplantations were started. This interval permitted maximum expression of rRIFN-γ induced class II antigens on the cell surface. Of each group, skin biopsies were taken for immunohistological staining.

Sensitization of the recipient To obtain highly sensitized recipients, WAG rats were treated 2 times by LEW skin grafting, 4 and 2 weeks before the current experiments.

Immunosuppression Cyclosporine A (CsA) was used at a dosage of 50 mg/kg at day 0 and day 2 after transplantation. CsA was dissolved in olive oil and administered by intramuscular injection.

Immunoperoxidase staining The monoclonal antibody OX-6 (Flow Laboratories, England) was used to demonstrate MHC class II antigen expression. After removal, skin biopsies were embedded in Tissue Tek (Miles Laboratories, Naperville, USA), snap-frozen in liquid nitrogen, and cut in 5 μm sections using a cryostat microtome (Bright Instrument Comp, Huntingdon, England) at -20°C. Frozen sections were mounted on to glass slides, allowed to air dry for 6 hours, fixed in acetone for 10 min and washed 3 times in PBS. The sections were exposed to OX-6 (1:100) at room temperature in a humidified atmosphere for 45 min. Peroxidase-conjugated rabbit anti-mouse immunoglobulin (Nordic, Tilburg, The Netherlands) was used as secondary step. Diaminobenzidine was used as chromogen. After each step the sections were washed 3 times in PBS. Finally, they were mounted in manilol.

Skin transplantation Full thickness skin transplantation was performed as follows: donor animals were anaesthesized with aether, the abdomen shaved and cleaned with PBS. Abdominal skin grafts, 1.5 cm in diameter, were resected and the panniculus of the grafts was removed with scissors. Subsequently, recipients were anaesthesized with aether, the dorsal thoracic area shaved, cleaned with alcohol and covered with adhesive tape. The taped area was lifted and a circular piece of skin, identical to the donor part, removed. The skin graft was fitted into the graft bed, which maintained shape and position due to the surrounding tape. A dressing of paraffin impregnated gauze and tissue paper was applied and covered with fixing tape wrapped around the thorax. Five (group V and VI) or 7 days (group I, II, III and IV) after transplantation, the dressing was removed. Graft survival was evaluated macroscopically. Complete necrosis of epidermal tissue was taken as the endpoint of graft survival.

Statistical analysis Fisher's test were used to analyse differences in survival times of skin grafts between distinct groups. Differences were considered to be significant at a $p < 0.05$.

Results

Induction of MHC class II antigen expression

In skin biopsies of rats treated with PBS, the presence of class II antigens was confined to dendritic-like cells scattered around in the dermis and occasionally in the epidermis (Fig. 9.1a). Vascular endothelial cells were negative in all skin biopsies taken from control animals. Skin biopsies of animals treated with rRIFN-γ (Fig. 9.1b) displayed a marked increase of MHC class II antigen expression. Induction of class II antigens was found on cells, located in the basal and spinous layers of the epidermis, around hair follicles and in the dermis. In the dermal layer class II antigens were expressed by dendritic-like cells and vascular endothelial cells. Antigen was expressed on the surface of cells as well as in the cytoplasma, suggesting that rRIFN-γ indeed induced the *de novo* synthesis of class II antigens and membrane-bound antigens were not acquired passively from other sources in the environment.

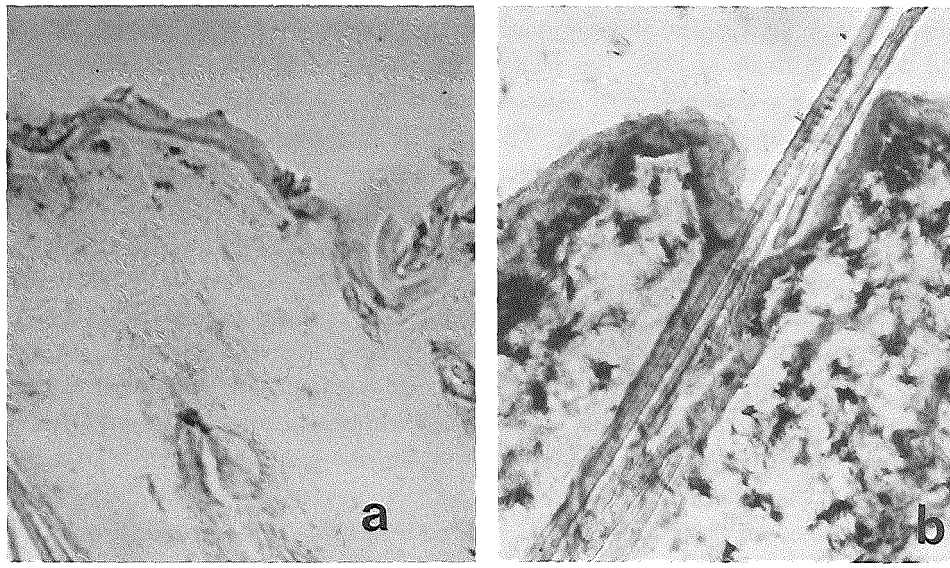

Figure 9.1. Photomicrograph of a skin specimen showing the dermal and epidermal layers of PBS (a) and rRIFN-γ (b) treated LEW rats; an increase in the number of MHC class II positive structures can be observed after rRIFN-γ treatment (immunperoxidase staining using anti-MHC class II monoclonal antibody OX-6). x 90.

Skin graft survival

In unmodified recipients transplantation of skin grafts enriched for MHC class II antigens resulted in graft survival times identical to those of allografted control skins (Table 9.1). In both groups skin grafts were rejected in 9-10 days. Enhanced class II expression on the moment of transplantation had a significant effect on graft rejection by sensitized animals. In the experimental (group IV; MST 5.3 ± 0.14) as well as control group (group III; MST 6.1 ± 0.10) a marked acceleration of skin graft rejection by sensitized recipients was found, when compared with the moment of rejection by unmodified recipients (group I; MST 9.5 ± 0.14). Skin grafts of rRIFN-γ treated donors were rejected more rapidly by sensitized recipients than those of controls. After removal of the dressing on the fifth postoperative day, complete rejection was found in 8 out of 12 skin grafts obtained from rRIFN-γ pretreated donors. Six days after grafting all skin transplants of rRIFN-γ treated donors were rejected. Rejection of skin grafts in the control group occurred on day 6 in 9 out of 10 animals, significantly later than in the experimental group ($p<0.001$; Fisher's test).

Recipients treated with CsA displayed a difference between the moment of rejection of skin grafts of PBS (group V) and rRIFN-γ (group VI) treated donors. It was found, that skin grafts from rRIFN-γ treated donors were rejected significantly earlier than those of PBS treated donors (MST 11.1 ± 0.23 *vs* 12.5 ± 0.42; $p<0.04$, Fisher's test).

Table 9.1. Survival time of pretreated skin grafts transplanted to modified recipients.

group	treatment		n	MST (days)	p
	donor	recipient			
I	PBS	-	14	9.5 ± 0.14	ns
II	rRIFN-γ	-	16	9.8 ± 0.14	
III	PBS	sens	10	6.1 ± 0.10	<0.001
IV	rRIFN-γ	sens	12	5.3 ± 0.14	
V	PBS	CsA	8	12.5 ± 0.42	<0.04
VI	rRIFN-γ	CsA	8	11.1 ± 0.23	

Skin grafts from PBS or rRIFN-γ treated LEW rats were transplanted to unmodified (I,II), sensitized (III,IV) and immunosuppressed (V,VI) WAG rats. rRIFN-γ was given by continuous i.v. infusion (1.5 x 10^5 U/kg/hr for 2 days). MST: mean survival time ± SEM.

