CYTOKINE DEPENDENCE OF B CELL MEMORY FORMATION

A study in mice with emphasis on IgG_1 and IgE

René van Ommen

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CYTOKINE DEPENDENCE OF B CELL MEMORY FORMATION

A study in mice with emphasis on IgG_1 and IgE

CYTOKINE AFHANKELIJKHEID VAN DE GEHEUGEN B CEL VORMING

Een studie in de muis met speciale aandacht voor IgG_1 en IgE

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CONTENTS

Chapter 1

General introduction

GENERAL INTRODUCTION

Mammals are born with virtually no self-generated immune protection. Immediately after birth they are more or less protected by antibodies that have crossed the placenta or are derived from the colostrum. This so called passive-immunization only holds for a limited period after birth. When the new-born is exposed to environmental antigens the immune-defense systems are gradually built up. This natural form of active immunization can be mimicked by a deliberate introduction of antigens into the host in order to provoke protective immune responses prior to a natural encounter with those antigens [Clark, 1991].

1.1 Antigens

Antigens can be classified into two categories, thymus independent (TI) and thymus dependent (TD) antigens [Katz and Benacerraf, 1972; Mond and Brunswick, 1987]. This classification of antigens is based on the ability or inability to evoke an immune response in athymic nude mice. From these experiments it was concluded that TI antigens can stimulate B cells without T cell interference, whereas TD antigens can only stimulate B cells in the presence of T cells. However, no such clear distinction can be made, as it was shown that rigorous T cell elimination significantly reduced *in vitro* immune responses to TI antigens as well [Parker, 1982; Mond et al., 1983; Endres et al., 1983; Mond and Brunswick, 1987]. These results suggest that B cells responding to TI antigens need T cells, or their products, for help in obtaining peak levels of the antibodies produced. Moreover, these results show that TI antigens are partly independent of T cell help, whereas TD antigens are completely dependent on T cells to evoke a humoral immune response [Vitetta et al., 1989].

TI antigens can be further subdivided in two categories based on their ability to evoke an immune response in CBA/N mice. TI type 1 antigens (e.g. TNP-LPS) can elicit an immune response in these mice, whereas TI type 2 antigens (e.g. TNP-ficoll) in similar mice do not elicit an immune response [reviewed in Savelkoul et al., 1988a]. Whereas immunological memory is generated after immunization with both TD and TI type 1 antigens, little or no memory formation takes place upon immunization with TI type 2 antigens [MacLennan et al., 1990].

1.2 Immunologic memory

Upon secondary exposure to an antigen, the immune system will react with the

generation of an antigen-specific immune response much more rapidly and will manifest itself much more strongly than upon the first exposure to the same antigen. This antigen-specific response is characterized by the appearance of immunoglobulin (Ig) classes other than IgM, that display a higher average binding affinity for the antigen they were originally raised to. These so called secondary immune responses are the result of pre-existing antigen-specific memory B and T cells, formed during the primary immune response [Vitetta et al.,1991; Gray, 1993]. This capacity of memory formation is one of the characteristics of the immune system.

Immunological memory is long-lived. Memory responses can be evoked months or years after an initial antigenic encounter [Celada, 1967; Celada, 1971]. The question is whether the B and T cells giving rise to immunological memory are also long-lived. It was suggested that B cell memory does not reside with very long-lived cells, but rather with clones that are maintained over long periods by continued stimulation with persisting antigen [Gray and Skarvall, 1988]. In the absence of antigenic stimulation, memory B cell clones have a half live of 2-3 weeks [Gray and Skarvall, 1988]. This indicates that memory B cells are deleted from the circulating pool within a few weeks unless they meet antigen again. Bromodeoxyuridine (BrdU) labelling experiments revealed that in the presence of antigen memory B cells do not divide and are not recruited from newly generated B cells over an extended period of more than 7 weeks [Schittek and Rajewski, 1990]. These results leave open the question whether resting, non-dividing, memory B cells require the persistence of antigen to sustain their longevity. They show that in the presence of antigen memory B cells can persist without cell division. The persistence of non-dividing memory B cells in the absence of antigen has not yet been demonstrated. Whereas T cells are necessary for the prolonged growth of activated B cells [Rajasekar et al., 1987], persistence of memory B cells has been reported in the absence of T cell help [Vieira and Rajewski, 1990]. This indicates that the persistence of memory B cells is independent of continuous, T cell dependent, recruitment of memory B cells. Nevertheless, memory B cells are only developed in germinal centers, and germinal centers are only formed when T cells are present, indicating the importance of T cells in early memory B cell development [Vitetta et al., 1991; Gray, 1993].

Recurrent activation of memory B cells, whether or not leading to cell division, seems to be necessary for their maintenance. Such recurrent activation could be provided by follicular dendritic cells (FDC). These cells form a structural network within B cell follicles located in the secondary lymphoid organs. During antibody responses antigen-antibody complexes are formed and deposited on the surface of FDC were they can persist for prolonged periods [Tew and Mandel, 1979; Tew et al., 1980; Van Rooijen,

1990]. B cells can directly be activated by antigen stored on FDC, whereas T cells cannot, because FDC cannot process bound antigen. However, it was shown that B cells can pick up antigen in the form of antigen particles called icosomes which are present on the FDC [Szakal et al., 1988; Kosco et al., 1988; Gray et al. 1991]. After processing the antigen, B cells can present the antigen in the context of major histocompatibility complex (MHC) class II to T cells [Gray et al., 1991]. Moreover, resting B cells become effective antigen-presenting cells (APC) for T cells, when interacting with FDC in a antigen-specific manner [Kosco-Vilbois et al., 1993]. Although only few T cells are found in germinal centers, the major part of the T cells present do have the same antigen specificity as the adjoining B cells [Fuller et al., 1993]. As *in vivo* studies have shown that B cells can stimulate memory, but not naive T cells [Ronchese and Hausmann, 1993], it is tempting to speculate that the majority of T cells in germinal centers are of the memory phenotype.

T cell memory is like B cell memory long-lived. It was shown that choriomeningitis virus specific $CD8^+$ T cells upon adoptive transfer in recipient mice persist in small numbers and comprise only a minor fraction of the total T cell population [Jamieson and Ahmed, 1989]. With respect to involvement of antigen in maintaining the longevity of memory T cells, however, there is still some debate. $CD8^+$ memory T cells specific for the minor histocompatibility male H-Y antigen [Gray and Matzinger, 1991] and viral antigens [Oechen et al., 1992] were reported not to persist in the absence of antigen. Similar observations were made for $CD4^+$ cells reactive to keyhole limpet hemocyanin (KLH) [Gray and Matzinger, 1991]. However, recently a study was published in which it was shown that $CD8^+$ memory T cells reactive to influenza virus can persist for 25 weeks in absence of antigen [Mullbacher, 1994]. In that study more T cells (5-fold) were adoptively transferred, which could explain the observed differences when compared to the study of Gray and Matzinger. The observation that 20-30% of the memory $CD4^+$ T cells, depending on the marker used to discriminate naive and memory T cells, and 40-60% of the memory CD8+ T cells do not incorporate BrdU over a 5 week period [Tough and Sprent, 1994] indicates that at least part of the memory T cells are resting cells that remain in interphase for a prolonged period.

1.3 Phenotypic characteristics of memory T cells

The development of memory T cells out of naive T cells is accompanied by a number of cell surface changes that may affect T cell triggering, interactions with B cells or other APC, and recirculation [Mackay, 1994]. These changes allow identification of these differentiation stages by means of monoclonal antibodies (mAb) directed to the surface markers involved (Table 1). Recent progress suggest that CD45 might be a useful

marker to distinguish naive from memory T cells. CD45 is a molecule expressed in different isoforms on all leucocytes as the result of alternative splicing [Thomas, 1989]. Acquisition of immunological memory is accompanied by changes in the expressed CD45 isoforms. Murine naive T cells express the B isoform of CD45, whereas memory T cells do not interact with anti-CD45RB antibodies [Dianzani et al., 1990]. In humans naive T cells are CD45RA positive, whereas memory T cells express CD45RO [Gray, 1993].

Table 1. Phenotypic characteristics of memory T and B cells

	T cells		B cells	
marker	naive	memory	naive	memory
CD45RB	high	low		
LFA-1	low	high		
ICAM-1	low	high		
CD44 (Pgp-1)	low	high	low	high
L-Selectin (MEL-14)	high	low	low	high
sIgD			+	-
PNA			high	low
J11D			high	low

For murine T cells it was shown that CD4, CD45 and CD3/TcR complex were physically linked in T cells of the memory phenotype, whereas in cells with the naive phenotype these molecules migrated independently from each other [Dianzani et al., 1990]. It was reported that CD45 is a tyrosine phosphatase which is important for the signal transduction after TcR triggering [Koretzky et al., 1990]. Therefore, the coupling of CD4, CD45, and CD3/TcR could explain the less stringent activation requirements observed for memory T cells when compared to naive T cells [Byrne et al., 1988; Ronchese and Hausmann, 1993; Croft et al., 1994]. Moreover, murine memory T cells express higher levels of adhesion molecules, such as CD44 (Pgp-1) [Butterfield et al., 1989; MacDonald et al., 1990], LFA-1 [Springer et al., 1987] and ICAM-1 [Prieto et al., 1989], but lower levels of the lymph node homing receptor, L-selectin (MEL-14) [Swain et al., 1991; Bradley et al., 1992], than naive T cells. The fact that naive $CD45RB^{high}$ T cells express higher levels of MEL-14 than memory $CD45RB^{low}$ T cells suggest that these cells have different patterns of recirculation [Bradley et al., 1992]. The down regulation of both CD45RB and MEL-14 which occurs in response to activation of $CD4^+$ T cells [Birkeland et al., 1989], represents a stable phenotypic change indicative of prior antigen experience [Bradley et al., 1992; Bradley et al., 1993]. Moreover, it was described that $CD44^+$ and $CD45RB^{low}$ memory T cells have higher metabolic rate, a larger cell size and a higher mean cellular protein and RNA content than T cells expressing the naive phenotype, suggesting that the memory T cells are in G1 phase of cell cycle (not necessarily cycling), rather than resting (out of cycle) in G0 [Stout and

Stuttles, 1992]. This could explain the response-ready state of antigen-experienced memory T cells. However, a naive phenotype does not necessarily mean that T cells did not experience antigen as was shown by Tough and Sprent [Tough and Sprent, 1994]. In this study evidence is presented that naive T cells can divide without losing their naive phenotype. In accordance with this study is the observation that upon *Leishmania major* infection primed $CD4^+$ T cells are found in the $CD45RB^{high}$ cell population [Powrie et al., 1994]. Moreover, the observation that in rats $CD45R^-$ and $CD45R^+$ $CD4^+$ T cells can interconvert, suggests that the CD45R marker is not static, but rather reflects the physiological status of the T cell [Bell and Sparshott, 1990]

Besides differences at the membrane level, naive and memory T cells differ with respect to the cytokines they produce upon stimulation *in vitro* [Mackey, 1994]. Moreover, different amounts of cytokines are produced by naive and memory T cells [Mackay, 1994]. Cytokines are proteins, that act as soluble mediators and regulators of immune responses. Their actions are performed by binding to specific receptors in a paracrine or autocrine fashion. Based on the recent literature, three stages of *in vivo* antigen stimulated T cells can be distinguished, that differ in the amount of cytokines they produce upon *in vitro* stimulation. It is thought that these different populations occur sequentially during ongoing immune responses *in vivo* and *in vitro*. When freshly isolated naive T cells are stimulated *in vitro* with KLH no cytokine synthesis is observed (Figure 1) [Bradley et al., 1993]. However, 5 days after priming with KLH primary effector T cells are formed that produced IL-2, IL-3, IL-4 and low, but detectable, levels of IFN-γ after antigen-specific stimulation (Figure 1) [Bradley et al., 1991; 1993]. *In vitro* it was shown that naive T cells needed 4 days of culture during which they proliferated and differentiated to the primary effector state characterised by the production of IL-4, IL-5, IFN-γ, IL-3, GM-CSF, and low levels of IL-2 [Swain et al., 1990a; Weinberg et al., 1990]. *In vivo* this primary effector state is seen as a transient population of $CD45RB^{low}$ and MEL-14 negative helper cells that secrete IL-2, IL-3, IL-4 and IFN-γ upon *in vitro* culturing [Bradley et al., 1991]. As a result of priming the frequency of KLH-specific T cells in the spleen increased 30-fold from $1/6x10^4$ to 1/2600 [Bradley et al., 1993]. Antigen-specific stimulation of these primed T cells, from mice that had been primed more than 6 weeks before, revealed a population that produced high levels of IL-2 and IL-3, and low, but detectable, levels of IL-4 and IFN-γ (Figure 1) [Bradley et al., 1992; 1993]. These so called resting memory T cells that were $CD45RB^{low}$ and $MEL\text{-}14^-$, after *in vivo* boosting gave rise to in a population of effector memory T cells that produced low levels of IL-2, IL-3 and high levels of IL-4 and IFN-γ as compared to the production by resting memory T cells upon subsequent *in vitro* antigen-specific stimulation (Figure 1) [Bradley et al., 1993]. As a result of boosting the frequency of antigen-specific T cells increased to 1/541, but this increase could

not entirely explain the higher levels of IL-4 found after culturing. This indicates that memory effector T cells also secrete more IL-4 per cell than primary effector cells [Bradley et al., 1993]. It has to be noted that the amount of IFN-γ produced by primary effector, resting memory, and memory effector T cells upon *in vitro* stimulation is lower than the produced IL-2, IL-3, and IL-4 levels under similar conditions [Bradley et al., 1993]. Moreover, the amount of IL-4 produced by primary effector cells is higher than the level secreted by resting memory T cells [Bradley et al., 1993]. IL-2 was reported to be essential for the generation of IL-4 synthesis [Powers et al., 1988]. On the other hand, it was found that IL-2 is not required for established IL-4 responses [Yang and HayGlass, 1993], indicating that IL-2 is involved in the expansion of IL-4 secreting cells and does not influence the production of IL-4 by *in vitro* restimulated T cells. However, both de novo and established IFN-γ responses are dependent on IL-2 [Yang and HayGlass, 1993].

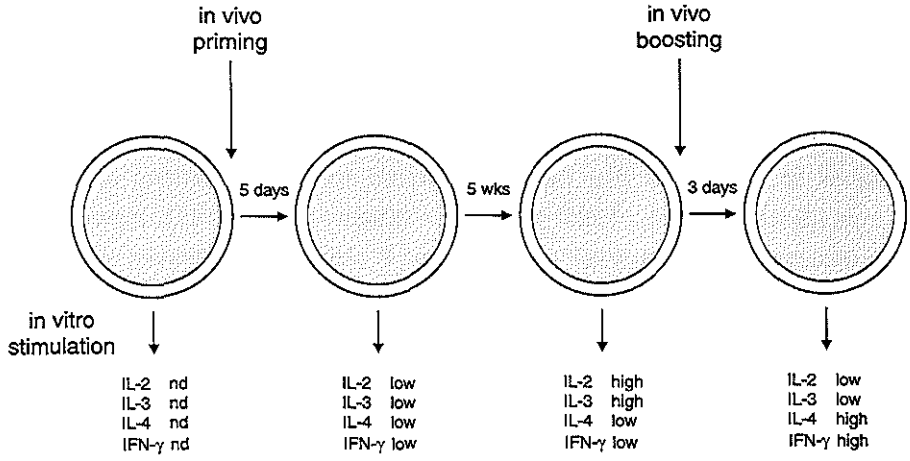

Figure 1. Cytokine production by naive T cells, primary effector T cells (5 days after *in vivo* priming), resting memory T cells (6 weeks after *in vivo* priming) and memory effector T cells (3 days after *in vivo* boosting) upon *in vitro* stimulation.

In general it can be concluded that both primary and memory effector T cells can produce IL-4 upon *in vitro* stimulation. However, the amount of IL-4 produced by effector memory T cells is much higher than the amount produced by naive T cells [Bradley et al., 1993].

During life time the number of memory T cells increases as a result of exposure to environmental antigens. This is illustrated by comparing T cells from young adult mice with T cells from aged mice. The $CD4^+$ T cell population from the aged mice is characterised by an increased frequency of cells representing the memory phenotype, $CD45RB^{low}$ and $Pgp\text{-}1^+$. Moreover, these cells produced more IL-4 than $CD4^+$ T cells

from the young adult mice upon stimulation with anti-CD3, whereas similar levels of IL-2 were seen [Ernst et al., 1990].

1.4 Phenotypic characteristics of memory B cells

Like memory T cells, memory B cells can be discriminated from naive cells on the basis of cell surface markers (Table 1). One of the most obvious characteristic of memory B cells is the expression of a surface immunoglobulin molecule other than IgM. Moreover, memory B cells express surface immunoglobulin molecules with a higher affinity for the antigen to which they were originally raised, as a result of somatic mutation [Berek and Ziegner, 1993]. This process, which takes place in the germinal center [Apel and Berek, 1990], is antigen driven and is called affinity maturation [Colle et al., 1990; Vitetta et al., 1991; Gray, 1993; Mackay, 1994]. Most of the B cells in mice co-express IgM and IgD on their cell surface. Upon antigenic challenge B cells switch to other isotypes than IgM and IgD [Coffman and Cohn, 1977]. Only surface IgD^- cells carried B cell memory as was shown by adoptive transfer experiments [Herzenberg et al., 1980]. It was shown that these cells also were specific for the antigen with which mice had been primed. Isolation of these antigen-specific memory B cells revealed, that they were IgG^+ cells [Yefenof et al., 1986] that had incorporated somatic mutations in their variable region genes [Siekevitz et al., 1987; McHeyzer-Wiliams et al., 1991]. However, somatic mutations also occurred in a small proportion of B cells that expressed IgM and IgD on their surface, indicating that IgD expression is not the primary marker to discriminate naive B cells from memory B cells [Gu et al., 1991; Schittek and Rajewski, 1992]. This was substantiated by former studies in which it was described that both the IgD^+ and the IgD^- cell population contain memory B cells [Zan-Bar et al., 1979; Lafrenz et al., 1981].

Other, non-immunoglobulin surface markers, that have been used to identify memory B cells are CD44, MEL-14 and peanut-agglutinin binding molecule (PNA). Memory B cells express high levels of CD44 (Pgp-1) [Camp et al., 1991]. Whereas early memory B cells, found in the germinal center express low-levels of L-Selectin (MEL-14) and high-levels of PNA, these cells in a later stadium express low-levels of PNA and high-levels of L-Selectin (MEL-14) [Coico et al., 1983; Kraal et al., 1988; Colle et al., 1990]. Another marker used to distinguish naive and memory B cells is the heat stable antigen (HSA), recognized by the mAb J11D. HSA is expressed on various hemopoietic cells. Little or no J11D expression is found on the surface of memory B cells responsible for secondary immunoglobulin responses [Bruce et al., 1981]. As a result of this, secondary IgG response were not influenced by depleting J11D cells. Moreover, the J11D expression on antigen-specific B cells decreased after immunization [Vitetta et al., 1991]. Both

$J11D^{low}$ and $J11D^{high}$ cells from primed mice could be stimulated to secrete IgM. However, the $J11D^{high}$ population turned out to be enriched in IgG-secreting cells [Yin and Vitetta, 1990]. This correlates with the observation that cells that express high densities for IgM also express high levels of J11D, whereas cells expressing high levels of surface IgG express low levels of J11D [Yin and Vitetta, 1990]. These $J11D^{low}$ B cells also contained somatically mutated immunoglobulin variable region genes [Linton et al., 1989], which also correlated with the memory B cell phenotype. The available data indicate that the HSA expression on B cells can be used to discriminate naive from memory B cells.

1.5 Mechanisms of memory B cell development

It is known that memory B cell development occurs in germinal centers [Gray and Leanderson, 1990; MacLennan et al., 1992; Tsiagbe et al. 1992]. However, the exact developmental pathway leading to memory B cells is currently not known. Three models could account for the formation of memory B cells. They could be derived from a precursor cell, as a result of unequal division giving rise to a memory cell and a plasma cell. Alternatively, it is possible that precursor cells differentiate either in a memory B cell or antibody forming plasma cell as a result of different micro-environments [Gray, 1993; MacKay, 1994]. The third possibility is that memory B cells and antibody forming plasma cells develop form different precursors [Linton et al., 1989; Klinman and Linton, 1990]. The conformity of these three mechanisms is that at some point the B cell lineages diverge, giving rise to plasma cells and memory B cells [Gray, 1993]. However, in the third proposed mechanism the two lineages already diverge before antigenic challenge, whereas in the other mechanisms B cells first have to meet antigen before they are committed to become memory B cells [Mackey, 1994].

Upon immunization with a thymus dependent antigen B cell proliferation occurs at two distinguishable sites in the spleen: the PALS associated foci and the germinal centers, respectively. Whereas isotype switching appears to occur in both the PALS associated foci and the germinal centers [Jacob et al., 1991a], memory B cell development only takes place in germinal centers [Gray and Leanderson, 1990; Maclennan et al., 1992; Tsiagbe et al. 1992]. The PALS associated foci are the first sites of B cell proliferation upon antigenic encounter in the T cell zone of the white pulp. Each focus is anatomically discrete and in situ genetic studies of Ig heavy chain genes from individual foci suggested that there is little or no cellular migration between the populations of adjacent foci [Jacob et al., 1991a; Jacob and Kelsoe, 1992]. The peak of the focus response is on day 10 post-immunization, thereafter this B cell response rapidly declines [Jacob et al., 1991a; Jacob and Kelsoe, 1992]. By day 4 after immunization the second

site of intense B cell proliferation appears, that initiates the germinal center formation [Jacob and Kelsoe, 1992]. The germinal center reaction reaches its maximum by day 10-12 of the primary response and remains stable until day 16 post immunization [Jacob et al., 1991a]. Five zones (follicular mantle, outerzone, apical light zone, basal light zone, and dark zone) can be identified on the basis of different phenotypes of residing follicular dendritic cells and B cells [Hardie et al., 1993]. Based on the percentage of cells undergoing mitoses or apoptosis it was concluded that the dark zone is a site of proliferation and probably also mutation, whereas the basal light zone is a site of selection [Hardie et al., 1993]. Both processes are important for affinity maturation that occurs selectively in germinal centers [Berek et al., 1991]. An increase in the number of somatic mutations can be observed already in the second week of a primary immune response. Most of these mutations occur in the genes coding for the complementarity determining regions, the parts of the antibody that are involved in antigen recognition. B cells proliferate without accumulation of somatic mutations, indicating that somatic mutations only occur during a limited period of time. The selectivity of this process is underlined by the occurrence of "key mutations" that occur upon immunization with a particular antigen and that increase the affinity a 10-fold. Two weeks following immunization, these key changes are found in practically all responding B cells [Berek and Ziegner, 1993]. The fact that the hypermutation mechanism starts to operate even prior to day 6 [Weiss et al., 1992], suggests a start of the hypermutation mechanism before isotype switching. Memory B cells that express an immunoglobulin molecule with increased affinity for the original antigen could enter for a second time the dark zone of the germinal center. Such entrance could occur via the outer zone allowing these B cells to undergo a second round of somatic mutation and affinity selection. However, currently no studies have been performed that could either prove or disprove this option.

A study, in which the clonal origin was assessed of antigen-specific B cells in the germinal centers and in the B cell foci outside the germinal centers, showed that there is a clonal relationship between these two B cell populations [Jacob and Kelsoe 1992; Jacob et al., 1992]. This indicated that these two populations were derived from one precursor after antigenic stimulation, thereby arguing against the theory of two precursor lineages existing before antigen-stimulation that was proposed by Linton et al. [Linton et al., 1989; Klinman and Linton, 1990]. However, it is also obvious that B cell populations sorted on the basis of J11D expression before antigenic stimulation can be subdivided in a $J11D^{high}$ population (80% of splenic B cells) giving rise to antibody forming cells after antigenic stimulation and a $J11D^{low}$ population that predominantly forms memory B cells upon antigenic stimulation [Linton et al., 1989; Klinman and Linton, 1990]. This was shown by immunizing SCID mice that had been repopulated

with J11D^{high} Ia^{+} and J11D^{low} Ia^{+} cells, respectively, in combination with T cells [Linton et al., 1989]. These results argue for a memory B cell commitment before antigenic stimulation. Moreover, in that study it was shown that hybridoma antibodies derived from spleen cells of SCID mice repopulated with J11D^{low} precursor cells, showed a rapid accumulation of somatic mutations [Linton et al., 1989]. The J11D^{low} population is also enriched for cells that give rise to germinal center formation, when transferred in SCID mice that normally do not express germinal centers [Linton et al., 1992]. At the moment, because of gaps in the knowledge of B cell precursor-products relationships, it is not possible to draw firm conclusions with respect to the developmental pathway leading to memory B cells.

The final differentiation to memory cells and antibody forming cells occurs at sites in the peripheral lymphatic tissues that are separated anatomically and functionally [Gray and Leanderson, 1990; MacLennan et al., 1992; Tsiagbe et al., 1992]. As mentioned before, B cell differentiation to memory B cell occurs in germinal centers, that are characterized by intense B cell proliferation. During this B cell proliferation somatic mutations of rearranged IgV genes and selection on the basis of antibody receptor affinity to the original antigen takes place [Jacob et al., 1991b; McHeyzer-Wiliams et al., 1993]. This process results in the formation of memory B cells that are characterised by antibody receptors possessing a higher affinity for the antigen than the naive B cells they are clonally derived of [Weiss and Rajewski, 1990]. On the contrary B cells proliferating in the peri-arteriolar lymphatic sheath do not undergo somatic mutations. These B cells develop in plasma cells [Jacob and Kelsoe 1992]. Although, both germinal center formation and the formation of antibody forming cell foci are strictly dependent on T cells, the number of T cells required differ, as was recently described [Vonderheide and Hunt, 1990; Streda and Cerny, 1994]. Moreover, the antibody cell foci developed in close conjunction to the surrounding T cells [Jacob et al., 1992]. It was described that the formation of antibody forming cells required more T cells than the development of germinal centers and memory B cells [Streda and Cerny, 1994]. Antigen-primed athymic nude mice contained enough T cells (<5% to 20% Thy-1^{+} cells) sufficient to help germinal centers and memory B cell development. This despite the fact that the T cells present have a abnormal phenotype [MacDonald et al., 1986; Kureg and Thomas, 1988; Kennedy et al., 1992] and an impaired lymphokine production [Kureg and Thomas, 1988; Abramson-Leeman et al., 1990]. However, they do not provide enough help to elicit antibody formation after immunization with a thymus-dependent antigen [Streda and Cerny, 1994]. These results suggest that germinal center and memory formation are less dependent on T cell help than antibody formation to thymus-dependent antigens.

1.6 T cell-dependent B cell activation

It was suggested by the classical experiments of Claman et al. [Claman et al., 1966] and Mitchell and Miller [Mitchel and Miller, 1968] that physical interactions between B cells and T cells are necessary for the induction of a humoral thymus dependent immune response [Vitetta, 1989]. Later it was shown that T cells in these responses could not be replaced by the cytokines they produced, indicating the necessity of a cognate interaction between B cells and T cells. Moreover, it was shown that an antigen-specific Th cell clone that could not produce cytokine was able to induce B cell activation [Bartlett et al., 1989]. For some years it was thought that the interaction between MHC class II antigen complex and the T cell receptor provided the contact help. However, recently a new activation molecule expressed on T cells was described, which is the ligand for the B cell differentiation molecule CD40 [Armitage et al., 1992; Noelle et al., 1992a]. This molecule turned out to be the key component of T cell induced B cell activation [Parker, 1993]. Presence of this activation molecule on the membranes of stimulated T cells was already suggested by experiments in which T cell membrane fragments were used to stimulate B cells in an antigen-nonspecific, MHC class II unrestricted manner [Brian, 1988; Hodgkin et al., 1990; Hodgkin et al., 1991; Noelle et al., 1991]. Neutralization of IL-2, IL-3, IL-4, IL-5, IL-6 or IFN-γ by monoclonal antibodies revealed that the activated T cell membrane associated activity was not mediated by membrane bound cytokines [Hodgkin et al., 1991; Noelle et al., 1991]. Moreover, antibodies directed to T and B cell membrane markers, Thy-1, CD23, B220, CD4 and ICAM-1 could not block the induced B cell activation [Hodgkin et al., 1991]. The necessity of T cell activation became clear by studies that used metabolically inactivated, resting T cells that formed conjugates with the B cells, but did not induce B cell activation, whereas anti-CD3 activated T cells after metabolic inhibition, still induced potent B cell activation [Noelle et al., 1989].

It was shown that the ligand of CD40, expressed on activated T cells was a 39-kd type II membrane protein in which the carboxyl terminus is extracellular [Armitage et al., 1992]. This protein, called gp39, turned out to be homologous to tumor necrosis factor α and ß [Hollenbaugh, et al., 1992]. It is expressed by activated $CD4^+$ cells both of the Th1 and Th2 type, but not by $CD8^+$ T cells, indicating its importance in determining the helper effector function [Roy et al., 1993]. Interestingly, upon anti-CD3 stimulation a consistently lower percentage of $CD45RB^{low}$ cells (memory T cells) expressed gp39 than did $CD45RB^{high}$ cells (naive T cells) under similar conditions. The amount of gp39 expressed by gp39 positive T cells of the memory phenotype, however, was 2-fold higher than the amount of gp39 expressed by gp39 positive T cells of the naive phenotype [Roy et al., 1993]. The expression of gp39 is transient, being detectable 4

hrs after activation and reaching a maximum expression level between 6 and 8 hrs and subsequently returning to baseline levels between 24 and 48 hrs post-activation. It has been suggested that CD40 itself is involved in gp39 down regulation, at least in part, by receptor mediated endocytosis [Yellin et al., 1994]. Immunohistological analysis of $gp39^+$ cells in the spleens revealed that after primary immunization with TNP-KLH $gp39^+$ cells could be detected at day 2 reaching a maximum by days 3 and 4 [Van den Eertwegh et al., 1993]. Upon boosting $gp39^+$ cells could already be detected by day 1, but reached their maximum by days 3 and 4. In this study it was also shown that gp39, IFN-γ, IL-2 and IL-4 are coordinately expressed [Van den Eertwegh et al., 1993]. The gp39 expressing T helper cells were not found in germinal centers or marginal zone of the spleen, whereas they did occur in outer peri-arteriolar lymphocyte sheaths and around the terminal arterioles in the spleen [Van den Eertwegh et al., 1993]. The finding that $gp39^+$ T helper cells were found in close proximity to antigen-specific antibody forming cells suggest that gp39 plays an important role in the specific-antibody production [Van den Eertwegh et al., 1993]

Besides a role in the activation of B cells, gp39 is also supposed to play an important role in the process of isotype switching in B cells. This results in B cells expressing other immunoglobulin molecules than IgM and IgD, which is also a major characteristic of memory B cells. It was shown that patients suffering from the X-linked hyper IgM syndrome, an immunodeficiency characterized by the absence of serum IgG, IgA and IgE, are impaired in the expression of gp39 as the result of a defect in the CD40 ligand gene [Aruffo et al., 1993; Korthauer et al., 1993; DiSanto et al., 1993; Allen et al., 1993; Callard et al., 1993]. For mouse B cells it was described that germ-line Cγ1 transcripts are induced by activated T cell membranes independent of IL-4 [Schultz et al., 1992], an event that is associated with isotype class-switching to IgG_1 [Esser and Radbruch, 1990]. This suggests that gp39 expressed on these membrane fragments acted like a switch factor for IgG_1. Studies in which the CD40-gp39 interaction were blocked after immunization with a thymus dependent antigen by administering a mAb directed to gp39, showed a markedly impaired primary antibody response. Similar results were found with respect to the secondary responses, indicating that gp39 has a profound role during thymus dependent immune responses [Foy et al., 1993]. The effect of anti-gp39 treatment could not be explained simply by anergy or deletion of responding T helper cells as it was shown that T helper cells of anti-gp39 treated mice could provide help upon adoptive transfer [Foy et al., 1993].

Besides CD40 and gp39 other membrane markers like B7 on B cells and CD28 and CTLA-4 on T cells are involved in the intimate contact of B cells and T cells [Parker, 1993]. CD28 is constitutively expressed on T cells whereas CTLA-4 and B7 are only

found on activated T cells and activated B cells, respectively [Linsley et al., 1990; Linsley et al., 1991; Freeman et al., 1991]. CTLA-4 is closely related to CD28, and is encoded by an adjacent highly homologous gene [Brunet et al., 1987], which synergizes with CD28 as a receptor for costimulatory signals on activated T cells [Linsley et al., 1992a]. A fusion protein of CTLA-4 and the constant domain of human IgG, called CTLA-4Ig, profoundly inhibits CD4 T cell responses to antigens *in vitro* and *in vivo* [Linsley et al., 1991; Linsley et al., 1992b; Liu et al., 1992]. The importance of an interaction of B cell B7 and T cell CD28 or CTLA-4 during T cell dependent immune responses was substantiated by experiments with transgenic mice that express the CTLA-4 human γ1 fusion protein in their serum. In these mice the T cell dependent antibody formation is profoundly inhibited. Germinal center formation is absent and correlates with an impaired isotype switching and reduced affinity maturation [Lane et al., 1994]. However, the priming of the $CD4^+$ T cells was not impaired. Hyperimmunized mice possessed increased numbers of antigen-specific $CD4^+$ T cells that were functional as was shown by frequency analysis in limiting dilution culture systems [Bianchi et al., 1987] and adoptive transfer in nu/nu mice [Ronchese et al., 1994a]. Alltogether these results suggest that the interaction of B7 with either CD28 or CTLA-4 is not necessary for the induction of T cell responses, but rather induces T cells to provide help for B cells, most likely by inducing gp39 expression on T cells. So B7 CD28 and CTLA-4 are not only involved in the intimate contact of B and T cells, but also play an important role in T cell dependent B cell activation.

An important issue is whether TD B cell responses require only the presence of B cells and T cells. This seems likely because it was shown that resting B cells *in vivo* can present antigen to T cells and efficiently induce an antibody response *in vivo* [Dennis et al., 1993]. The antigen-specific antigen uptake by B cells is 100 to 10,000-fold more efficient than non-specific antigen-presentation, indicating that presentation of antigen by antigen-specific B cells accounts at least in part for the specificity of the antibody response [Parker, 1993]. B cells, however, are not an absolute requirement for T cell activation. It was shown that T cells can be primed by KLH in mice with severe combined immunodeficiency that are reconstituted with T cells alone. Upon *in vitro* stimulation these T cells produced IL-2, indicating that they are functional [Sunshine et al., 1991]. Proliferation is essential for T cells to differentiate from precursor into functional effector cells that possess helper activities [Swain et al., 1991]. *In vivo* studies have shown that resting small B cells are unable to induce naive T cells to proliferate [Ronchese and Hausmann, 1993]. This indicates that other types of APC are necessary to activate naive T cells. Such other APC could be dendritic cells or macrophages, because these types of cells can directly activate naive T cells [Janeway, 1989]. Once exposed to antigen, these now called memory T cells can be activated in an

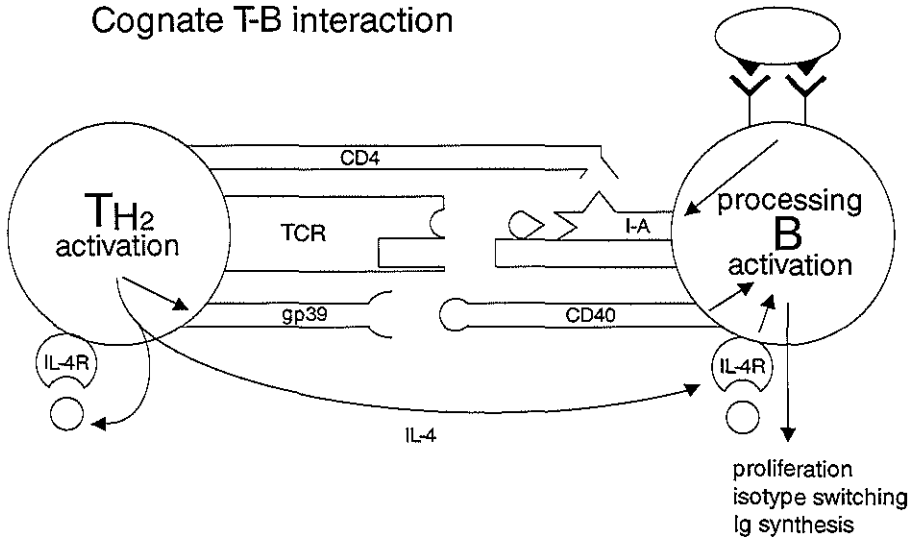

Figure 2. Schematic view of a cognate T-B cell interaction. Antigen captured by B cell membrane bound Ig molecules is internalized, processed and presented in the context of MHC class II molecules to the T cell. Recognition of the T cell epitope MHC class II complex by the T cell receptor and CD4 leads to T cell activation, resulting in expression of the T cell activation molecule gp39. Binding of gp39 to the on B cells constitutively expressed CD40 molecule leads to B cell activation. Moreover, it triggers the T cell to secrete cytokines, resulting in B cell proliferation, isotype switching and immunoglobulin synthesis.

antigen dependent manner by resting B cells [Ronchese and Hausmann, 1993; Croft et al., 1994]. One of the important aspects of the T helper cell B cell interaction is the receptor directed polar release of helper cytokines in the immunological synapse (Figure 2) resulting in high concentrations of these cytokines at the B cell membrane [Poo et al., 1988; Kupfer et al., 1991].

1.7 T helper cell subsets

$CD4^+$ helper T cells can be divided in at least two effector populations based on their cytokine production profile. T helper type 1 (Th1) cells that produce IL-2 and IFN-γ, but not IL-4, IL-5, IL-6 and IL-10, and T helper type 2 (Th2) cells that produce IL-4, IL-5, IL-6 and IL-10, but not IL-2 and IFN-γ [Mosmann and Coffman, 1989; Mosmann et al., 1991]. Cytokines are involved in the regulation of the different Th subsets. IFN-γ inhibits the proliferation of Th2 clones, whereas it does not influence the cytokine production by these cells. It also inhibits the proliferation of Th2 cell clones exposed to IL-2 and IL-4, whereas the proliferation and cytokine production by Th1 cell clones is not affected [Gajewski and Fitch, 1988; Gajewski et al., 1989; Gajewski and Fitch, 1991]. These results indicate that IFN-γ serves as an immune regulatory molecule through which Th1 cells can interfere with the clonal expansion and effector function of Th2 cells. On the contrary, IL-4 inhibited the production of IL-2 and IFN-γ by naive

$CD4^+$ and $CD8^+$ T cells that were stimulated with soluble CD3 in combination with APC [Tanaka et al., 1993]. A similar inhibition was observed when naive T cells from T cell receptor transgenic mice were stimulated with antigen and APC in the presence of IL-4 [Tanaka et al., 1993]. However, not all T helper cell clones can be classified within the Th1 and Th2 subsets according to their cytokine secretion pattern. Cells exist that secrete both Th1 and Th2 cytokines [Firestein et al., 1989; Gajewski et al., 1989; Street et al., 1990; Mosmann et al., 1991]. These cells, termed T helper type 0 (Th0) cells, could be the common precursor of Th1 and Th2 cells, or could be a third effector T helper cell population [Firestein et al., 1989; Torbett et al., 1990; Rocken et al., 1992a]. Beside the capacity to regulate Th1 cytokine production, IL-4 directs the development of Th0 cells to Th2 cells resulting in an increased IL-4 production [Swain et al., 1990b; Swain, 1991; Abehsira-Amar et al., 1992]. Whereas proliferation of both Th1 and Th2 clones in the absence of TCR ligation can be stimulated by exogenous IL-2, only Th2 clones proliferate in response to exogenous IL-4 [Frenandez Botran et al., 1988].

Recently, IL-12 was described as a cytokine that has powerful effect on the IFN-γ production by Th1 cells. IL-12 is produced by monocytes, macrophages and B cells and is required for optimal IFN-γ production both *in vivo* and *in vitro* [Trinchieri, 1993]. Moreover, it was shown that IL-12 is an obligatory factor for the generation of Th1 cells. Naive T cells from mice transgenic for an ovalbumin-specific TCR can be induced to develop in Th1 cells when ovalbumin is given in combination with IL-12, whereas they develop in Th2 cells in the presence of IL-4 [Hsieh et al., 1993]. In this study, it was also shown that IL-4 overrules IL-12. The IL-12 production can be regulated by the Th2 cytokine IL-10, as it was shown that IL-10 impaired the ability of splenic and peritoneal APC to stimulate cytokine synthesis by Th1 clones [Fiorentino et al., 1991]. Under similar conditions IL-10 did not impair the stimulation of Th2 cells. Taken together, the balance of IL-4 and IL-12 determines the differentiation of Th0 cells into either Th1 or Th2 cells [Trinchieri, 1993]. During this process IFN-γ has a positive feedback by enhancing the IL-12 production by monocytes and macrophages, whereas IL-4 and IL-10 have a negative feedback by inhibiting the production of IL-12 [Trinchieri, 1993]. Moreover, a synergism of IL-12 and IFN-γ in inducing anti-CD3 stimulated naive $CD4^+$ T cells to develop in T cells with the Th1 phenotype has been reported [Schmitt et al., 1994]. In that study it was shown that TGF-ß inhibits the IL-12 induced Th1 development of naive $CD4^+$ T cells in the absence of accessory cells. Besides cytokines, T cell receptor/CD3 ligation was reported be important for the development of Th2 cells. Freshly isolated and polyclonally activated $CD4^+$ T cells developed via Th0 intermediates in T cells of the Th2 phenotype when besides IL-2 a mAb directed to either CD3 or the T cell receptor was added [Rocken et al., 1992b]. This result can be

explained by the recent observation that Th2 cells may arise when Th0 intermediates are stimulated via the T cell receptor in the absence of a costimulatory signal. This stimulation results in "anergized" Th0 cells in which the Th1 characteristics are downregulated, resulting in T cells that share several properties with Th2 cells [Gajewski et al., 1994].

Studies that used established Th1 and Th2 cell clones revealed that the proliferation of these cells is optimally stimulated by different APC. Whereas Th2 cell clones optimally proliferate upon stimulation with purified splenic B cells, Th1 cells need stimulation with adherent spleen cells for maximum proliferation [Gajewski and Fitch, 1991; Gajewski et al., 1991]. However, a similar study also using established Th1 and Th2 cell clones showed that exogenous IL-1 is necessary for B cells to stimulate maximum Th2 cell clone proliferation, whereas Th1 cell clones, stimulated with either peritoneal macrophages or splenic B cells, do not need IL-1 for maximum proliferation [Chang et al., 1990]. On the contrary, cytokine secretion by Th1 and Th2 cell clones stimulated with either peritoneal macrophages or splenic B cells is not dependent on IL-1 [Chang et al., 1990]. In the presence of IL-2 B cells are able to secrete IL-1 [Hawrylowicz et al., 1989], suggesting a crucial role of IL-2 in the stimulation of Th2 cells by B cells. B cells, however, are not an absolute requirement for the *in vivo* priming of both IL-4 and IFN-γ producing cells. This was found by examining SCID mice adoptively transferred with either T cells or T and B cells. It was therefore concluded that dendritic cells suffice as APC for the *in vivo* priming of T cells producing either IL-4 or IFN-γ [Ronchese et al., 1994b].

Studies that used Th1 and Th2 cell clones to stimulate B cells *in vitro* independent of their antigen-specificity revealed that the different cytokine synthesis patterns correlated with different effects on the isotype-switching in B cells [Coffman et al., 1988; Stevens et al., 1988]. All used murine Th2 cell clones were excellent helpers of B cell proliferation and immunoglobulin production. When using Th2 cell clones for B cell stimulation, IgM and IgG_1 accounted for most of the antibody produced, but the IgE response, which was relatively substantial, was the most striking of all [Coffman et al., 1988; Stevens et al., 1988]. The IgG_{2a} levels, however, were generally lower than levels of the other isotypes produced. Conversely, Th1 cell clones induce significant B cell activation and proliferation, but less than 5% of the immunoglobulin production induced by Th2 cell clones [Coffman et al., 1988]. Addition of anti-IFN-γ significantly enhanced the immunoglobulin production by B cells stimulated with Th1 cell clones, but the best effect was seen when IL-2 was administered in combination with anti-IFN-γ resulting in increases of 10% to 50%. Th1 cell clones under optimal conditions stimulated B cells to secrete 20- to 50-fold more IgG_{2a} than do Th2 cell clones, whereas almost no IgG_1 and

IgE production was observed [Coffman et al., 1988; Stevens et al., 1988]. These findings substantiate the dichotomy of Th1 and Th2 clones with respect to both cytokine production and helper-activities. However, both Th1 and Th2 cell clones are effective in inducing B cell memory and affinity maturation upon adoptive transfer in nude mice, or with unprimed B cells in irradiated recipients [Rizzo et al., 1992].

1.8 Immunoglobulin isotype switching

During B cell proliferation immunoglobulin heavy chain class switching can take place at any time point. However, at two stages switching occurs with remarkably high frequencies. First, upon a functional V_HDJ_H rearrangement and the formation of a functional V_LJ_L gene, B cells express a complete IgM molecule on their membrane. During this event B cells are formed that express both IgM and IgD immunoglobulin molecules on their membrane that express a similar antigen specificity [Moore et al., 1981; Tucker, 1985]. These so called naive B cells are in G0 stage of the cell cycle. Secondly, upon antigenic stimulation these resting B cells change into B cells that proliferate, differentiate into immunoglobulin secreting plasma cells and memory cells, and switch to the expression of other constant heavy chain gene expression [Gray, 1993; Mackay, 1994].

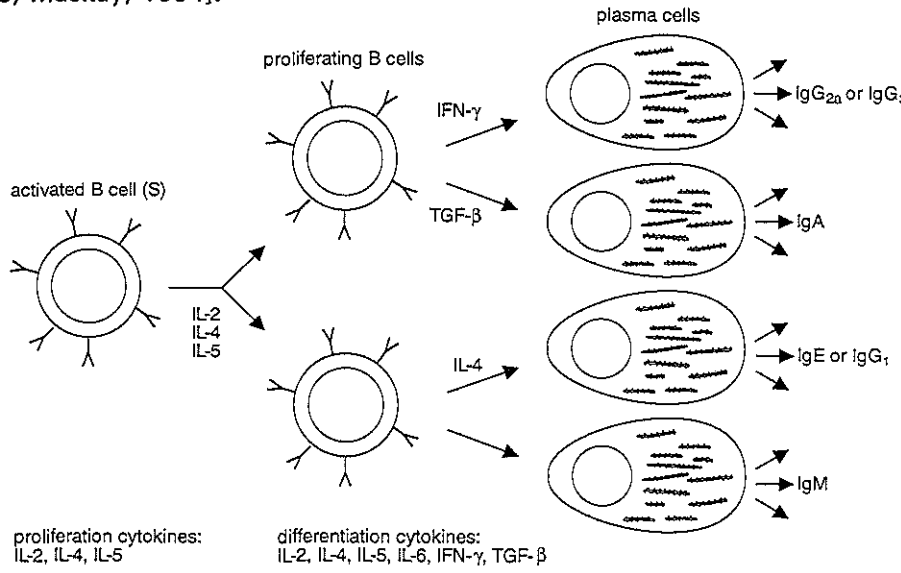

Figure 3. Cytokines involved in B cell proliferation and differentiation into clones of IgM, IgG_{2a}, IgG_3, IgE, IgG_1 or IgA secreting plasma cells.

The switching of sIgM and sIgD positive B cells to the expression of other isotypes is regulated by cytokines (Figure 3). In vitro studies with murine B cells revealed the cytokines that play an important role in the process of isotype switching. IL-4 selective-

ly stimulates the production of both IgG_1 and IgE [Coffman and Carty, 1986; Lebman and Coffman, 1988], whereas the production of both these isotypes is inhibited by IFN-γ [Snapper and Paul, 1987]. IFN-γ, however, induces in vitro IgG_{2a} production by B cells [Snapper and Paul, 1987] and transforming growth factor ß (TGF-ß) and interleukin-5 (IL-5) are involved in IgA production [Coffman et al., 1989; Sonoda et al., 1989]. The role of IL-4 and IFN-γ on the IgG_1, IgE and IgG_{2a} production has been confirmed in vivo [Finkelman et al., 1990].

It is currently thought that TGF-ß stimulates the isotype switch to IgA as it was shown that this cytokine induced sIgA negative cells to express sIgA [Lebman et al., 1990], whereas IL-5 is thought to enhance the IgA production by IgA positive cells. Recently, it was shown that relatively low levels of TGF-ß are necessary for the secretion of all IgG isotype subclasses and IgE by LPS stimulated B cells [Snapper et al., 1993]. It was suggested that TGF-ß regulate these responses in a autocrine fashion.

1.9 Molecular aspects of immunoglobulin isotype switching

In mice the genes encoding for the 8 different heavy-chain isotypes are located in the C_H locus on chromosome 12. They are clustered at the 3' end of the variable-region gene segment in the order 5'-Cμ-Cδ-$C\gamma_3$-$C\gamma_1$-$C\gamma_{2b}$-$C\gamma_{2a}$-Cε-Cα-3'. The entire locus spans approximately 200 kb [Shimizu et al., 1982]. During the process of isotype switching a fully assembled and expressed V_H gene is coupled to a new C_H gene. As a result of this, antibodies retain the same antigen specificity, but have different effector functions [reviewed in Coffman et al., 1993].

Three mechanisms, during which DNA recombination occurs, have been proposed for isotype switching: mitotic interchromosomal recombination between homologous chromosomes, unequal sister chromatid exchange and looping-out and deletion (Table 2) [Harriman et al., 1993]. The specific recombination products that are expected to occur after sister chromatid exchange, however were rarely found [Wabl et al., 1985]. Unequal recombination between homologs seems also unlikely, because the V and C region allotypes of a given immunoglobulin are both encoded by one homologue as was shown in mice based on serology [Gearhart et al., 1980]. Nevertheless this process seems to occur at some low frequency as a result of which it can explain the "back switches" that occur at low frequencies in myeloma cell lines [Radbruch et al., 1980]. Circular DNA, consisting of the intervening regions, that occurs as recombination products after looping out and deletion recombination have been isolated [Iwasato et al., 1990; Matsuoka et al., 1990; Von Schwedler et al., 1990]. Based on these result it is currently believed that looping out and deletion is the major recombination event leading

to isotype switching [Coffman et al., 1993; Harriman et al., 1993].

The constant region genes, except Cδ, are at the 5' site flanked by switch regions that play an important role in the class switch recombination event. These switch regions vary in length from 1 kb (Sε) to 10 kb ($S\gamma_1$) and are composed of tandem repeats of pentameric sequences, like GAGTC [Gritzmacher, 1989; Esser and Radbruch 1990; Coffman et al., 1993; Harriman et al., 1993]. They share homology with each other, especially the Sμ, Sε and Sα [Gritzmacher, 1989]. The Cδ gene that is not flanked at the 5' site by a typical switch region is expressed by alternative RNA splicing [Gritzmacher, 1989]. It was found that all recombination breakpoints could be detected in the switch regions which indicated that the switch regions are indeed used as targets for recombination [Iwasato et al., 1990].

Table 2. Ig class switch recombination

DNA rearrangement
- recombination between homologs
- unequal sister chromatid exchange
- looping out and deletion
no DNA rearrangement
- differential splicing of long-transcripts
- trans-splicing of germline transcripts

Moreover, it was described that recombination breakpoints are focused at the switch nuclear protein (SNIP) and switch nuclear A protein (SNAP) binding sites [Kenter et al., 1993]. During the process of looping out and deletion two switch regions are combined followed by cutting which results in four free DNA ends [Harriman et al., 1993]. Relegation of these free DNA ends can result in the original configuration (no switch), in an inversion of the looped out sequences (loss of H chain expression) [Jack et al., 1988], or deletion of the looped sequences from the chromosome (switch), resulting in switch circles [Iwasato et al., 1990; Matsuoka et al., 1990; Von Schwedler et al., 1990]. DNA replication seems to be important in the process of isotype switching as it was shown by Chu et al. that inhibition of DNA replication in B cells with aphidicolin inhibited isotype switching [Coffman et al., 1993]. The fact that B cell mitogens like LPS and anti-IgM antibodies stimulate isotype switching, supports the idea that DNA replication is associated with isotype class-switch recombination [Coffman et al., 1993].

1.10 Regulation of isotype switching to IgG_1 and IgE

The process of heavy chain class switch recombination is not a stochastic event, but is directed to a particular or limited number of constant heavy chain genes by cytokines [Finkelman et al., 1990]. Studies which used mice that could not express IL-4 as a result of gene targeting, clearly demonstrated that IL-4 is obligatory for the isotype

switch to IgE, whereas it is not obligatory for the isotype switch to IgG_1 [Kuhn et al., 1991]. IL-4, however, did enhance the production of IgG_1, as was shown by neutralizing IL-4 [Vitetta et al., 1985] and by addition of IL-4 containing T helper supernatants [Coffman and Carty, 1986]. But less IL-4 is required for obtaining peak IgG_1 responses than for peak IgE responses upon LPS stimulation of B cells *in vitro* [Snapper et al., 1988a]. The restricted period of 1-3 days after LPS stimulation during which IL-4 enhances the IgG_1 and IgE production pointed to the involvement of IL-4 in the process of isotype switching to and not enhancing the production of these two isotypes, as it acts 1 to 2 days before the onset of the immunoglobulin production in these cultures [Coffman and Carty, 1986, Snapper et al., 1988a]. Moreover, it was shown that IL-4 significantly increased the precursor frequency of cells that gave rise to IgG_1 and/or IgE secreting cells [Bergstedt-Lindqvist et al., 1988; Savelkoul et al., 1988b; Lebman and Coffman, 1988]. The role of IL-4 as switch inducing factor is substantiated by functional studies that indicated that IL-4 induces alterations in the $S\gamma_1$ region [Berton and Vitetta, 1990] and accumulation of germline γ_1 and ε transcripts [Rothman et al., 1988; Stavnezer et al., 1988; Berton et al., 1989; Esser and Radbruch, 1990; Gerondakis, 1990]. These events are directly associated with isotype class switching [Jung et al., 1993; Xu et al., 1993]. Moreover, the increase in frequency of cells expressing γ_1 germline transcripts correlated with the increase in cells secreting IgG_1 [Goodman et al., 1993]. IFN-γ, however, inhibits the expression of both γ_1 and ε germline transcripts induced by LPS and IL-4, whereas it enhances the γ_{2a} germline transcript expression [Berton et al., 1989; Severinson et al., 1990]. This is in concordance with the *in vivo* observation that IFN-γ profoundly inhibited the by goat anti-mouse IgD induced IgG_1 and IgE response, whereas it enhanced the IgG_{2a} response [Finkelman et al., 1988a]. The induction of γ_1 and ε germline transcripts by IL-4 supports evidence for a recombination mechanism in which a nonspecific S region recombination complex derives its specificity by the accessibility of the different switch regions instead of recombination induced by switch region-specific recombinases [Coffman et al., 1993]. This indicates that switch inducing factors do not regulate the expression of switch-region specific recombinases, but rather regulate the accessibility of specific switch-regions for recombination.

The ε germline transcript initiates 2 kb upstream from the $S\varepsilon$ region sequence. It does not contain a VDJ gene segment or another switch region sequence [Gerondakis, 1990]. A similar structure was described for γ2b and α germline transcripts [Lutzker and Alt, 1988; Radclive et al., 1990]. These sequences contain a stop codon that prevents them from being fully transcribed [Lutzker and Alt, 1988; Stavnezer et al., 1988; Berton et al., 1989; Gerondakis, 1990]. Therefore they were formally termed "sterile transcripts". During an in vivo immune response in which mice were injected

with goat-anti-mouse-IgD, known to elicit a strong polyclonal increase in serum IgE [Finkelman et al., 1990], germline ε transcripts expression preceded productive ε transcript expression. Moreover, inhibition of the IL-4 activity by an antibody directed to the IL-4 receptor decreased the induction of both germline and productive ε transcripts, indicating that the relation between germline transcripts and isotype switching also holds in vivo [Thyphronitis et al., 1993]. A direct effect of ε germline transcripts on isotype class switching was demonstrated using a mutant cell line that constitutively transcribed the ε locus, resulting in constitutive ε germline expression. These cells were able to switch to IgE in the absence of IL-4, whereas the parental cell line required IL-4 to switch to IgE [Xu et al., 1993]. Moreover, it was described that B cell-specific activator protein (BSAP), a nuclear transcription factor, binds to a conserved region upstream of the initiation site of the ε germline transcript, were it acts as an inducer of the ε germline expression. IL-4 induces the expression of two presently uncloned nuclear proteins, that bind downstream of the BSAP-binding site to the initiation site of the ε germline transcripts. The nonhistone chromosomal protein HMG-1(Y) binds to this same region. The IL-4 induced nuclear proteins are indistinguishable from the nuclear factors that bind to the BRE sequence within the IL-4-inducible enhancer at the MHC Aα locus [Coffman et al., 1993]. Collectively, these results suggest an active role of ε germline expression, instead of being a side effect, during the process of isotype switching to IgE.

Not only IL-4, but also Th2 cells were reported to induce the γ_1 germline transcript expression in B cells [Berton and Vitetta, 1992]. Th2 cells induced a steady state level of γ_1 germline transcripts as compared to the expression of these transcripts in B cells upon LPS stimulation in the presence of IL-4. These results also suggested an important role for cognate interactions between activated T cells and B cells besides cytokines in the induction of γ_1 germline transcript expression. This finding was further extended by the observation of Schultz et al. that membrane fragments of activated Th1 cells induced γ_1 germline transcript expression in the absence of IL-4, whereas germline ε transcripts were only found when IL-4 was also present [Schultz et al., 1992]. However, a similar study undertaken by Noelle et al. revealed that no germline γ_1 transcripts could be detected when B cells were stimulated with membrane fragments of activated Th1 cells in absence of IL-4 [Noelle et al., 1992b].

An important role for the germline transcripts in isotype class switching might be a direct interaction with the recombination enzyme complex or the switch region chromatin, thereby rendering the switch region accessible for recombination [Coffman et al., 1993]. Germline transcripts could also act as a substrate for RNA trans-splicing which could explain the occurrence of cells expressing both $sIgG_1$ and sIgE on their membrane

that are found upon stimulating B cells in vitro with LPS in the presence of IL-4 [Snapper et al., 1988b]. Another mechanism that could account for the existence of cells expressing two different isotypes is alternative splicing of long RNA transcripts, encoding V_HDJ_H and multiple constant heavy chain genes in germline configuration, which does also lead to isotype switching without DNA recombination (Table 2) [Harriman et al., 1993]. Differential splicing of this long nuclear RNA transcript encoding specific constant heavy chain genes to V_HDJ_H coding RNA could result in the expression of different immunoglobulin molecules, in a manner similar to that described for the observed co-expression of sIgM and sIgD [Moore et al., 1981]. Examination of switch circles isolated from mice upon *Nippostrongylus brasiliensis* infection revealed the possibility that B cells switched to IgG_1 by looping out and deletion can switch further to IgE by the same mechanism (Figure 4) [Yoshida et al., 1990].

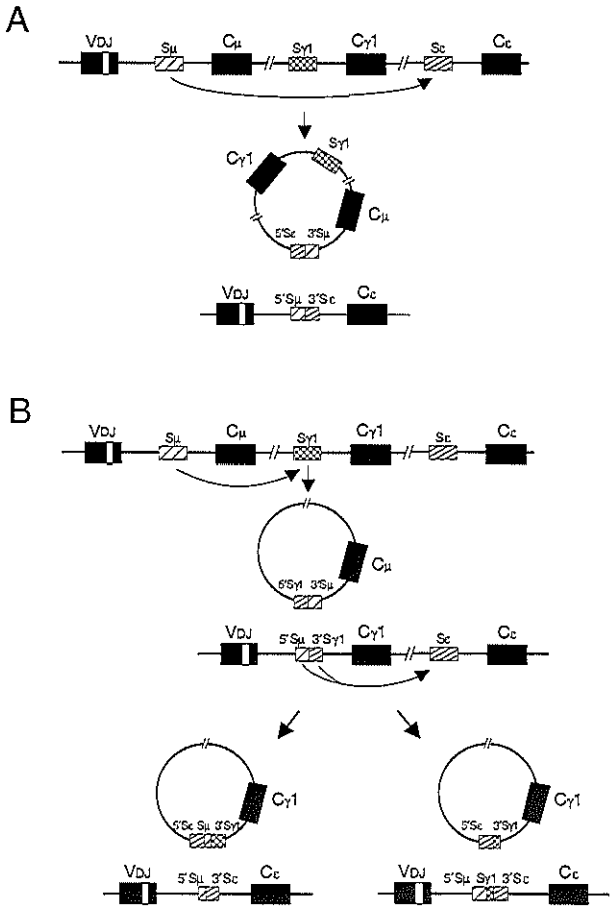

Figure 4. Schematic diagram of direct (A) and sequential (B) isotype switch recombination at the DNA level to the expression of IgE. During the direct isotype switch (A) one switch circle is generated as result of a recombination between Sμ and Sε. During sequential isotype switching two switch circles are formed. One is the result of a recombination between Sμ and $S\gamma_1$ and the second is the result of a recombination between $S\mu\gamma_1$ and Sε. During the latter switch two types fo switch circles are formed that only differ with respect to the recombined S region.

This so called sequential isotype switching can explain why during the process of isotype switching to IgE many B cells are $sIgG_1$ and sIgE double-positive [Snapper et al.,

1988b]. Other studies showed that sequential isotype switching, during which B cells switch from IgM to IgE with IgG_1 positive B cells as intermediates, indeed occurred upon stimulating B cells with LPS in the presence of IL-4 [Siebenkotten et al., 1992; Mandler et al., 1993]. In these studies it was found that isotype switching to IgE predominantly occurs through sequential isotype switching [Siebenkotten et al., 1992; Mandler et al., 1993]. Moreover, cytoplasmic staining revealed IgG_1 and IgE double-positive B cells, suggesting co-secretion of IgG_1 and IgE by $sIgG_1$ sIgE double-positive B cells [Mandler et al., 1993]. Only limiting dilution experiments will reveal whether cells exist that co-secrete IgG_1 and IgE, however so far such studies have not been published. Sequential isotype switching also occurs in human B cells. It was reported that these cells stimulated in the presence of IL-4 switch from IgM to IgE via IgG_4, the homologue of murine IgG_1 [Mills et al., 1992; Jabara et al., 1993; Zhang et al., 1994]

1.11 Introduction to the experimental work

B cells switched to IgG_1 or IgE rapidly loose their sIgM as was shown by analyzing B cells upon *in vivo* immunization with goat-anti-mouse-IgD [Snapper and Finkelman, 1990]. Moreover, it was shown that all secreted IgE originated from sIgE expressing B cells [Snapper and Finkelman, 1990]. Similar results were found when B cells were cultured with LPS in the presence of IL-4 [Snapper et al., 1988b; Mandler et al., 1993]. In concordance with the sequential isotype switching model was the observation that $sIgG_1$ and sIgE double-positive and sIgE single-positive B cells produced similar amounts of IgE, whereas the $sIgG_1$ and sIgE double-positive B cells produced less IgG_1 than the $sIgG_1$ single-positive B cells [Mandler et al., 1993]. The existence of sIgE positive B cells could explain why secondary IgE responses, like primary IgG_1 responses, are less dependent on IL-4 [Heusser et al., 1989; Katona et al., 1991], although it has been shown for secondary IgE responses to TNP-KLH, using neutralizing antibodies directed to IL-4, that IL-4 does sustain these IgE responses *in vivo* [Finkelman et al., 1988b].

The aim of the work presented in this thesis was to improve the insight into the involvement of cytokines, especially IL-4, in the formation of memory B cells switched to IgG_1 and IgE and the dependence on cytokines of these B cells to become IgG_1 and IgE secreting cells upon secondary encounter with the antigen. During the course of this study sequential isotype switching was reported to be the major recombination mechanism leading to B cells that secrete IgE upon primary immunization [Siebenkotten et al., 1992; Mandler et al., 1993]. We questioned whether sequential isotype switching, during which $sIgG_1$ positive B cells switch to sIgE positive B cells with $sIgG_1$ sIgE double-positive B cells as intermediates, also plays a major role during secondary IgG_1 and IgE responses.

In order to obtain systemically elevated IL-4 levels *in vivo* we implanted alginate encapsulated CV-1 cells that were stably transfected with the murine IL-4 gene (CV-1/IL-4 cells). This treatment had previously been shown to be sufficient to transform IgE non-responder SJA/9 mice into IgE high-responder mice [Savelkoul et al., 1991]. The effect of the thus modulated IL-4 level *in vivo* was studied both at the B cell and T cell level. Prolonged IL-4 treatment was carried out after TNP-KLH immunization, and the effects of this treatment were determined on the total IgG_1, IgG_{2a} and IgE, and antigen-specific IgG_1 and IgE serum levels. Adoptive transfer experiments in which spleen cells from control treated or IL-4 treated mice were transferred to lethally irradiated non-treated control mice revealed the effect of prolonged IL-4 treatment on the antigen-specific memory formation for IgG_1 and IgE. Transferring combinations of B cells and T cells from either control or IL-4 treated mice gave insight into the effects of IL-4 on antigen-specific memory B and T cell development. The effects on the cytokine production profile of T cells was examined both at the mRNA expression and the production level. The number of switched B cells in spleens of IL-4 and control treated mice were determined phenotypically by FACScan analysis and functionally by stimulating B cells in an isotype-specific fashion using rabbit-anti-mouse-Ig molecules and a rabbit-Ig-specific murine Th2 clone [Tony and Parker, 1985]. These studies provided insight into the process of sequential isotype switching during secondary IgG_1 and IgE responses. Moreover, we studied the cytokine dependence of memory B cells to become IgG_1 and/or IgE secreting plasma cells, by neutralizing cytokines *in vivo* using antibodies or alginate encapsulated hybridoma cells. Our method used to elevate the *in vivo* IL-4 level also allowed to investigate the long term effect of a predetermined period of systemically elevated IL-4 on the IgG_1 and IgE production *in vivo*, since stopping the implantation of alginate encapsulated IL-4 producing cells abrogated the exogenous IL-4 production after two weeks.

1.12 References

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Chapter 2

Modulation of systemic cytokine levels by implantation of alginate encapsulated cells

H.F.J. Savelkoul, R. van Ommen, A.C.T.M. Vossen, E.G. Breedland, R.L. Coffman[1] and A. van Oudenaren

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[1] Department of Immunology, DNAX Research Institute, Palo Alto, CA, USA

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Modulation of systemic cytokine levels by implantation of alginate encapsulated cells

Huub F.J. Savelkoul *[a], René van Ommen [a], Ann C.T.M. Vossen [a], Elvera G. Breedland [a], Robert L. Coffman [b], Adri van Oudenaren [a]

[a] *Department of Immunology, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, Netherlands.*
[b] *Department of Immunology, DNAX Research Institute, Palo Alto, CA, USA*

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Abstract

The availability of cell lines that are transfected with IL-4, IL-5 and IFN-γ cytokine genes permits the prolonged in vivo delivery of functional cytokines in relatively large doses for the modulation of specific immune responses. Often the transfected cells are xenogeneic or allogeneic to the experimental animal and have to be encapsulated in such a way that no cellular response by the host will be induced. Alginate has proven to be a simple matrix for encapsulating cells under mild conditions suitable for in vivo implantation. Encapsulated cells express the transfected IL-4 gene for at least 14 days after in vivo implantation and were shown to be functional during that period by modulating ongoing IgE responses. The application of adherent growing transfected cells permits dose-response titrations and provides an easy method for local and systemic cytokine delivery. Alternatively, hybridoma cells can be encapsulated and the secreted antibody monitored in the serum. It was found that no host immune response was triggered by alginate encapsulated cells. The efficiency of treatment by encapsulated hybridoma cells was shown to be equivalent to that of injecting purified antibodies.

Key words: Alginate encapsulation; Cytokine transfected cell line; Hybridoma

1. Introduction

The immunoregulatory role of cytokines in immune responses is well established. A special interest has developed in the modulation of immune responses in experimental animal model systems by treatment with cytokine-neutralizing monoclonal antibodies (mAb) or recombinant cytokines in vivo. This can be achieved by repeated injections of relatively large amounts (mg) of purified mAb. Alternatively, multiple injections with several μg of purified recombinant cytokines have to be given each day to induce a detectable effect. Major drawbacks to the application of these techniques are the large amounts of highly purified mAb or recombinant cytokines required, the need to use large scale purification procedures (with problems of yield, recovery, and endotoxin contamination of biologically active mate-

* Corresponding author.

Abbreviations: IL, interleukin; IFN-γ, interferon-γ; mAb, monoclonal antibody.

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rial) and the short biological half life of most cytokines (minutes) in the circulation that necessitate multiple injections each day.

IL-4 is obligatory for the induction of IgE synthesis as evidenced by its ability to induce the expression of ϵ germline transcripts (Rothman et al., 1988; Yoshida et al., 1990), the inhibition of parasite-induced IgE responses by neutralizing antibodies against IL-4 (Finkelman et al., 1990), the absence of IgE responses in nematode-infected mice made IL-4 deficient by gene targeting (Kuhn et al., 1991) and the hyperproduction of IgE in IL-4 transgenic mice (Tepper et al., 1990; Muller et al., 1991).

The availability of cell lines that are transfected with cytokine genes enables the in vivo application of functional cytokines in doses sufficient to modulate specific immune responses (Lee et al., 1986; Yokota et al., 1987; Karasuyama and Melchers, 1988). The implantation of these cell lines in vivo can be used as an alternative to cytokine transgenic animals without the potential drawbacks of developmental disturbances (Tepper et al., 1990; Muller et al., 1991). However, the transfected cells will often be xenogeneic or allogeneic to the experimental animal and have to be encapsulated in such a way that no cellular response by the host will be induced. Encapsulation in alginate provides a simple, suitable method for immobilizing viable cells under mild conditions for in vivo implantation (Kupchik et al., 1983; Kierstan and Coughlan, 1985; Bucke, 1987).

This study describes the combination of adherent growing cytokine gene transfected cell lines and alginate encapsulation for the in vivo modulation of the murine IgE response.

2. Materials and methods

2.1. Mice

Female BALB/c, SJA/9 and SJL mice were bred and kept in our own colony and used at an age of 12–16 weeks. Animals were kept in light-cycled rooms and had free access to acidified water and food. The microbial status of the mice fulfilled the standard of 'specific pathogen free V' according to the criteria of the Dutch Veterinary Inspection, as described in the law on animal experiments.

2.2. Antibodies

Rat monoclonal IgG1 antibodies specific for murine IL-4 (11B11, Ohara and Paul, 1987), IL-5 (TRFK 5, Schumacher et al., 1988) IFN-γ (XMG 1.2, Cherwinski et al., 1987) and *E. coli* β-galactosidase (GL 113, used as an isotype control, Chatelain et al., 1992) were obtained by purification from the supernatants of stable hybridoma subclones grown in defined RPMI 1640 medium supplemented with pyruvate (0.1 M), L-glutamine (4 mM), antibiotics (100 U/ml penicillin and 50 μg/ml streptomycin) and 1% (v/v) Nutridoma SP (Boehringer, Mannheim, Germany). The supernatants were concentrated approximately 20 times by 10000 Da ultrafiltration (Amicon, Danvers, MA). The mAb were purified by saturated (20% w/v) sodium sulphate precipitation and dialysis into 50 mM sodium acetate buffer, pH 4.9. After centrifugation at 10000 rpm to remove precipitated albumin the antibodies were further purified by affinity chromatography on a 10 ml bed volume of Immunopure protein G (Pierce, Rockford, IL) using the same acetate buffer as loading buffer and 0.1 M glycine-HCl, pH 2.5 as elution buffer. After neutralization with 1 M Tris, the sample was dialysed into phosphate buffered saline, concentrated by ultracentrifugation and filter sterilized through a 0.22 μm Millex GV filter (Millipore, Bedford, MA).