Discussion

The induction of MHC class II antigen expression during allograft rejection is thought to play a significant role in graft rejection. In skin, these antigens are mainly found on dendritic-like cells, defined as the bone-marrow derived Langerhans's cells, located in the dermal layer. Normally, rat epidermal skin cells do not express class II antigens. However, they can be induced *de novo* on epidermal skin cells (keratinocytes), as was first reported by Lampert (12) and Mason(13). They showed that keratinocytes of rats suffering from graft-versus-host disease express MHC class II antigens and demonstrated that these antigens were produced by the keratinocytes themselves and were not passively acquired from other positive cells such as the Langerhans's cells (14). It has also been demonstrated that keratinocytes of skin allografts express class II antigens during skin graft rejection (15). Today, it is well documented that IFN-γ, released by activated T cells, induces the expression of MHC class II antigens (6,7).

In the present study we demonstrated that IFN-γ treatment leads to *de novo* MHC class II antigen expression on keratinocytes of normal rat skin. Expression was found on keratinocytes located in the basal layers of the epidemis, around hair follicles, and on dendritic-like cells scattered around in the dermis. These findings are in agreement with those reported by Steiniger *et al.* (16). In addition, they studied the disappearance of rRIFN-γ induced class II antigens *in vivo* and noted that the expression on keratinocytes remained detectable at least untill the sixth day after induction.

We studied the immunologic relevance of induced class II antigen expression using an allogeneic skin transplantation model. The experimental results demonstrate that enhanced class II antigenicity of skin grafts has no effect on the moment of graft rejection by unmodified recipients. However, skin grafts obtained from rRIFN-γ treated donors were rejected significantly more rapidly by sensitized as well as weakly immunosuppressed recipients. It follows, that enhancement of class II antigen expression on skin grafts may correlate with increased immunogenicity. It is known that CsA suppresses class II antigen expression. Skin grafts of rRIFN-γ treated donors, enriched for class II antigens, may have annulled this CsA effect and provided immunologic stimuli, *i.e.* class II antigens, that surpassed the CsA-induced immunosuppression of the affector pathway.

The shortened survival times of rRIFN-γ treated skin grafts transplanted to sensitized recipients may be attributed to a more powerful effector pathway of rejection due to the presence of primed effector cells directed against class II antigens. It is well documented that CD4 T cells may function as effector cells and exert direct cytotoxicity to allografted cells expressing class II antigens (17,18). rRIFN-γ might also have increased MHC class I antigen expression on skin grafts, thereby stimulating CD8 T cells mediated rejection. Similar findings have been

reported by others studying pancreatic islet allograft immunogenicity and rejection after IFN-γ treatment (19,20).

In contrast to skin graft rejection by modified recipients, rejection by unmodified recipients was not changed by rRIFN-γ pretreatment. This observation is in agreement with findings in an allogeneic heart transplantation model in rodents. These findings suggest that the amount of donor MHC class II antigens normally present within a graft suffices to stimulate CD4 T cells to a maximum if the recipient's immune system is not compromized in one way or the other (*i.e.* sensitization or immunosuppression).

In conclusion, the results of the current experiments indicate that upregulation of allogeneic MHC class II antigen expression is of minor importance to the unmodified rejection process; however, if the recipient's immune system is modified by sensitization or suppression, upregulation of MHC class II antigen expression can accelerate immune responses.

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Chapter 10

SUCCESSFUL TREATMENT OF COLON CANCER IN RATS WITH rRIFN-γ

Abstract

The antiproliferative activity of rRIFN-γ was tested against a weakly immunogenic colon adenocarcinoma (CC531) *in vitro* and *in vivo*. Tumor cell suspensions of CC531 were incubated with different dosages of rRIFN-γ to assess a direct antiproliferative effect. No antiproliferative effects of rRIFN-γ were found after a three-days incubation period.

In vivo antiproliferative activities were tested in a subrenal capsule assay (SRCA) and a liver metastases model. In the SRCA, intravenous treatment with rRIFN-γ at a dosage of $5x10^5$ U/kg/day for 5 consecutive days, starting at the day of tumor implantation, did result in a significant inhibition of tumor growth ($p < 0.05$). In the liver metastases model the same treatment schedule with rRIFN-γ, started at the day of CC531 tumor cell injection into the portal vein, significantly inhibited the development of liver metastases ($p < 0.001$).

In conclusion, rRIFN-γ can exert an impressive anti-tumour activity *in vivo*, while direct cytotoxicity *in vitro* is absent. Indirect mechanisms, such as changes of target cells or host immune functions, may be responsible for the inhibition of tumor growth and dissemination by rRIFN-γ.

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Introduction

The lymphokine IFN-γ is produced by mitogen or antigen-stimulated T lymphocytes (1,2). Compared to IFN-α and -ß several differences in biologic activity have been reported, particularly in relation to its immunomodulating and antiproliferative properties. Evidence from *in vitro* and *in vivo* studies suggests that IFN-γ may have a much greater antitumor effect than either IFN-α or -ß (3,4). IFN-γ has been reported to exert a direct cytotoxic effect on certain tumor cell lines (5,6). In addition, a number of immunological functions influenced by IFN-γ may synergize with this direct cytotoxic action. It has been demonstrated that IFN-γ can enhance monocyte cytotoxicity (7,8,9), macrophage activity and natural killer (NK) cell activity (10,11). Furthermore, IFN-γ can enhance the expression of surface antigens on tumor cells (12,13) and on cells of the host immune system, *i.e.* macrophages (14,15,16), which may facilitate tumorcell lysis. Despite the well-recognized antiproliferative effects of IFN-γ *in vitro*, the mechanism(s) of its action *in vivo* are still poorly understood.

Recent advances in molecular biology and recombinant DNA technology have resulted in the production of highly purified rat IFN-γ. The present study was undertaken to evaluate the antitumor activity of this preparation *in vitro* and *in vivo*, using a rat colon adenocarcinoma (CC531), which was previously found to be susceptible to treatment with immune response modifiers (17,18,19).

Materials and methods

Animals Male rats of the inbred WAG strain were used. The animals were bred under specific pathogen-free conditions, weighed about 200 g and were 10-12 weeks old.

Tumor Tumor CC531 is a 1,2 dimethylhydrazine-induced, moderately differentiated adenocarcinoma of the colon, originating from a male WAG rat (17). The tumor is transplantable in syngeneic animals and is weakly immunogenic as determined by *in vivo* challenge-protection experiments according to the method of Prehn and Main (20). In the experiments presented here, the tumor was in its 19th passage. The tumor is also maintained in tissue culture as a stationary cell line in RPMI-1640 medium (Gibco, UK), supplemented with 10% fetal calf serum (FCS). Tumor cell suspen-sions were prepared from culture monolayers by trypsinization for 2 min and resus-pension in fresh medium.

IFN-γ Recombinant DNA-derived rat IFN-γ (rRIFN-γ) was kindly provided by Dr. P.H. van der Meide. Details of the cloning, expression and purification have been reported elsewhere. The preparation used in the current experiments contained 4 x 10^6 U per mg protein and had a purity of 98%. The antiviral units were estimated by

determining, in serial dilutions, the protective effect of rRIFN-γ against vesicular stomatitis virus infection of rat fibroblast monolayers in a microtiter assay.

Experimental design To assess whether rRIFN-γ had a direct antiproliferative effect on tumor CC531, cells were harvested from culture by trypsinization. After washing 10^5 cells in 4 ml RPMI-1640 with 10% FCS were pipetted into 35mm tissue culture plates (Costar). One ml of RPMI 1640, containing 2000, 4000 or 8000 units of rRIFN-γ was added and the plates were incubated at 37°C. After 3 days the number of cells per plate was counted in a microcell counter (TAO, Japan) and the percentage of living cells determined by Trypan blue dye exclusion. Each dose-experiment was in triplicate. In the control plates only RPMI 1640 was added.

Subrenal capsule assay The first *in vivo* model used was the subrenal capsule assay (SRCA), which was performed as follows. WAG rats (5 animals per group) were anesthized with ether, a median laparotomy was performed and both kidneys exposed. A small incision was made in the renal capsule and a tumor cube of 6-8 mg, obtained from the 19th passage, was implanted and pushed up to the upper kidney pole. The animals were killed one week later and the tumors removed and weighed.

Liver metastases model The second *in vivo* model was a liver metastases model. Artificial liver metastases were evoked by injection of 5 x 10^5 tumor cells into the portal vein. Under ether anesthesia the portal vein was exposed via an abdominal midline incision and 0.5 ml of the cell suspension was injected using a 0.4 x 12 mm needle. The rats were laparotomized 30 days after tumor cell inoculation and the number of liver nodules visible at the surface of the liver lobes, inspected *in situ*, was counted. Each experimental group contained 6-7 animals. In the liver metastases experiments and the SRCA, rRIFN-γ was administered i.v. at a daily dose of 5 x 10^5 U/kg in a volume of 0.5 ml for 5 consecutive days, starting at the day of tumor implantation.

Statistics Experimental data were analyzed using Student's *t* test. Differences were considered to be significant at $p<0.05$.

Results

As shown in Table 10.1 CC531 tumor cells were not susceptible to treatment with rRIFN-γ *in vitro*. None of the concentrations of rRIFN-γ used resulted in inhibition of cell proliferation. The number of cells counted after a 3 days incubation period was similar in all cases. There was no difference in the percentage of dead cells between control and experimental groups. Table 10.2 shows the effect of rRIFN-γ on tumor CC531 as measured in the SRCA. It was found that treatment with rRIFN-γ led to a significant inhibition of tumor growth ($p<0.05$). The mean tumor weight of the

Table 10.1. Susceptibility of CC531 tumor cells to direct cytotoxicity of rRIFN-γ

rRIFN-γ	n	Dead cells
inactivated	15.0 ± 3.2 x 10^5	9.6 ± 0.6
2000 U/well	14.6 ± 3.5 x 10^5	13.0 ± 3.4
4000 U/well	14.4 ± 3.2 x 10^5	12.6 ± 4.6
8000 U/well	14.8 ± 2.4 x 10^5	8.0 ± 3.6

10^5 tumor cells were cultured with rRIFN-γ for 3 days; the total number was counted (n: M ± SD) and the percentage of dead cells was determined by means of trypan blue (dead cells: % ± SD). Each dose-experiment was performed in triplicate.

Table 10.2. Effect of rRIFN-γ on growth of tumor CC531 in a subrenal capsule assay.

Treatment	n	Tumor weight (mg ± SD)	p
PBS	10	30.6 ± 7.2	<0.05
rRIFN-γ	10	18.5 ± 6.5	

rRIFN-γ therapy (5 x 10^5 U/kg/day, i.v.) was given for 5 days, starting on the day of implantation. Each group contained 5 animals of which both kidneys were used for tumor implantation.

control rats amounted to 30.6 ± 7.2 mg and to 18.5 ± 6.5 mg in the group treated with rRIFN-γ. The results obtained with rRIFN-γ in the liver metastases model in two separate experiments are shown in Table 10.3. An impressive inhibition of tumor development as a result of treatment with rRIFN-γ was observed; in both experiments the difference between the control and rRIFN-γ group was highly significant ($p<0.001$). In experiment I, 5 animals from the control group having more than 60 nodules were killed on the day of inspection, the remaining 2 animals with 24 liver metastases and all animals from the rRIFN-γ treated group were kept alive. The controls survived for 52 and 55 days; the rRIFN-γ treated animals survived for 88, 91, 95, 95 and more than 100 days (2x), respectively.

Table 10.3. Effect of rRIFN-γ on CC531 tumor growth in a liver metastases model.

	Treatment	Number of liver metastases
Exp I	PBS	24, 24, <60, <60, <60, <60, <60, <60
	rRIFN-γ	0, 0, 0, 1, 3, 3, 7
Exp II	PBS	4, 9, 20, 20, <60
	rRIFN-γ	0, 0, 0, 1, 2, 10

Liver metastases were evoked by injection of 5 x 10^5 CC531 tumor cells into the portal vein. rRIFN-γ (5 x 10^5 U/kg/day, i.v.) was given for 5 days, starting on the day of cell injection. Metastases were counted after 30 days.

Discussion

In a previous communication we reported on the susceptibility of tumor CC531 for virus-induced rat IFN-α/ß in the SRCA and the failure to treat artificial liver metastases with this preparation (17). The results from the present study indicate that rRIFN-γ has impressive antitumor capacity in both *in vivo* models. In contrast, rRIFN-γ, in the dosages used, had no effect on CC531 *in vitro*. Consequently, it is likely that tumor inhibition evoked by rRIFN-γ *in vivo* was indirect and due to activation of the immune system of the host, either or not combined with an enhanced susceptibility of the tumor cells. Such an enhanced sensitivity has recently been demonstrated for monocyte-mediated cytolysis by Feinman (22). Alternatively, increased destruction of tumor cells by rRIFN-γ activated monocytes, independent of an effect on target cells has been reported repeatedly (7,15,23-25). Enhancement of NK cell activity may have been a second mechanism for the antitumor activity of rRIFN-γ. Improved NK-mediated cytotoxicity by rRIFN-γ has been reported earlier by others (10,11). NK cells are known to be most effective against a small tumor emboli (26,27), which may provide a likely explanation for the observed discrepancy between the moderate effect of rRIFN-γ in the SRCA, where tumor cubes were used, and the marked effect in the liver metastases model. An additional explanation for the surprisingly high effectiveness of rRIFN-γ in the liver model hinges on an important antitumor role of Kupffer cells. Recent findings by Pearson *et al. (28)*, also obtained in a rat liver metastases model, suggest that the activity of Kupffer cells may determine the outcome of metastases growth in the liver for a great deal. Kupffer cell stimulation by Corynebacterium parvum significantly reduced the number of liver metastases, whereas depression of Kupffer cell-activity by silica, gadolinium and

injection of rRIFN-γ within 24 hours leads to an increased expression of class II histocompatibility antigens on Kupffer-like cells in the liver, indicating the presence of high-affinity receptors for rRIFN-γ and rapid post-receptor action on cell metabolism (unpublished results). Analogous to the effects of IFN-γ on macrophages, namely increased class II antigen expression associated with improved phagocytosis (8,15), it may also have the capacity to enhance the antitumor activity of Kupffer cells in addition to the increased antigen expression observed.