2.3. Cell lines

The above mentioned hybridoma cell lines were kind gifts of Dr. R.L. Coffman (DNAX Research Institute, Palo Alto, CA). Cultures of these hybridoma cell lines were aiso used for alginate encapsulation. Stable transformants of the monkey CV1 cell line were obtained by transfecting the murine IL-4 or IL-5 gene and placing the gene under control of the SV40 promotor. Alternatively CHO-Ki cells were stably transfected with the murine IFN-γ gene. All the transfected cell lines were kind gifts of Dr. N. Arai

(DNAX). These adherent cell lines were grown on Cytodex 3 beads (Pharmacia, Uppsala, Sweden) in Dulbecco's modified medium (DMEM) containing 10% FCS, glutamine and antibiotics. Cells were kept in exponentional growth phase by splitting the cultures every 3 days. The TNP-specific mouse IgE secreting IgELA2 cell line was a kind gift of Dr. M. Wabl (Rudolph et al., 1981).

2.4. Alginate encapsulation

Cultured transfected cells grown on Cytodex beads were washed extensively with cold sterile saline and mixed with two parts of a sterile 1.2% alginate solution (FMC, Valiensbaek Strand, Denmark) that was warmed to room temperature. The mixture was transferred into a syringe of suitable size equipped with a 23–27 gauge needle depending on the application (refer results section). Next, the mixture was injected into a fresh solution of 80 mM $CaCl_2$ (prepared in water) at room temperature with continuous gentle mixing. The capsules were washed with cold saline and a 1 ml volume containing 2×10^6 cells (or other cell concentrations as indicated in the results section) was injected intraperitoneally (i.p.) or subcutaneously (s.c.) as indicated. Alternatively, cultured hybridoma cells could also be encapsulated using 27 gauge needles. The capsules could be recovered for periods up to 18 days after injection by peritoneal washing. The cells could be isolated by incubating the capsules for 5 min at 37°C with a 1% trypsin-EDTA mixture (Gibco, Grand Island, NY), thereby establishing that these capsules remained intact in the mouse and that the encapsulated cells were viable for this period in vivo and were still able to produce cytokines in vitro after their recovery.

2.5. Assays

Mice were immunized with 10 μg TNP-KLH adsorbed on alum i.p.. Total serum IgE levels were measured by an isotype-specific ELISA as described previously (Coffman and Carty, 1986). TNP-specific IgE was quantitated in a modified sandwich ELISA. Plates were coated with the same rat monoclonal anti-mouse IgE antibodies (EM95, 2 μg/ml) and incubated overnight at 4°C with diluted serum samples. TNP modified with alkaline phosphatase (kind gift of Dr. A.J.M. van den Eertwegh, MBL-TNO, Rijswijk, Netherlands) was then added. The ELISA was further developed using Sigma 104 phosphatase substrate (Sigma). The detection limit of this ELISA was 0.2 ng/ml. IL-4, IL-5 and IFN-γ were measured by a sandwich ELISA as described previously (Schumacher et al., 1988; Cherwinski et al., 1987; Chatelain et al., 1992). Hybridoma protein produced by encapsulated hybridoma cells was measured in a rat IgG1-specific ELISA by coating plates with 4 μg/ml of a purified rat IgG1-specific mouse monoclonal antibody Mr G1P (clone MRG 1; Pharmingen, San Diego, CA) and incubating with appropriate dilutions of serum. Subsequently, 0.5 μg/ml of a biotin-conjugated mouse anti-rat IgL (κ chain) monoclonal antibody (clone MRK 1; Pharmingen), a 1/1000 dilution of a streptavidin horseradish peroxidase conjugate (Jackson Immunological Laboratory, Westgrove, PA) and the substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS; Sigma, St. Louis, MO) were added. A purified rat IgG1 (clone R3 34; Pharmingen) was used as an isotype-specific standard. The detection limit of this ELISA was 1.25 ng/ml of rat IgG1 in mouse serum and there was no cross-reactivity with murine IgG1. Substituting the coat with an *E. coli* β-galactosidase-specific rat IgG1 mAb (GL113) revealed no signal due to idiotypic activity. In order to detect rat IgG1-specific immune responses in the treated mice, serum dilutions were incubated on ELISA plates coated with 4 μg/ml of rat IgG1 (11B11). For the detection of peroxidase-labelled goat anti-mouse IgM or IgG antibodies (Southern Biotechnology Associates, Birmingham, AL) were used at a 1/1000 dilution. The derived serum concentrations were expressed as arithmetic means ($\pm$1 SD).

2.6. Reverse type PCR analysis of IL-4 gene expression

Cells were collected and resuspended in 0.5 ml of 4 M guanidinium thiocyanate (GTC; Fluka, Buchs, Switzerland) and either analysed directly

or frozen at −70°C until further analysis. Total cellular RNA was isolated after making up the volume of the sample to 2.5 ml with GTC according to the method of Chirgwin et al. (1979). Gradient centrifugation was performed on an equal volume of 5.7 M cesium chloride for 18 h in an ultracentrifuge (Sorvall-Du Pont, Newtown, CT). After ethanol precipitation, 1 μg RNA was used in a reverse transcriptase reaction, modified from the procedure of Krug and Berger (1987). Briefly, 0.01 U oligo$(dT)_{15}$ (Pharmacia, Uppsala, Sweden) was added to a final volume of 14 μl and both RNA and oligo$(dT)_{15}$ were heated for 3 min at 85°C. Then the oligo-primed RNA was added to a mixture containing 1 × avian myoblastoma virus reverse transcriptase buffer (50 mM Tris-HCl, pH 8.3; 50 mM KCl; 10 mM $MgCl_2$; 1 mM dithiothreitol; 1 mM EDTA (ethylenediamine tetraacetate disodium dihydrate salt); 1 μg/ml bovine serum albumin), 1 mM deoxy nucleotide triphosphate (dNTP), 4 mM sodium pyrophosphate, 40 U RNAsin (Promega, Madison, WI) and 5 U avian myoblastoma virus reverse transcriptase (Boehringer Mannheim, Germany). This mixture was incubated for 1 h at 39°C. From this cDNA mixture 5% was used in a PCR reaction. To this end, the cDNA was mixed with 1 × Taq buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% (w/v) gelatin), 0.2 mM dNTP, sense and anti-sense primers ($OD_{0.2}$) and 1 U Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). For amplification, 35 cycles (1 min at 94°C for denaturation, 2 min at 55°C for annealing and 3 min at 72°C for primer extension) were performed, using a Perkin-Elmer Cetus DNA thermal cycler. For all samples, an IL-4 sense and anti-sense primer set and a sense and anti-sense primer set for the HPRT (hypoxanthine phosphatidyl ribosyltransferase) housekeeping gene were used.

2.7. Staining

Cell suspensions were stained with anti-I-A^d (clone 39-10-8, Pharmingen, San Diego, CA) mAb as described previously (Knulst et al., 1991). Cells were analysed using a flow cytofluorometer (FACScan, Becton Dickinson, Mountain View, CA).

3. Results

3.1. Cytokine transfected cells or hybridoma cells can be encapsulated

Alginate permits the encapsulation of both non-adherent hybridoma cells and adherent cytokine gene transfected cell lines. The latter cells can be grown on Cytodex beads and subsequently encapsulated in alginate as shown in Fig. 1. The difference in the cell source determines the minimum size of the needle employed in the encapsulation procedure, namely 25 or 27 gauge for hy-

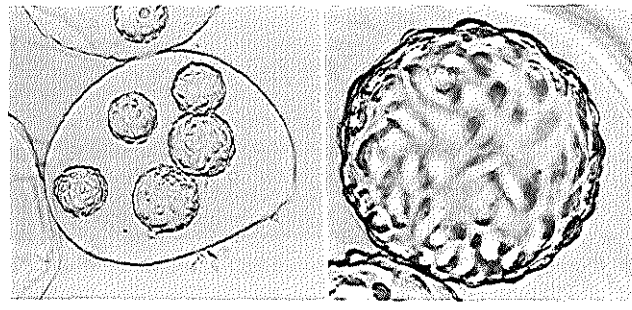

Fig. 1. *A*: photograph showing beads covered with IL-4 transfected CV1 cells encapsulated in alginate (40 × magnification). *B*: detail of a Cytodex bead fully covered with CV1/IL-4 cells (100 × magnification).

bridoma cells or 23 gauge for cells grown on beads (data not shown). The use of beads allows the exact dosage of cells implanted in vivo after encapsulation to be determined, since cells adhere to the beads only. Fully occupied beads grown with transfected CV1/IL-4 cells contained on the average 500 live cells per bead as determined by Trypan Blue exclusion after stripping the cells by incubation for 5 min in trypsin EDTA. Using a 23 gauge needle, capsules comprising, on average, five beads per capsule were obtained. This necessitates implantation of roughly 4000 beads per mouse in order to administer 2×10^6 cells.

To allow a sufficient degree of gelatination and hardening to occur, the capsules were incubated for some time in a $CaCl_2$ solution. This treatment was not detrimental to the viability of the encapsulated cells. In order to determine the optimal incubation time, XMG 1.2 cells were encapsulated and the capsules were incubated for various periods in the $CaCl_2$ solution after which the capsules were dissolved with trypsin-EDTA. The viability of the cells was determined by trypan blue exclusion. The results (Table 1), showed that viability during incubation times of 1 min up to 15 min did not differ significantly. In subsequent experiments the capsules were incubated for 10 min.

Table 1
Effect on cell viability of hardening of alginate capsules in $CaCl_2$

Time (min)	Viability (%)
0	82
1	60
2	62
5	50
10	44
15	50

XMG1.2 cells encapsulated in alginate were maintained in fresh 80 mM $CaCl_2$ solution prepared in water. Viability is expressed relative to the starting cell population.

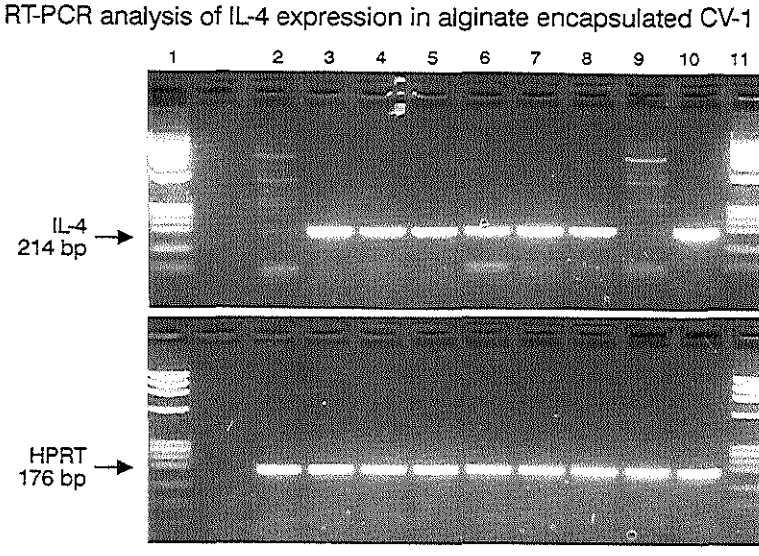

Fig. 2. Alginate encapsulated CV1/IL-4 cells (2×10^6) were implanted (i.p.). Capsules were washed out of the peritoneum on the days indicated and cells were detached from the beads. Total RNA was extracted, cDNA was prepared and reverse type PCR analysis was performed using an IL-4 primer set (*A*) or HPRT primer set (*B*). Lane 1: base pair marker (Phi X174 RF DNA HaeIII digest); lane 2: mock transfected control; lanes 3–8: CV1/IL-4 cells washed out on days 0, 3, 5, 7, 10 or 14; lane 9: mock transfected control; lane 10: positive control (CDC 35 T cell clone); lane 11: bp marker.

3.2. Encapsulated cells secrete cytokines or antibodies in vivo

The in vivo production of cytokines by encapsulated cytokine-transfected cells cannot be measured in the serum due to the abundant presence of inhibitors, such as soluble cytokine receptors. Alternatively, despite the fact that cytokine is continuously released in vivo at a single time point, not enough IL-4 may be present in the serum to be detected in our assay. This could be the result of a low secretion rate, analogous to IL-6 (Shirai et al., 1993). Therefore, we analysed the mRNA expression of IL-4 by encapsulated CV1/IL-4 cells that were washed out of the peritoneal cavity on various days after implantation. The results (Fig. 2) show that even up to 14 days after implantation IL-4 mRNA could still be detected. After day 14 the level of expression of both IL-4 mRNA and HPRT mRNA slowly declined suggesting increasing cell death.

In order to determine whether encapsulated hybridoma cells were still able to secrete their mAb, 2×10^6 TNP-specific mouse IgE secreting IgELA2 hybridoma cells were injected into BALB/c mice. Serum analysis of total and TNP-specific IgE showed up to a 10-fold increase in total IgE level after 6 days due to a 100-fold increase in TNP-specific IgE antibodies (Table 2). The serum IgE level remained elevated for up to 32 days, although gradually declining after 14 days.

The injected hybridoma cells carried the H-2^d haplotype that is compatible with the haplotype of the BALB/c mice used. Hence there was rapid development of peritoneal tumors accompanied by the formation of ascites fluid. This suggests that upon leakage or breaking of the alginate capsules, the hybridoma cells are able to induce ascites formation. However, as shown in Table 2, ascites formation occurred, relatively late (more than 14 days) after implantation of encapsulated hybridoma cells.

Table 2
TNP-specific IgE levels in the serum of IgELA2 hybridoma-bearing mice

Day	IgE (μg/ml)		Ascites incidence
	Total	TNP-specific	
0	1.11 ± 0.42	< 0.005	0/10
6	4.29 ± 1.13	1.44 ± 0.37	0/10
14	8.45 ± 1.69	5.36 ± 1.21	2/10
22	8.63 ± 1.24	6.54 ± 1.15	6/10
32	2.86 ± 0.87	1.04 ± 0.17	10/10

BALB/c mice were implanted i.p. with 2×10^6 TNP-specific IgELA2 cells encapsulated in alginate. Serum IgE levels, expressed in μg/ml, are arithmetic means $\pm$ 1 SD ($n = 10$).

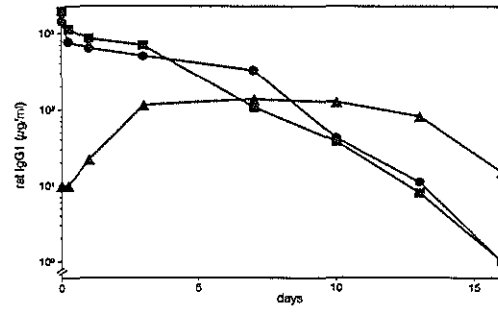

Fig. 3. Kinetics of clearance of rat IgG1 from mouse serum. BALB/c mice were injected with either 1.5 (squares) or 2 mg (circles) purified XMG 1.2 mAb or implanted i.p. with 2×10^6 encapsulated cells (triangles). Serum levels of rat IgG1 were monitored by specific ELISA. Results are expressed as means ($n = 5$).

Repeating the experiment in SJL mice, peritoneal cells were harvested on days 3, 5 and 7 after implantation and the non-encapsulated cells were stained for the H-2^d haplotype. Subsequent FACScan analysis failed to reveal significant amounts ($> 5\%$) of free hybridoma cells (data not shown). We therefore assume that leakage of actively secreting cells from the capsule is not significant.

3.3. Kinetics of the in vivo production by encapsulated cells

To determine the rate at which rat IgG1 appeared in the serum after implantation of encapsulated hybridoma cells, kinetic experiments were performed. As shown in Fig. 3, significant levels of rat IgG1 were detected in the serum after 3 days, peaking around day 8, and lasting for at least 13 days. For comparison, mice were injected

once with either 1.5 or 2 mg of purified XMG 1.2 (rat IgG1) antibodies (Fig. 3). In these cases a steadily declining serum level of rat IgG1 was observed. From the data obtained the rate of disappearance ('biological half life') of rat hybridoma IgG1 in mouse serum in vivo was estimated to be of the order of 4.5 days. This figure was calculated from the data obtained after injection of purified hybridoma protein as well as from the data obtained after implantation of encapsulated hybridoma cells. In addition, in the case of rat IgG1 production by encapsulated hybridoma cells in vivo, the synthetic rate as determined by the increase in serum level was estimated to be of the order of 1.5 days. As is evident from Fig. 3, implantation of 2×10^6 encapsulated hybridoma cells provided a serum level of rat IgG1 that was lower than that obtained by injecting 1.5 mg purified IgG1. On the other hand the serum levels of rat IgG1 were much more prolonged after implantation than after injection of purified rat IgG1. Collectively, these results suggest that implantation of alginate encapsulated cytokine transfected cells or hybridoma cells provides an efficient method for cytokine or antibody treatment in vivo for periods of up to 14 days.

3.4. Dose-response titration of encapsulated cells

In order to determine the amount of hybridoma protein present in the serum of mice implanted with various doses of encapsulated cells, dose titrations were performed. To this end, encapsulated rat IgG1-secreting hybridoma cells specific for the cytokine IL-5 were implanted. The appearance of rat IgG1 in the serum of these mice was determined by ELISA and the persistence of this rat Ig was studied. As shown in Fig. 4*A*, the injection of 2×10^6 encapsulated cells resulted in the appearance in the serum of hybridoma protein at concentrations up to 100 μg/ml. These levels were reached after 7 days and lasted for up to 21 days, after which they steadily declined. A dose of 3×10^3 cells resulted in a 1000-fold lower serum level of hybridoma protein that did not show clear kinetics and was at the limit of detection of the ELISA. The other doses tested resulted in intermediate serum levels of rat IgG1. The maximum increase in rat IgG1 levels in the serum occurred between days 7 and 21, suggesting that the implanted cells were still actively secreting the hybridoma protein (Fig. 4*B*).

3.5. Implantation sites of encapsulated cells

All of the results described above were obtained by i.p. implantation of encapsulated cells. This site can easily accommodate 1 ml samples. Moreover, multiple implantations of 1 ml samples can be performed i.p. when using 2 week intervals. For applications in which a single implantation is to be used the s.c. site may be preferable.

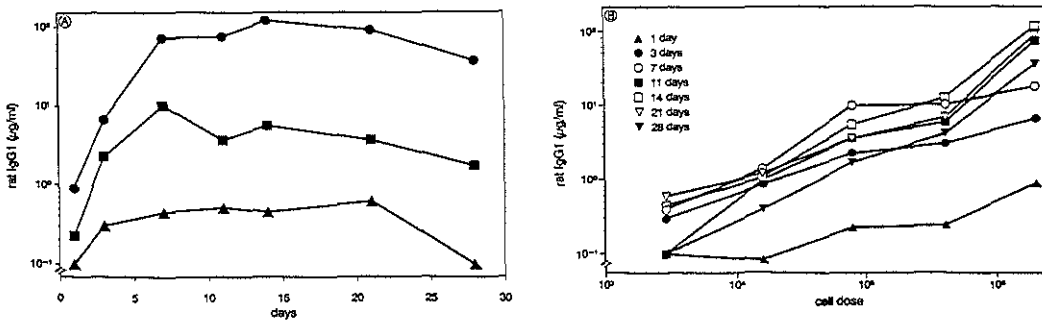

Fig. 4. Kinetics of cell dose-dependent rat IgG1 production. BALB/c mice were implanted i.p. with encapsulated TRFK 5 cells and rat IgG1 production was monitored by ELISA. *A*: serum levels of hybridoma protein at various intervals after implantation of alginate encapsulated cells. Doses of 2×10^6 (circles), 8×10^4 (squares) and 3×10^3 (triangels) encapsulated TRFK5 cells were used. *B*: serum levels of hybridoma protein at various doses of the alginate encapsulated cells. Serum levels were determined 1, 3, 7, 11, 14, 21 and 28 days after implantation as indicated. Results are expressed as mean serum levels ($n = 5$).

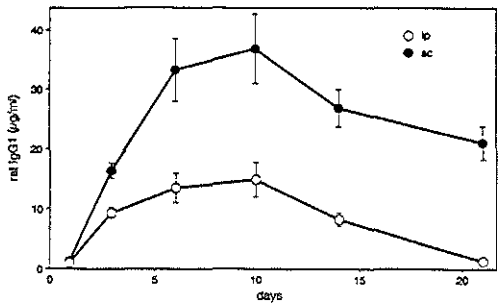

Fig. 5. Effect of implantation site on the production of hybridoma protein. BALB/c mice were implanted i.p. (open symbols) or s.c. (closed symbols) with 2×10^6 encapsulated 11B11 cells. Rat IgG1 levels were monitored by a specific ELISA. Results are expressed as mean serum levels (±1 SD) ($n = 10$).

To analyze efficacy in terms of long term antibody production, both sites of implantation were compared. To this end, 2×10^6 11B11 cells were encapsulated and implanted either i.p. or s.c.. Again, rat IgG1 levels were monitored. Fig. 5 shows that s.c. implantation resulted in significantly higher and more sustained antibody production than i.p. implantation. The s.c. site was therefore considered to be superior for single implantation and cells were found to remain active for periods up to 1 month (data not shown). The i.p. site, on the other hand, was found to be more suitable for multiple injections (every 14 days) which permits cytokine or antibody treatments over prolonged periods of time.

3.6. Mouse anti-rat response after implantation of encapsulated cells

Since mice can be treated for long periods of time with encapsulated hybridoma cells that provide low but persistent levels of hybridoma protein, a host anti-rat response could be expected to occur. In order to investigate the occurrence of such a response, BALB/c mice were immunized with 10 mg TNP-KLH on alum i.p. 3 months later mice were boosted with a similar dose of antigen. During the whole immunization period the mice were treated every other week with 2×10^6 encapsulated 11B11 cells implanted i.p. In total the mice received seven repeated injections. As is evident from Fig. 6, only a minor rat IgG1-specific IgM response developed during the booster response. No IgG anti-rat IgG1 response could be detected in these mice (data not shown). Monitoring of the serum revealed around 10 μg/ml of rat IgG1 was present in the mouse serum (data not shown).

Table 3
Cytokine-dependent modulation of primary IgE responses by encapsulated cells

Strain	TNP-KLH immunization	Encapsulated cells	Antibody treatment	Anti-TNP IgE (ng/ml)	
				Day 0	Day 14
BALB/c	–	–	–	15	18
	+	–	–	20	1947
	+	CHO/IFN-γ	–	18	21
	+	CHO/IFN-γ	XMG1.2	17	2354
	+	11B11	–	32	28
	+	–	11B11	25	27
	+	Capsules only	–	29	32
SJA/9	+	–	–	32	25
	+	CV-1/IL-4	–	19	2048
	+	CV-1/IL-4	GL113	19	2123
	+	CV-1/IL-4	11B11	21	20
	+	CV-1/IL-5	–	16	20
	+	GL113	–	20	29

Mice were immunized (i.p.) with 10 μg TNP-KLH in alum on day 0. Mice were treated with 2×10^6 alginate encapsulated cells implanted i.p. and/or with 2 mg of purified XMG1.2 or GL113 or 10 mg of 11B11 mAb i.p. Serum levels were determined by TNP-specific IgE ELISA. Results are reported as arithmetic means ($n = 5$).

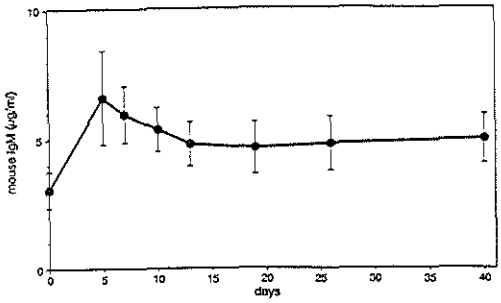

Fig. 6. Mouse anti-rat response after implantation of rat IgG1-secreting hybridoma cells. BALB/c mice were implanted with 2×10^6 encapsulated 11B11 cells i.p. Mouse IgM anti-rat IgG1 responses were determined in an ELISA procedure. Results are expressed as means $\pm$ 1 SD ($n = 5$).

3.7. Modulation of IgE formation in vivo by encapsulated cells

In order to test the possibilities of modulating in vivo immune responses, e.g., IgE formation, mice were immunized and implanted with encapsulated fibroblast cells transfected with cytokine genes or hybridoma cells. IgE high responder BALB/c or non-responder SJA/9 mice were immunized with 10 μg TNP-KLH on alum i.p. These mice were implanted i.p. with encapsulated CV1 cells transfected with the IL-4 or the IL-5 gene or with CHO cells transfected with the IFN-γ gene. Subsequently, these mice were treated with 10 or 2 mg of purified anti-IL-4 or anti IFN-γ mAb i.p. respectively, doses widely shown to be sufficient to block IL-4 or IFN-γ-mediated effects in vivo. As an isotype control, mice were treated with GL113 mAb. The resulting TNP-specific IgE levels in the serum were analysed by ELISA. As shown in Table 3, the resulting TNP-specific IgE response in BALB/c mice could be blocked by IFN-γ secreting cells, anti-IL-4 secreting cells or purified anti-IL-4 mAb. Blocking the activity of IFN-γ by treatment with purified anti-IFN-γ mAb resulted in a marked increase in TNP-specific IgE to levels higher than those obtained by TNP-KLH immunization alone. SJA/9 mice could overcome the defective IgE production following implantation with IL-4-transfected cells, and the subsequent IgE production could be blocked by anti-IL-4, suggesting an IL-4-mediated IgE production. The results in Table 3 also show that implanting 2×10^6 11B11 or XMG 1.2 hybridoma cells resulted in in vivo effects that were at least comparable to those obtained by injecting 2 mg of XMG 1.2 or even 10 mg of 11B11 per mouse. These doses have been widely shown to be sufficient to block cytokine mediated effects in vivo. Moreover, these results show that injection of encapsulated empty Cytodex beads or IL-5 transfected CV1 cells did not lead to IgE production in SJA/9 mice.

4. Discussion

This study has shown that alginate encapsulation of cytokine transfected cells offers an easy way to establish a constant systemic level of cytokine in vivo for prolonged periods of time. Moreover, the use of adherent growing transfected fibroblasts in combination with Cytodex beads provides an exact dosage of implanted cells and allows precise dose-response relationships to be defined. Although hybridoma cells can also be encapsulated effectively in alginate, growth will continue until limited by physical parameters, such as charge repulsion between the net negative surface of cells and the negatively charged carbohydrate groups of the alginate (Haug and Smidsrod, 1967). The leakage of entrapped cells out of the capsules is undetectable, at least to day 7. Therefore, cells released from the capsules resulting from breaking or dissolving the capsule will not remain viable and thus such cells will not contribute to the observed serum levels of hybridoma protein. The maximum numbers of cells that can be encapsulated is largely determined by the low gel strength of the capsules and by certain diffusion restrictions; mainly for oxygen (Cheetham et al., 1979). Determination of an exact dosage of implanted hybridoma cells is virtually impossible, since the implanted capsules permit further proliferation until limited by physical constraints. On the other hand, the activity of implanted hybridoma cells can be measured easily by monitoring the serum for the presence of

the secreted antibodies. As shown here, the rate of disappearance of rat IgG1 delivered in this way is roughly 4.5 days, which is an estimate of the biological half-life and equivalent to the half life estimated by the injection of purified rat mAb (Wawrzyneczak et al., 1992). Recently, an alternative method was described to deliver some cytokines for periods up to 9 days by complexing low doses of cytokine with either neutralizing antibodies or soluble receptors (Sato et al., 1993). A potential problem arising from the implantation of hybridoma cells for in vivo antibody production, certainly for anti-cytokine antibodies, is cytokine production by the hybridoma cells themselves. As has been described, hybridoma cells can secrete relatively large amounts of IL-6 and although in this study rat hybridomas were used in mice, there can be sufficient cross-reactivity to induce interfering unknown bystander effects (Van Damme et al., 1987).

As shown here, the implanted capsules stay intact and the cells continue to secrete their cytokine for up to 3 weeks. Furthermore, using an ELISA specific for soluble mouse-anti-rat IgG1 antibodies does not reveal a detectable anti-rat response in the host triggered by encapsulated cells. The ELISA employed, however, would not be able to detect anti-rat antibodies complexed to the rat hybridoma protein. Such complexes could consist of anti-isotypic antibodies or anti-idiotypic antibodies specific for rat IgG1. The use of an irrelevant rat IgG1 for coating did not reveal any signal in the serum of treated mice, suggesting that either large complexes are formed (due to anti-isotypic antibodies) in the serum or substantial amounts of anti-idiotypic antibodies are present that cannot be detected. The serum levels of rat IgG delivered by alginate encapsulated hybridomas were relatively low (mostly ≤ 25 μg/ml), and this IgG was only induced in the absence of an adjuvant. Although the serum level of rat Ig was sufficient to block the specific ELISA, we consider the induction of an anti-rat Ig response unlikely within the limitations of our detection technique. Moreover, the decrease in serum levels of rat IgG1 after 14 days was reflected in the parallel deterioration of the alginate capsules and the increased death of hybridoma cells. This makes it unlikely that a mouse anti-rat response (both idiotypic and isotypic) would be responsible for the decrease in rat IgG serum levels.

Complexes of cytokines with anti-cytokines have been shown to increase the half life of cytokines in the serum (Sato et al., 1993). Anti-cytokine antibodies (delivered by alginate capsules) should be active in their depleting capacity for periods of more than 2 weeks but this has not been observed. Moreover, the injection of rat purified IgG1 antibodies did not induce an anti-rat immune response despite the initial high doses of antibody present. Since no adjuvant was used, the serum half life of 4.5 days ensured a rapid decline to levels comparable to those in alginate implanted mice. In another study (Van Ommen et al., submitted) mice were treated at bi-weekly intervals with 10 mg purified 11B11 per mouse for a period of 3 months. In these mice no significant rat IgG1-specific IgG response could be detected during this treatment and the rat IgG1 serum levels never exceeded 25 μg/ml. This makes it unlikely that an induced immune response would be missed using the ELISA. When other anti-mouse cytokine rat IgG mAbs were used, anti-rat IgG1 responses were not detected. This was probably due to the fast clearance of both uncomplexed and cytokine-complexed antibodies.

A medium for the entrapment of living cells should cause as little trauma to the cells as possible. There should, ideally, be no shock to the cells from change of temperature, chemical environment and osmotic pressure. Entrapment in calcium alginate meets all these criteria. Alginate, extracted with sodium hydroxide from *Macrocystis pyrifera*, consists of a copolymer of β-D-mannopyranosyl uronate and α-L-gulopyranosyl uronate linked by $(1 \rightarrow 4)$-glycosidic linkages. When exposed to divalent ions such as Ca^{2+}, an inert three-dimensional polymer gel network is formed with relatively large interconnected interstitial spaces (Bucke, 1987). The average diameter of the capsules (approximately 0.5–5 mm), is limited by the size of the needle and the pressure applied to the syringe. The main disadvantages of alginate capsules are (1) they are rapidly disrupted by chemicals capable of chelat-

ing calcium ions, and (2) the potential limitation of oxygen transfer into the capsule (Familetti and Fredericks, 1988; Hashimoto and Shirai, 1990). When comparing various implanted hybridoma cells, it appears that at maximum production these cells yield systemic levels of 10–100 μg/ml of rat IgG. This points to a limited production potential, possibly due to a lack of oxygen or reduced diffusion of nutrients and waste products.

IgE responses are dependent on the presence and activity of functional IL-4 as the most prominent IgE switch inducing factor. IFN-γ, being a major antagonist of IL-4, plays an important role in the downregulation of the IgE production (Finkelman et al., 1988; Mosmann and Coffman, 1989a). It has been suggested that the ratio of IL-4 to IFN-γ determines the ability to produce IgE in vivo (Snapper and Paul, 1987; Mosmann and Coffman, 1989b). Thus, implantation in IgE high-responder BALB/c mice of either cytokine transfected cells or hybridoma cells provides an effective model system to modulate the IgE response based on influencing the balance between essential cytokines in vivo. As is evident from the results (Table 3), the IgE response induced by immunization with TNP-KLH can effectively be inhibited by implantation of IFN-γ producing CHO/IFN-γ cells. Subsequent neutralization with purified anti-IFN-γ mAb (XMG 1.2) induced complete reversal of this inhibition. Implantation of anti-IL-4 secreting hybridoma cells (11B11) or injection of purified anti-IL-4 mAb resulted in a similar degree of inhibition to that observed with implanted CHO/IFN-γ cells. These findings are in complete agreement with the hypothesis that the relative amounts of functional IL-4 and IFN-γ are sufficient to determine the IgE response in vitro. IgE non-responder SJA/9 mice, on the other hand, do not react to TNP-KLH immunization with the formation of TNP-specific IgE. Following infection with the helminth parasite *Nippostrongylus brasiliensis* it has previously been shown that implantation of alginate encapsulated CV-1/IL-4 transfected cells was able to restore IgE formation to levels comparable to those in BALB/c mice (Savelkoul et al., 1991). Here, we were able to show that a similar treatment restored the formation of TNP-specific IgE. This SJA/9 model clearly shows that implantation of CV-1/IL-4 cells yields sufficient functional IL-4 to induce the formation of IgE. Collectively, these results illustrate the potential applicability of implantation of alginate encapsulated cytokine transfected cells in the modulation of immune responses in vivo.

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Chapter 3

The effect of IFN-γ, alum and complete Freund adjuvant on TNP-KLH induced IgG_1, IgE and IgG_{2a} responses in mice

R. van Ommen, A.E.C.M. Vredendaal, M. de Gooyer, A. van Oudenaren and H.F.J. Savelkoul

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SUMMARY

Adjuvants are considered to play an important role in directing the isotype and amount of antibodies produced upon immunization by conducting the development of either Th-1 or Th2 cells upon T cell stimulation. This is based on the different cytokine production patterns that were observed after in vitro restimulation of T cells isolated from mice immunized with antigen either adsorbed on alum or emulsified in complete Freund adjuvant (CFA). However, other studies suggest that primarily the type of antigen determines which isotypes are produced and to what extent. In these studies, however, IgE was not determined. Therefore, we examined whether alum and CFA influenced the amount and/or ratio of IgG_1, IgE and IgG_{2a} produced after TNP-KLH immunization. We found similar levels of IgG_1, IgE and IgG_{2a} antibodies upon immunization with TNP-KLH either adsorbed on alum or emulsified in CFA. Moreover, we found that administration of IFN-γ in combination with TNP-KLH adsorbed on alum did not increase the amount of IgG_{2a} produced. IFN-γ treatment resulted in an increased IL-6 and decreased IFN-γ production by spleen cells upon Con A stimulation, whereas it did not change the IL-4 production in similar conditions. The presented results suggest that upon immunization with TNP-KLH high IL-4 levels are produced, resulting in an antibody response that is dominated by IgG_1, independent of the adjuvant employed. The IL-4 inducing property of TNP-KLH is substantiated by the finding that repeated immunization of mice with TNP-KLH, without adjuvant, increases the serum total IgE level. The presented data imply that the carrier part of TNP-KLH preferentially results in Th2 cell activity after which the adjuvant merely enhances the antibody responses generated.

INTRODUCTION

Helper T cells (Th) play an important role in antigen-induced humoral immune responses[1]. At least two effector subpopulations of murine Th cells, differentiated according to the spectrum of cytokines produced, have been reported[2, 3]. Th1 cells exclusively produce IL-2 and IFN-γ, whereas Th2 cells exclusively produce IL-4, IL-5 and IL-10, but not IL-2 and IFN-γ[3]. It has been described that adjuvant, used to facilitate the induction of antigen-specific immune responses, plays an important role in determining which T cell subset will be activated *in vivo*[4-5]. Immunization of mice with either ovalbumin (OVA) or ragweed pollen extract in the presence of alum resulted in T cells that produced IL-2, IL-4 and IFN-γ upon *in vitro* restimulation with the same antigen. On the contrary, immunization with either OVA or ragweed pollen extract in the presence of complete Freund adjuvant (CFA) resulted in T cells that produced IL-2 and IFN-γ, but not

IL-4 upon *in vitro* restimulation[6]. The amount of IFN-γ produced by T cells from mice primed with antigen emulsified in CFA and cultured in the absence of antigen was markedly higher than the production of IFN-γ in similar cultures of T cells from mice primed with antigen adsorbed on alum[6].

Based on these results it was suggested that alum augments preferentially Th2 mediated responses, whereas CFA augments preferentially Th1 mediated responses. Antigen adsorbed on alum would thus be expected to induce IgG_1 and IgE responses, since IL-4 is involved in the process of isotype switching to IgG_1 and IgE[7, 8]. Upon immunization in the presence of CFA, the same antigen would be expected to elicit a preferential IgG_{2a} response, since IFN-γ has been reported to enhance IgG_{2a} production[7, 8]. Moreover, IL-4 inhibits IgG_{2a} production, whereas IFN-γ decreases both the IgG_1 and IgE synthesis[9], indicating that the balance between these two cytokines determines the amount and isotype of antibodies that will be produced.

We have recently shown that primary TNP-specific IgE responses are completely dependent on IL-4 and can be largely inhibited by IFN-γ, whereas secondary antigen-specific IgE responses are partially IL-4 independent and can hardly be inhibited by IFN-γ in a concentration that does inhibit the primary TNP-specific IgE response[10].

In the literature contradictory results have been described concerning the effects on the isotype production profile and concentration of antibody depending on the type of adjuvant employed. Moreover, immunization with different antigens (HSA, FITC, cytochrome c) showed different effects on the isotype production profile and amount of antibodies when administered either adsorbed on alum or emulsified in CFA[11-13]. However, in these studies the IgE isotype was not determined. The presence of this isotype, however, is indicative for the balance between IL-4 and IFN-γ *in vivo*.

It was reported by Beck et al. that immunization with OVA in alum resulted in higher levels of antigen-specific IgE than when administered in CFA[14]. However, the observed antigen-specific serum IgG_1 and IgG_{2a} responses were not influenced when using either alum or CFA as adjuvant. These authors suggested that primarily the type of adjuvant determined the resulting IgE production[14]. From other studies it can be concluded that the type of antigen plays, next to the type of adjuvant, a determining role in the production of other isotypes than IgE[11-13].

We studied the effect of alum and CFA as adjuvant on the antigen-specific IgG_1 and IgG_{2a} and the total IgE antibody production after TNP-KLH immunization. Moreover, we examined the effect of continuous presence of systemic IFN-γ on the isotypes produced

after immunization with TNP-KLH adsorbed on alum. Besides the effects on the primary response we determined the influence of adjuvant on the antigen-specific IgE memory formation.

MATERIALS AND METHODS

Mice

Female BALB/c and C57BL/6 mice were bred and maintained at the Department of Immunology of the Erasmus University. All mice were at an age of 12-16 weeks at the start of the experiments. They were held in light-cycled rooms and had access to acidified water and pelleted food ad libitum. The microbiological status of the mice fulfilled the standard of "specific pathogen free V" according to the criteria of the Dutch Veterinary Inspection, as described in the law on animal experiments. The experiments were approved by the Animal Experiments Committee of the Erasmus University.

Immunization

KLH (Pierce, Rockford, IL, USA) was trinitrophenylated to a level of 25 TNP residues per 10^5 Da of KLH (as determined spectrophotometrically) by using trinitrobenzenesulphonic acid (Eastman Kodak, Rochester, NY, USA)[15]. Mice were injected with 0.2 ml containing either 10 μg or 100 μg TNP-KLH adsorbed on 2 mg alum. Alternatively, either 10 μg or 100 μg TNP-KLH emulsified in 0.2 ml CFA was injected i.p., or 10 μg TNP-KLH in saline was injected every 2 days till day 14, as indicated in the results section.

Cytokine treatment

Mice immunized with 100 μg TNP-KLH adsorbed on alum were implanted i.p. with 2 x 10^6 CHO/IFN-γ cells encapsulated in alginate on day -1, 14 and 28 as described previously for CV-1/IL-4 cells[15-17]. The CHO/IFN-γ cells were a kind gift of Dr. N. Arai (DNAX Research institute, Palo Alto, CA, USA). Empty beads encapsulated in alginate were used as control for the IFN-γ treatment. No immunological effects were observed as result of this treatment in all experiments.

Isotype specific ELISA

Total serum IgE, IgG_1 and IgG_{2a} levels were measured by isotype-specific ELISA as described previously[15]. Detection limits for the IgE, IgG_1 and IgG_{2a} ELISA were 0.5

ng/ml, 0.2 ng/ml and 0.3 ng/ml, respectively. TNP-specific IgG_1 and IgE were determined as previously described[15], with 0.2 ng/ml and 1 ng/ml as detection limit in the ELISA, respectively. TNP-KLH-specific IgG_{2a} was measured by direct ELISA. Plates were coated with TNP-KLH (3 μg/ml), blocked with 1% BSA and incubated with the appropriate dilutions of serum. Subsequent steps were biotin-conjugates GAM/IgG_{2a} (Southern Biotechnology, Birmingham, AL, USA), SA-HRP (Jackson Immunoresearch, West Grove, PA, USA) and the substrate 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) (Sigma, St. Louis, MO, USA). Purified TNP-specific IgG_{2a} was used for the standard curve in this antigen-specific assay. The detection limit of this ELISA was 0.4 ng/ml.

Con A stimulation of splenocytes

Spleen cells at 2 x 10^6/ml were cultured in RPMI 1640 medium supplemented with 10% heat inactivated FCS, 2mM glutamine, 0.1 M pyruvate, 100 IU/ml penicillin, 50 μg/ml streptomycin, 50 μM 2-mercapto-ethanol in 6 replicate wells of a 24 wells flat-bottom plate (1 ml/well) with 10 μg/ml Concanavalin A (Sigma). After 48 hours culture supernatants were harvested and stored at -70^0 C before use.

Determination of cytokines

IL-4, IL-6, IL-10 and IFN-γ were determined in ELISA as previously described[18-21]. The detection limits of the ELISA were 0.2 ng/ml, 1.5 U/ml, 3 U/ml and 0.2 ng/ml, respectively.

Statistical analysis

Differences between groups were analyzed using the Student's *t-test*. Values of $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

Mice were immunized i.p. with 100 μg TNP_{25}-KLH either adsorbed on 2 mg alum or emulsified in CFA (Table 1). On the days indicated serum was collected and antigen-specific IgG_1 and IgG_{2a} and total IgE were determined in isotype-specific ELISA. Serum total IgE and TNP-specific IgG_1 and IgG_{2a} peak levels were not different whether TNP-KLH was given adsorbed on alum or emulsified in CFA (Table 1). Primary immunization of BALB/c mice with 100 μg TNP-KLH either absorbed on alum or emulsified in CFA

provoked a response dominated by a 25-fold increase in TNP-specific IgG_1 and resulting in a peak level in the serum of 1.5 mg/ml. Even a proportional higher increase was observed for TNP-specific IgG_{2a} upon immunization with 100 μg TNP-KLH, reaching a maximum serum level of 54 μg/ml when administered emulsified in CFA, and 78 μg/ml when adsorbed on alum (Table 1). With this dose of TNP-KLH employed the primary total IgE (Table 1) and antigen-specific IgE (data not shown) responses were only marginal and did not differ between the two adjuvants used. These results show that immune responses induced by TNP-KLH immunization, either adsorbed on alum or emulsified in CFA, do not differ in the isotypes and amount of antibodies produced. Immunization of C57BL/6 mice with 100 μg TNP-KLH adsorbed on alum resulted in an isotype response pattern similar to that in BALB/c mice, and also dominated by TNP-specific IgG_1. The peak levels of the isotypes produced, however, were consistently 2-fold lower (Table 1).

Table 1 Ig-isotype distribution of IgG_1, IgE and IgG_{2a} antibodies after immunization of BALB/c and C57BL/6 mice with TNP-KLH either on alum or in CFA

mice	adjuvant	isotype*	day 0	day 14
BALB/c	alum	IgE	1.6±0.3	3.0±0.5
		anti-TNP IgG_1	64±14	1544±59
		anti-TNP IgG_{2a}	<0.4	78±15
BALB/c	CFA	IgE	1.2±0.2	2.0±0.4
		anti-TNP IgG_1	58±4	1485±68
		anti-TNP IgG_{2a}	2.0±0.1	54±9
C57BL/6	alum	IgE	0.1±0.01	1.2±0.4
		anti-TNP IgG_1	3±0.1	929±130
		anti-TNP IgG_{2a}	<0.4	19±3

BALB/c or C57BL/6 mice were immunized i.p. either with 100 μg TNP-KLH adsorbed on alum, or with 100 μg TNP-KLH in CFA as indicated. *Total IgE and TNP-specific IgG_1 and IgG_{2a} serum levels were determined. Results are presented as arithmetic mean ± SEM in μg/ml (n=5).

It has been reported that immunization with antigen emulsified in CFA induces T cells that predominantly produce IFN-γ, and do not secrete IL-4[6]. Therefore, since IFN-γ inhibits the IL-4 induced IgE synthesis[7, 8] lower levels of IgE after immunizing BALB/c mice with TNP-KLH in CFA would be expected, than when alum was used as adjuvant. Such an inhibition was not observed (Table 1), suggesting that CFA did not induce enough IFN-γ to significantly inhibit the TNP-KLH induced IgE production. Therefore, we next studied the effect of continuous presence of IFN-γ on total and antigen-specific IgG_1, IgG_{2a} and IgE synthesis induced by immunization with 100 μg TNP-KLH adsorbed on alum. To ensure a continuous systemic presence of IFN-γ we implanted CHO cells that were stably transfected with the murine IFN-γ gene and encapsulated in alginate, in the

peritoneal cavity of BALB/c mice 1 day before and 14 days after antigenic challenge[15-17]. Surprisingly, under these conditions IFN-γ augmented both the serum total IgG_1 and IgE levels, whereas it did not influence the serum total IgG_{2a} levels (Table 2). However, IFN-γ treatment markedly decreased the TNP-specific IgG_1 and IgE response, but did not influence the TNP-specific IgG_{2a} response, at day 14 (data not shown).

In the literature, BALB/c mice were described as IgE high-responder mice, whereas C57BL/6 mice were described as IgE low-responder mice[22]. Therefore, we next studied the effect of immunization with TNP-KLH adsorbed on alum in the presence or absence of exogenous IFN-γ on the IgG_{2a} production in C57BL/6 mice. In these mice, however, immunization with TNP-KLH adsorbed on alum in the presence of exogenous IFN-γ also did not result in increased serum total IgG_{2a} levels (data not shown). Nevertheless, the lower IgE production found in these mice upon TNP-KLH immunization (Table 1) suggests a lower expression level of IL-4 and/or higher level of IFN-γ, than in BALB/c mice.

The encapsulated IFN-γ producing cells secreted enough biologically active IFN-γ to enhance IgG_{2a} synthesis, as was shown by implanting IFN-γ producing cells in unimmunized naive BALB/c mice. The serum total IgG_{2a} levels significantly increased in these mice as result of IFN-γ treatment (Table 2). Moreover, when alum without antigen was given in the presence of IFN-γ, a further enhancement of the IgG_{2a} synthesis was observed (Table 2). Alum alone did not induce IgG_{2a} production. These results suggest that an adjuvant like alum augments ongoing immune responses, in which the isotypes produced are determined by the cytokines elicited upon antigenic stimulation.

Together these results suggest that upon immunization with TNP-KLH excessive IL-4 and less, or strongly inhibited levels of IFN-γ are induced. As a result, no further increase in the serum total IgG_{2a} level was observed, when IFN-γ treatment was added to TNP-KLH immunization adsorbed on alum (Table 2). The unexpected effects of the IFN-γ treatment with respect to the increased serum total IgG_1 and IgE can not easily be explained. It is possible that the amount of exogenously produced IFN-γ was too low to inhibit the IL-4 induced isotype switching to IgG_1 and IgE. These two isotypes are coupled through the process of sequential isotype switching[23]. Alternatively, IFN-γ levels could be high enough to support polyclonal bystander activation by increasing the MHC class II expression on macrophages and dendritic cells, resulting in enhanced antigen presentation[24-26]. It is known that IFN-γ decreases the IL-4 induced upregulation of MHC class II molecules on B cells[1]. Therefore, it is possible that the observed inhibition of the antigen-specific IgG_1 and IgE response by IFN-γ is the result of a down regulation of MHC class II molecules on the B cells, that are essential for antigen-specific T-B cell interactions[27].

Table 2 Ig-isotype distribution of IgG_1, IgE and IgG_{2a} antibodies after immunization of BALB/c mice with TNP-KLH in the presence or absence of exogenous IFN-γ

A

	IgE (μg/ml)		IgG_1 (mg/ml)		IgG_{2a} (mg/ml)	
TNP-KLH/Alum	-	IFN-γ	-	IFN-γ	-	IFN-γ
day 0	1.2±0.3	1.2±0.3	1.5±0.2	1.5±0.2	0.9±0.1	0.9±0.1
day 14	1.2±0.2	11.4±1.3	3.8±0.2	3.3±0.4	1.1±0.4	1.2±0.1
day 51	1.7±0.3	61.0±7.4*	3.5±0.1	11.7±1.2*	1.5±0.1	1.4±0.2

B

	IgG_{2a} (mg/ml)		
no antigen	alum	alum/IFN-γ	IFN-γ
day 0	1.0±0.1	1.0±0.1	0.8±0.1
day 14	0.7±0.1	1.9±0.3	1.4±0.2
day 37	0.9±0.1	3.0±0.2**	1.7±0.3**

BALB/c mice were injected with either 100 μg TNP-KLH adsorbed on alum (A), or alum alone (B). 2 x 10^6 CHO/IFN-γ cells encapsulated in alginate, or empty beads encapsulated in alginate were implanted i.p. on day -1, 14 and 28. Serum total IgE, IgG_1 and IgG_{2a} levels were determined. Results are expressed as arithmetic mean ± SEM (n=5). Statistical evaluation using students *t-test*: * values were compared between control and IFN-γ treated mice ($p<0.05$), ** values were compared relative to day 0 ($p<0.05$).

We next studied whether the endogenous cytokine production profile of spleen cells had changed as a result of IFN-γ treatment. Therefore, the cytokine production by Con A stimulated spleen cells from control and IFN-γ treated mice 1 day after the last of ten control or IFN-γ administrations was determined. Similar amounts of IL-4 were detected upon culturing spleen cells from control or IFN-γ treated mice, whereas no IL-10 production could be detected (Table 3). The IL-6 production by spleen cells from IFN-γ treated mice was significantly increased when compared to the IL-6 production by spleen cells from control treated mice. However, the IFN-γ production slightly decreased, in similar culture conditions, as a result of IFN-γ treatment (Table 3), suggesting a negative feedback by IFN-γ itself. The increased IL-6 production in combination with unchanged IL-4 production suggested an increase in the number of activated macrophages and monocytes. These results substantiate our opinion that IFN-γ, under the conditions used, merely acts on these types of cells.

Table 3 Cytokine production profile of splenocytes 1 day after the last of 10 IFN-γ administrations

Treatment	IL-4 (ng/ml)	IL-6 (U/ml)	IL-10 (U/ml)	IFN-γ (ng/ml)
Control	0.3±0.01	5.1±1.4	< 3	1.0±0.1
IFN-γ	0.4±0.03	10.5±1.5*	< 3	0.5±0.1*

Spleen cells (2 x 10^6/ml) pooled from either two control treated or two IFN-γ treated BALB/c mice 1 day after the last of ten control or IFN-γ administrations were cultured with Con A (10 μg/ml) for 48 hours in six replicate wells. The supernatants were harvested from these wells and individually tested for cytokine production. The results are represented as arithmetic mean ± SD (n=6). * Statistical evaluation using students *t-test* $p < 0.05$

The preferential induction of IL-4 expression *in vivo* by TNP-KLH immunization became clear by injecting BALB/c mice repeatedly with 10 μg TNP-KLH in saline, that is every 2 days till day 14. This immunization scheme resulted in 1.89 μg serum total IgE, 4-fold higher than the saline control (Figure 1). Primary IgE production was determined because of its total dependence on IL-4 [15, 28, 29]. Immunization with this dose of 10 μg TNP-KLH adsorbed on alum resulted in a 20-fold increase of the serum total IgE level at day 14 (Figure 1). These results substantiate that TNP-KLH itself already induces IL-4 production reflected by elevation of the total serum IgE levels, whereas alum, used as adjuvant, merely enhances the antigen-induced antibody response. Similar primary IgE responses were found upon immunization with TNP-KLH either adsorbed on alum, or in CFA (Table 1).

We next studied the effect of adjuvant on the memory formation for IgE, because it is possible that formation of memory B cells for IgE is more dependent on the ratio of IL-4 and IFN-γ than the primary IgE production. Therefore, BALB/c mice were immunized i.p., either with 10 μg TNP-KLH adsorbed on alum, or emulsified in CFA. This dose of TNP-

KLH was used because it elicits a significant primary serum total IgE response (Figure 1)[28, 30]. Three months later all mice were boosted with 10 μg TNP-KLH adsorbed on alum. On day 7, both total and antigen-specific IgE serum levels were determined in pooled sera of 4 mice (Figure 2). Under these conditions it did not make a difference whether alum or CFA was used as adjuvant during priming. Upon boosting with 10 μg TNP-KLH adsorbed on alum similar levels of both antigen-specific and total IgE were produced at day 7 by mice primed either with TNP-KLH adsorbed on alum or emulsified in CFA (Figure 2). It was therefore concluded that also the memory formation for IgE is not influenced by alum and CFA.

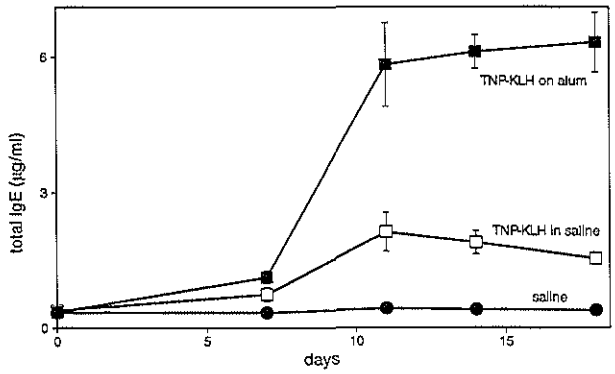

Figure 1. BALB/c mice were immunized with 10 μg TNP-KLH in saline □ or saline alone ● every two days till day 14, or immunized once with 10 μg TNP-KLH adsorbed on alum at day 0 ■. Serum total IgE levels were determined on the days indicated. Results are expressed in μg/ml as arithmetic mean ± SEM (n=5).

Collectively, our results show that when using TNP-KLH as antigen, the resulting production of IgG_1, IgE and IgG_{2a} is not dependent on the adjuvant employed. CFA and alum rather act as enhancement factors for ongoing antigen-induced antibody responses, in which the carrier part of the antigen determines the isotypes produced. Upon using KLH as carrier, apparently an abundant IL-4 production is generated by carrier-specific Th2 cells which is not easily counteracted by IFN-γ. Recently, it has been described that IL-4 production is dependent on the type of antigen and the duration of antigenic stimulation[31]. It is possible that repeated antigenic stimulation of T cells, by immunization with TNP-KLH every 2 days for 14 days, induces IL-4 production resulting in IgE synthesis. When antigen is given once, the use of adjuvant could result in an antigen-depot, resulting in repeated antigenic-stimulation and subsequent IL-4 production. This indicates that IL-4 can be induced by antigen, independent of the type of adjuvant, resulting in an antibody response dominated by IgG_1.

It has been described by Fox that immunization with a protein antigen either given adsorbed on alum or emulsified in CFA induced similar T cell proliferation and cytokine production[13]. Moreover, in this study it was shown by limiting dilution analysis that comparable frequencies of antigen-specific T cells are induced by the antigen, indepen-

dent of the adjuvant used. These data support our view that it is most likely the carrier part of antigen that preferentially activates the development of either Th1 or Th2 effector cells, after which the adjuvant merely enhances the antibody responses generated. We can not exclude the possibility that other types of antigen in combination with either alum or CFA will result in different isotype patterns. However, at the moment no such studies are available with respect to the type of antibodies tested in our study.

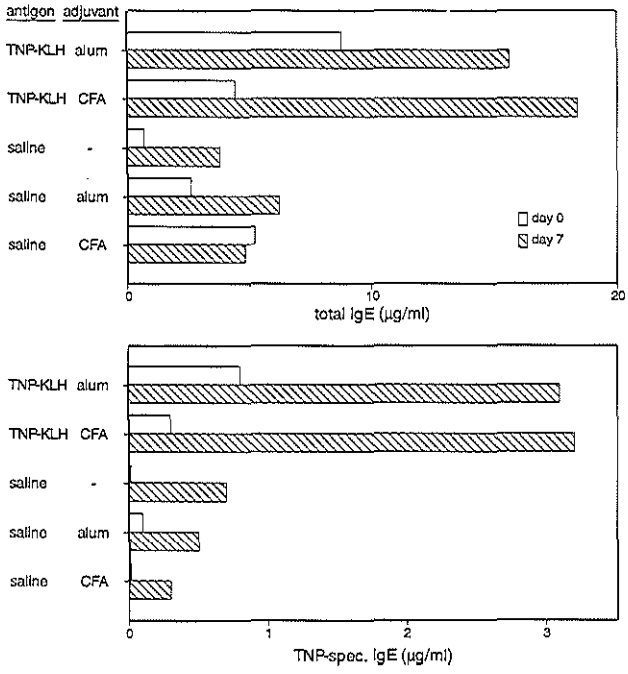

Figure 2. BALB/c mice were primed i.p. with 10 µg TNP-KLH either adsorbed on alum or emulsified in CFA as indicated. As a control alum or CFA was injected in the absence of antigen. Three months later all mice were boosted with 10 µg TNP-KLH adsorbed on alum i.p. On day 0 and day 7 after booster total and antigen-specific serum IgE levels were determined in pooled sera of 4 mice. Results are expressed in µg/ml as arithmetic mean of 3 ELISA determinations.

Acknowledgments

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Chapter 4

Prolonged *in vivo* IL-4 treatment inhibits antigen-specific IgG_1 and IgE formation

R. van Ommen, A.E.C.M. Vredendaal, and H.F.J. Savelkoul

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Prolonged *In Vivo* IL-4 Treatment Inhibits Antigen-Specific IgG_1 and IgE Formation

R. VAN OMMEN, A. E. C. M. VREDENDAAL & H. F. J. SAVELKOUL

Department of Immunology, Erasmus University, Rotterdam, The Netherlands

van Ommen R, Vredendaal AECM, Savelkoul HFJ. Prolonged *In Vivo* IL-4 Treatment Inhibits Antigen-Specific IgG_1 and IgE Formation. Scand J Immunol 1994;40:1-9

IL-4 is obligatory for primary IgE responses, whereas primary IgG_1 and secondary IgE responses are partially IL-4 independent. To investigate the effect of IL-4 on the antigen-specific memory formation for these isotypes, BALB/c mice were treated after primary TNP-KLH immunization with recombinant IL-4 for a period of 4 months. This prolonged presence of a high IL-4 level resulted in increased serum levels of total IgG_1 and IgE, whereas total IgG_{2a} did not change. The expression of CD23, but not $I\text{-}A^d$, increased on the splenic B cells. IL-4 treatment did not affect the IL-4 production by Con A stimulated spleen cells, whereas it did decrease the IFN-γ production. In the same mice the TNP-specific IgG_1 and IgE serum levels, however, were decreased. Similar results were found when the antigen was continuously present during the IL-4 treatment. Furthermore, it was shown that IL-4 decreased the formation of IgG_1 and IgE memory cells. These results point to different effects of IL-4 in regulating antigen-specific and bystander responses.

R. van Ommen, Department of Immunology, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

INTRODUCTION

IL-4 is a pleiotropic lymphokine, produced mainly by activated T cells, which has a number of activities on B cells [1]. For example IL-4 is known to enhance proliferation of B cells in response to soluble anti-Ig [2, 3]. Other effects of IL-4 are the increased expression of MHC class II [4–7] and CD23 ($Fc_\epsilon RII$) on resting B cells [8–10]. IL-4 also stimulates non-lymphoid haematopoietic cells, especially macrophages and mast cells [1, 11]. IL-4 is obligatory for IgE synthesis, whereas it has an enhancing effect on IgG_1 production. Functional studies indicate that IL-4 is a 'switch inducing' factor. It alters the chromatine structure of the $S\gamma_1$ region [12] and induces accumulation of germline γ_1 and ϵ transcripts [13–16]. Treatment of mice with neutralizing antibodies to IL-4 or IL-4 receptor completely inhibits the production of IgE, while the IgG_1 response to various antigens is only marginally affected [17–19]. Similarly IgE responses are undetectable in nematode infected mice which are made IL-4 deficient by gene targeting. IgG_1 can be detected in these mice, but the level is only one-sixth that of control mice [20].

Abbreviations: GAM, goat-anti-mouse; KLH, keyhole limpet hemocyanin; Ra, rat; RaAM, rat-anti-mouse; RARa, rabbit-anti-rat; RAM, rabbit-anti-mouse; SA-HRP, horse radish peroxidase-conjugated streptavidin; TD, thymus-dependent.

All the mentioned effects of IL-4 are inhibited by IFN-γ [1, 8, 10, 21]. On the other hand, IFN-γ is directly involved in stimulating IgG_{2a} synthesis, which can be inhibited by IL-4 [22–24]. Thus, the relative presence of functional IL-4 and IFN-γ determines whether and how much IgE can be produced after optimal stimulation. IgE memory responses are partially IL-4 dependent and thereby differ from primary responses that are completely dependent upon the presence of IL-4. It was hypothesized that this might be due to memory B cells that had already switched to IgE expression during the primary response [19].

The aim of this study was to investigate the effect of IL-4 on the antigen-specific memory formation for IgG_1 and IgE. To this end, BALB/c mice were treated continuously with recombinant IL-4 after primary TNP-KLH immunization for a period of 4 months. We used a method for cytokine administration that allowed persistent IL-4 levels for a prolonged period of time [25]. In this report we demonstrate that prolonged presence of a high IL-4 level increases the background level of IgG_1 and IgE, whereas IgG_{2a} is not influenced. On the contrary, IL-4 decreases the TNP-specific IgG_1 and IgE responses. Adoptive transfer experiments revealed also an inhibition of the formation of TNP-specific IgG_1 and IgE memory-B cells by IL-4. These results indicate that the level of functional IL-4 directs the balance between

the production of polyclonal and antigen-specific IgG_1 and IgE in TD immune responses.

MATERIALS AND METHODS

Mice. Female BALB/c mice were bred and maintained in the animal facilities of our own department. All mice were at an age of 12–16 weeks at the start of the experiments. Mice were held in light-cycled rooms and had access to acidified water and pelleted food *ad libitum*. The microbiological status of the mice fulfilled the standard of 'specific pathogen free V' according to the criteria of the Dutch Veterinary Inspection, as described in the law on animal experiments.

Immunization and anti-IL-4 treatment. KLH (Pierce, Rockford, IL, USA) was trinitrophenylated to a level of 25 TNP residues per 10^5 kDa of KLH (as determined spectrophotometrically) [26] by using trinitrobenzenesulphonic acid (Eastman Kodak, Rochester, NY, USA). Mice were injected with 0.2 ml containing 10 or 100 μg TNP-KLH adsorbed on 2 mg alum i.p.

IL-4 was neutralized by 10 mg rat MoAb specific for IL-4 (11B11) [27]. Rat MoAb specific for *E. coli* β-galactosidase (GL113) [25] was used as an IgG_1 isotype control.

IL-4 treatment. Mice were implanted i.p. with 2×10^6 CV-1/IL-4 cells encapsulated in alginate every 2 weeks as described earlier [25]. The monkey CV-1 cells were stably transfected with the murine IL-4 gene under control of the SV40 promotor. These cells were a gift of Dr N. Arai (DNAX Research Institute, Palo Alto, CA, USA). Briefly, cells were grown on Cytodex 3 beads (Pharmacia, Uppsala, Sweden). Fully covered beads were harvested after 2 days of culture and washed three times with sterile saline. The volume of the pellet was determined and 1 volume of saline followed by 2 volumes of a 1.2% sterile solution of cellprep alginate (FMC Bioproducts, Rockland, PA, USA) were combined in a syringe. This suspension was squirted through a 25-gauge needle into a fresh 80 mM $CaCl_2$ solution. The encapsulated cells were washed three times with saline and 2×10^6 encapsulated cells in 1 ml were injected i.p. with a 19-gauge needle. Empty beads encapsulated in alginate were used as control for the IL-4 treatment. No immunological effects were observed after injection of alginate encapsulated beads.

Adoptive transfer of spleen cells. Spleens of contol treated and IL-4 treated mice were removed under aseptic conditions and single cell suspensions were prepared. Ten million cells were transferred via the tail vein into naive recipients. The recipients had been sub-lethally irradiated [6 Gy] with a Caesium-137 source (Gammacell 40, Atomic Energy of Canada, Ottawa, Canada) 1 day before cell transfer. All reconstituted mice were immunized with 10 μg TNP-KLH adsorbed on alum i.p. immediately after transfer.

Isotype-specific ELISA. Total serum IgE and IgG_1 levels were measured by isotype-specific ELISA as described previously [21]. Detection limits for the IgE and IgG_1 ELISA were 0.5 ng/ml and 0.2 ng/ml, respectively. TNP-KLH-specific IgG_1 was measured by direct ELISA. Plates were coated with TNP-KLH (3 μg/ml), blocked with 1% BSA and incubated with the appropriate dilutions of serum. Subsequent steps were biotin-conjugates GAM/IgG_1 (Southern Biotechnology, Birmingham, AL, USA), SA-HRP (Jackson Immunoresearch, West Grove, PA, USA) and the substrate 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) (Sigma, St. Louis, MO, USA). The detection limit of this ELISA was 0.2 ng/ml. TNP-specific IgE was quantified in a modified sandwich ELISA. Plates were coated with monoclonal RaAM/IgE (EM95, 2 μg/ml) [28] and incubated overnight at 4°C with diluted serum samples. TNP modified with alkaline phosphatase (kind gift of Dr A. J. M. van den Eertwegh, MBL-TNO, Rijswijk, The Netherlands) was used as second step. The ELISA was further developed by using Sigma 104 phosphatase substrate (Sigma). The detection limit of this ELISA was 1 ng/ml. Highly purified monoclonal anti-TNP antibodies of the appropriate isotypes were used for the standard curves in both antigen-specific assays.

In order to determine total serum IgG2a, plates were coated with GAM/IgG2a (Southern Biotechnology) blocked, washed and incubated with the appropriate dilutions of serum. The assay was further developed by applying biotinylated RaAM/IgG2a (Pharmingen, San Diego, CA, USA) as second step, SA-HRP and ABTS substrate. The detection limit was 0.3 ng/ml. Murine IgG_{2a} was used for the standard curves.

Flow cytometric analysis. Cells (2.5×10^5) were incubated on ice for 30 min with the appropriate MoAb, either as undiluted culture supernatant or carefully titrated purified MoAb followed by a triple wash with PBS containing 1% BSA and 0.1% azide. After using unconjugated MoAb, another 30 min incubation was performed with a conjugated specific second-step MoAb on ice. After a triple wash the cells were taken up in isotonic fluid and analysed on a FACSCAN analyser (Becton Dickinson, Mountain View, CA, USA). A life gate was used to gate out rare dead cells and erythrocytes.

The following unconjugated rat-anti-mouse MoAbs were used as undiluted culture supernatants: MT4 (CD4, clone H129.19, Ra IgG_{2a}), Lyt-2 (CD8, clone 53-6.7, Ra IgG_{2a}), B220 (CD45RA, clone RA3-6B2, Ra IgG_{2a}), Thy-1 (clone 59-AD2.2, Ra IgG_{2a}), CD3 (clone KT3, Ra IgG_{2a}), CD45 (clone 30-G12, Ra IgG_{2a}), F4/80 Ag (clone F4/80, Ra IgG_{2b}) and anti-granulocyte (clone RB6-8C5, Ra IgG_{2b}). All these MoAb's were kindly provided by Professor W. van Ewijk from our department. The following MoAbs were optimally titrated RaAM/I-A^d-FITC (Pharmingen) at 10 μg/ml, RaAM/CD23 (Pharmingen) at 10 μg/ml, GAM/Ig-PE (CLB, Amsterdam, The Netherlands, 1/50 diluted), and RARa/IgG, $F(ab')_2$-fragments-FITC conjugated (Cappel/Organon Technika, Oss, The Netherlands, 1/100 diluted).

Con A stimulation of splenocytes. Spleen cells (2×10^6/ml) were cultured in RPMI 1640 medium supplemented with 10% heat inactivated FCS, 2 mM glutamine, 0.1 M pyruvate, 100 IU/ml penicillin, 50 μg/ml streptomycin, 50 μM 2-mercapto-ethanol in four replicate wells of a 24-well flat-bottom plate (1 ml/well) with 10 μg/ml Concanavalin A (Sigma). After 48 h culture supernatants were harvested and stored at −70°C before use.

Determination of cytokines. IL-4, IL-10 and IFN-γ were determined in ELISA as described previously [29–31]. IL-4 was also determined in a CT.4S bioassay (kind gift of Dr L. Nagelkerken, IVEG-TNO, Leiden) with a detection limit of 0.05 μg/ml [32]. The detection limits of the ELISAs were, 0.2 ng/ml, 3 U/ml and 0.2 ng/ml, respectively. IL-6 was determined in a bioassay using B9 indicator cells as described [33]. The detection limit of this bioassay was 0.1 U/ml.

RESULTS

Secondary IgE responses are partially IL-4 independent

Mice immunized with 10 μg TNP-KLH developed a primary total IgE response that reached a level of 3.7 μg/ml at day 14. Secondary immunization at 3 months after priming resulted in a total IgE response with a maximum level of 8.9 μg/ml at

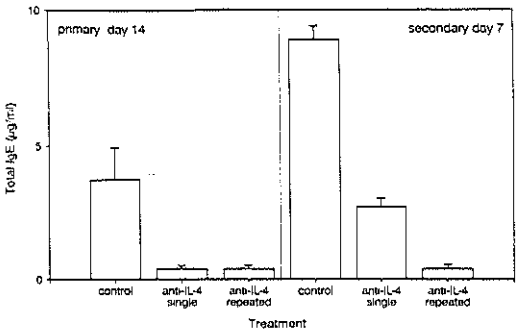

Fig. 1. Primary and secondary serum peak IgE responses in presence and absence of IL-4. Mice were primed and boosted with 10 μg TNP-KLH adsorbed on alum and were injected with either 10 mg GL113 (rat IgG_1 isotype control) or 10 mg 11B11 (anti-IL-4) once, on the day of immunization, or every 2 weeks starting at primary immunization till the end of the secondary response. Results are expressed as arithmetic mean ± SEM ($n = 5$).

day 7. The primary IgE response could be completely blocked by anti-IL-4 treatment indicating the absolute requirement of IL-4 in the induction of a primary IgE response (Fig. 1). Anti-IL-4 treatment at the start of a secondary immune response did not completely inhibit the secondary IgE response. At day 7 a maximum IgE level of 2.7 μg/ml, 30% of the control situation, was observed. On the other hand, mice that were continuously treated with anti-IL-4 did not show primary or secondary serum IgE responses (Fig. 1).

Phenotypic analysis of IL-4 treated mice

In order to investigate the effects of IL-4 on secondary TNP-specific IgG_1 and IgE responses mice were treated continuously with IL-4 for 4 months after primary immunization with 100 μg TNP-KLH. Treatment did not result in any symptoms of morbidity in all experiments. After this period the cellular composition of the spleen of control and IL-4 treated mice was analysed by flow cytometry. The results (Table 1) show that prolonged IL-4 treatment consistently reduced the absolute numbers of splenic $CD3^+$ (by 20%) and Thy-1 (by 15%) T cells as compared to control treated mice. Staining for CD4 and CD8 showed that this decrease was primarily due to a decrease in the number of $CD4^+$ cells. IL-4 did not influence the absolute number of $B220^+$ and sIg^+ cells. On the other hand, the absolute numbers of macrophages ($F4/80^+$) and granulocytes increased by 300% and 75%, respectively. Prolonged IL-4 treatment apparently led to disturbances in cell numbers of the main cell populations in the spleen. The alterations in cell populations and their localization was confirmed by immunohistological stainings of spleen sections of IL-4 and control treated mice (data not shown).