In conclusion, the present study has shown that rRIFN-γ exerts a marked antiproliferative effect on tumor CC531, especially in a situation where a low tumor burden was involved. The finding that rRIFN-γ had no effect on the tumor *in vitro* suggests that the antitumor activity *in vivo* was indirect and probably mediated by an activated immune system.

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Chapter 11

ANTITUMOR ACTIVITY OF rTNF ON COLON CANCER IN RATS IS PROMOTED BY rRIFN-γ; TOXICITY IS REDUCED BY INDOMETHACIN

Abstract

The activity and toxicity of rMuTNF, alone or combined with rRIFN-γ, was tested in rats bearing a weakly immunogenic colon adenocarcinoma, implanted subcutaneously (s.c.) or under the renal capsule. A single intravenous (i.v.) injection of 40μg/kg of rMuTNF was lethal to non-tumor bearing rats in 3 to 5 hr, whereas 10μg/kg was not. Doses of 4μg/kg rMuTNF were well tolerated when given daily for one week. The most prominent toxic feature of rMuTNF was hemorrhagic colitis which could be alleviated by intraperitoneal (i.p.) pretreatment with indomethacin (10mg/kg). rMuTNF (10 mg/kg) injected s.c. into tumors led to retardation of growth in 20% of the rats. Combined with $4x10^5$ U/kg rRIFN-γ, which on its own had no effect, response was found in 50% of tumors with 2 out of 20 tumors showing regression. Combined treatment with rMuTNF (2μg/kg) and indomethacin (10mg/kg) had an effect similar to that of rMuTNF alone (25 ± 9mg vs 24 ± 7mg). Under protection of indomethacin the rMuTNF dose could be increased to 40μg/kg, however, without increase of antitumor effects.

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Introduction

TNF is a protein mainly produced by activated macrophages, originally demonstrated in the serum of animals treated with Bacillus Calmette-Guerin and endotoxin (1). TNF has been functionally defined by its cytostatic and cytolytic properties against certain tumor cell types *in vitro* and by its its capacity to cause hemorrhagic necrosis of animal and xenografted human tumors *in vivo* (2). TNF belongs to a group of molecules with related structure exhibiting various biological activities, reminiscent of the interferon system (3). Lymphotoxin, also designated as TNFß, which is a product of activated lymphocytes (4), belongs to the TNF family, whereas cachectin, produced by macrophages (5), is identical to TNF. Under physiological conditions TNF most likely plays a regulatory role during infection (6). This notion is also evidenced by the finding that TNF can enhance the phagocytic capacity and antibody-dependent cytotoxicity of neutrophilic granulocytes and can stimulate their adherence to endothelial cells (7,8). It has also been shown that TNF can induce the expression of MHC class II antigens on macrophages (9). The antitumor effects of TNF may be partly related to these effects of TNF on neutrophils and macrophages.

Recently, recombinant human and mouse TNF have become available and their antitumor capacities clearly demonstrated (10-13). In some tumor models the effect of TNF was markedly enhanced by simultaneous administration of interferons, notably IFN-γ (13-15).

We have previously reported an antiproliferative capacity of IFN and IFN-inducers using a transplantable colon adenocarcinoma in inbred rats (16,17). In the present study this clinically relevant tumor model was employed to determine the therapeutic capacity and toxicity of rMuTNF given alone or in combination with rRIFN-γ.

Materials and methods

Animals Male rats of the inbred WAG strain were used. The animals were bred under specific pathogen-free conditions, weighed 200-250 g and were 10-12 weeks old.

Tumor A 1,2 dimethylhydrazine-induced, moderately differentiated colon adenocarcinoma (CC531), transplantable in syngeneic WAG rats, was used (16). The tumor is weakly immunogenic as determined by the immunization-challenge method of Prehn and Main (18). In the present experiments the tumor was in its 18th passage.

Subcutaneous implantation Under ether anesthesia tumor cubes measuring 2mm were implanted in the left flank of WAG rats. Tumor growth was assessed by measuring with calipers the two largest perpendicular diameters at regular time intervals. The average diameter was taken as measure of tumor size.

Subrenal capsule assay (SRCA) WAG rats were anesthetized with ether, a median laparotomy was performed and both kidneys exposed. A small incision was made in the renal capsule and a tumor cube of 6-8mg was implanted and pushed up to the upper kidney pole. The animals were killed after one week and the tumors in both kidneys were enucleated and weighed.

TNF Details of the production of recombinant DNA-derived murine TNF (rMuTNF) have been published elsewhere (19). The preparation used was over 99% pure, contained 72 x 10^6 units per mg protein and less than 50ng endotoxin per mg protein.

IFN-γ Recombinant DNA-derived rat IFNγ (rRIFN-γ) was a gift from Dr. P.H. van der Meide (ITRI-TNO, Rijswijk, The Netherlands). Details of cloning, expression and purification of rRIFN-γ have been published by Dijkema *et al.* (20). The preparation used in the present experiments contained 10^6 units per mg protein and had a purity of 25%.

Indomethacin Indomethacin was obtained as a powder, dissolved in PBS and injected intraperitoneally (i.p.) at a dose of 10mg/kg body weight in a volume of 0.5ml.

Experimental design In the first series of experiments non-tumor bearing rats were treated i.v. with different dosages of rMuTNF (1-1000μg/kg) in order to assess its toxicity. In follow-up studies rMuTNF was combined with indomethacin in order to abrogate major side effects. Each experimental group contained at least three animals. To study the effect of intralesional injected rMuTNF the subcutaneous tumor model was used. Pieces of tumor CC531 were implanted in the flank of WAG rats. After 20 days, animals bearing tumors with a diameter between 1-1.5cm were randomized into three groups of 10 animals each. After randomization, intratumoral injections were given of 40μg/kg rMuTNF, alone or in combination with 4 x 10^5 U/kg rRIFN-γ, on days 0, 10 and 15. The injected volume always amounted to 0.1ml. The experiments were performed 2 times.

Previously, we studied the effect of rRIFN-γ on tumor growth using the same CC531 model (21). Since no antitumor effects were found at a dosage of 4 x 10^5 U/kg rRIFN-γ, this experiment was not repeated in the present study.

The SRCA was used to investigate the possible interference of indomethacin with the antiproliferative capacity of rMuTNF. It was also employed to see whether the therapeutic effect of rMuTNF could be enhanced by increasing the dose under indomethacin protection. Doses of 8 or 40μg/kg rMuTNF were given i.v. on days 0, 2 and 4. Indomethacin was given i.p. at a dosage of 10mg/kg, 1 hr before the administration of rMuTNF. The assay was read 1 week after tumor implantation. Each experimental group contained 5-6 animals.

Statistical analysis Results were analyzed using Chi-square and Student's *t* test. Differences were considered to be significant at $p<0.05$.