In IL-4 treated mice, no IL-4 could be detected in the serum as determined by both IL-4 specific ELISA and bioassay (data not shown). In order to measure IL-4 driven phenomena other than IgE induction, the expression of CD23 and $I\text{-}A^d$ on lymphocytes was studied by flow cytometric analysis. To this end spleen cells were stained with anti-$I\text{-}A^d$ and anti-CD23 antibodies at day 1, 2 and 3 after last CV-1.IL-4 administration (Table 2). FACSCAN analysis showed that in the IL-4 treated group the number of CD23 middle and high expressing lymphocytes gated on the basis of forward scatter/side scatter plot was increased at the expense of CD23 low expressing lymphocytes. In the spleens of IL-4 treated mice, the $I\text{-}A^d$ expression was increased particularly in the intermediate expressing lymphocytes at the expense of the low expressing population. No change in $I\text{-}A^d$ expression was observed in the high expressing lymphocyte subpopulation (Table 2).

To analyse the effects of IL-4 on the $I\text{-}A^d$ and CD23 expression of B cells double-stainings with GAM/Ig and

Table 1. Phenotype of splenocytes after prolonged IL-4 treatment

Antigen	Positive cells		Number of cells ($\times 10^7$)	
	Control	IL-4	Control	IL-4
B220	30.4	26.1	3.8	3.5
sIg	25.8	27.9	3.2	3.8
Thy-1	52.7	41.3	6.6	5.6
CD3	52.7	39.3	6.6	5.3
CD4	33.8	25.6	4.2	3.5
CD8	14.5	11.5	1.8	1.6
CD45	85.1	74.6	10.6	10.1
F4/80	0.9	3.1	0.1	0.4
Granulocyte	3.4	5.4	0.4	0.7

Surface marker expression evaluated by FACSCAN analysis of total spleen cells from control or IL-4 treated mice. Cell suspensions were pooled from two mice. Rare dead cells and erythrocytes were gated out. The results are representative for three independent experiments.

Table 2. Effect of prolonged IL-4 treatment on CD23 and $I\text{-}A^d$ expression of lymphocytes

Treatment	Ag	Expression					
		Low		Middle		High	
		Per cent	MCF	Per cent	MCF	Per cent	MCF
Control	CD23	61	146	13	282	26	493
IL-4	CD23	42	161	21	281	36	546
Control	$I\text{-}A^d$	51	163	18	313	31	549
IL-4	$I\text{-}A^d$	35	178	32	309	33	551

Percentage of splenic lymphocytes gated on the forward scatter/side scatter plot. MCF is mean channel fluorescence. Cell suspensions were pooled from two mice. The results are representative for five independent experiments.

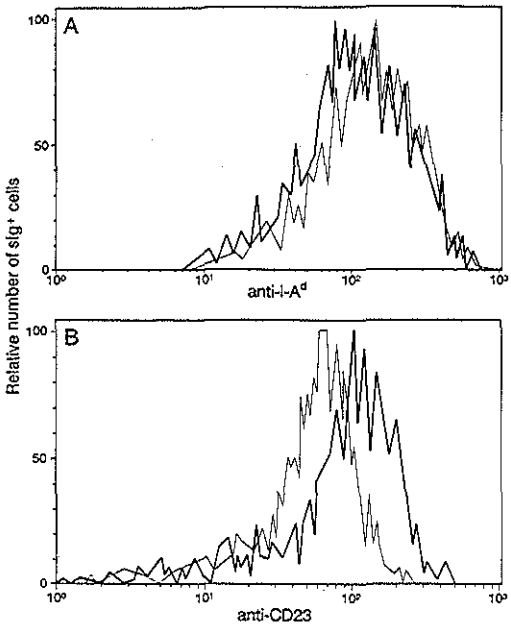

Fig. 2. Expression of CD23 and I-A^d on B cells on day 1 after the last of 10 IL-4 treatments. (A) Histogram representing staining of sIg^+ spleen cells with anti-I-A^d antibody. (B) Histogram representing staining of sIg^+ spleen cells with anti-CD23 antibody. The thick line represents the staining of cells from IL-4 treated mice and the thin line that of control mice. Cell suspensions from two mice were pooled. The results are representative for three individual experiments.

respectively anti-I-A^d and anti-CD23 were done. This study revealed that B cells from control treated mice were already positive for I-A^d. Apparently IL-4 treatment did not result in a further increase of this expression, whereas expression of CD23 on these B cells showed a significant increase (Fig. 2). The number of intermediate I-A^d positive B cells increased as a result of prolonged IL-4 treatment, as was also seen after staining of lymphocytes with anti-I-A^d.

IL-4 treatment reduces the basal IFN-γ production in the spleen

The influence of prolonged treatment on the cytokine production profile was determined by measuring the production of IL-4, IL-6, IL-10, and IFN-γ in cultures of Con A stimulated spleen cells at day 1 after the last injection of encapsulated CV-1/IL-4 cells. At that time, IL-4 treated mice displayed elevated total IgE and total IgG_1 serum levels. In IL-4 treated mice the concentration of IFN-γ was reduced 4.5-fold from 1.71 ng/ml in the sup of control mice to 0.38 ng/ml the sup of IL-4 treated mice. No such reduction was observed for the cytokines IL-4, IL-6, and IL-10 as shown in Table 3. Similar results were obtained at days 2 and 3 after the last of 10 CV-1/IL-4 administrations (data not shown).

Table 3. Cytokine profile of splenocytes 1 day after the last of 10 IL-4 administrations

Mice	IL-4 (ng/ml)	IL-6 (U/ml)	IL-10 (U/ml)	IFN-γ (ng/ml)
Control	0.24	701	19.2	1.71
IL-4	0.22	632	21.2	0.38

Spleen cells (2×10^6 ml) pooled from two mice were cultured with Con A (10 μg/ml) for 48 h in four replicate wells. The supernatants harvested from these wells were pooled prior to cytokine analysis. 1 U IL-10 is approximately 1 pg.

Primary TNP-specific IgG_1 and IgE responses are inhibited by IL-4 treatment

Previous studies have demonstrated the involvement of IL-4 in IgE and IgG_1 responses *in vivo* and *in vitro*. In order to study the effect of continuous IL-4 treatment during primary immunization on the antigen-specific and total serum IgG_1 and IgE responses, BALB/c mice were immunized with either 100 μg TNP-RIgG or 100 μg TNP-KLH. Mice were treated with IL-4 by injecting alginate encapsulated CV-1/IL-4 cells every 2 weeks. This treatment was carried out for 4 months. During this period the serum levels for IgG_1 and IgE were monitored. Immunization with 100 μg TNP-KLH led to a response of approximately 2 mg total IgG_1, both in control and IL-4 treated mice at day 14. The total IgE response at this time point in control and IL-4 treated mice was 0.5 and 1.5 μg/ml, respectively. As a result of the IL-4 treatment an increase in total serum IgG_1 was observed during the 4 months of treatment: control mice exhibited an IgG_1 plateau level of 3.2 mg/ml whereas IL-4 treated mice displayed an IgG_1 level of 13.7 mg/ml (Fig. 3A). The total IgE levels in the serum of IL-4 treated mice immunized with 100 μg TNP-KLH displayed a more profound increase (from 1.0 to 12.3 μg IgE/ml during the first month), which reached a plateau of 153.6 μg IgE/ml after 4 months of IL-4 treatment (Fig. 3B). In the same period, the total serum IgE levels in control mice increased to 3.8 μg IgE/ml (Fig. 3B).

In order to determine whether the increase in serum IgG_1 was antigen-specific, a TNP-specific IgG1 ELISA was performed. IL-4 treatment resulted in a seven-fold decrease of TNP-specific IgG_1 at day 86 of treatment (Fig. 3C). A similar decrease was seen for TNP-specific IgE (Fig. 3D). No increase in total serum IgG_{2a} was observed after immunization with TNP-KLH and prolonged IL-4 treatment (data not shown).

Similar results were obtained when mice were immunized with 100 μg TNP-RIgG. Total serum IgE of control mice stayed at a level of 1.8 μg/ml whereas in IL-4 treated mice it

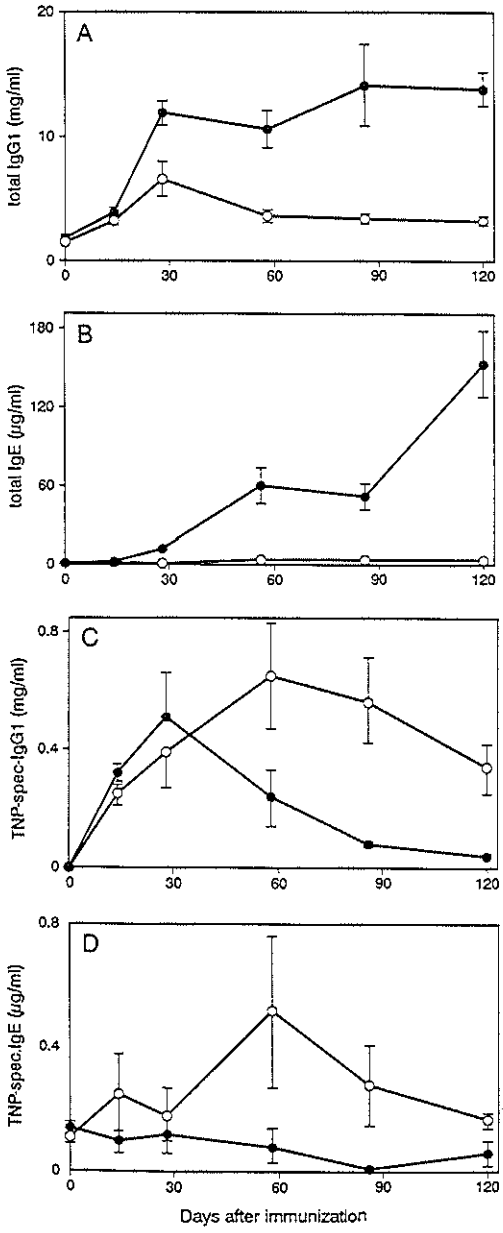

Fig. 3. Effect of IL-4 on total and TNP-specific IgE and IgG_1 levels. Mice were immunized with 100 µg TNP-KLH adsorbed on alum followed by a control treatment (○) or a prolonged IL-4 treatment (●) every 2 weeks. Serum levels of total IgG_1 (A), and IgE (B), and TNP-specific IgG_1 (C) and IgE (D) serum levels were determined by ELISA, and are expressed as arithmetic mean±SEM ($n = 5$).

reached a level of 29.0 µg/ml after 4 months treatment (data not shown). The IgG_1 serum levels were significantly elevated by IL-4 treatment (after control treatment 2.1 mg/ml and IL-4 treatment 14.5 mg/ml) (data not shown).

Treatment of mice with encapsulated CV-1/IL-4 cells every 2 weeks, without immunization with TNP-KLH, resulted also in high levels of total serum IgG_1 and IgE, respectively 17.9 mg/ml and 57.4 µg/ml at day 70 (data not shown). These levels are comparable with the levels of total serum IgG_1 and IgE that occur after treatment with CV-1/IL-4 cells in combination with immunization with TNP-KLH. IL-4 treatment alone increased the serum levels for TNP-specific IgG_1 from 22 µg/ml (day 0) to 93 µg/ml (day 70) (data not shown). These results indicate that prolonged IL-4 treatment leads to a strong polyclonal activation of B cells, part of which are specific for TNP.

IL-4 inhibits the hyperexpression of TNP-specific IgG_1 and IgE induced by repeated antigen exposure

In order to investigate whether the inhibition of the TNP-specific IgG_1 and IgE responses by IL-4 was the result of a lack of antigen, the IL-4 treatment was repeated under conditions of repeated antigen exposure. To this end mice received a primary immunization with 100 µg TNP-KLH on alum on day 0 and starting at day 14 100 µg TNP-KLH in saline every 2 weeks in conjunction with a control- or IL-4 treatment. Repeated antigen exposure itself resulted in high levels of total serum IgG_1 (20.0 mg/ml). Moreover, because of this repeated antigenic exposure practically 100% of this serum IgG_1 was antigen-specific at day 56. IL-4 treatment of mice subjected to continuous antigen exposure resulted in 15.5 mg/ml total serum IgG_1, with only 56% being TNP-specific (Fig. 4, upper part). IL-4 treatment resulted in a higher serum level of total IgE (65.9 µg/ml) when antigen was continuously present (after control treatment: 8.5 µg/ml). The TNP-specific fraction, however, decreased to 0.4% after IL-4 treatment (after control treatment 21%; Fig. 4, lower part).

No differences in the amount of KLH-specific IgE were found both in control and IL-4 treated mice, when using KLH modified with horseradish peroxidase instead of TNP modified with alkaline phosphatase in ELISA (data not shown).

Mice with elevated serum IgG_1 and IgE levels still respond to a booster immunization

To determine whether IL-4 treated TNP-KLH immunized BALB/c mice with elevated serum IgE and IgG_1 levels could still respond to a secondary immunization, such mice were boosted with 10 µg TNP-KLH. This dose of TNP-KLH is known to give an optimum IgE response. On day 7, an increase in TNP-specific serum IgG_1 was observed which reached a plateau of approximately 2.6 mg/ml at day 21. This plateau did not differ significantly for the IL-4 treated and control groups (Fig. 5A). The TNP-specific IgE levels also did not differ between the control and IL-4 treated group

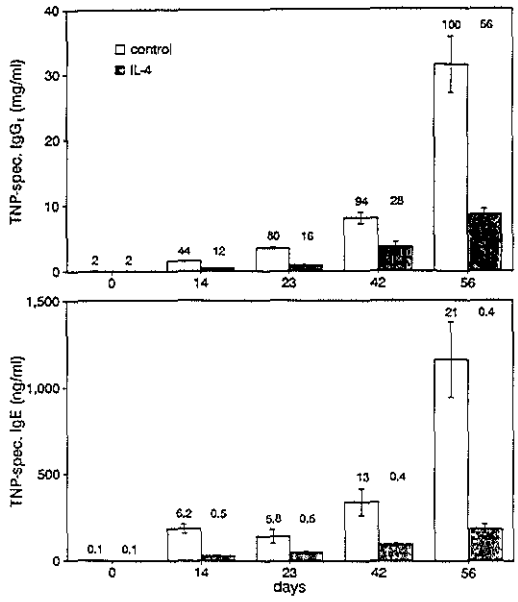

Fig. 4. Mice were i.p. immunized with 100 μg TNP-KLH adsorbed on alum on day 0 followed by injection of 100 μg TNP-KLH in saline every 2 weeks. The control group received a control treatment every 2 weeks, whereas the IL-4 group received CV-1/IL-4 cells encapsulated in alginate every 2 weeks. Results are expressed as arithmetic mean ± SEM (n = 5). The numbers in the Fig. represent the percentage TNP-specific serum Ig versus total serum Ig.

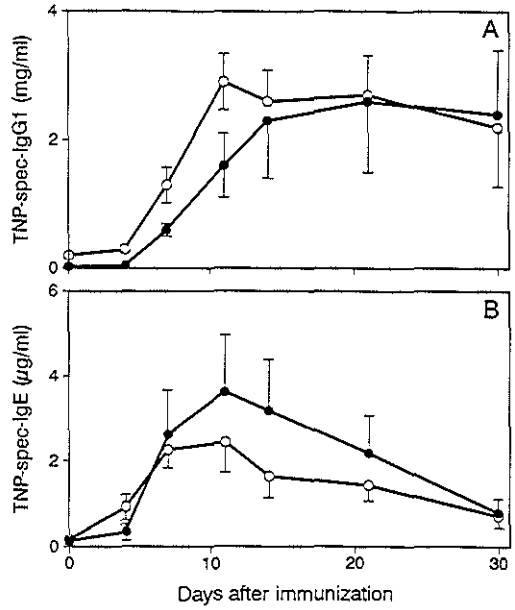

Fig. 5. Effect of IL-4 treatment on the TNP-specific secondary IgG_1 and IgE response. Primed IL-4 treated (●) and control treated mice (○) were boosted with 10 μg TNP-KLH adsorbed on alum on days 0, 3 months after priming. Serum levels of TNP-specific IgG_1 (A) and IgE (B) were determined by ELISA and are expressed as arithmetic mean ± SEM (n = 5).

(Fig. 5B). Comparable results were found after TNP-RIgG booster immunization (data not shown). Moreover, IL-4 treatment during priming did not result in an increase or decrease of total and TNP-specific serum IgG_{2a} after booster immunization (data not shown). Thus IL-4 treatment did not disturb the antigen-specific isotype regulation during a secondary immune response. These results also indicate that TNP-specific IgE can be detected in a large pool of total serum IgE using our TNP-specific IgE ELISA.

Continuous IL-4 treatment inhibits the TNP-specific IgG_1 and IgE memory formation

In order to investigate the influence of continuous IL-4 on the TNP-specific IgG_1 and IgE memory formation an adoptive transfer system was used. Irradiated recipient mice were reconstituted with TNP-KLH primed spleen cells from control and IL-4 treated mice isolated on day 0 and 3 months after the last of nine injections of encapsulated CV-1/IL-4 cells. All recipient mice were immunized with TNP-KLH immediately after reconstitution. On days 7, 9 and 12 the TNP-specific IgG_1 and IgE serum levels were determined. In two independent experiments, the TNP-specific IgG_1 and IgE levels in mice reconstituted with spleen cells from IL-4 treated mice were significantly lower than in mice reconstituted with spleen cells from control mice (Table 4). Prolonged IL-4 treatment of the donors apparently reduced the TNP-specific IgG_1 and IgE memory formation. No differences were seen in the percentages of $B220^+$ and $Thy\text{-}1^+$ spleen cells between IL-4 treated- and control mice 3 months after the last injection of encapsulated CV-1/IL-4 cells (data not shown). So the difference in the percentage of T cells in the spleens from IL-4 treated mice at day 0 and three months after the last administration of CV-1/IL-4 cells did not influence the IgG_1 and IgE production after adoptive transfer (Table 4). The total IgE levels at day 9 did not differ between mice adoptively transferred with spleen cells from control treated and IL-4 treated mice in both experiments. This indicates that the differences in TNP-specific IgE levels are not influenced by differences in total IgE levels.

DISCUSSION

The main finding from this study is that prolonged IL-4 treatment after primary immunization with a TD antigen led to a strong increase in polyclonal but not in antigen-specific IgG_1 and IgE formation. This was based on the

Table 4. TNP-specific serum IgG_1 and IgE responses after adoptive transfer of spleen cells from control and IL-4 treated mice

	TNP-spec. IgG_1 (mg/ml)			TNP-spec. IgE (μg/ml)		
	7	9	12	7	9	12
A						
Control	1.2 ± 0.1	4.4 ± 1.0	8.0 ± 1.7	52.9 ± 8.3	80.1 ± 12.7	50.5 ± 10.5
IL-4	0.4 ± 0.1	2.0 ± 0.4	3.9 ± 0.8	20.6 ± 4.8	55.1 ± 16.0	38.9 ± 9.6
B						
Control	0.5 ± 0.05	2.8 ± 0.3	6.7 ± 0.7	21.6 ± 0.4	87.6 ± 14.0	61.9 ± 6.7
IL-4	0.1 ± 0.02	0.9 ± 0.5	1.6 ± 0.4	7.0 ± 1.7	33.4 ± 6.8	14.6 ± 1.6

Irradiated mice (6 Gy) were reconstituted with 1×10^7 TNP-KLH primed spleen cells from control and IL-4 treated mice at the end of the treatment (A) and 3 months after treatment (B). All reconstituted mice were boosted with 10 μg TNP-KLH on day 0. Serum levels of TNP-specific IgG_1 and IgE were determined on days 7, 9 and 12. Results are represented as arithmetic mean ± SEM ($n = 5$).

finding that TNP-specific IgG_1 and IgE responses were consistently reduced upon treatment with IL-4. The IgG_{2a} response was not influenced, indicating that the observed effects of IL-4 were specific for the IgG_1 and IgE isotypes. The decrease in the TNP-specific IgG_1 and IgE responses by IL-4 is accompanied by a decrease in the TNP-specific memory formation for these isotypes as is seen in the adoptive transfer experiments. The question remained whether IL-4 exerted its effect directly on the B cells or indirectly via other cell types, such as T cells.

It has been suggested that IL-4, when persistently present, can inhibit the IL-4 receptor expression on T cells [34]. Since IL-4 can act as a proliferation factor of activated T cells [35] it is tempting to speculate that IL-4 receptor down-regulation can lead to decreased T cell proliferation, resulting in a reduced availability of T cells. Indeed, we did find a decrease in the number of T cells in the spleens of IL-4 treated mice (Table 1), and we cannot exclude that the observed decrease in $CD4^+$ T cells of IL-4 treated mice is to some extent due to a (selective) inhibition of antigen-specific T_h2 cells. However, no difference in the IL-4 production by spleen cells after Con A stimulation was observed as a result of the IL-4 treatment.

On the other hand, the possibility of an indirect effect of IL-4 on the B cells seems unlikely, because the TNP-specific IgG_1 and IgE responses in the adoptive transfer experiments were not influenced by differences in the percentage of T cells in the spleen cell samples of IL-4 treated mice used to reconstitute the irradiated mice (Table 4). These results indicate that a more likely explanation for the observed phenomena is a direct negative effect of IL-4 on the antigen-specific B cell consistent with the findings of Asano *et al.* [36]. Furthermore, the adoptive transfer results show that the observed inhibition of the TNP-specific memory formation for IgG_1 and IgE by IL-4 is a long term effect as the same degree of inhibition is found 3 months after the last administration of IL-4 (Table 4).

No differences were observed between the TNP-specific secondary IgG_1 and IgE responses in IL-4 and control treated mice, indicating that the isotype-specific regulation in IL-4 treated mice was obviously not disturbed. This despite the fact that these IL-4 treated mice had total IgG_1 and IgE levels that were increased five-fold and 12-fold, respectively over control mice. In the adoptive transfer experiments a decrease in the TNP-specific memory formation resulting from the IL-4 treatment was observed. These adoptive transfer experiments were carried out to study the TNP-specific memory responses in a more isolated way, without the possible interference of residual systemic effects induced in the treated donor mice. Furthermore, the resulting TNP-specific IgE responses were much larger in this system and therefore potential differences between IL-4 and control treatment became visible.

The markedly elevated bystander responses, that is responses to other antigens than TNP-KLH, in IL-4 treated mice could be the result of enhanced differentiation or prolonged survival of pre-activated B cells [37, 38]. As it is shown in Figs 1 and 4, immunization with TNP-KLH adsorbed on alum induces enough endogenous IL-4 to mount an IgE and IgG_1 response. The effect of CV-1-secreted IL-4 may be enhancement of responses to 'e.g., environmental antigens' that do normally not induce levels of IL-4 high enough to result in IgE or IgG_1 responses, possibly by reversing the Fc receptor-mediated inhibition of B-cell activation [39]. This could lead to activation of B cells which can subsequently switch to IgG_1 and IgE, causing high levels of these isotypes.

The high polyclonal IgG_1 and IgE levels themselves could have a negative effect on TNP-specific IgG_1 and IgE formation. The high level of polyclonal IgG_1 alone could possibly also inhibit the TNP-specific IgE response, by inhibiting the TNP-specific IgG_1 levels, as it is described that the IgG_1 response is coupled to the IgE response [40].

The lack of sufficient antigen and/or antigen-specific helper T cells for TNP-specific B cells when IL-4 is excessively present could also be an explanation for the observed inhibition of TNP-specific IgG_1 and IgE. Both would lead to a limited cognate T–B cell interaction that is obligatory for antigen-specific antibody responses against TD antigens [41].

However, our results indicate that a lack of antigen is not the explanation for decreased antigen-specific antibody responses since IL-4 also inhibits TNP-specific IgG_1 and IgE responses when antigen is repeatedly injected.

Our results indicate that antigen and IL-4 should be in balance for maximum antigen-specific IgG_1 and IgE responses. Disturbance of this balance by administration of IL-4 leads to an inhibition of the antigen-specific responses together with an elevation of the bystander responses. On the other hand, antigen-specific responses could need locally present antigen in conjunction with the crucial amount of IL-4. So in general, antigen-specific and bystander responses are differentially regulated. These results extend the *in vitro* findings of Asano *et al.* [36] who reported an inhibitory effect of high doses of antigen on the antigen-specific IgG_1 response. This inhibition was found to be mediated by IL-4, which exerted its effect only on the cognate pathway.

Besides the effect on immunoglobulin secretion mediated by prolonged IL-4 treatment we observed that the number of splenic T cells slightly decreased, largely due to a decrease in $CD4^+$ cells as was also seen in some IL-4 transgenic mice [42]. The IgE and IgG_1 hyperproduction that we observed in our IL-4 treated mice was also seen in some IL-4 transgenics [42, 43]. In all described IL-4 transgenic mice the MHC class II expression on B cells increased [43, 44]. We could not detect an increase in $I\text{-}A^d$ expression on the splenic B cells of IL-4 treated mice. However, B cells of IL-4 treated mice showed an increased CD23 expression. This might be due to the increased IL-4 level, but could also be the result of the elevated serum IgE levels [8–10]. All of the observed phenomena could be explained by different levels of expression of IL-4 in transgenic and IL-4 treated mice.

The observed effects on total serum IgG_1 and IgE levels, and CD23 expression on the splenic B cells in mice that were repeatedly treated with IL-4 provide evidence for the presence of increased levels of functional IL-4 *in vivo*. This occurred despite the fact that no IL-4 could be detected in the circulation of such IL-4 treated mice. Previous studies have shown that injection of 2×10^6 alginate encapsulated CV-1/IL-4 cells resulted in a consistent IL-4 production for periods up to 2 weeks. This treatment proved to be sufficient to transform IgE non-responder SJA/9 mice into IgE high-responder mice [25]. Treatment of mice with the IL-5 producing CV-1/IL-5 cell line did not result in any of the effects described in this article, although eosinophilia occurred (data not shown).

As a result of prolonged IL-4 treatment the IFN-γ production by Con A stimulated spleen cells decreased, while the production of the T_h2 cytokines (IL-4, IL-6 and IL-10) was not affected (Table 3). IL-4 treatment apparently did not dysregulate the endogenous production of T_h2 cytokines. A possible explanation for the decreased IFN-γ production by the IL-4 treatment could be a decrease in the development of T_h1 cells from T_h0 cells [45–49]. Another possibility is a direct effect of the applied IL-4 on the IFN-γ production by $CD4^+$ and/or $CD8^+$ T cells. For the human system this has been described by Vercelli *et al.* [50] who reported a decreased IFN-γ production and mRNA expression in mixed lymphocyte cultures and Con A stimulated peripheral blood mononuclear cells as a result of exposure to IL-4. It might be that IL-4 decreases the transcription and/or stability of IFN-γ mRNA.

Our results underline the peculiar effects of IL-4 on the proliferative and isotype switching capacity of B cells. Moreover, they point to differences in the effect of IL-4 on antigen-specific and bystander responses. This indicates that caution has to be taken when IL-4 administration is considered to increase the induction of IgG_1 or IgE specific for a particular antigen.

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Chapter 5

Prolonged *in vivo* IL-4 treatment decreases the TNP-specific memory formation for IgG_1

R. van Ommen and H.F.J. Savelkoul

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INTRODUCTION

IL-4 is a pleiotropic lymphokine, produced mainly by activated T-cells, which has a number of activities on B-cells[1]. It is obligatory for IgE synthesis, and has an enhancing effect on the IgG_1 production[2]. Whereas it is impossible to detect IgE in nematode infected mice which are made IL-4 deficient by gene targetting, IgG_1 can be detected, but the level is only one-sixth that of control mice[3]. The aim of this study was to investigate the effect of prolonged IL-4 treatment on the total and antigen-specific serum IgG_1 levels. Furthermore, we investigated the effect of such treatment on the memory formation for IgG_1. To that end, BALB/c mice were treated for a period of three months with IL-4 after primary TNP-RIgG immunization. We used a method for cytokine administration that allowed persistent IL-4 levels for a prolonged period of time[4].

MATERIALS AND METHODS

Mice: Female BALB/c mice were bred and maintained at the Department of Immunology of the Erasmus University. All mice were at an age of 12-16 weeks at the start of the experiments. They were held in light-cycled rooms and had access to acidified water and pelleted food ad libitum. The microbiological status of the mice fulfilled the standard of "specific pathogen free V" according to the criteria of the Dutch Veterinary Inspection, as described in the law on animal experiments.

IL-4 treatment: Mice were implanted i.p. with 2×10^6 CV-1/IL-4 cells (kind gift of Dr. N. Arai, DNAX Research Institute, Palo Alto, CA, USA) encapsulated in alginate every two weeks as described earlier [4,5].

Immunization: Rabbit-IgG (RIgG) (Sigma Chemical Co., St. Louis, MO, USA) was trinitrophenylated to a level of 25 TNP residues per 10^5 Da of RIgG (as determined spectrophotometrically) by using trinitrobenzenesulphonic acid (Eastman Kodak, Rochester, NY, USA). Mice were injected with 0.2 ml PBS containing either 10 or 100 µg TNP-RIgG adsorbed on 2 mg alum i.p.

Isotype specific ELISA: Total and TNP-specific IgG_1 levels were measured by isotype-specific ELISA as described previously[5], both with a detection limit of 0.2 ng/ml.

ELISA-spot assay: Nitrocellulose bottomed 96-wells Multiscreen HA plates (Millipore Co., Bedford, MA, USA) were coated with GAM/IgG_1, 1 µg/ml (Southern Biotechnology, Birmingham, AL, USA), and blocked with PBS containing 1% BSA. The plates were then incubated with spleen cell samples for four hours in a humidified and vibration free 5% CO_2 incubator. Plates were washed once with 0.05% Tween 20 in distilled water and twice with PBS containing 0.1% BSA and 0.05% Tween 20. Subsequently, the

plates were treated as in a normal ELISA. Development was done by using AEC substrate which was prepared by dissolving 25 mg of 3-amino-9-ethyl carbazole (AEC) (Sigma, St. Louis, MO, USA) in 2 ml dimethylformamide, followed by addition of 95 ml of 0.05 M acetate buffer, pH 5.0 and 40 µl of 30% H_2O_2. The substrate solution was filtered (0.2 µm). Developed plates were dried and the red spots were enumarated under low magnification 10x using a dissecting microscope (Stemi SV 6, Zeiss, Oberkochen, FRG) equipped with a coaxial reflected light source.

Preparation of B cells: Splenic B cells were prepared from control and IL-4 treated mice. T cells were cytotoxically eliminated by treatment of the spleen cells with anti-Thy-1.2 (clone F7D5; Serotec, Oxford, U.K.) and low-tox guinea pig complement (Cederlane, Hornby, Ontario, Canada) in a two-step procedure at 0^0 and 37^0 C, respectively.

T-B cell culture: CDC35, an I-A^d restricted rabbit-Ig-specific murine Th2 clone[6], a kind gift of Dr. D.C. Parker, was maintained by 2-weekly stimulation with irradiated BALB/c spleen cells (30 Gy) and 50 µg/ml rabbit-IgG (RIgG) (Sigma) in complete RPMI 1640 medium supplemented with 10% heat inactivated FCS, 2 mM glutamin, 0.1 M pyruvate, 100 IU/ml penicillin, 50 µg/ml streptomycin, 50 µM 2-mercapto-ethanol and 20 IU/ml IL-2. Cells were washed prior to culture with B cells and viability was assessed by trypan blue exclusion. Routinely, viability was >98%. T-cell depleted spleen cells at 2.5 x 10^5 cells /ml were cultured in 8 replicate wells of flat-bottom microtiter plates together with 5 x 10^4 cells/ml of irradiated CDC35 cells (30 Gy) in 0.2 ml of complete RPMI 1640 medium at 5% CO_2 and 37^0 C. The following antigens were used for stimulation: 30 ng/ml TNP-RIgG, 10 ng/ml TNP-KLH + 10 ng/ml RIgG, 10 ng/ml RIgG, 10 ng/ml RAM/IgE, or 10 ng/ml RAM/IgG_1. After 5 days of culture, cells were harvested for ELISA-spot assay and supernatants for ELISA.

RESULTS

Prolonged IL-4 treatment increases total serum IgG_1 levels

Previous studies have demonstrated the involvement of IL-4 in IgG_1 responses *in vivo* and *in vitro*[2]. In order to study the effect of continuous IL-4 treatment during primary immunization on the booster IgG_1 response, mice were immunized with 100 µg TNP-RIgG. Subsequently, mice were treated with recombinant IL-4 by injecting alginate encapsulated CV-1/IL-4 cells every two weeks. This treatment was carried out for three months. During this period the serum level for total IgG_1 increased to a plateau level of 14.5 mg/ml, whereas in control treated mice the level of total serum IgG_1 stayed at 2.1 mg/ml (Figure 1).

To determine if mice with elevated total serum IgG_1 levels were at a plateau for this

isotype or still could respond on a secondary immunization, mice were boosted with 10 μg TNP-RIgG. In both groups it was possible to evoke a secondary response with normal kinetics. The total serum IgG_1 level is increased 1.7 mg/ml in the control treated group and 2.6 mg/ml in the IL-4 treated group, between day 4 and 7 (Figure 1).

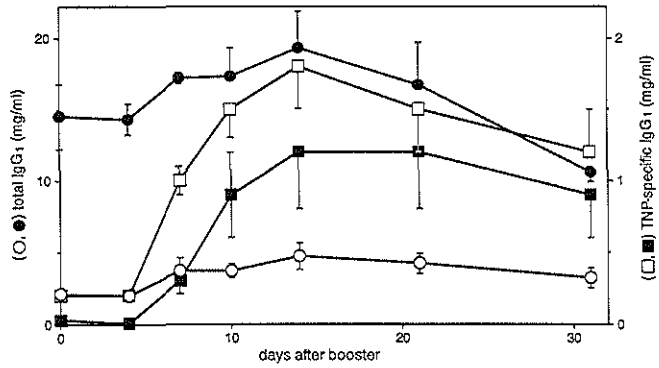

Figure 1. Effect of IL-4 on total and TNP-specific IgG_1 serum levels. Mice were immunized with 100 μg TNP-RIgG adsorbed on alum followed by a control treatment (open symbols) or a prolonged IL-4 treatment (closed symbols) every two weeks. Serum levels of total and TNP-specific IgG_1 were determined by ELISA and are expressed as arithmetic mean ± SEM (n = 5).

In IL-4 treated mice the memory TNP-specific IgG_1 response was 29% of the response seen in control treated mice on day 7 (Figure 1). These data suggest that the TNP-specific IgG_1 memory formation is decreased by prolonged IL-4 treatment.
Booster immunization did not result in an increase of serum IgG_{2a} levels in control and IL-4 treated mice (data not shown). This indicates that IL-4 treatment does not disturb the isotype regulation during a secondary immune response.

Continuous IL-4 treatment increases the number of IgG_1 and/or IgE positive-B cells

A cognate T-B cell culture system previously described[6] was used to determine whether prolonged IL-4 treatment had resulted in an increase of secondary B cells expressing IgG_1 and/or IgE. In this system T cell depleted splenocytes (T cells << 2%) were stimulated with antigen and CDC35 cells, a Th2 cell-line specific for Rabbit IgG. RAM/IgE and RAM/IgG_1 were used to selectively stimulate ε^+ and $\gamma1^+$ B cells. TNP-RIgG was used to stimulate antigen-specific B cells, whereas TNP-KLH and RIgG served as controls.
After five days of culture the number of IgG_1-secreting cells was determined in an ELISA-spotassay. Stimulation with RAM/IgG_1 in the presence of exogenous IL-4 (100 U/ml) revealed an 4.5 fold increase in the number of IgG_1 spot-forming cells (SFC) when compared to control treated mice (Figure 2). A similar increase was seen when no exogenous IL-4 was added during the culture period, although the numbers of IgG_1-SFC

in absence of exogenous IL-4 during the culture were lower than when IL-4 was present. These two observations indicate that IL-4 can act as a proliferation factor in this *in vitro* culture system.