Results

Toxicity studies

A single i.v. injection of 1000, 400 or 40μg/kg of rMuTNF was uniformly lethal within 3-5 hr. The animals became diarrheic within 1 hr, blood appearing in the feces rapidly thereafter. At autopsy, the most prominent finding was hemorrhagic enteritis which was mainly restricted to the colon. A single dose of 8μg/kg was well tolerated and could be repeated on alternate days without leading to hemorrhagic colitis. Dosages of 4μg/kg could be given daily. The lethal effect of 40μg/kg rMuTNF could be abolished by pretreating animals with 10mg/kg indomethacin i.p., 1 hr before injection of rMuTNF. Animals receiving this combination developed only mild diarrhea; this treatment could be repeated 3 times on alternate days. However, it repeatedly led to excessive wasting and a weight loss of 25% within one week was not exceptional.

Effect of intratumoral rMuTNF on subcutaneous tumor growth

The growth curves of the tumors treated by intratumoral injection with rMuTNF, alone or in combination with rRIFN-γ, are shown in Figure 11.1. The mean tumor diameter on day 21 was 2.7 $\pm$ 0.4cm for the controls, 2.4 $\pm$ 0.3cm for the group treated with rMuTNF and 1.9 $\pm$ 0.8cm for the group treated with rMuTNF and rRIFN-γ. The difference between the latter group and the controls at this particular point (day 21) was significant ($p<0.01$; chi-square). Remarkably, some tumors were clearly inhibited in growth, whereas others hardly showed any response. This was found in both experimental groups. Intratumoral injection of rMuTNF produced only mild toxicity in some animals and this was not increased when rMuTNF was combined with rRIFN-γ.

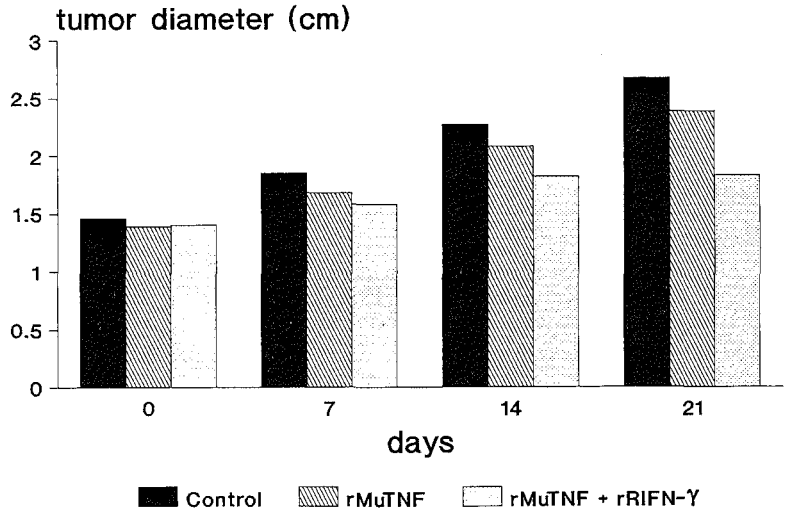
Table 11.1. Effect of intratumoral injection of rMuTNF and rRIFN-γ on tumor CC531.

Treatment	Progression	Arrest	Regression
Controls	100%	0%	0%
rMuTNF	80%	20%	0%
rMuTNF+rRIFN-γ	50%	40%	10%

Treatment was started 3 weeks after s.c. tumor implantation when the tumors were between 1 and 1.5 cm in diameter. rMuTNF was given at a dose of 10 μg and rRIFN-γ at a dose of 10^5 units on day 0, 10 and 15. Each group contained 20 rats.

Using the same treatment protocol, the study was repeated with 20 animals per group. The results are summarized in Table 11.1. They are grouped according to the effect of one or more intratumoral injections on tumor growth, *i.e.* progression, arrest

Figure 11.1. Effect of intratumoral injections with rMuTNF (10μg) and rRIFN-γ (10^5 U) on CC531 tumor growth. Twenty days after s.c. tumor implantation treatment was started (day 0, 10 and 15). Controls were injected with PBS. Each group consisted of 10 animals. On day 21 there was a significant difference between the control group and the group treated with rMuTNF and rRIFN-γ ($p<0.01$).



or regression. If a tumor stopped growing for at least one week this was classified as "arrest". "Regression" indicates that tumor size was reduced by 50% or more. The results indicate that treatment with rMuTNF led to arrest of growth in 20% of the cases but not to tumor regression. Only combined treatment of rMuTNF and rRIFN-γ gave rise to regression. In one case the tumor seemed to have disappeared completely; however, it recurred after 14 days and was insensitive to further treatment. The combined treatment with rMuTNF and rRIFN-γ had an overall response rate of 50%, whereas rMuTNF alone had a response rate of 20% ($p<0.001$; chi-square).

Combined effect of rMuTNF and indomethacin

In the course of the toxicity studies mentioned above, it was found that indomethacin could alleviate the toxic side-effects of rMuTNF. The SRCA was used to investigate whether the combined administration of these agents would interfere with the antiproliferative potential of rMuTNF. The results are shown in Table 11.2. Indomethacin alone had no effect on tumor growth, whereas treatment with rMuTNF produced a significant inhibition of tumor development ($p<0.001$). The combined administration of both agents had an effect similar to that of treatment with rMuTNF

Table 11.2. Effect of indomethacin and rMuTNF on tumor CC531.

Treatment	Tumor weight	p
Controls	48 ± 13	
Indomethacin	40 ± 8	ns
rMuTNF	24 ± 7	<0.001
Indomethacin + rMuTNF	25 ± 9	<0.001

rMuTNF was given i.v. at a dose of 8μg/kg on days 0, 2 and 4 in a SRCA. Indomethacin (10mg/kg) was given i.p. 1 hr before rMuTNF. After 1 week, tumors were enucleated and weighed. Groups consisted of 6 animals, from each of which both kidneys were used. Tumor weight in mg: mean ± SD

alone, indicating that indomethacin neither abrogated nor potentiated the antitumor activity of rMuTNF.

Antiproliferative effects of high doses of rMuTNF

Pretreatment with indomethacin allows rMuTNF to be administered in doses which would have been lethal when given alone. The SRCA was employed to investigate whether such high dosages would lead to a stronger inhibition of tumor growth. The results are shown in Table 11.3. A dose of 40μg/kg appeared to produce the same antiproliferative effect as a dose of 8μg/kg.

Table 11.3. Effect of different doses of rMuTNF on tumor CC 531.

Treatment	Tumor weight	p
Controls	45 ± 8	
8 μg/kg rMuTNF	33 ± 5	<0.001
40 μg/kg rMuTNF	34 ± 11	<0.05

Treatment with rMuTNF was given on days 0, 2 and 4 in a SRCA. After 7 days tumors were enucleated and weighed. Each group contained 5 animals, from each of which both kidneys were used. Tumor weight in mg: mean ± SD.

Discussion

Our study has shown that rMuTNF has antitumor activity against a rat colon adenocarcinoma when administered by intratumoral or intravenous injection. Local administration was only moderately effective but could be significantly improved when

stration was only moderately effective but could be significantly improved when rMuTNF was combined with rRIFN-γ. These results are in agreement with earlier observations on the *in vivo* effects of partially purified and rMuTNF (11,22).

The toxicity of rMuTNF was a serious problem and was higher than anticipated on basis of results obtained in nude mice (13). A dose of 25μg rMuTNF was not toxic for nude mice, whereas in our experiments a 10μg dose was lethal for a 250g rat. This susceptibility was not a particular feature of the WAG strain used in the current experiments; pilot studies performed using the inbred BN rat strain gave similar results (data not shown). In both strains the predominant pathological finding was a severe hemorrhagic colitis, shock being the most likely cause of death. It has been shown that TNF can induce the production of prostaglandin E2 in cultured fibroblasts (23). This observation, together with the finding that TNF has a marked effect on endothelial cells (24,25) led us to the hypothesis that the toxic effects of rMuTNF were due to exaggerated production of prostaglandins by vascular cells with the ensuing modulation of platelet aggregation and vascular tonus. Indirect evidence that this hypothesis might be correct is provided by our results obtained with the prostaglandin-inhibitor indomethacin. The toxic side-effects of rMuTNF were considerably reduced by pretreatment with indomethacin and mortality did not occur. However, the excessive wasting of animals treated with high doses of rMuTNF was not abolished by treatment with indomethacin. It is quite possible that the wasting was due to the cachectin-like activity of rMuTNF. Cachectin is able to suppress the activity of lipoprotein-lipase in adipocytes and is identical to TNF (5). It has been suggested that some of the metabolic disturbances, including cachexia, seen in cancer patients, may be due to the activity of endogenously produced TNF (3).

Indomethacin, at the dosage used, had no effect on growth of tumor CC531. Inhibition of intestinal rat tumors by indomethacin has been reported by several authors; however, other workers did not observe such an effect (26-28). Indomethacin did not interfere with the antitumor activity of rMuTNF and allowed relatively high doses of rMuTNF to be given. However, increasing the dose did not lead to an improvement of the results. Balkwill *et al.*, using human tumor xenografts in nude mice, have reported similar results for tumors that were sensitive to TNF treatment at a low dose (13). Increasing the dose did not lead to a better antiproliferative effect for these tumors. In contrast, tumors which were resistant to TNF became responsive when the dose was increased.

The synergistic activity of rMuTNF and rRIFN-γ could be expected from the results of other studies in which both cytokines were used (11,13). Different mechanisms may contribute to this effect. There may be a direct cytotoxic effect which is reinforced by using both cytokines. Fransen *et al.* (14) demonstrated the synergism of rMuTNF with IFN-γ on different cell lines *in vitro*. It has been demonstrated that IFN-γ can enhance the expression of tumor necrosis factor receptors on various cell types (29); this

may render cells more susceptible to TNF treatment. Furthermore, IFN-γ may augment the production of endogenous TNF in response to endotoxin by enhancing TNF-gene transscription and mRNA translation (30,31). Although we did not find a stronger antiproliferative response to higher doses of rMuTNF, an extended period of treatment with a low dose may certainly be more effective. Finally, the synergistic activity of rMuTNF and rRIFN-γ may be explained by indirect mechanisms of action exerted by host cells. IFN-γ has been shown to activate macrophages, thereby enhancing tumoricidal activity (32) and other defensive functions (21,33). Previously, we could not find an antitumor effect of lower dosages of rRIFN-γ in the same model (21). It is conceivable that the dose of rRIFN-γ used was near a threshold level that can be exceeded by adding another cytokine, such as rMuTNF.

Our results provide further evidence for the potential of TNF in tumor therapy. Improvement of the therapeutic efficacy of TNF is likely to be obtained by the combined administration of TNF with IFNs, especially IFN-γ. Our present results and those of others clearly point in the direction of such a multimodality approach in which there may be a place for indomethacin to limit toxicity.

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PART C

GENERAL DISCUSSION AND SUMMARY

GENERAL DISCUSSION

The experimental work with rRIFN-γ in rodents, presented in this thesis, has clearly demonstrated that this cytokine can exert pleiotropic activities *in vivo*. rRIFN-γ was found to stimulate the activity of NK cells, T cells and macrophages (Chapter 4), to increase MHC class II antigen expression on various cell types (Chapter 5), and to inhibit tumor growth, alone (Chapter 10) or in combination with TNF (Chapter 11). The results of these experiments are in agreement with reports on the effects of rIFN-γ in mice and man (1).

The feasibility to manipulate graft rejection with rRIFN-γ was studied. It was found that low dosages of rRIFN-γ had no effect on the outcome of graft survival, while high dosages were associated with serious toxicity and mortality limiting the use of rRIFN-γ. Hemodynamic and immunological changes due to the surgical procedure and blood loss might have amplified rRIFN-γ toxicity (2).

In the physiological process of inflammation, lymphoid cells are capable to focus their secretory apparatus towards the site of immunostimulation before cytokines are released, thereby producing high concentrations of cytokines at the centre of immune responses (3,4). It remains to be determined whether these high cytokine concentrations can be achieved adequately in the pericellular environment by systemic administration of cytokines.

In man the limitations of systemic treatment by intravenous infusion have been reported repeatedly. Clinical studies with IFN-γ (1), IL-2 (5) and TNF (6) have demonstrated serious dose-limiting toxicities, such as hypotension and pulmonary insufficiency. As discussed below new techniques of cytokine administration must be designed that reduce toxicity, while maintaining efficacy. In addition, the availability of monoclonal antibodies against cytokines may help to clarify and to manipulate immune reactions.

rRIFN-γ induces MHC class II antigen expression on different cell types, including interstitial dendritic-like cells and endothelial cells. These cells have the capacity to stimulate allogeneic lymphocytes by presentation of MHC class II antigens (7,8). We found that a significant increase in the number of dendritic and endothelial cells expressing MHC class II antigens in a graft had no effect on rejection by unmodified recipients. Thus enhancement of MHC class II antigen expression within a graft does not imply increased immunogenicity. Apparently, the normal amount of MHC class II antigen expressing cells within a graft meets the requirements to activate allogeneic helper T-cells in an unmodified rejection process most sufficiently. However, in those settings where the immune system of the recipient is modified, *e.g.* the afferent

pathway suppressed by CyA or the efferent pathway stimulated by sensitization, an increase of allogeneic MHC class II antigens accelerates rejection. In case of CyA immunosuppression the increase of allogeneic MHC class II antigens may annul the effect of CyA which inhibits the production of lymphokines responsible for MHC antigen expression (9). If the recipient is sensitized, effector mechanisms may be facilitated by an increase of allogeneic MHC class II antigen expression on target cells. Clearly, MHC class II antigens play a role in the process of antigen recognition and rejection. However, in our experiments it was not possible to manipulate the rejection process with rRIFN-γ, although a significant increase of MHC class II antigen expression was found.

In this thesis also the antitumor activity of rRIFN-γ was studied. It was found that rRIFN-γ treatment could reduce the number of liver metastases in a colon adenocarcinoma model, while rRIFN-γ had no effect on tumor growth *in vitro*. These findings strongly suggest that the antitumor effect of rRIFN-γ was caused by indirect mechanisms, such as activation of host effector cells (described in Chapter 4). It has been demonstrated that NK cells that reside in liver and lung have the capacity to reduce the take of tumor cells (10). In our experiments the antitumor effect of rRIFN-γ may indeed be explained by stimulation of NK cell cytotoxicity.

rRIFN-γ and rTNF were found to have a synergistic antitumor effect on colon adenocarcinoma in a subcutaneous model. *In vitro* studies have indicated additional effects of both cytokines. Several mechanisms may explain this finding. In addition to augmentation of direct cytotoxicity, IFN-γ may induce the expression of TNF-receptors on tumor cells, increase activation of effector cells stimulated by TNF and induce the release of other cytokines that act synergistically with TNF (for review see ref. 4).