The increase of $\gamma 1^+$ positive B cells was reflected in the IgG_1 production level. The production of IgG_1 after 5 days of culture of T depleted spleen cells from IL-4 treated mice stimulated with RAM/IgG_1 in the presence of exogenous IL-4 was 4.5 fold higher than when cells from control mice were stimulated. In the absence of IL-4 during the culture period an 13.6 fold increase was observed. No increase in the number of IgG_1-SFC was seen after 5 days of culture with Th2 cells and RAM/IgE when comparing cells from control and IL-4 treated mice (Figure 2), whereas in the same culture more IgG_1 was produced by spleen cells from IL-4 treated mice (Figure 2).

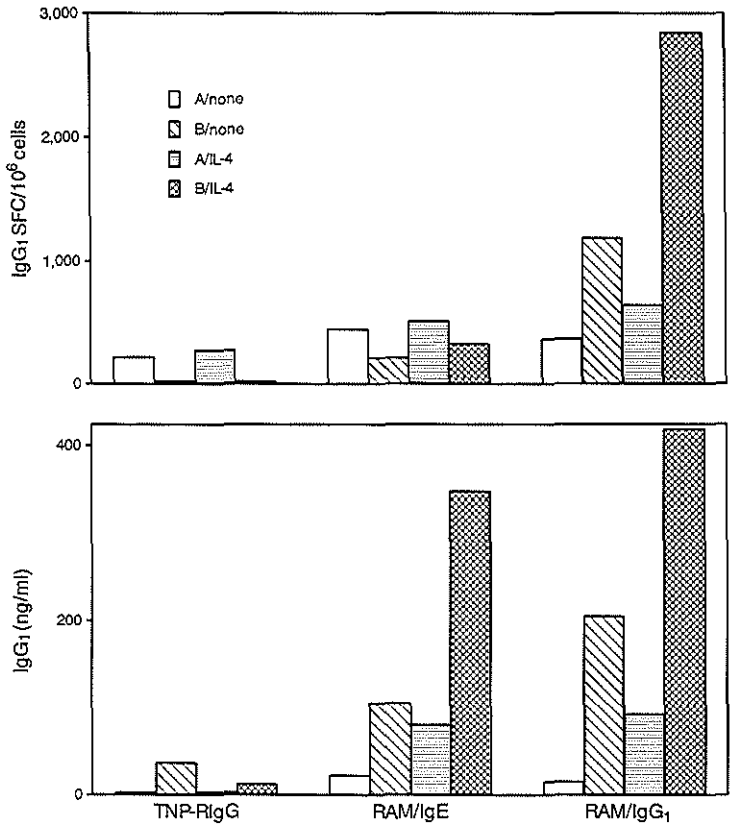

Figure 2. IL-4 treatment increases the number of IgG_1 secondary B cells in the spleen. Thy-1 depleted spleen cells from control treated mice (A) and IL-4 treated mice (B) were cultured with irradiated (30 Gy) CDC35 cells in the presence of either 30 ng/ml TNP-RIgG, 10ng/ml RAM/IgE or 10 ng/ml RAM/IgG_1. The number of IgG_1 spot forming cells (SFC) per 10^6 cells and the cumulative IgG_1 production were determined on day 5 by ELISA-spot assay and ELISA, respectively.

A possible explanation for this phenomenon is that during the five days of culture ε^+ B cells that were probably also $\gamma_1{}^+$, switch completely to ε^+ B cells that are no longer

capable of producing IgG_1. This might be the reason that they were not detected in the ELISA-spotassay for IgG_1. IL-4 treatment of mice reduced the number of TNP-specific IgG_1 secreting cells. No IgG_1 secreting cells were found after five days culture of T cell depleted splenocytes of IL-4 treated mice when TNP-RIgG is used as the antigen, whereas in the same situation IgG_1 producing cells were found when cells were used from control treated mice (Figure 2). Prolonged IL-4 treatment also reduced the production of antigen specific IgG_1 as was observed by stimulating cells with RAM/IgG_1 (data not shown).

DISCUSSION

The main finding from this study is that prolonged IL-4 treatment after primary immunization with a T cell dependent antigen led to a strong increase in polyclonal but not in antigen-specific IgG_1. The observed effects of IL-4 were specific for IgG_1 as it was shown that the serum IgG_{2a} levels were not influenced by IL-4 treatment. The IgG_1 memory formation specific for TNP decreased as a result of prolonged IL-4 treatment, resulting in a decreased TNP-specific IgG_1 respons after booster. On the contrary, the pool of secondary B cells already switched to IgG_1 increased. The use of a culture system in which B cells were stimulated polyclonally in an isotype-specific fashion, independent of their antigenic specificity, allowed this conclusion.

Part of these B cells turned out to be also IgE positive, in that they could be stimulated in a T-B cell culture system by RAM/IgE to produce IgG_1. Such cells have been described by Snapper et al.[7] and are the intermediate cell type in the process of sequential isotype switching from IgM via IgG_1 to IgE[8,9]. Other mechanisms which allow the existence of $\gamma_1\varepsilon$ double-positive B cells are alternative splicing of long nuclear RNA and trans-splicing[10]. In both mechanisms switching occurs without DNA recombination.

An explanation for the markedly increased polyclonal IgG_1 production in IL-4 treated mice could be enhanced differentiation of pre-activated B cells as described for IL-6[11] or prolonged survival of pre-activated B cells allowing enhanced clonal outgrowth[12]. Another explanation could be the release of the Fc receptor-mediated inhibition of B cell activation by IL-4[13]. This may lead to activation of B cells which can subsequently switch to IgG_1 causing high levels of IgG_1. This high level of polyclonal IgG_1 could possibly inhibit the TNP-specific IgG_1 levels by overruling this antigen-specific response.

Altogether these results indicate that caution has to be taken when IL-4 administration is considered to increase the production of IgG_1 specific for a particular antigen.

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Chapter 6

Secondary IgE responses *in vivo* are predominantly generated via $\gamma_1\epsilon$-double positive B cells

R. van Ommen, A.E.C.M. Vredendaal and H.F.J. Savelkoul

Scand. J. Immunol, in press.

SUMMARY

We have recently developed a model in which mice were treated with IL-4 after primary TNP-KLH immunization, resulting in elevated total serum IgG_1 and IgE levels, but decreased antigen-specific levels and memory formation for these isotypes. In this report, we describe that the former observed effects of IL-4 are mediated at the B cell and not the T cell level. Major changes occurred in the $\gamma_1\varepsilon$-double positive B cell population which is increased as a result of IL-4 treatment. Moreover, it is shown that $\gamma_1\varepsilon$-double positive B cells can develop *in vitro* out of γ_1-positive primed B cells and that these double-positive cells can differentiate into IgG_1- and IgE- secreting cells. Existence of $\gamma_1\varepsilon$-double-positive memory B cells can explain differences in cytokine dependence of TNP-specific memory IgG_1 and IgE responses found after adoptively transferring primed spleen cells into irradiated naive recipients. Whereas the IL-4 independent TNP-specific memory IgG_1 responses could be blocked efficiently by neutralizing IL-5 and IL-6, TNP-specific memory IgE responses were virtually not susceptible to such treatment. These IgE responses were also not susceptible to IFN-γ, used in doses that could inhibit the primary IgE response. Inhibition of the TNP-specific memory IgG_1 response by neutralizing IL-5 and IL-6 is accompanied by a 10 fold increase of the IL-4 independent TNP-specific IgE memory response. These data indicate that secondary IgE responses primarily result from B cells that are either switched to IgG_1, or are double positive for IgG_1 and IgE, thereby suggesting a minor role for ε-single positive B cells in secondary IgE responses.

INTRODUCTION

IL-4 was found to be obligatory for the induction of IgE synthesis as based on its ability to induce the expression of ε-germline transcripts [1, 2], the inhibition of parasite-induced IgE responses by neutralizing antibodies against IL-4, [3] the absence of IgE responses in nematode-infected mice that were made IL-4 deficient by gene targeting [4] and the IgE hyperproduction in IL-4 transgenic mice [5-7]. Although IL-4 induces accumulation of germline transcripts for γ_1 [8, 9], which correlates with an increase in IgG_1-secreting cells [10], it is not required for IgG_1 synthesis as was shown in IL-4 deficient mice [4].

Functional studies indicate that IL-4 is a "switch inducing" factor. It alters the chromatin structure of the $S\gamma_1$ region [9] and induces accumulation of germline γ_1 and ε transcripts [1, 8, 11, 12]. The induction of γ_1 germline transcripts is inhibited by IFN-γ [8],

which also inhibits the *in vivo* production of IgG_1 and IgE [13]. IL-4 and IFN-γ act reciprocally in the determination of IgG_1 and IgE responses [14], whereas IL-5 seems to act synergistically with IL-4 in that it specifically enhances the accumulation of productive γ_1 and ε transcripts [15]. IL-5 and IL-6 are both cytokines involved in the maturation of B cells to become Ig-secreting plasma cells [16, 17]. However, we have recently shown that primary and secondary antigen-specific and total IgG_1, but not IgE responses are dependent on IL-6 to obtain peak levels [18].

Like IgG_1 responses, secondary IgE responses are partially IL-4 independent [18-21], although IL-4 seems to be still important in sustaining *in vivo* IgE responses [22]. The IL-4 independent component of a secondary IgE response developed only when IL-4 was present during the primary response [3, 19], indicating that such response was based on B cells that have already switched to IgE. Such cells were, although in low frequency, found after immunization and can produce IgE upon subsequent culturing [23].

Recently, evidence for sequential isotype switching from μ to ε via γ_1 was reported [2, 24]. This isotype switching mechanism could explain why during the process of switching to IgE many cells express both γ_1 and ε on their membranes [25]. It was further suggested that individual $\gamma_1\varepsilon$ double positive B cells can co-secrete these two isotypes [24].

Most of the studies on the involvement of IL-4 in IgE responses used mAb to neutralize IL-4. However, recently IgE regulation in IL-4 transgenic mice was described [5-7]. These mice are exposed to high levels of IL-4 during development which can lead to aberrant situations. Therefore, we used an alginate encapsulation method in which an IL-4 producing cell line is encapsulated and subsequently implanted in the peritoneal cavity of normal developed mice, to modulate *in vivo* IL-4 levels [19]. The aim of this study was to investigate the effect of thus modulated IL-4 levels on the memory formation for IgG_1 and IgE responses *in vivo* with respect to B and T cells. It was found that the previously observed IL-4 induced decreased antigen-specific memory formation for IgG_1 and IgE [19] was mediated at the B cell and not the T cell level. Flow cytometry revealed that IL-4 exerted its effect mainly on the pool of $\gamma_1\varepsilon$-double positive B cells. It was found *in vitro* that these $\gamma_1\varepsilon$-double positive B cells developed out of γ_1-positive B cells. Moreover, data are presented that suggest that $\gamma_1\varepsilon$-double positive B cells can either develop into IgG_1- or IgE-secreting cells, leaving open the question if both isotypes are produced at the same time by one B cell. *In vivo* the existence of those cells three months after priming is indirectly shown by differences in cytokine requirement of memory B cells. This cytokine dependence is not changed by IL-4 treatment during the memory formation, indicating that IL-4 mediates its effects on pools of B

cells, not inducing intrinsic changes in B cells leading to different cytokine requirements.

MATERIALS AND METHODS

Mice

Female BALB/c mice were bred and maintained at the Department of Immunology of the Erasmus University. All mice were at an age of 12-16 weeks at the start of the experiments. They were held in light-cycled rooms and had access to acidified water and pelleted food ad libitum. The microbiological status of the mice fulfilled the standard of "specific pathogen free V" according to the criteria of the Dutch Veterinary Inspection, as described in the law on animal experiments. The experiments were approved by the Animal Experiments Committee of the Erasmus University.

Immunization

KLH (Pierce, Rockford, IL, USA) was trinitrophenylated to a level of 25 TNP residues per 10^5 Da of KLH (as determined spectrophotometrically) [26] by using trinitrobenzenesulphonic acid (Eastman Kodak, Rochester, NY, USA). Mice were injected with 0.2 ml containing either 10 or 100 μg TNP-KLH adsorbed on 2 mg alum i.p. as indicated in the Results section.

Cytokine treatment

Mice immunized with 100 μg TNP-KLH adsorbed on alum were implanted i.p. with $2x10^6$ CV-1/IL-4 cells encapsulated in alginate every two weeks for a period of four months [19,27,28]. The CV-1/IL-4 cells were a kind gift of Dr. N. Arai (DNAX Research institute, Palo Alto, CA, USA]. Empty beads encapsulated in alginate were used as control. No immunological effects were induced by this treatment in all experiments. This control is sufficient, since it has been shown that the IL-5 producing cell line CV-1/IL-5 encapsulated in alginate could not restore the impaired IgE synthesis in SJA/9 mice (data not shown). Encapsulated CV-1/IL-4 cells *in vivo* produce IL-4 for a period of at least two weeks [28]. The IFN-γ treatment consisted of two injections of 25 μg of purified IFN-γ dialysed and diluted in PBS + 10 mM cysteine per day on days 1 to 5 of the primary response. During the secondary response two injections of 40 μg per day were given i.p. on days 1 to 3.

Adoptive transfer of spleen cells

Spleens of control treated and IL-4 treated mice were removed under aseptic conditions and single cell suspensions were prepared. Spleen cells ($1x10^7$) were transferred via the tail vein into naive recipients. The recipients had been sublethally irradiated (6 Gy) with a Cesium-137 source (Gammacell 40, Atomic Energy of Canada, Ottawa, Canada) 1 day before cell transfer. All reconstituted mice were i.p. immunized with 10 µg TNP-KLH adsorbed on alum immediately after transfer.

Anti-cytokine treatment

Mice were treated *in vivo* by i.p. injection of neutralizing antibodies directed to IL-4 (11B11, rat IgG_1, 10 mg/mouse) [29], IL-5 (TRFK-5, rat IgG_1, 2 mg/mouse) [30], IL-6 (20F3, rat IgG_1, 2 mg/mouse) [31], IFN-γ (XMG1.2, rat IgG_1, 10 mg/mouse) [32] alone or in combination. Rat mAb specific for E. coly ß-galactosidase (GL113) [27] was used as an IgG_1 isotype control. The mAb were purified from culture supernatants by protein G affinity chromatography [33]. These doses of antibody have been widely shown to be sufficient to neutralize the respective cytokine activities in a variety of systems.

Isotype specific ELISA

Total serum IgE, IgG_1 and IgG_{2a}, levels were measured by isotype-specific ELISA as described previously [19,34]. Detection limits for the IgE, IgG_1 and IgG_{2a} ELISA were 0.5 ng/ml, 0.2 ng/ml and 0.3 mg/ml, respectively. TNP-specific IgG_1 and IgE serum levels were determined as previously described [19], with 0.2 ng/ml and 1 ng/ml as detection limit in the ELISA, respectively.

ELISA-spot assay

Nitrocellulose bottomed 96-wells Multiscreen HA plates (Millepore Co., Bedford, MA, USA) were coated with either EM95 [35], 2 µg/ml, or goat-anti-mouse IgG_1, 1 µg/ml (Southern Biotechnology), and blocked with PBS containing 1% BSA. The plates were then incubated with spleen cell samples for four hours in a humidified and vibration free 5% CO_2 incubator. Plates were washed once with 0.05% Tween 20 in distilled water, to remove the cells and twice with PBS containing 0.1% BSA and 0.05% Tween 20. Subsequently, the plates were treated as in a normal ELISA. Development was done by using AEC substrate which was prepared by dissolving 25 mg of 3-amino-9-ethyl carbazole (AEC) (Sigma) in 2 ml dimethylformamide, followed by addition of 95 ml of 0.05 M acetate buffer, pH 5.0 and 40 µl of 30% H_2O_2. The substrate solution was

filtered (0.2 μm) to remove particulate matter. Developed plates were dried and the red spots were enumerated under low magnification (10x) using a dissecting microscope (Stemi SV 6, Zeiss, Oberkochen, FRG) equipped with a coaxial reflected light source.

Preparation of B cells and T cells

Splenic B cells were prepared from control and IL-4 treated mice. T cells were cytotoxically eliminated by treating spleen cells with anti-Thy-1.2 (clone F7D5; Serotec, Oxford, U.K.) and low-tox guinea pig complement (Cederlane, Hornby, Ontario, Canada) in a two-step procedure at 0°C and 37°C, respectively [36]. For the preparation of T cells, B cells and granulocytes were eliminated by a similar procedure using anti-B220 (clone RA3-6B2) and anti-Granulocyte (clone RB6-8C5). Both mAb were kindly provided by Professor Dr. W. van Ewijk from our department. Viable cells were isolated by flotation on Histopaque 1119 (Sigma Chemical Co., St. Louis, MO, USA). The percentage of residual Thy-1$^+$-cells was < 2% and B220$^+$-cells < 5% as determined by flow cytometry.

T-B cell culture

CDC35, an I-A^d restricted rabbit-Ig specific Th2 clone [37], a kind gift of Dr. D.C. Parker, was maintained by stimulating, every two weeks, with irradiated BALB/c spleen cells (30 Gy) and 50 μg/ml rabbit IgG (RIgG) (Sigma) in complete RPMI 1640 medium, RPMI 1640 medium supplemented with 10% heat inactivated FCS, 2 mM glutamine, 0.1 M pyruvate, 100 IU/ml penicillin, 50 μg/ml streptomycin, 50 μM 2-mercapto-ethanol and 20 IU/ml IL-2. Prior to culture with B cells the CDC35 cells were washed and viability was assessed by trypan blue exclusion. Routinely, viability was >98%.

T-cell depleted spleen cells at 2.5x10^5 cells /ml were cultured in 8 replicate wells of flat-bottom microtiter plates together with 5x10^4 cells/ml of irradiated CDC35 cells (30 Gy) in 0.2 ml of complete RPMI 1640 medium at 5% CO_2 and 37°C. The following antigens were used for stimulation: 30 ng/ml TNP-RIgG, 10 ng/ml TNP-KLH + 10 ng/ml RIgG, 10 ng/ml RIgG, 10 ng/ml rabbit-anti-mouse IgE (RAM/IgE) [38], or 10 ng/ml rabbit-anti-mouse IgG_1 (RAM/IgG_1) [39], 10 ng/ml rabbit-anti-mouse $IgG_{2a/2b}$ (RAM/$IgG_{2a/2b}$) (Nordic Immunology, Tilburg, the Netherlands). These rabbit-anti-mouse isotype specific antibodies were tested extensively for isotype specificity. After 5 days of culture, cells were harvested for ELISA-spot assay and supernatants for ELISA. For large scale experiments, essentially similar cultures were performed at 2 ml in 24 wells flat-bottom plates. On day 4 of the culture cells were harvested and stained for FACScan analysis.

FACScan

Cells ($2.5x10^5$) were incubated on ice for 30 min with the appropriate mAb, either as undiluted culture supernatant or optimally titrated purified mAb followed by a triple wash with PBS containing 1% BSA and 0.1% azide. After using unconjugated mAb, another 30 min incubation was performed with a conjugated-isotype-specific second-step mAb on ice. After a triple wash the cells were taken up in isotonic fluid and analyzed on a FACScan analyzer (Becton Dickinson, Mountain View, CA, USA). A life gate was used to gate out rare dead cells and erythrocytes. The following mAb were used for staining: biotin-conjugated goat-anti-mouse IgG_1 (Southern Biotechnology) at 12.5 µg/ml, rat-anti-mouse IgE (Pharmingen, San Diego, CA, USA) at 10 µg/ml, rat-anti-mouse B220 (clone RA3-6B2) and rat-anti-mouse Thy-1 (clone 59-AD2.2) both as undiluted culture supernatant. rabbit-anti-rat IgG, F(ab')2-fragments-FITC conjugated (Cappel/Organon Technika, Oss, the Netherlands, 1/100 diluted), and R-Phycoerythrin conjugated streptavidin (Caltag, San Francisco, CA, USA, 1/20 diluted) were used as second step reagents. To prevent cytophilic binding of the immunoreagents to FcγR staining was performed after mild acid treatment in the presence of 2% normal goat serum (Dako A/S, Glostrup, Denmark). Stainings with rabbit-anti-rat IgG, F(ab')2-fragments-FITC conjugated (Cappel/Organon), and R-Phycoerythrin conjugated Streptavidin (Caltag) alone were used as control.

Acid treatment

To remove cytophilic bound immunoglobulin molecules, spleen cells were treated with 0.05 M acetate buffer (pH 4.0) containing 0.085 M NaCl, 0.005 M KCl, and 1% FCS for 1 min on ice as described by Kumagai et al. [40]. To neutralize the acid to the range of pH 7.2, PBS supplemented with 0.1 M Hepes (Gibco Life Technologies Ltd, Paisley, Scotland) was added to the suspension. Next the cell suspension was underlaid with FCS. The cells were pelleted and washed two times with HBSS. No decrease in viability was observed as determined by trypan blue exclusion.

Statistical analysis

Differences between groups were analyzed using the Student's *t-test*. Values of $p<0.05$ were considered significant.

RESULTS

IL-4 inhibits memory formation of TNP-KLH specific B cells but not T cells

In a previous study we have shown that prolonged IL-4 treatment induced decreased TNP-specific IgG_1 and IgE responses after primary immunization, that was accompanied by a decreased memory formation for these two isotypes [19]. In order to determine whether IL-4 exerted this inhibitory effect on the TNP-KLH-specific B cells, T cells or both, we reconstituted irradiated mice with TNP-KLH-primed B cells from control treated mice and TNP-KLH-primed T cells from IL-4 treated mice and vice versa. The B cells (>98% $B220^+$ cells) and T cells (>95% Thy-1^+ cells) were isolated three months after the last of 10 IL-4 or control administrations to exclude direct effects of exogenously IL-4, for it is to be expected that no exogenously IL-4 is produced at this time point. Furthermore, at this time point no differences were found in the percentage and numbers of T and B cells between control and IL-4 treated mice (data not shown).

Irradiated control mice reconstituted with primed B cells from IL-4 treated mice displayed after immunization with TNP-KLH significant lower TNP-specific IgG_1 and IgE levels when compared to mice reconstituted with primed B cells from control mice. These levels were not influenced by using either T cells from control or IL-4 treated mice (Figure 1). Apparently, T cells from IL-4 treated mice were not able to inhibit or amplify memory B cells from control treated mice to give rise to TNP-specific IgG_1 or IgE production. Moreover, T cells from control mice failed to amplify the induction of TNP-specific B cells from IL-4 treated mice to produce TNP-specific IgG_1 or IgE levels comparable to those of control treated mice. These results suggest that IL-4 exerted its inhibitory effect on the TNP-specific memory formation for IgG_1 and IgE at the B cell level, not influencing the memory T cell development.

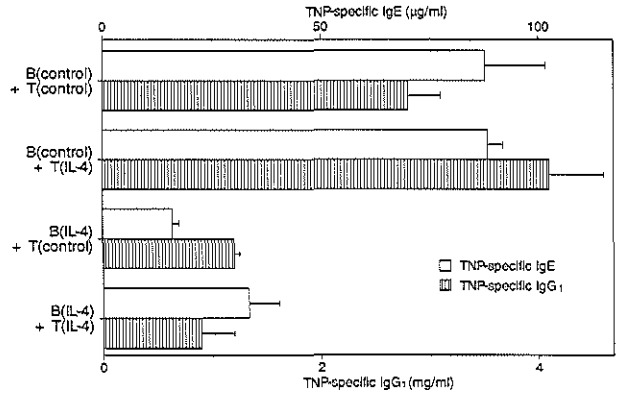

Figure 1. Irradiated mice (6 Gy) were reconstituted with combinations of $4x10^6$ purified TNP-KLH primed B cells and $4x10^6$ purified TNP-KLH primed T cells from control and IL-4 treated mice as indicated. All reconstituted mice were boosted with 10 µg TNP-KLH at day 0. TNP-specific IgG_1 and IgE serum levels were determined at day 9. Results are represented as arithmetic mean ± SEM (n=4-6). TNP-specific IgG_1 is expressed in mg/ml and TNP-specific IgE is expressed in µg/ml.

$\gamma_1\varepsilon$-double positive B are increased in IL-4 treated mice after 10 IL-4 administrations

To study the effect at the B cell level more precisely, we examined the number of switched B cells in spleen of control and IL-4 treated mice by FACScan analysis. At day 2 and 3 after the last of 10 administrations the number and percentage of γ_1-single, ε-single and $\gamma_1\varepsilon$-double positive B cells were determined (Table 1). On these two days only the number of $\gamma_1\varepsilon$ double-positive B cells were significantly increased (2 fold) in the IL-4 treated mice when compared to control mice. No major differences were seen when comparing the number of γ_1-single or ε-single positive B cells in spleens of IL-4 treated mice and control mice at day 2 and 3 after the last of 10 administrations. Both in control and IL-4 treated mice 40% of the γ_1-positive B cells in the spleen were also μ-positive (data not shown). These results show that IL-4 treatment caused its effect mainly by increasing the pool of $\gamma_1\varepsilon$-double positive B cells.

Table 1. Number of switched B cells in spleens of control and IL-4 treated mice

	control treated		IL-4 treated	
sIg expression	2	3	2	3
γ_1-single$^+$	58740 (53)	69300 (63)	59500 (54)	89600 (60)
ε-single$^+$	178 (0.2)	59 (0.1)	85 (0.1)	51 (0.03)
$\gamma_1\varepsilon$-double$^+$	4450 (4.0)	5742 (5.2)	8500 (7.7)	8192 (5.5)

Surface marker expression evaluated by FACScan analysis of total spleen cells from control and IL-4 treated mice at day 2 and 3 after the last of 10 alginate administrations. Cell suspensions were pooled from two mice. Rare dead cells and erythrocytes were gated out. Results are the absolute number of positive cells ($x10^3$). Number of lymphocytes per spleen based on the forward scatter/ side scatter plot were $1.1x10^8$ in control mice both on day 2 and 3. In spleens of IL-4 treated mice the number of lymphocytes were $1.1x10^8$ and $1.5x10^8$ on day 2 and 3, respectively. Numbers in parentheses represent percentage of lymphocytes.

$\gamma_1\varepsilon$-double positive B cells can develop in either IgG_1 or IgE secreting cells

To investigate if the increase in $\gamma_1\varepsilon$-double positive B cells would result in increased numbers of IgG_1- and IgE- secreting cells after isotype-specific stimulation we used a cognate T-B cell culture system [37]. In this system T cell depleted splenocytes from control and IL-4 treated mice were stimulated with antigen and CDC35 cells, a Th2 cell-line specific for rabbit IgG (RIgG). In this culture system rabbit-anti-mouse IgE (RAM/IgE), and rabbit-anti-mouse IgG_1 (RAM/IgG_1) were used to selectively stimulate ε- and γ_1-positive B cells through their membrane bound isotypes. TNP coupled to RIgG (RIgG-TNP) was used to stimulate TNP-specific B cells, whereas TNP-KLH and RIgG served as controls for the *in vitro* stimulation. Both TNP-KLH and RIgG were used as a control because stimulation by these two antigens gave a measurement of non-cognate activati-

on of B cells in this system.

In all experiments this non-cognate activation leading to IgG_1 and IgE producing cells at day 5 of the culture was less than 3 % of the number of IgG_1 or IgE producing cells that developed after specific stimulation. Addition of IL-4 (100 IU/ml) to the cultures resulted in an 2 fold increase of this non-cognate activation (data not shown). Moreover, addition of IL-4 failed to increase the number of IgG_1- and IgE- secreting cells after 5 days of culture, when compared to the situation in which no IL-4 was added (data not shown). This indicated that the CDC35 cells themselves produced enough IL-4 after cognate-stimulation to give rise to IgG_1- and IgE-secreting cells.

Kinetic studies had shown that a culture period of five days was optimal for the development of IgG_1- and IgE-secreting cells, when B cells were used from primed mice (data not shown). After 5 days of culture the numbers of IgG_1- and IgE-secreting cells were determined by ELISA-spot assay. Results representative for two experiments showed that upon stimulation of T cell depleted spleen cells from IL-4 treated mice with the Th2 cell line in the presence of RAM/IgG_1 >2 fold more IgG_1-secreting cells were formed as compared to the situation in which splenic B cells from control treated mice were used (Figure 2C). A similar increase was seen when RAM/IgE was used as antigen in these cultures (Figure 2C).That these differences were not caused by an increased proliferation of T cell depleted spleen cells from IL-4 treated mice is shown in Figure 2A. The number of IgE-secreting cells was dramatically increased when spleen cells were used from with IL-4 treated mice in combination with RAM/IgE and to a lesser extent with RAM/IgG_1 as antigen (Figure 2B). These data suggested an increase in B cells already switched to IgG_1 (>2 fold) and IgE (7 fold) as a result of prolonged IL-4 treatment. When rabbit-anti-mouse $IgG_{2a/2b}$ (RAM/$IgG_{2a/2b}$) was used as stimulating antigen 5585 IgG_1 and 288 IgE-secreting cells per culture of T cell depleted spleen cells of control treated mice on day 5 were found. Upon culturing T cell depleted spleen cells of IL-4 treated mice with RAM/$IgG_{2a/2b}$ 4296 IgG_1 and 169 IgE-secreting cells per culture were found on day 5. This result makes clear that the observed differences in IgG_1- and IgE- secreting cells were not caused by anti-Ig reactivity or FcR mediated effects of RAM/IgG_1 and RAM/IgE. Otherwise, similar increases would have been found upon culturing with RAM/$IgG_{2a/2b}$.

The increase in IgG_1- and IgE- secreting cells is supported by the cumulative immunoglobulin levels produced during 5 days of culture. When stimulating ε-positive B cells in the pool of T cell depleted spleen cells from control mice using RAM/IgE, 56 ng/ml of IgE was produced. During the same period cells from IL-4 treated mice produced 217 ng/ml IgE (Table 2). As result of the IL-4 treatment an increase in the IgG_1 production

was also seen when the γ_1-positive B cells of the T cell depleted spleen cells were stimulated with RAM/IgG_1 (19 μg/ml when cells were used from control mice and 29 μg/ml when cells were used from IL-4 treated mice) (Table 2). Only marginal increases of IgG_{2a} production were found when T cell depleted spleen cells from IL-4 treated mice instead of control treated mice were stimulated with RAM/IgG_1 or RAM/IgE (Table 2). Addition of IL-4 to the cultures did not markedly increase the production of the measured isotypes (Table 2).

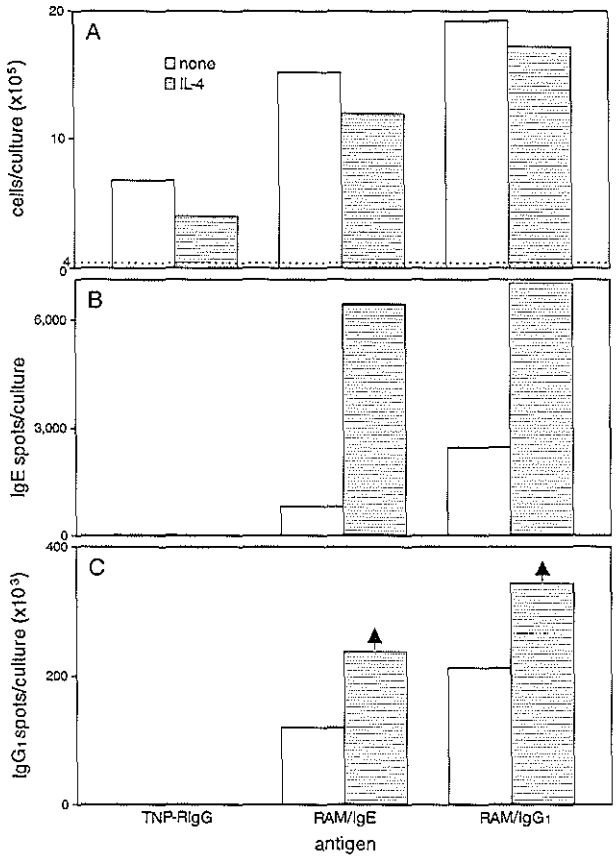

Figure 2. IL-4 treatment increases the number of to IgE and IgG_1 switched B cells in the spleen as compared to control mice. T cell depleted spleen cells were cultured with irradiated (30 Gy) CDC35 cells in the presence of either 30 ng/ml TNP-RIgG, 10 ng/ml RAM/IgE or 10 ng/ml RAM/IgG_1. The total number of cells, the dashed line indicates the maximum number of cells in cultures stimulated with TNP-KLH and RIgG (A), and the numbers of IgE- (B) and IgG_1-producing cells (C) per culture (average number per eight pooled wells of 200 μl) were determined at day 5.

Collectively, these results point to a $\gamma_1\varepsilon$-double positive B cell population that can produce both IgG_1 and IgE upon stimulation via their isotype, and that is increased as result of IL-4 treatment.

Table 2. Isotype levels in the supernatants of secondary T-B cell cultures *in vitro*

	control		IL-4 treated	
isotype	RAM/IgE	RAM/IgG_1	RAM/IgE	RAM/IgG_1
		no IL-4 added to the cultures		
IgE (ng/ml)	56	279	217	426
IgG_1 (µg/ml)	10	19	11	29
IgG_{2a} (ng/ml)	130	160	183	313
		IL-4 (100 IU/ml) added to the cultures		
IgE (ng/ml)	147	286	322	406
IgG_1 (µg/ml)	13	18	15	25
IgG_{2a} (ng/ml)	96	86	255	175

$2.5x10^5$ /ml T cell depleted spleen cells from control and IL-4 treated mice and $5x10^4$ /ml irradiated (30 Gy) CDC35 cells were cultured for 5 days in 8 wells of 200 µl in the presence of 10 ng/ml RAM/IgE or RAM/IgG_1. Results represent supernatant levels as determined by ELISA.

γ_1-positive secondary B cells can undergo sequential isotype switching to $\gamma_1\varepsilon$-positive B cells

We next studied whether $\gamma_1\varepsilon$-double positive B cells, besides *in vivo*, also developed during the *in vitro* T-B cell cultures. Therefore, we examined the percentage of switched B cells in the pool of $B220^+$ cells at days 0 and 4 upon culturing in the presence of antigen and CDC35 cells by flow cytometry (Table 3). To rule out a significant contribution of cytophylic IgG_1 or IgE to the estimation of the percentage γ_1-positive and ε-positive cells, cells were treated with acid before staining. This procedure has previously shown to remove all cytophilically FcR bound isotypes [40]. Moreover, all stainings were carried out in the presence of 2% normal goat serum, to prevent aspecific cytophilic binding of the immunoreagents to FcγR. At day 0 the percentages of ε-positive, γ_1-positive and $\gamma_1\varepsilon$-double positive B cells were determined and these cells were stimulated with RAM/IgG_1 or RAM/IgE. At day 0, no differences were seen in percentages of ε-positive and $\gamma_1\varepsilon$-double positive B cells when comparing control mice and IL-4 treated mice. IL-4 treatment had increased the percentage of γ_1-positive B cells by 16%. By day 4 the percentage of γ_1-positive B cells decreased 13% when RAM/IgG_1 was used as antigen. At the same time the fraction of ε-positive B cells in this culture showed a 3-fold increase. Most of these ε-positive B cells, were $\gamma_1\varepsilon$-double positive. These results argue for a sequential switch in these cultures in which γ_1-positive B cells stimulated by RAM/IgG_1 and Th2 cells switch to ε-positive B cells with $\gamma_1\varepsilon$-double positive B cells as an intermediate stage.

Table 3. Surface Ig phenotype of B cells in secondary T-B cell cultures *in vitro*

		sIg				
	stimulation	ε total	ε^*	$\gamma_1\varepsilon$	γ_1 total	γ_1^*
day 0						
control	-	3	0	4	84	80
IL-4 treated	-	3	2	1	100	99
day 4						
control	RAM/IgG_1	2	1	1	82	81
IL-4 treated	RAM/IgG_1	9	2	7	87	80
control	RAM/IgE	0	0	1	87	86
IL-4 treated	RAM/IgE	4	2	2	78	76

ε^* = total $sIgE^+ - \gamma_1\varepsilon$ γ_1^* = total $sIgG_1^+ - \gamma_1\varepsilon$

Results are expressed as percentages of $B220^+$ B cells in cultures with RAM/IgE or RAM/IgG_1 as stimulating antigen. $2.5x10^5$ /ml T cell depleted spleen cells and $5x10^4$ /ml irradiated (30 Gy) CDC35 cells were cultured in 10 wells of 2 ml.