Importantly, the current experiments have demonstrated that combinations of cytokines, such as IFN-γ and TNF, may act synergistically in the treatment of malignancies. However, toxic side-effects may be reinforced too. As shown in our model, local administration by intralesional injection reduces cytokine toxicity. As this technique has its limitations for application in patients with disseminated cancer, new techniques must be developed to administer cytokines locally at the centre of antitumor reactions. Several techniques are currently being investigated. One of these is focused on genetic engeneering of tumor infiltrating lymphocytes (TIL) to increase their cytokine production. Assuming that these manipulated cells will home preferentially at the tumor site, locally high concentrations of cytokines may be induced. However, the feasibility of this technique remains to be determined. Local administration may also be achieved by the use of cytokine-containing liposomes, alone or in combination with monoclonal antibodies directed against target cells. Further, treatment schedules are being designed that combine the use of exogenous recombinant cytokines with the application of biological response modifiers, agents

that are able to induce endogenous cytokine production. And last but not least molecular biology technologies may improve the molecular design of cytokines and produce new molecules with increased specific activity (11).

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SUMMARY

IFN-γ was one of the first cytokines produced by recombinant DNA technology. After the production of recombinant DNA derived mouse and human IFN-γ, recombinant DNA derived rat IFN-γ became available. With this species-specific cytokine it has become possible to study biological effects of IFN-γ in rats. Compared to the mouse model, the rat model is more suitable to study activities of IFN-γ in tumor and organ transplantation experiments, since these experiments are technically far less demanding in rats. In this thesis multiple biological effects of rRIFN-γ are studied in transplantation and tumor experiments in rats.

In Chapter 1 a brief introduction on immune reactions is given with emphasis on cell signalling.

A review on IFN-γ is presented in Chapter 2. Several aspects of IFN-γ are being discussed, varying from the molecular biology to clinical studies.

In Chapter 3 the objectives of investigation are presented.

In Chapter 4 various immunoregulatory activities of rRIFN-γ *in vivo* are demonstrated. rRIFN-γ significantly increases the activity of NK cells, T cells and peritoneal phagocytes. High doses of rRIFN-γ were toxic to rats leading to deterioration of their condition. This finding may reflect the flu-like symptoms observed in patients treated with IFN-γ. The rapid physiologic clearance of IFN-γ is well-documented. Considering the toxicity of high doses of IFN-γ, this rapid clearance mechanism may protect the organism against side-effects of its own products.

In Chapter 5 the capacity of rRIFN-γ to induce MHC class II antigen expression on various cell structures is demonstrated. Different rat strains showed similar patterns of induction, except for endothelial cells. Induction of MHC class II antigen expression on endothelial cells was found in LEW, but not in BN or WAG rats.

Although rRIFN-γ was found to stimulate various immune parameters, rRIFN-γ treatment of recipients had no effect on graft rejection (Chapter 6). Administration of high doses of rRIFN-γ to transplant recipients immediately after operation led to a high mortality (75%).

Since MHC class II antigens are known to be involved in helper T cell activation, the relevance of increased MHC class II antigen expression on allografts prior to transplantation was studied. Pretreatment of BN rats with rRIFN-γ increased the number of dendritic-like cells within cardiac grafts before transplantation to LEW recipients (Chapter 7); in addition, pretreatment with rRIFN-γ of LEW rats increased MHC class II antigen expression on dendritic-like cells as well as on endothelial cells before transplantation to WAG rats. However, in none of these experiments pretreatment with rRIFN-γ had an effect on acute graft rejection. These findings indicate

that the normal amount of MHC class II antigens within a graft suffices to activate T cells to a maximum level.

Experiments were performed in which the host immune system was manipulated prior to transplantation of skin grafts of rRIFN-γ pretreated donors (Chapter 9). Recipients were treated either by short-term immunosuppression (CsA) or by sensitization (donor specific skin transplantation). In both experiments accelerated rejection of rRIFN-γ pretreated skin grafts was found. These findings may be explained by alterations in the afferent as well as the efferent pathway of the host immune responses. MHC class II antigen enriched grafts may have stimulated helper T cells in weakly immunosuppressed recipients and cytotoxic T cells in sensitized recipients. Thus, upregulation of MHC class II antigen expression does not affect rejection by unmodified recipients, but accelerates rejection by immunomodulated recipients.

It was found that rRIFN-γ inhibited tumor growth *in vivo*, while no direct antiproliferative effects of rRIFN-γ were demonstrated *in vitro* (Chapter 10). This finding suggests that indirect antitumor mechanisms are activated by rRIFN-γ, such as those described in Chapter 4. The outcome of these experiments supports the concept of immunotherapy, which is based on the assumption that stimulation of the host's immune system may lead to elimination of tumor cells.

TNF is a cytokine of which immunoregulatory and antitumor activities have been identified. Combined local treatment with rRIFN-γ and TNF was found to have a synergistic antitumor effect (Chapter 11). Further studies on treatment schedules and routes of administration have to be performed to determine the strongest antitumor activity of various combinations of cytokines.

In Chapter 12 a general discussion is presented. It is emphasized that systemic toxicity due to increased IFN-γ serum concentrations limits its use to manipulate immune reactions at a local level. Therefore, new techniques must be developed by which it will be possible to increase local concentrations of IFN-γ without inducing significant elevations of serum levels. With the availability of rRIFN-γ the rat model may offer perspectives for further cytokine research.

SAMENVATTING

Cytokines zijn eiwitten die met name geproduceerd worden door cellen van het immuun systeem. Verschillende cytokines zijn geidentificeerd en door recombinant DNA technieken beschikbaar gemaakt voor onderzoek. Interferon-γ was een van de eerste cytokines dat in grote hoeveelheden werd geproduceerd en waarvan, *in vitro*, meerdere immunoregulerende en antitumor eigenschappen werden vastgesteld. Het belang van IFN-γ voor immunoregulerende en antitumor processen *in vivo* is echter niet duidelijk. Dankzij de ontwikkeling van recombinant ratte-IFN-γ (rRIFN-γ) is het mogelijk geworden de funkties van dit cytokine bij de rat te analyseren. In dit proefschrift worden de resultaten van een dergelijke analyse gepresenteerd.

In het eerste hoofdstuk worden recente ontwikkelingen op het gebied van de cellulaire immunologie en de recombinant DNA technologie beschreven. Het belang van deze ontwikkelingen voor klinische vormen van immunomodulatie, zoals in de transplantatie-geneeskunde en in de oncologie, wordt toegelicht. Tot op heden vormt het aspecifieke karakter van therapie gericht op immunomodulatie een belangrijke tekortkoming. Een beter inzicht in de funktie en het werkingsmechanisme van cytokines zou mogelijkheden kunnen bieden voor selektieve manipulatie van het immuunsysteem. In het eerste hoofdstuk wordt een korte inleiding gegeven in de immunologie, waarbij de verschillende celtypen en cytokines die betrokken zijn bij immunologische reakties nader worden gedefiniëerd.