Such sequential switching was not seen for B cells from control treated mice, indicating that IL-4 is important for the commitment of B cells to undergo sequential switching. In cultures in which T cell depleted spleen cells from control treated mice were stimulated with RAM/IgE the percentage of γ_1-positive B cells decreased, but no difference was seen in the percentage of ε-positive and $\gamma_1\varepsilon$-double positive B cells. These results suggest that stimulated $\gamma_1\varepsilon$-double positive B cells differentiate to IgG_1- and/or IgE-secreting cells that do not express sIg, and are therefore no longer detectable by flow cytometry.

$\gamma_1\varepsilon$-double positive B cells can explain the different cytokine requirements of memory IgG_1 and IgE responses

We have shown in adoptive transfer experiments that TNP-specific IgG_1 and IgE memory responses were not differentially influenced by T cells from control and IL-4 treated mice (Figure 1). Moreover, it was shown that $\gamma_1\varepsilon$-double positive B cells can develop both in IgG_1- and IgE- secreting cells *in vitro* (Figure 2). Therefore, we next studied the cytokine dependence of memory B cells to become IgG_1- or IgE- secreting cells *in vivo*.

Table 4. Influence of cytokines on TNP-specific secondary IgG_1 and IgE responses after adoptive transfer

	TNP-specific IgG_1		TNP-specific IgE	
treatment	day 9	day 12	day 9	day 12
control	2847±333	6720±697	87.6±14	61.9±67
+αIL-4	1376±293	5366±584	4.5±1.0	9.1±1.7
+αIL-4+αIL-5	2183±554	4127±1070	8.5±0.4	12.4±1.5
+αIL-4+αIL-6	1934±513	3999±463	6.6±1.0	5.8±0.7
+αIL-5+αIL-6	1483±150	2553±362	92.8±8.9	65.0±7.7
+αIL-4+αIL-5+αIL-6	1593±286	2332±361	110.4±12.4	57.1±7.2
irradiation control	66±12	35±0	0.015±0.011	0.005±0.001

Irradiated mice (6 Gy) were reconstituted with $1x10^7$ TNP-KLH primed spleen cells from control treated mice. Antibody treatment: control (GL113, 4 mg/mouse i.p.); αIL-4 (11B11, 10 mg/mouse i.p.); αIL-5 (TRFK-5, 2 mg/mouse i.p.); and αIL-6 (20F3, 2 mg/mouse i.p.). All reconstituted mice were boosted with 10 μg TNP-KLH adsorbed on alum i.p. Results are represented in μg/ml as arithmetic mean ± SEM (n=5).

To this end, irradiated mice were reconstituted with TNP-primed spleen cells from control treated mice (Table 4). Neutralizing the presence of IL-4 by anti-IL-4 mAb in these mice did not reduce the production of TNP-specific IgG_1, whereas it did partially reduce the production of TNP-specific IgE (Table 4). This production of TNP-specific IgE was 1820 fold higher than the IgE levels found in immunized non-reconstituted irradiated mice, and is 15% of the TNP-specific IgE level in reconstituted mice that received control mAb. This indicated that the TNP-specific IgE response after immunization of irradiated mice reconstituted with spleen cells from control treated mice was at least in part (15%) IL-4 independent. Using neutralizing mAb against IL-5 and IL-6 TNP-specific IgG_1 and IgE memory responses were analyzed in reconstituted recipient mice. The TNP-specific IgE memory responses turned out to be IL-5 and IL-6 independent, whereas the TNP-specific IgG_1 production was markedly decreased after these treatments. Moreover, these results also suggest a additive IL-5 and IL-6 dependence of the TNP-specific IgG_1 production during memory responses. In the presence of these neutralizing antibodies the complete TNP-specific IgE response was IL-4 independent. This may indicate that γ_1-positive and/or $\gamma_1\varepsilon$-double positive TNP-specific memory B cells, that are blocked to differentiate to IgG_1-secreting cells in the absence of IL-5 and IL-6 [18], can develop in IgE-secreting cells independently from IL-4.

As it was shown that IL-4 can commit B cells to undergo sequential isotype switching by which $\gamma_1\varepsilon$-double positive cells are formed, we also examined the cytokine requirement of TNP-specific memory B cells from IL-4 treated mice to produce IgG_1 and IgE. As stated before, no differences were seen in the percentages of splenic T and B cells between control and IL-4 treated mice at this time point. It was found that even prolonged IL-4 treatment for four months after primary immunization did not induce differences in the cytokine dependence of the TNP-specific memory B cells generated

(Table 5). IL-4 treatment reduced the generation of TNP-specific IgG_1 and IgE memory cells, but no differences were seen in the cytokine requirement for secondary TNP-specific IgG_1 and IgE responses in irradiated recipient mice reconstituted with either spleen cells from control or IL-4 treated mice. These results indicate that IL-4 treatment did not induce intrinsic changes in the TNP-specific memory B cells with respect to their cytokine dependence to become IgG_1 or IgE secreting plasma cell.

Table 5. Effect of prolonged IL-4 treatment on the cytokine dependence of TNP-specific memory B cells

	days after transfer		
treatment	7	9	12
		TNP-specific IgG_1	
control	130±22	925±348	1612±394
+αIL-4	77±20	515±101	1949±379
+αIL-5+αIL-6	79±12	517±74	808±123
+αIL-4+αIL-5+αIL-6	72±10	493±71	609±87
		TNP-specific IgE	
control	7.0±1.7	33.4±6.8	14.6±1.6
+αIL-4	0.2±0.04	2.7±0.6	4.0±1.2
+αIL-5+αIL-6	2.9±0.7	33.8±7.7	20.1±2.5
+αIL-4+αIL-5+αIL-6	3.6±1.5	29.0±7.8	17.2±4.5

Irradiated mice (6 Gy) were reconstituted with $1x10^7$ TNP-KLH primed spleen cells from IL-4 treated mice. Antibody treatment: control (GL113, 4 mg/mouse i.p.) αIL-4 (11B11, 10 mg/mouse i.p.); αIL-5 (TRFK-5, 2 mg/mouse i.p.); and αIL-6 (20F3, 2 mg/mouse i.p.). All reconstituted mice were boosted with 10 μg TNP-KLH adsorbed on alum i.p. Results are represented in μg/ml as arithmetic mean ± SEM (n=5).

Memory B cells are less dependent on IL-4 and resistant to IFN-γ to become IgE secreting cells *in vivo*

To more fully determine the role of cytokines in a non-adoptive transfer model, we immunized naive mice and mice primed three months before with TNP-KLH during which either IL-4 was neutralized, or IFN-γ administered. The effect of neutralizing IL-4 on TNP-specific memory IgE responses had already been described [22], although in that study mice were boosted already 3 or 6 weeks after priming. However, for IFN-γ comparable studies were not described.

Immunization of mice with 10 μg TNP-KLH resulted in a primary TNP-specific IgE response starting at a level $<<$ 0.03 μg/ml at day 0 and reaching a level of 0.48 μg/ml at day 14. This increase could be blocked completely by neutralizing IL-4, indicating the IL-4 requirement of the primary TNP-specific IgE response. This anti-IL-4 treatment was

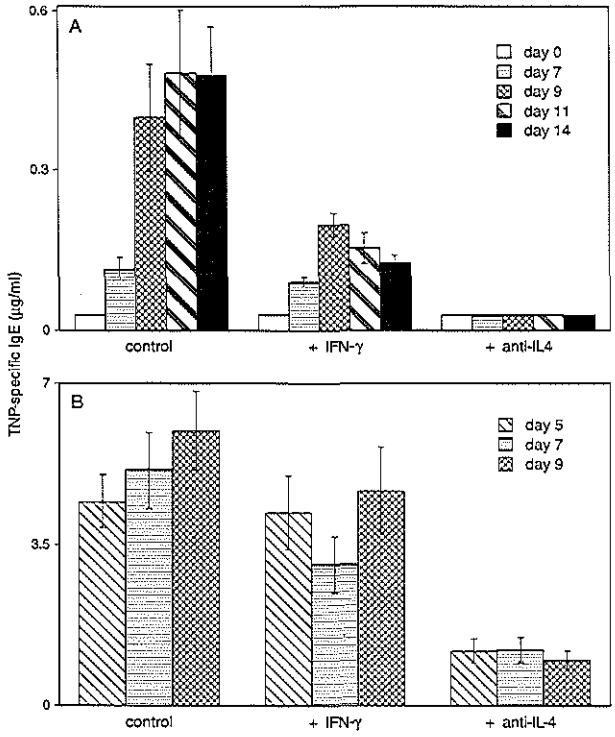

Figure 3. Effect of anti-IL-4 and IFN-γ on the primary (A) and secondary (B) TNP-specific IgE response. Mice were primed and boosted with 10 µg TNP-KLH adsorbed on alum followed by either a control, anti-IL-4 or IFN-γ treatment as described in the Materials and Methods section. Serum levels of TNP-specific IgE are expressed as arithmetic mean ± SEM (n=5).

more efficient in blocking the TNP-specific IgE response than was IFN-γ which inhibited the response up to 27% (Figure 3A). On the contrary, the secondary IgE response could only be inhibited up to 24% at day 7 by treatment with anti-IL-4, suggesting a partial IL-4 independence of this response (Figure 3B). TNP-KLH immunization in combination with a control treatment gave rise to 5.1 µg/ml TNP-specific IgE at day 7 as compared to 1.2 µg/ml that occurred in the presence of anti-IL-4. Moreover, this secondary TNP-specific IgE response could not be inhibited to any significant extent by IFN-γ treatment (Figure 3B), pointing to the minor role that IFN-γ plays during the secondary IgE response.

DISCUSSION

In this paper we show that the effects of prolonged IL-4 treatment during memory formation after priming with TNP-KLH, that we have previously described [19], are primarily mediated at the B cell level, leaving the T cells unchanged. Major changes occur in the $\gamma_1\varepsilon$-double positive B cell population which is increased significantly as a

result of IL-4 treatment. Moreover, *in vitro* T-B cell cultures in which primed B cells were stimulated in an isotype-specific manner by rabbit-anti-mouse isotype antibodies and a rabbit Ig-specific Th2 cell clone suggested the existence of a $\gamma_1\varepsilon$-double positive B cell population that can develop into both IgG_1- and IgE-secreting cells.

The number of B cells switched to IgG_1 and IgE increased *in vivo* as result of prolonged IL-4 treatment. This was found both at the membrane level and by stimulating B cells *in vitro* independent of their antigen-specificity but isotype-specific with rabbit anti-mouse isotype-specific antibodies, that were tested extensively for isotype specificity, and a rabbit Ig-specific Th2 cell clone. Moreover, it was found that upon stimulating γ_1-positive cells IgE-secreting cells could be detected on day 5 of culture. These IgE-secreting cells can either originate from γ_1-positive cells that have undergone sequential isotype switching [2, 24], or can be the result of pre-existing $\gamma_1\varepsilon$-double positive B cells [25] that differentiate to IgE-secreting cells after stimulation. When ε-positive B cells are isotype-specifically stimulated both IgG_1- and IgE-secreting cells are found that are most likely the result of $\gamma_1\varepsilon$-double positive cells that have the ability to differentiate either into IgG_1- or IgE-secreting cells. This result implicates that B cells exist that express IgE on their membrane, but have not yet undergone sequential isotype switching. Moreover, they show that these B cells can be stimulated via this membrane IgE to develop in IgG_1-secreting cells. An other possibility could be that these $\gamma_1\varepsilon$-double positive B cells upon stimulation can develop in cells that co-secrete these isotypes, an option for which evidence has been presented by Mandler et al. [24]. In all cases more IgG_1- and IgE-secreting cells per culture were found after culturing T cell depleted spleen cells of IL-4 treated mice than upon culturing T cell depleted spleen cells of control treated mice. These results are not caused by anti-Ig activity of the rabbit-anti-mouse isotype specific antibodies used because opposite effects were found upon culturing with $RAM/IgG_{2a/2b}$ as antigen. It has been described by Snapper et al. that Thy-1 positive B cells are highly enriched in IgE-secreting cells [41]. In this study we stimulated Thy-1 depleted spleen cells and determined the number of B cells that became IgG_1- and IgE-secreting cells. Therefore, Thy-1 depletion will not influence the numbers of IgE-secreting cells that were found as result of stimulation. In fact, Thy-1 depletion could have had a positive effect in lowering the background levels of cells that already secrete IgE independent of specific stimulation.

FACScan analysis revealed that upon IL-4 treatment the pool of $\gamma_1\varepsilon$-double positive cells is profoundly increased *in vivo* at days 2 and 3 after IL-4 administration. On the contrary no major changes occurred the pool of ε-single positive B cells by IL-4 treatment, suggesting that the previously found effect that IL-4 increased the total IgE serum levels [19] was mediated by increasing the pool of $\gamma_1\varepsilon$-double positive B cells. The

results of both the *in vitro* T-B cell culture system and the *in vivo* adoptive transfer experiments provide evidence for such double-positive B cells from which IgE-secreting cells originate through sequential isotype switching which has been reported by Yoshida et al. [2]. These authors provided molecular evidence for a sequential class switching from μ to ε via γ_1 [2]. This could explain why during the process of switching to IgE in the LPS system 75% of the ε-positive B cells coexpress γ_1, representing 9% of the total B cell population [25]. In our study further evidence is provided for such a sequential class switching detectable at the membrane level. When selectively stimulated, isotype-specific via γ_1, with a rabbit Ig-specific Th2 clone and RAM/IgG_1 antibody as antigen, the number of γ_1-positive splenic B cells decreased while there was an increase in the percentage of $\gamma_1\varepsilon$-double positive B cells from 1 to 7% of all $B220^+$ B cells. This was only seen when B cells were obtained from mice that were repeatedly treated with IL-4. We therefore conclude that IL-4 commits γ_1-positive B cells to undergo a sequential switch to ε-positive B cells. However, it is also possible that IL-4 increases B cell populations resulting in the possibility to detect sequential isotype switching at the membrane level. A successive DNA deletion could explain the simultaneous expression of $sIgG_1$ and sIgE, if the γ_1 mRNA expression is stable and functional even after the switch to ε. Other mechanisms which allow the existence of $\gamma_1\varepsilon$-double positive cells are the alternative splicing of long nuclear RNA and trans-splicing [42]. In both mechanisms switching occurs without DNA recombination.

When studying lipopolysaccharide-activated murine B cells, IL-4 induced IgE class switching occurred predominantly through sequential isotype switching [24]. In this system it was also shown that LPS stimulation in the presence of IL-4 resulted in $\gamma_1\varepsilon$-double positive cells that could produce IgG_1 as well as IgE. Recently, evidence for a successive class switching was reported in the human system by comparing $S\mu$-$S\varepsilon$ junctions in IL-4 treated human B lymphoblastoid cells [43]. These composite switch regions contained $S\gamma$ sequences, indicating that sequential switching had occurred. The presented data from our study show that sequential isotype switching is also likely to occur during secondary responses. It is shown that γ_1-positive primed B cells upon stimulation develop in $\gamma_1\varepsilon$-double-positive B cells that can differentiate in both IgG_1- and IgE-secreting cells.

The fact that memory B cells can undergo sequential isotype switching makes it possible that they are not tightly controlled by T cells. This could be the reason that the TNP-specific IgG_1 and IgE responses in irradiated control mice are not influenced by using T cells from control or IL-4 treated mice. Using an adoptive transfer system, we studied the cytokine dependence of TNP-specific secondary IgG_1 and IgE responses. Such system was chosen, because it is known that secondary IgE responses are

preferentially enhanced after transferring spleen cells in irradiated control mice [19, 44]. It was found that IL-5 and IL-6 were necessary for maximum *in vivo* TNP-specific IgG_1 responses. IL-4 treatment during the primary response did not chance this cytokine dependence. These results with respect to IgG_1 are in line with the observation that IL-4 can induce membrane bound IgG_1 on B cells, whereas IL-2 and IL-5 are necessary for subsequent IgG_1 secretion [45]. By using the adoptive transfer system it was clearly shown that the TNP-specific secondary IgE response is partially IL-4 independent in that it could not be completely inhibited by neutralizing antibodies against IL-4. This IL-4 independent IgE level is even higher than the IL-4 independent IgE response in boosted mice (Figure 3B). Surprisingly, in the absence of IL-5 and IL-6 no inhibition of the secondary TNP-specific IgE response by anti-IL-4 mAb was detectable. A possible explanation for these results is that TNP-specific IgG_1 memory B cells do not mature to IgG_1-secreting plasma cells in the absence of IL-5 and IL-6, since these two cytokines are likely to be involved in this process [16-18]. The γ_1-positive, or $\gamma_1\varepsilon$-double positive B cells that are arrested could subsequently switch to become ε-positive B cells for which they need less IL-4 or eventually can switch independently of the presence of IL-4. These B cells do not appear to be dependent on IL-5 and IL-6 to become IgE-secreting cells [18]. Other factors that induce or potentiate secretion of IgE like IL-13 in the human system [46], and that are not analyzed in our study could be involved in the development of IgE-secreting cells. Although it has not yet been ascertained that IL-13 induces IgE secretion by mouse B cells it might be possible that under certain circumstances IL-13 induces B cells to produce IgE.

Recently, it has been described that neutralizing anti-IL-4 antibodies, when complexed with IL-4, can serve as reservoirs for long-term (at least 9 days) delivery of cytokines *in vivo* [47]. It is possible that in the absence of IL-5 and IL-6 a persistent low dose of IL-4, as a result of IL-4-anti-IL-4 complexes, is sufficient to allow differentiation of γ_1-positive and/or $\gamma_1\varepsilon$-double positive B cells in IgE-secreting cells. The presence of additional IL-4 in combination with neutralization of IFN-γ did not increase the TNP-specific secondary IgG_1 and IgE response (data not shown). This indicates that the endogenous production of IL-4 was already sufficient to result in a maximum response in irradiated mice reconstituted with spleen cells from control treated mice.

We next studied the dependence on IL-4 and the susceptibility to IFN-γ of primary and secondary TNP-specific IgE responses. With respect to IFN-γ no studies have been described in which its role in memory IgE responses is established. We found that the primary TNP-specific IgE response is completely IL-4 dependent and can be inhibited extensively by IFN-γ whereas the secondary TNP-specific IgE response is partially IL-4 independent and can not be inhibited by IFN-γ in a concentration that does inhibit the

primary TNP-specific IgE response. The IL-4 independent part of the IgE response is probably the result of memory B cells already switched to IgE, whereas the IL-4 dependent part of the response can result from B cells switched to IgG_1, which need IL-4 to switch further to IgE. Finkelman et al. described that memory IgE responses for TNP-KLH are like primary responses dependent on IL-4 [22]. This discrepancy with our results is most likely the result of different resting periods between priming and boosting used in these studies. Using sheep red blood cells as thymus dependent antigen it was found that only 12 weeks after priming the maximum memory formation for IgG_1 was achieved [48]. It is quite possible that during the three weeks after priming memory B cells switched to IgE are not formed to an extent that allowed detection of IgE in the absence of IL-4.

Overall, these results make clear that sequential isotype switching not only plays an important role in the generation of primary, but also secondary IgG_1 and IgE responses. Such sequential isotype switching, most likely, causes the differences in cytokine requirements that are observed when examining unprimed naive B cells versus memory B cells to become IgG_1- or IgE-secreting cells. Furthermore, it is shown that also *in vivo* the IgE inducing capacity of IL-4 is mediated through the generation of $\gamma_1\varepsilon$-double positive cells *in vivo*, that can be stimulated to become both IgG_1 and IgE secreting cells.

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Chapter 7

Prolonged *in vivo* IL-4 treatment leads to persistent IgE production that is IL-4 independent

R. van Ommen, A.T.J. Bianchi[1], V.E.C.J. Schijns[2], and H.F.J. Savelkoul

Submitted for publication

[1] DLO-Central Veterinary Institute, Lelystad, the Netherlands

[2] Department of Infectious Diseases and Immunology, Veterinary Faculty, University of Utrecht, the Netherlands

SUMMARY

It was previously shown that memory IgE responses, like IgG_1 responses, are partially IL-4 independent. For the induction of the IL-4 independent part of a memory IgE response IL-4 had to be present after primary immunization. When mice were treated with IL-4 for a prolonged period, elevated serum total IgE levels were observed, that turned out to be persistent. They stayed at a high level even for four months after the last IL-4 administration. Subsequent neutralization of IL-4 did not influence this persistently elevated serum total IgE level indicating its IL-4 independence. Mice with persistent IgE could still develop protective immunity against a viral infection after vaccination, although their virus-specific IgG_{2a} antibody production was profoundly impaired. Cultures in which B cells from mice with persistent IgE were stimulated with membrane fragments of activated Th1 cells revealed a B cell population that did not need IL-4 to produce IgE. Part of this IgE production turned out to be gp39 independent, whereas the IgG_1 production was completely gp39 dependent. The number of $\gamma_1\varepsilon$-double positive B cells was increased in the spleen of IL-4 treated mice whereas almost no ε-single positive B cells could be detected three months after last IL-4 administration. This suggested that the IL-4 independent persistent IgE serum level in IL-4 treated mice is the result of a $\gamma_1\varepsilon$-double positive B cell population.

INTRODUCTION

IL-4 is a cytokine that is obligatory for the induction of IgE responses, as was clearly shown by the absence of IgE responses in nematode infected mice that were made IL-4 deficient by gene targeting (1). These mice mount an IgG_1 response which indicates that IL-4 is not required for the production of this isotype (1).

Functional studies have indicated that IL-4 is a "switch inducing factor". It alters the chromatin structure of the $S\gamma_1$ region (2) and induces accumulation of germline γ_1 and ε transcripts (3, 4, 5, 6, 7), events that are associated with isotype class switching (8, 9). IFN-γ inhibits the induction of γ_1 germline transcripts (5) and the *in vivo* production of IgG_1 and IgE, whereas it induces IgG_{2a} synthesis (10, 11). IL-4 inhibits the IFN-γ induced IgG_{2a} synthesis, indicating that IL-4 and IFN-γ act reciprocally with respect to isotype regulation (10).

Secondary IgE responses differ from primary IgE responses with respect to their IL-4 dependence. Primary IgE responses can be completely inhibited by neutralizing IL-4,

whereas 30% of the secondary IgE response was found to be IL-4 independent by this treatment (12, 13). However, presence of IL-4 during the primary response is obligatory for the IL-4 independent secondary IgE response (12, 13), suggesting that this response is resulting from B cells already switched to IgE (14). Such cells are found after immunization, and can produce IgE upon subsequent culturing (15).

We have previously reported that prolonged *in vivo* IL-4 treatment resulted in an increased number of B cells switched to IgG_1 and IgE (16). In that study, it was shown that the increased population of ε-positive B cells was also positive for γ_1. These cells normally occur during the process of switching to IgE, and are most likely the result of sequential isotype switching (17). Moreover, it was suggested that these $\gamma_1\varepsilon$-double positive B cells can co-secrete IgG_1 and IgE (17).

The aim of this study, was to investigate the long term effect of a predetermined period of systemically elevated IL-4 level on IgG_1 and IgE formation *in vivo*. Studies like this, became possible by implanting an IL-4 producing cell-line encapsulated in alginate in two weeks intervals in normally developed mice during a predetermined period (12, 18, 19). Such studies are not feasible in mice that are genetically modified to obtain systemically elevated IL-4 levels (20, 21, 22).

We found that the total serum IgG_1 and IgE levels, that increased as a result of the prolonged IL-4 treatment, stayed at this high level even three months after the last of 10 IL-4 administrations. Moreover, it was found that the persistent elevated total serum IgE level could not be inhibited by neutralizing IL-4, indicating its IL-4 independence.

A viral infection model was used to study whether mice with persistent IgE could still develop protective immunity against a lethal challenge with a virulent virus strain after vaccination with a non-virulent virus strain (23). This model was chosen, because it is known that most virus induced responses are restricted to the IgG_{2a} isotype subclass (24, 25), which is related to IFN-γ produced by Th1 cells (10, 26).

By stimulating splenic B cells from mice with persistent IgE with T cell membrane fragments of activated Th1 cells a B cell population was found that did not need IL-4 to produce IgE. Moreover, it was observed that part of these B cells did produce IgE independently of an interaction with the CD40 ligand gp39. This membrane protein, which is expressed on activated T cells (27) has been described to be crucial in thymus dependent immune responses (28) and is associated with immunoglobulin class switching (29-32). Such population of cells was not found in the pool of splenic B cells from control treated mice which exhibited normal levels of IgE. The IgG_1 production by

splenic B cells from both control and IL-4 treated mice turned out to be completely dependent on an interaction between gp39 and CD40. Overall, our results indicate that temporarily elevated systemic IL-4 levels have profound effects on the IgE immune status by generating a B cell population that does not need IL-4 to produce IgE, and in part can be stimulated by activated Th1 cell membrane fragments independently of gp39.

MATERIALS AND METHODS

Mice

Female BALB/c mice were bred and maintained at the Department of Immunology of the Erasmus University. All mice were at an age of 12-16 weeks at the start of the experiments. Mice were held in light-cycled rooms and had access to acidified water and pelleted food ad libitum. The microbiological status of the mice fulfilled the standard of "specific pathogen free V" according to the criteria of the Dutch Veterinary Inspection, as described in the law on animal experiments. The experiments were approved by the Animal Experiments Committee of the Erasmus University.

Immunization

KLH (Pierce, Rockford, IL, USA) was trinitrophenylated to a level of 25 TNP residues per 10^5 Da of KLH (as determined spectrophotometrically) by using trinitrobenzenesulphonic acid (Eastman Kodak, Rochester, NY, USA) (12). Mice were injected with 0.2 ml containing 100 μg TNP-KLH adsorbed on 2 mg alum i.p.

Viral infection

Mice were immunized i.p. with 10^7 p.f.u. of a PRV TK^- mutant of the NIA-3 strain and 28 days later challenged i.p. with 100 x LD_{50} doses of the naive NIA-3 strain (TK^+) (1 LD_{50} dose is 70 p.f.u.) as described previously (23).

Cytokine treatment

Mice immunized with 100 μg TNP-KLH adsorbed on alum were implanted i.p. with 2 x 10^6 CV-1/IL-4 cells encapsulated in alginate every two weeks for a period of three months as described previously (12, 18, 19). The CV-1/IL-4 cells were a kind gift of Dr. N. Arai (DNAX Research institute, Palo Alto, CA, USA). Empty beads encapsulated

in alginate were used as control for the IL-4 treatment. No immunological effects were observed by this treatment in all experiments.

Adoptive transfer of spleen cells

Spleens of control treated and IL-4 treated mice were removed under aseptic conditions and single cell suspensions were prepared. 1 x 10^7 spleen cells were transferred via the tail vein into naive recipients. The recipients had been sublethally irradiated (6 Gy) with a Cesium-137 source (Gammacell 40, Atomic Energy of Canada, Ottawa, Canada) 1 day before cell transfer. All reconstituted mice were i.p. immunized with 10 µg TNP-KLH adsorbed on alum immediately after transfer.

Anti-cytokine treatment

Mice were treated *in vivo* by i.p. injection of neutralizing antibodies directed to IL-4 (11B11, rat IgG_1, 10 mg/mouse) (33), or were implanted with $2x10^6$ alginate encapsulated 11B11 cells i.p. (34). The rat IgG_1 production by these 11B11 cells was determined in the serum by using a rat IgG_1 specific ELISA as described previously (19).

Isotype specific ELISA

Total serum IgE, IgG_1 and IgG_{2a} levels were measured by isotype-specific ELISA as described previously (12). Detection limits for the IgE, IgG_1 and IgG_{2a} ELISA were 0.4 ng/ml, 0.2 ng/ml and 0.3 ng/ml respectively. PRV-specific IgG_{2a} levels were determined by ELISA. Plates were incubated overnight at 4^oC with inactivated PRV in $NaHCO_3$ (0.05 M, pH 9.6), washed with water and staturated with 1% BSA in PBS. Two-fold serum dilutions (100 µg/well; in PBS containing 0.05% Tween 80 and 0.1% BSA) were added and incubated for 1 h at 37^oC. Subsequently plates were incubated with a peroxidase-coupled goat anti-mouse IgG_{2a} (Southern Biotechnology, Birmingham, AL, USA) for 1 h at 37^oC. Plates were developed using tetramethylbenzidine as substrate at room temperature. After 10 min the reaction was stopped with 2 M sulphuric acid and read at an optical density of 450 nm. The titer was defined as the reciprocal of the highest dilution at which the absorbance was equal to 2 times the background level.

Preparation of B cells

Splenic B cells were prepared from control and IL-4 treated mice. T cells were cytotoxically eliminated by treatment of the spleen cells with anti-Thy-1.2 (clone F7D5; Serotec, Oxford, U.K.) and low-tox guinea pig complement (Cederlane, Hornby, Ontario, Canada)

in a two-step procedure at 0° and 37° C, respectively (34). Viable cells were isolated by flotation on Histopaque 1119 (Sigma Chemical Co., St. Louis, MO, USA). The percentage of residual Thy-1$^+$-cells was < 2% as determined by flow cytometry.

T cell membrane stimulation of B cells

T cell depleted spleen cells at 2.5 x 10^5 cells/ml were cultured in flat-bottom microtiter plates together with 100 μg/ml T cell membrane fragments of activated H66 cells (Th1 clone), a kind gift of Dr. P.D. Hodgkin (35, 36) in 0.2 ml complete RPMI 1640 medium supplemented with 10% heat inactivated FCS, 2 mM glutamine, 0.1 M pyruvate, 100 IU/ml penicillin, 50 μg/ml streptomycin, 50 μM 2-mercaptoethanol, 1.75 ng/ml IL-5 and 5 μg/ml anti-IFN-γ (XMG 1.2) (37), as described previously (34). Recombinant murine IL-5 was purified from the culture supernatant of a stable transfected CV-1/IL-5 cell line (kind gift of Dr. N. Arai) by affinity chromatography on anti-IL-5 (TRFK-5) (38) immobilized on Affigel-10 (Bio-Rad Laboratories Inc., Hercules, CA, USA). The cultures contained 1/10 diluted MR1 supernatant (anti-gp39; a kind gift of Dr. E. Claassen), 35 ng/ml purified IL-4, 10 μg/ml anti-IL-4 (11B11) (33), or 1/2 diluted CDC35 culture supernatant in combinations as indicated in the results section. CDC35 cells (Th2 clone) were a kind gift of Dr. D.C. Parker (39). Cultures which did not contain T cell membrane fragments of activated H66 cells served as controls. After 5 days of culture at 5% CO_2 and 37° C supernatants were harvested for ELISA.

Flow cytometric analysis

2.5 x 10^5 cells were incubated on ice for 30 min with the appropriate mAb, either as undiluted culture supernatant or carefully titrated purified mAb followed by a triple wash with PBS containing 1% BSA and 0.1% azide. After using unconjugated mAb, another 30 min incubation was performed with a conjugated specific second-step mAb on ice. After a triple wash the cells were taken up in isotonic fluid and analyzed on a FACScan analyzer (Becton Dickinson, Mountain View, CA, USA). A life gate was used to gate out rare dead cells and erythrocytes.

The following mAb were used for staining: goat-anti-mouse IgG_1-biotin (Southern Biotechnology, Birmingham, AL, USA) at 12.5 μg/ml, rat-anti-mouse IgE (Pharmingen, San Diego, CA, USA) at 10 μg/ml, rat-anti-mouse B220 (clone RA3-6B2) and rat-anti-mouse Thy-1 (clone 59-AD2.2) both as undiluted culture supernatant. Rabbit-anti-rat IgG, F(ab')2-fragments-FITC conjugated (Cappel/Organon Technika, Oss, The Netherlands, 1/100 diluted), and R-Phycoerythrin conjugated Streptavidin (Caltag, San Francisco, CA, USA, 1/20 diluted) were used as second step reagents. To prevent cytophilic binding of

the immunoreagents to FcγR staining was performed after mild acid treatment in the presence of 2% normal goat serum (Dako A/S, Glostrup, Denmark). Stainings with rabbit-anti-rat IgG, F(ab')2-fragments-FITC conjugated (Cappel/Organon), and R-Phycoerythrin conjugated Streptavidin (Caltag) alone were used as control.

Acid treatment

To remove FcR bound immunoglobulin molecules, spleen cells were treated with 0.05 M acetate buffer (pH 4.0) containing 0.085 M NaCl, 0.005 M KCl, and 1% FCS for 1 min on ice as described by Kumagai et al. (40). To neutralize the acid to the range of pH 7.2, PBS supplemented with 0.1 M Hepes (Gibco Life Technologies Ltd, Paisley, Scotland) was added to the suspension. Next the cell suspension was underlayed with FCS. The cells were pelleted and washed two times with HBSS. No decrease in viability was observed as determined by trypan blue exclusion.

RNA isolation and reverse transcription

RNA was isolated by guanidium thiocyanate and purified by gradient centrifugation on 5.7M $CsCl_2$ for 18 hrs in an ultracentrifuge (Sorvall-Du Pont, Newtown, CT, USA) as described earlier (41). Contaminating genomic DNA was removed by using DNase I (BRL, Gaithersburg, MD). After ethanol precipitation 1 μg of total RNA was reversed transcribed as follows; 0.01 U oligo $(dT)_{12\text{-}18}$ (Pharmacia, Uppsala, Sweden) was added to 14 μl H_2O containing 1 μg isolated total RNA and heated for 3 min at 85 °C. Subsequently, the mixture was chilled on ice and the now oligo (dT) primed RNA was reverse transcribed in 20 μl mixture containing 50mM TRIS-HCl, pH 8.3; 50 mM KCl; 10 mM $MgCl_2$; 1mM dithiotreitol; 1 mM EDTA (ethylenediamine tetra acetate, disodium dihydrate salt); 1 μg/ml nuclease-free BSA (Pharmacia); 1 mM dATP; 1 mM dTTP; 1 mM dGTP; 1 mM dCTP (Pharmacia); 4 mM Na pyrophosphate; 40 U RNAse inhibitor (Promega, Madison, WI, USA) and 5 U avian myoblastoma virus reverse transcriptase (Boehringer Mannheim, Germany) for 1 hour at 39 °C.

Semiquantitative PCR

The cDNA from the reverse transcription reaction was ^{2}log diluted in DEPC treated H_2O. The separate dilutions were each subjected to PCR as described (42). For amplification 35 cycles (1 min at 94^0 C for denaturation, 2 min at 55^0 C for primer annealing and 3 min at 72^0 C for primer extension) were performed using a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). Primer sets for IL-4 resulting in a 180 bp product (42), IL-10 resulting in a 420 bp product (43), IFN-γ resulting in a 245 bp product (43) and

HPRT (hypoxanthine phosphatidyl ribosyltransferase) a house keeping gene transcript resulting in a 176 bp product (44) were used. Reverse transcribed DNase I treated CDC35 RNA and D1.1 RNA were used as positive control for Th2 and Th1 cytokines, respectively. CDC35 and D1.1 are I-A^d restricted rabbit-Ig specific Th2 and Th1 clone, respectively. Both were a kind gift of Dr. D.C. Parker and were maintained as previously described (39). After amplification the reaction products were electrophorated in 2% SeaKem LE Agarose (FMC BioProducts, Rockland, ME, USA). After staining with ethidium bromide the gels were photographed, and the photographs were subsequently scanned with a model 620 Video Densitometer (Bio-Rad Laboratories Inc., Hercules, CA, USA). The reverse transcribed mRNA amount in the two cDNA preparations were compared in the linear part of the cDNA dilution curve, and were expressed as dilution of cDNA to obtain a similar optical density (34).

Con A stimulation of splenocytes

Spleen cells at 2 x 10^6/ml were cultured in RPMI 1640 medium supplemented with 10% heat inactivated FCS, 2mM glutamine, 0.1 M pyruvate, 100 IU/ml penicillin, 50 µg/ml streptomycin, 50 µM 2-mercapto-ethanol in 4 replicate wells of a 24 wells flat-bottom plate (1 ml/well) with 10 µg/ml Concanavalin A (Sigma). After 48 hours culture supernatants were harvested and stored at -70^0 C before use.