In het tweede hoofdstuk wordt een literatuuroverzicht gegeven van IFN-γ. De ontdekking van interferonen en de indeling in een drietal klassen (IFN-α, -ß en -γ) wordt beschreven. Uiteenlopende aspecten van IFN-γ worden besproken, variërend van de moleculaire biologie tot de eerste resultaten van klinische onderzoeken.

In het derde hoofdstuk worden de doelstellingen van de verschillende experimenten genoemd.

De eerste experimenten waren gericht op het aantonen van de immunomodulerende effecten van rRIFN-γ bij de rat (Hoofdstuk 4). Behandeling met rRIFN-γ leidde tot haematologische veranderingen, waaronder een reduktie van het aantal perifere bloedcellen en beenmergcellen. Wij stelden vast dat rRIFN-γ leidt tot een toename van de aktiviteit van NK cellen, fagocyterende cellen en T cellen.

Drie rattestammen werden gebruikt om het effect van rRIFN-γ op de expressie van MHC klasse II antigenen te bestuderen (Hoofdstuk 5). Bij alle rattestammen werd in meerdere organen een sterke toename van klasse II positieve, dendrietachtige interstitiële cellen gevonden; in organen van LEW-ratten werd tevens induktie van MHC klasse II antigenen op endotheel van grote bloedvaten gezien. Deze bevinding stemt

overeen met de variatie in induktiepatronen die worden waargenomen bij acute afstotingen.

Het effect van rRIFN-γ op het proces van afstoting werd bestudeerd in een harttransplantatie model (BN hart in een WAG of BN hart in een LEW ontvanger), zoals beschreven Hoofdstuk 6. Harttransplantatie werd gevolgd door behandeling van de ontvanger met rRIFN-γ. Deze behandeling had geen effect op het afstotingsproces; bij toediening van hoge doseringen rRIFN-γ werd een ernstige morbiditeit en mortaliteit (tot 75%) geconstateerd.

Wij stelden vast dat rRIFN-γ de expressie van MHC klasse II antigenen kan induceren (Hoofdstuk 5). Deze antigenen spelen een belangrijke rol in de stimulatie van CD4 T cellen (T helper cellen). Via donor-voorbehandeling met rRIFN-γ werd de expressie van MHC klasse II antigenen versterkt, alvorens een harttransplantatie werd uitgevoerd. Deze voorbehandeling leidde tot een significante toename van interstitiele cellen met MHC klasse II antigen expressie in het hart van BN-ratten; bij LEW-ratten werd bovendien *de novo* induktie van MHC klasse II antigenen op vaatendotheel gevonden. Transplantatie van dergelijke voorbehandelde harten naar ratten van een andere stam had echter geen effect op het beloop van het acute afstotingsproces (Hoofdstuk 7 en 8). Deze bevindingen suggereren dat in een normaal transplantaat reeds voldoende MHC klasse II antigenen aanwezig zijn om in een niet voorbehandelde ontvanger een afstotingsreaktie volledig in gang te zetten.

In de volgende experimenten werd de immuunstatus van de ontvanger gemanipuleerd (Hoofdstuk 9). In een huidtransplantatie model (LEW huid in een WAG ontvanger) werd het effect van rRIFN-γ donor voorbehandeling bestudeerd na sensitisatie of immunosuppressie van de ontvanger. In beide modellen werd een significante versnelling gevonden van het afstotingsproces, hetgeen het belang aangeeft van een toename van MHC klasse II antigenen voor de gemodificeerde afstoting. Mogelijk vormt de toename van MHC klasse II antigenen in het immunosuppressie-model een sterkere immunogene stimulus voor CD4 T cellen in de afferente component van de afstoting; in het sensitisatie-model kan een toename van alloantigenen het aantal target cellen verhogen, hetgeen een sterkere stimulus vormt voor reeds aanwezige effector cellen.

Antitumor effecten van rRIFN-γ werden *in vitro* en *in vivo* bestudeerd (Hoofdstuk 10). De groei van een celsuspensie van het colon adenocarcinoom CC531 kon *in vitro* niet worden beinvloed door behandeling met rRIFN-γ. In het diermodel werd echter wel een antitumor effect gevonden. rRIFN-γ behandeling van WAG-ratten met een CC531 tumor-implantaat onder het nierkapsel leidde tot een significante remming van tumorgroei. In een levermetastasen model werd vastgesteld dat behandeling met rRIFN-γ, gestart na injektie van CC531 tumor cellen in de vena portae, leidde tot een significante vermindering van het aantal metastasen. Deze bevindingen suggereren dat het antitumor effect van rRIFN-γ op indirekte wijze tot stand kan komen,

mogelijk door veranderingen aan de target-cellen en/of door veranderingen in de afweer van de gastheer tegen tumorcellen, zoals die van de Kuppfer cellen of NK cellen (vgl. Hoofdstuk 5).

Tot slot werd het effect van rRIFN-γ in combinatie met TNF bestudeerd in het colon adenocarcinoom model. Door injektie van de verschillende cytokines in subcutaan geimplanteerde CC531 tumoren kon een synergistisch effect van TNF en rRIFN-γ worden aangetoond (Hoofdstuk 11).

Hoofdstuk 12 bevat een algemene discussie over de resultaten van de verschillende experimenten. De bevindingen over de immunomodulerende en antitumor eigenschappen van IFN-γ in het rattemodel stemmen overeen met die uit studies in muizenmodellen en in de mens. Het is duidelijk dat IFN-γ slechts één van de vele cytokinen is die een rol spelen in immunologische reakties en dat manipulatie met IFN-γ alleen geen mogelijkheden biedt tot beinvloeding van acute afstotingsreakties. Onderzoek met monoclonale antilichamen tegen IFN-γ kan nadere informatie geven over de rol van dit cytokine in de regulatie van immunologische processen. Ten aanzien van de antitumor werking van IFN-γ zal verder onderzoek verricht moeten worden naar de mogelijkheden van lokale toediening en verhoging van de antitumor effectiviteit door combinatie met andere middelen, zoals cytokines (TNF) of chemotherapeutica. Het in dit proefschrift beschreven model biedt goede mogelijkheden voor verdere studies op dit gebied.

APPENDICES

List of abbreviations

APC	antigen presenting cell
cpm	counts per minute
CD	cluster of differentiation
CO_2	carbon dioxide
Con A	concanavalin A
^{51}Cr	^{51}chromium
CTL	cytotoxic T lymphocyte
DNA	desoxyribonucleic acid
FCS	fetal calf serum
H-2	locus of the MHC in mice
i.p.	intraperitoneal
i.v.	intravenous
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LT	lymphotoxin
MAF	macrophage activating factor
MHC	major histocompatibility complex
MIF	migration inhibiting factor
μCi	microcurie
n	number of observations
NK	natural killer
P	level of significance
PBL	peripheral blood lymphocyte
PBS	phosphate buffered saline
PEC	peritoneal exudate cell
PHA	phytohaemagglutinin
PLt	platelet
RBC	red blood cell
RNA	ribonucleic acid
RT-1	rat MHC haplotype
s.c.	subcutaneous
SD	standard deviation
SEM	standard error of the mean
SRBC	sheep RBC
TCR	T cell receptor
TNF	tumor necrosis factor
U	unit
WBC	white blood cell

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Nawoord

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Curriculum vitae

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