Determination of cytokines

IL-4, IL-6, IL-10 and IFN-γ were determined in ELISA as previously described (37, 45, 46, 47). The detection limits of the ELISAs were, 0.2 ng/ml, 1.5 U/ml, 3 U/ml and 0.2 ng/ml, respectively.

RESULTS

Persistence of IgG_1 and IgE levels in IL-4 treated mice

We have previously described that, continuous IL-4 treatment for a prolonged period of time resulted in a 7-fold increase of total serum IgG_1 and a 15-fold increase of total serum IgE by the end of the treatment, as compared to control treated mice (12). The aim of this study was to examine the long term effects of a deliberate period of systemically present IL-4 in TNP-KLH primed mice. To this end, total serum IgG_1 and IgE levels were determined up to four months after the last of 10 administrations of IL-4 producing cells encapsulated in alginate (Table 1). In other studies, we have shown that

IL-4 mRNA is present in encapsulated cells recovered out of the peritoneum of mice injected with encapsulated CV-1/IL-4 cells two weeks before (19), suggesting that up to 14 days encapsulated CV-1/IL-4 cells produce IL-4. Moreover, the amount of IL-4 has proven to be functional (18). However, when the similar procedure is carried out 18 days after implantation no IL-4 mRNA can be detected (19). This indicated that after two weeks there is a rapid decline in the amount of IL-4 mRNA expression in CV-1/IL-4 cells encapsulated in alginate, resulting in an abrogated IL-4 production at day 18. Therefore, we decided to implant cells every 14 days, to ensure a continuous IL-4 production. Peritoneal washes from these mice, 90 days after the last of 10 IL-4 administrations, did not reveal any evidence for intact capsules, living CV-1/IL-4 transfected cells, or IL-4 mRNA expression (data not shown). From these studies it is obvious that four months after the last administration of CV-1/IL-4 cells encapsulated in alginate no exogenous IL-4 production can be expected. However, at this time the total IgG_1 and IgE serum levels were still markedly increased in IL-4 treated mice as compared to control treated mice (Table 1). Total serum IgE levels were at a level of 34 μg/ml, comparable with the IgE level on the day of the last IL-4 administration. In the same period the serum IgG_1 levels had decreased 2.3-fold, from 14 to 6 mg/ml (Table 1), but were still elevated as compared to the levels seen in control treated mice (2 mg/ml). Even 7 months after the last IL-4 administration the serum IgE level was still high (48.9 μg/ml). These results suggested a long lasting effect of a period with systemically elevated IL-4 on total serum IgG_1 and IgE. Both in control and IL-4 treated mice the TNP-specific IgG_1 and IgE serum levels had returned to baseline levels four months after the last of 10 control or IL-4 administrations (data not shown).

Table 1. Persistence of serum IgG_1 and IgE levels in serum of mice four months after the last of 10 IL-4 administrations

isotype	treatment	day 0	fold increase	day 120	fold increase
IgG_1	control	2±0.1	7	2±0.1	3
IgG_1	IL-4	14±2		6±0.6	
IgE	control	2±0.3	15	2±0.1	17
IgE	IL-4	29±4		34±7	

Serum levels of IgG_1 and IgE were determined on day 0 and day 120 after the last of 10 IL-4 administrations. Results are expressed as arithmetic mean ± SEM (n=3-5), IgG_1 in mg/ml and IgE in μg/ml.

Cytokine production profile in mice with persistent IgE

We reported previously that prolonged IL-4 treatment resulted in a downregulation of the IFN-γ production by spleen cells after Con A stimulation, whereas the IL-4, IL-6 and

IL-10 production was not influenced (12). We questioned whether such intrinsic changes in the cytokine production level of the spleen cells from IL-4 treated mice accounted for the observed persistent IgE serum level. Therefore, the cytokine production profile after Con A stimulation of spleen cells from control and IL-4 treated mice was determined three months after the last of 10 IL-4 administrations (Table 2). No differences were seen in the IFN-γ production between spleen cells from control and IL-4 treated mice after Con A stimulation. However, the IL-4 and IL-6 production by Con A stimulated spleen cells from IL-4 treated mice significantly increased. IL-10, like IL-4 and IL-6 produced by Th2 cells, turned out not to be elevated in the supernatant of Con A stimulated spleen cells of IL-4 treated mice. These results indicated that more IL-4 is produced upon stimulating spleen cells obtained from IL-4 treated mice when compared to control treated mice three months after the last of 10 IL-4 administrations. However, increased cytokine production after *in vitro* stimulation does not necessarily reflect an increased cytokine production *in vivo*.

Table 2. Cytokine production profile of splenocytes three months after the last of 10 IL-4 administrations

Treatment	IL-4 (ng/ml)	IL-6 (U/ml)	IL-10 (U/ml)	IFN-γ (ng/ml)
Control	0.73±0.06	10.2±0.41	4.0±0.6	6.1±0.3
IL-4	1.48±0.11	15.8±1.6	5.3±0.4	5.6±0.9

Spleen cells (2 x 10^6/ml) pooled from two mice were cultured with Con A (10 μg/ml) for 48 hours in six replicate wells. The supernatants were harvested from these wells and individually tested for cytokine production. The results are represented as arithmetic mean ± SD (n=6).

Cytokine mRNA expression in mice with persistent IgE

To examine the *in vivo* cytokine production by spleen cells of mice with elevated serum IgE, we determined the cytokine mRNA expression in spleen cells three months after the last of 10 IL-4 administrations. Splenic total RNA was isolated and genomic DNA was removed by DNase I treatment before the reverse transcription reaction (Figure 1). After reverse transcription, using oligo dT as a primer to give cDNA from mRNA, semi-quantitative PCR was performed as previously described (34). HPRT mRNA expression was used as a control to standardize the cDNA preparations to be tested (Figure 1). This used semi-quantitative PCR reaction was based on dilutions of the reverse-transcribed mRNA that were subjected to PCR with the appropriate primers. No major differences were seen in the IL-4 mRNA expression, whereas the IFN-γ mRNA markedly increased in the spleens of mice with persistent serum IgE levels (Figure 2). IL-10 mRNA could not be detected in the spleen of untreated mice whereas mRNA for this cytokine could readily be detected in IL-4 treated mice with persistent IgE (Figure 2).

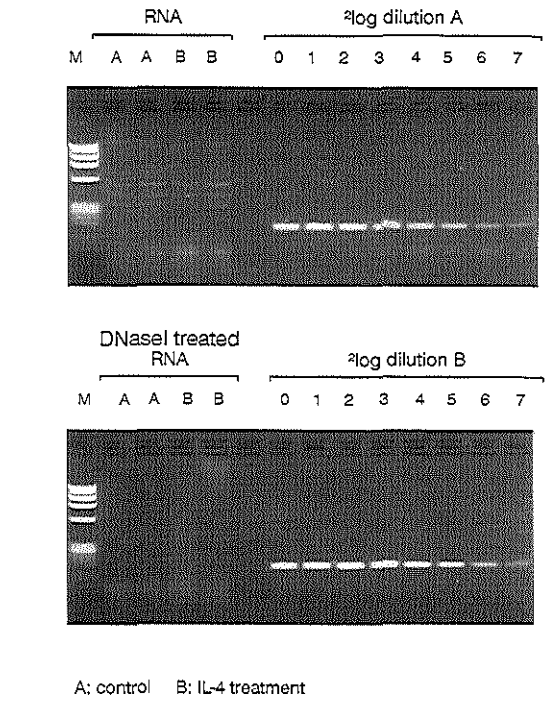

Figure 1. mRNA expression for HPRT in reverse transcribed DNase I treated total RNA preparations from spleens of control (A) and IL-4 treated mice (B) three months after the last control or IL-4 treatment. Lanes 0 to 7 represent dilutions of cDNA in the PCR reaction as indicated. Non-reverse transcribed RNA and DNase I treated RNA were used as negative control in the PCR reaction. M = marker (PhiX174 RF DNA-Hae III Digest).

No increased IFN-γ serum levels (4 ng/ml in IL-4 treated mice vs. 3 ng/ml in control treated mice) were observed in mice that exhibited elevated IFN-γ mRNA expression in their spleen and persistent high IgE levels in their serum. Moreover, neither IL-4 nor IL-10 could be detected in the serum of both IL-4 and control treated mice (data not shown). Overall, these results show that the observed persistent IgG_1 and IgE serum levels are not caused by an increased spontaneous IL-4 production. Furthermore, they show that the increased IFN-γ mRNA expression is not reflected in increased IFN-γ serum levels, that could influence the IgG_1 and IgE production.

The persistent IgE level is IL-4 independent

To further study the involvement of IL-4 in sustaining the high total serum IgE levels, IL-4 treated mice with increased serum IgG_1 and IgE levels were treated with neutralizing

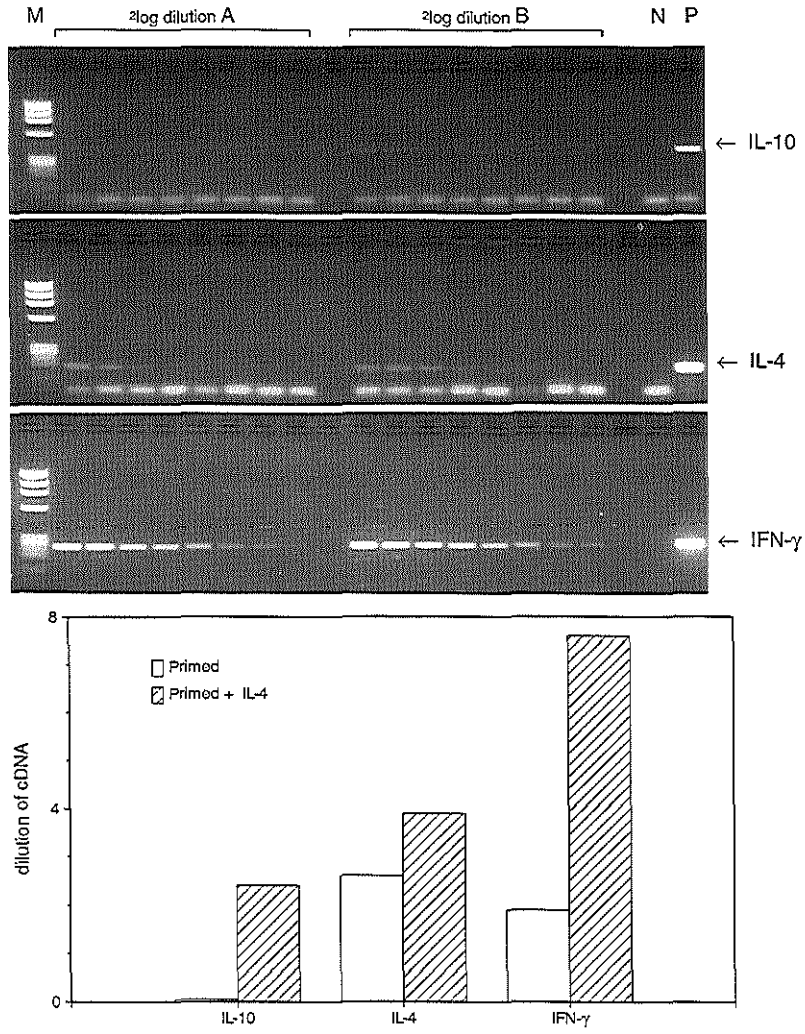

Figure 2. mRNA expression (Fig. 2A) for IL-10, IL-4 and IFN-γ in reverse transcribed DNase I treated total RNA preparations from spleens of control (A) and IL-4 treated mice (B) three months after the last of 10 control or IL-4 treatments. 8 ^{2}log dilutions of cDNA of control (A) and IL-4 treated mice (B) were subjected to PCR as indicated. M=marker (PhiX174 RF DNA-Hae III Digest), N=negative control and P=positive control. Histogram (Fig. 2B) represents the semi-quantitatively determined IL-10, IL-4 and IFN-γ mRNA expression.

antibody directed to IL-4 in a dose (10 mg/mouse) that completely neutralizes IL-4 induced effects. Total serum IgE levels were not significantly inhibited by this treatment, indicating their IL-4 independence (Figure 3). To rule out the possibility that a continuous blocking of IL-4 is required to decrease the total serum IgE level, mice were implanted with 11B11, an anti-IL-4 producing cell line, encapsulated in alginate. Even with a systemic level of 23.3 μg/ml rat IgG_1 in serum of treated mice for a period of 17 days, the serum IgE level did not decrease (Figure 3). These results substantiate that

the maintenance of the IL-4 induced high IgE serum levels is IL-4 independent. The total serum IgG_1 level in IL-4 treated mice was also not influenced by neutralizing IL-4 antibodies (data not shown).

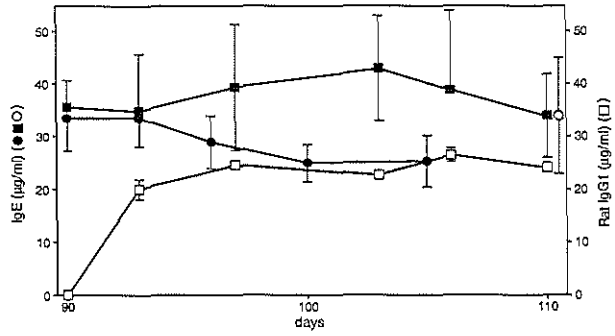

Figure 3. Effect of anti-IL-4 on the persistent IgE level in IL-4 treated mice. On day 90 after the last of 10 IL-4 administrations, mice were either i.p. injected with purified anti-IL-4 ●, or i.p. implanted with an alginate encapsulated anti-IL-4 producing cell line (11B11) ■. Serum levels of total IgE were determined by ELISA in all mice. Rat IgG_1 serum levels were determined in mice that were implanted i.p. with encapsulated 11B11 cells □. ○ represents the total IgE serum level in IL-4 treated mice with persistent IgE at day 110 after the last of 10 IL-4 administrations that received a control treatment starting at day 90. Results are expressed as arithmetic mean ± SEM (n=5).

IL-4 and gp39 independent IgE formation by B cells from IL-4 treated mice

The *in vivo* results suggested that B cells from IL-4 treated mice do not need IL-4 to produce IgE. To further analyze this contingency, an *in vitro* culture was performed in which B cells from control and IL-4 treated mice three months after the last alginate administration were stimulated with T cell membrane fragments from activated Th1 cell clones in the presence or absence of IL-4. Membranes of activated Th1 cell clones were used since these preparations do not contain traces of IL-4. Furthermore, it had been shown that membrane fragments of activated Th1 cells can stimulate B cell proliferation and prepare B cells for cytokine-induced differentiation to secrete Ig independent from antigen (36). Possible traces of IFN-γ present in the membrane preparations were neutralized by the addition of anti-IFN-γ antibody during the culture. IL-5 was added to allow differentiation of activated B cells (36, 48). Cultures which did not contain IL-4 or CDC35 culture supernatant, were supplemented with anti-IL-4 antibody to neutralize any possible traces of IL-4. Control experiments without T cell membrane fragments showed that the anti-IL-4 antibody did not have any stimulating effect on the B cells with respect to IgG_1 and IgE production (Table 3).

Table 3. Spontaneous IgG_1- and IgE- secretion by B cells from control and IL-4 treated mice

addition	IgG_1 (ng/ml)		IgE (ng/ml)	
	control	IL-4	control	IL-4
-	438±36	450±21	0.4±0.5	25±3.7
IL-4	330±40	701±38	<0.4	49±9.4
CDC35sn	295±21	530±38	0.4±0.3	25±4.6

Thy-1 depleted spleen cells of control and IL-4 treated mice, 3 months after the last of 10 treatments, were cultured in the absence of T cell membrane fragments of activated Th1 cells, to measure the spontaneous Ig production by activated B cells. The cultures were performed in the absence or presence of IL-4. To the cultures that did not contain IL-4 (purified or CDC35sn), anti-IL-4 was added. At day 5 the supernatants were harvested, and the total IgG_1 levels determined. Results are expressed as arithmetic mean ± SD (n=4). sn = culture supernatant.

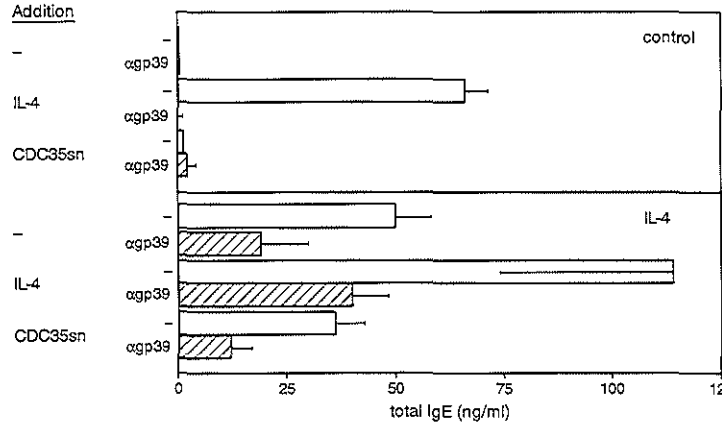

Figure 4. Thy-1 depleted spleen cells of control and IL-4 treated mice, 3 months after the last of 10 treatments, were stimulated with T cell membrane fragments of activated Th1 cells in the presence or absence of IL-4 and anti-gp39 (MR-1). To the cultures that did not contain IL-4 (purified or CDC35 sn), anti-IL-4 was added. At day 5 the supernatants were harvested and the total IgE levels determined. Results corrected with negative control (production in the absence of activated T cell membrane fragments) are expressed as arithmetic mean ± SD (n=4). sn = culture supernatant.

No IgE formation was found when B cells from control treated mice were stimulated with T cell membranes in the absence of IL-4, whereas B cells from IL-4 treated mice in similar conditions produced 50 ng/ml IgE (Figure 4). This amount of IgE was comparable to that produced by stimulated B cells from control treated mice when IL-4 (35 ng/ml) was added to the culture. The CDC35 culture supernatant did neither contain enough IL-4 (< 35 ng/ml) to help B cells from control treated mice to produce IgE, nor to enhance the IgE production by B cells from IL-4 treated mice, after T cell membrane stimulation (Figure 4). Upon culturing B cells from IL-4 treated mice addition of either anti-IL-4 or CDC35 culture supernatant did not result in altered IgE production over a 5 day period. This indicates again that anti-IL-4 itself does not have stimulating properties. Similar

results were found for IgG_1. No IgG_1 production by B cells from control treated mice was found upon culturing in the absence of IL-4, whereas in similar culture conditions IgG_1 production did occur upon culturing B cells from IL-4 treated mice (Figure 5). In the presence of IL-4 B cells from control treated mice produced 0.9 mg/ml more IgG_1 than B cells of IL-4 treated mice (Figure 5), whereas in similar conditions more IgE was produced by B cells from IL-4 treated mice than by B cells from control treated mice (114 ng/ml vs. 66 ng/ml) (Figure 4).

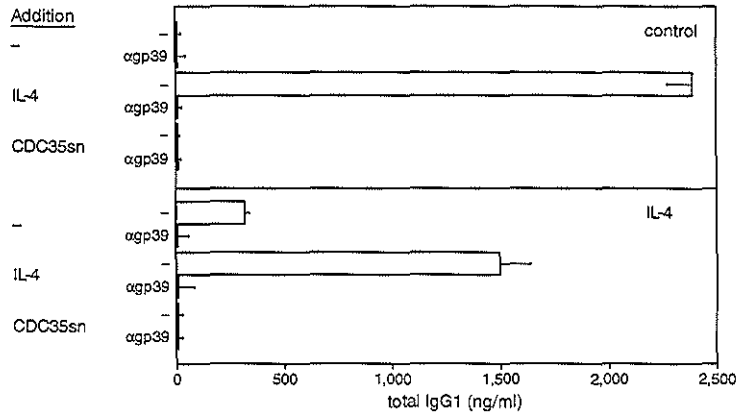

Figure 5. Thy-1 depleted spleen cells of control and IL-4 treated mice, 3 months after the last of 10 treatments, were stimulated with T cell membrane fragments of activated Th1 cells in the presence or absence of IL-4 and anti-gp39 (MR-1). To the cultures that did not contain IL-4 (purified or CDC35 sn) anti-IL-4 was added. At day 5 the supernatants were harvested, and the total IgG_1 levels determined. Results corrected with negative control (production in the absence of activated T cell membrane fragments) are expressed as arithmetic mean ± SD (n=4). sn = culture supernatant.

It was noted that the spontaneous IgG_1 production by B cells from IL-4 treated mice in the absence of activated T cell membrane fragments was 2-fold higher than using B cells from control treated mice in similar conditions (0.3 mg/ml and 0.7 mg/ml IgG_1, respectively), when IL-4 was present during the 5 days of culture (Table 3). Spontaneous IgE production displayed even a 10-fold increase. B cells from control treated mice produced 0.4 μg/ml IgE in the absence of activated T cell membrane fragments, but in the presence of IL-4 in the culture. In similar culture conditions B cells from IL-4 treated mice three months after the last IL-4 administration produced 49 μg/ml IgE (Table 3). When IL-4 was absent during the culture lower levels of spontaneously produced IgG_1 and IgE were found. In these culture conditions no difference was observed with respect to the IgG_1 production by B cells from control and IL-4 treated mice, whereas with respect to IgE still a 5-fold increase was observed (Table 3). These results suggested that in the spleen of IL-4 treated mice more activated B cells are present than in the spleen of control treated mice, even three months after the last of 10 IL-4 administrations. These B cells can use either IL-4, IL-5, or both to develop into IgG_1 or

IgE secreting cells that produce these isotypes independently of activated T cell membranes.

Blocking studies with the MR1, a mAb directed to the CD40 ligand gp39, showed that the IgE production by B cells from control treated mice was completely gp39 dependent, whereas B cells from IL-4 treated mice were able to produce IgE independent of gp39 when stimulated with T cell membrane fragments (Figure 4). The IgG_1 production by B cells from both IL-4 and control treated mice could be inhibited completely by the addition of anti-gp39 antibody (Figure 5).

The culture supernatants were harvested at day 5 of the culture and corrected for the spontaneous IgE production in the absence of activated T cell membrane fragments, to ensure that the IgE was produced by secondary B cells. Other experiments revealed that upon culturing with T cell membrane fragments, IgE production corrected for spontaneously produced IgE (in the absence of T cell membrane fragments) by naive B cells could only be detected after 6 days of culture (data not shown).

The number of $\gamma 1\varepsilon$ double-positive cells is increased in the spleen of IL-4 treated mice three months after last IL-4 administration

The IL-4 and gp39 independent IgE formation by B cells of IL-4 treated mice could be the result of maturing B cells already switched to IgE. To study this, the phenotypes of splenic B cells from IL-4 treated and control mice were examined by FACScan analysis at days 2 and 3, 3 months and 1 year after the last IL-4 administration (Figure 6, Table 4). To rule out a significant contribution of cytophilically FcR bound isotypes cells were treated with mild acid (40). To prevent aspecific cytophilic binding of the immunoreagents to the empty FcR, all stainings were carried out in 2% normal goat serum. Analysis was carried out on lymphocytes gated on the forward scatter/side scatter plot. The quadrant settings were based on the single stainings for γ_1-positive B cells and ε-positive B cells (Figure 6). On all days tested no significant differences were seen in the number of γ_1-single positive B cells in the spleens of control treated and IL-4 treated mice after the last of 10 IL-4 or control administrations (Table 4). On all these time points, almost all detectable ε-positive B cells were positive for γ_1 as well. Moreover, these numbers of $\gamma_1\varepsilon$-double positive B cells were significantly higher in IL-4 treated mice when compared to control treated mice at all time points examined (Table 4), suggesting that the IL-4 independent IgE production originates from this B cell population.

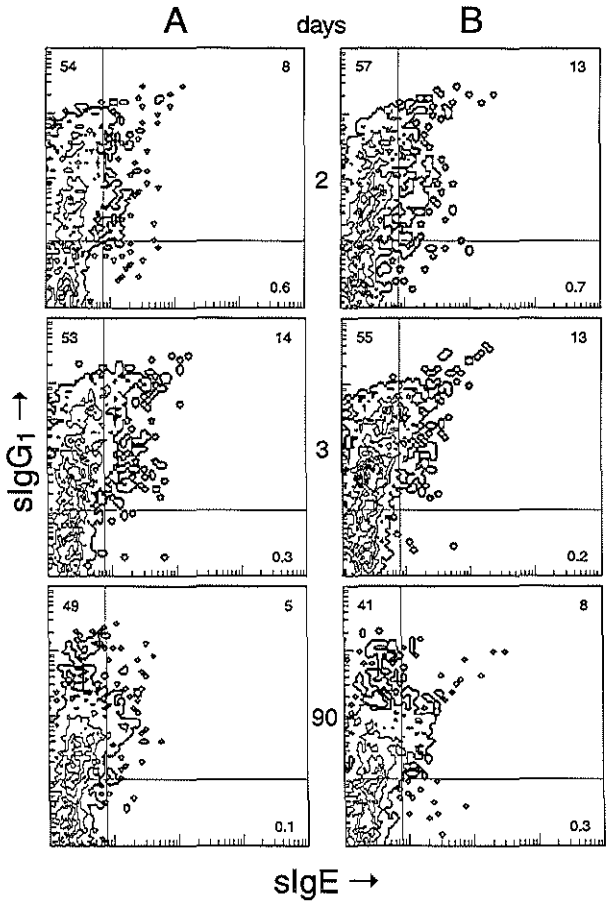

Figure 6. Contour plots of the $sIgG_1$ and sIgE expression on lymphocytes from control (A) and IL-4 treated mice (B) at days 2, 3 and 90 after the last control or IL-4 administrations. Lymphocytes were gated on forward scatter/side scatter plot. Percentages of lymphocytes are indicated within the quadrants.

Table 4. Number of switched B cells in spleens of control and IL-4 treated mice

sIg expression	2 days	3 days	3 months	1 year
		Control		
γ_1-single$^+$	546	697	700	892
ε-single$^+$	6	4	2	0
γ_1ε-double$^+$	84	170	74	78
		IL-4		
γ_1-single$^+$	578	980	667	934
ε-single$^+$	8	3	5	0
γ_1ε-double$^+$	119	224	138	136

Surface marker expression evaluated by flow cytometric analysis of total spleen cells from control and IL-4 treated mice at 2 days, 3 days, 3 months and 1 year after the last of 10 control or IL-4 administrations. Cell suspensions were pooled from two mice. Rare dead cells and erythrocytes were gated out. Lymphocytes gated on the forward scatter / side scatter plot were analyzed. Results are the absolute number of positive cells ($x10^5$) per spleen.

Mice with persistent IgE can be vaccinated against a lethal virus infection

We next studied whether mice with persistently elevated IL-4 independent IgE levels, were perturbed in their potential to mount a Th1 mediated immune response. Therefore we studied a virus induced response, which is related to IFN-γ production and IgG_{2a} formation (24, 25). At day 0, mice were primed with 10^7 p.f.u. of a non-virulent PRV TK^- mutant of the NAI-3 strain, because in other studies we have shown an increase in the IgG_{2a} levels after PRV infection (data not shown). At day 28 mice were challenged with 100x the LD_{50} dose of virulent NIA-3 strain. All mice, except 3 out of 4 control mice at 10 months after last control treatment, that were primed with the non-virulent virus strain survived the challenge with the virulent strain. In contrast, all non-primed mice succumbed to infection with this strain (Table 5). Apparently, the memory formation necessary to survive a challenge with 100x the LD50 dose of NAI-3 strain is not disturbed in mice with persistently elevated IgE. No increase in the total serum IgG_{2a} could be detected in both control and IL-4 treated mice at day 21 after priming with the non-virulent NAI-3 TK^- mutant, whereas naive mice showed an increase in the serum IgG_{2a} level from 0.9 mg/ml to 1.7 mg/ml (Figure 7).

Table 5. Mice with persistent IgE are not disturbed in developing protective immunity against a viral infection

treatment	days after administration	vaccination	challenge	survival
control	46	+	+	5/5
IL-4	46	+	+	5/5
control	208	+	+	4/4
IL-4	208	+	+	4/4
control	314	+	+	*1/4
IL-4	314	+	+	4/4
none		-	+	0/10
none		+	+	10/10

At day 46, 208 or 314 after the last of 10 control or IL-4 administrations mice were vaccinated with a PRV TK^- mutant of the NIA-3 strain (10^7 p.f.u.). 28 days after vaccination mice were challenged with 100 x LD_{50} of the native NIA-3 strain (TK^+). At day 7 after challenge number of survival/infected animals was scored.

*Animals were already in a bad condition before challenge.

Challenge with the virulent strain did not result in further increases of the total IgG_{2a} serum levels in all examined mice (Figure 7). Moreover, no changes in the IFN-γ serum levels were detected, neither at day 7 and 14 after priming, nor at day 7 after challenge with the two virus strains (data not shown). We also examined the total IgG_1 serum levels after priming and challenge, but did not find changes with respect to the levels

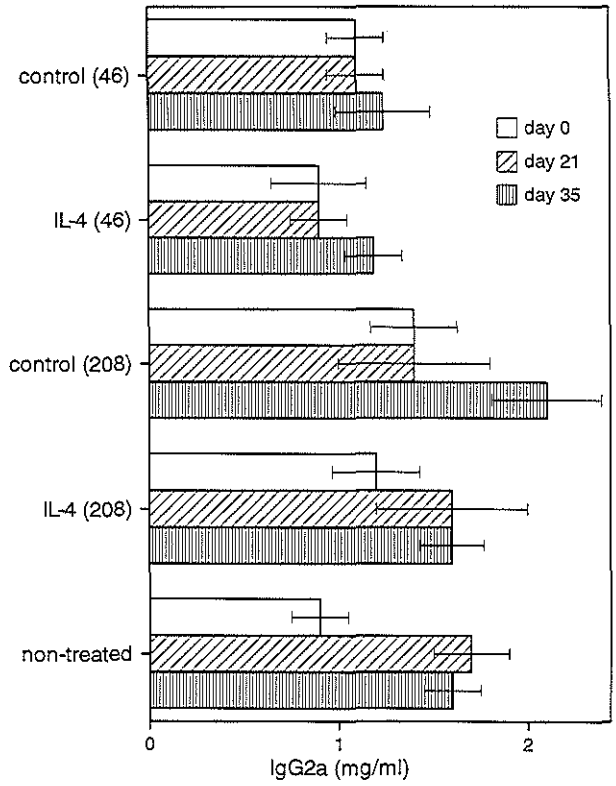

Figure 7. Effect of a viral infection on the total serum IgG_{2a} level in control and IL-4 treated mice 46 and 208 days after the last of 10 treatments. At day 0 mice were i.p. infected with the PRV TK^- mutant of the NIA-3 strain and at day 28 the mice were challenged with 100LD50 of the native NIA-3 strain (TK^+). Results are exrpressed as artithmetic mean ± SEM (n=4-5).

observed before either immunization (data not shown). Examination of the virus-specific IgG_{2a} serum levels revealed that at day 21 after priming a significant increase in virus-specific IgG_{2a} could be observed in control treated mice that had been primed on day 46 or 208 after the last control administration (Table 6). However, no virus-specific IgG_{2a} could be detected in the serum of IL-4 treated mice at day 21 after priming.

Table 6. PRV-specific IgG_{2a} titers in mice vaccinated at 46 and 208 days after the last of 10 control or IL-4 treatments

treatment	days after administration	day 0	day 21 days after vaccination	day 35
control	46	<2000	24000±10119	24000±5060
IL-4	46	<2000	<2000	<2000
control	208	<2000	14500±6500	24000±4619
IL-4	208	<2000	<2000	9500±7500
none		<2000	16400±4750	40000±8000

At day 46 or 208 after the last of 10 control or IL-4 administrations mice were vaccinated (day 0) with a PRV TK^- mutant of the NIA-3 strain (10^7 p.f.u.). 28 days after vaccination mice were challenged with 100 x LD_{50} of the native NIA-3 strain (TK^+). Titers were determined as described in the materials and methods and are expressed as arithmetic mean ± SEM (n=4-5).

Challenge with 100x the LD50 dose of NAI-3 strain induced a low level of virus-specific IgG_{2a} in mice vaccinated at 208 days after the last IL-4 administration. Under similar conditions no virus-specific IgG_{2a} could be detected in IL-4 treated mice that had been primed and subsequently challenged 46 days after the last of 10 IL-4 administrations. Priming and challenge of non-treated control mice with the virus stains resulted in slightly increased levels of virus-specific serum IgG_{2a} as compared to control treated mice. Collectively, these data demonstrate that the generation of virus-specific IgG_{2a} production is profoundly inhibited in IL-4 treated mice, even when the mice are vaccinated at 46 and 208 days after the last IL-4 administration. The drop in virus-specific IgG_{2a} production in the Th2 biased IL-4 treated mice, however, does not impair the vaccination-induced antiviral memory formation necessary for protective immunity.

DISCUSSION

In this study we show that persistent IgE serum levels that are IL-4 and in part also CD40 ligand independent are induced by prolonged IL-4 treatment. We have previously shown that IL-4 treatment of mice resulted in elevated serum levels for total IgG_1 and IgE, but not IgG_{2a}(12) and IgM (unpublished observations), accompanied by increased numbers of splenic $\gamma_1\varepsilon$-double positive B cells (16). These results suggested that the $\gamma_1\varepsilon$-double positive cells were responsible for the elevated total serum IgE serum levels. Such $\gamma_1\varepsilon$-double positive cells were also found in B cell cultures stimulated with LPS in the presence of IL-4. Moreover, it was shown that in these conditions such cells were formed as the result of sequential isotype switching (17). In this study also co-secretion of IgG_1 and IgE by the same cells was described. Our studies indicate the *in vivo* existence of this cell type and its upregulation by prolonged *in vivo* IL-4 treatment. Other studies in which the *in vivo* IL-4 levels were modulated systemically by generating IL-4 transgenic mice reported different observations with respect to IgE. These differences were most likely the result of differences in the *in vivo* IL-4 expression (21, 49). A study in which long term *in vivo* IL-4 expression is induced by using retroviral-mediated gene transfer showed that the serum IgG_1 levels increased 3-fold, whereas no increase in serum IgE was observed as a result of this treatment (22). This indicates that in our model enough IL-4 was present to result in a 7-fold increased serum total IgG_1 levels and a 15-fold increased serum total IgE levels (12). Moreover, in a previous study we clearly showed that the serum total IgG_{2a} level did not increase as a result of the prolonged IL-4 treatment, indicating the isotype specificity of the used approach. We were next interested in the possible effects of a restricted period of high IL-4 on the IgG_1 and IgE serum levels. Such question can be addressed in our model, because IL-4 can be elevated during a predetermined period, whereas in other described models of

high IL-4 no shut off of the IL-4 production can be achieved.

The total serum IgG_1 and IgE production was still elevated four months after the last of 10 IL-4 administrations (1 administration every 2 weeks). The elevated total serum IgE levels four months after the last of 10 IL-4 administration were as high as the levels observed immediately after ending the treatment. The total serum IgG_1 levels were also still increased four months after the last of 10 IL-4 administrations, but were declined when compared to the levels immediately after the last IL-4 administration. In other studies (19), we have shown that CV-1/IL-4 cells encapsulated in alginate can be recovered out of the peritoneum till day 18, but that IL-4 mRNA can only be detected in the recovered encapsulated cells till day 14. After day 14 there is a rapid decline in detectable IL-4 mRNA, resulting in no detection at day 18. Moreover, it is highly unlikely that xenogenic CV-1/IL-4 cells can persist out of the alginate capsules, indicating that the elevated IgG_1 and IgE serum levels can not be simply explained by persisting CV-1/IL-4 cells.

Next, we investigated whether prolonged IL-4 treatment induced changes in the cytokine production profiles of spleen cells that would still be present three months after the last IL-4 administration. Con A stimulation of spleen cells revealed that the maximal IL-4 and IL-6 production was increased three months after the last of 10 IL-4 administrations, 1 administration every 2 weeks, whereas no difference was seen for the IFN-γ production. Measurement of the cytokine production by spleen cells upon Con A stimulation one day after the last of 10 IL-4 administrations showed decreased IFN-γ, but similar IL-4, IL-6, and IL-10 productions as compared to control treated mice (12). This indicates that changes did occur in the T cell population during the three months after the last of 10 IL-4 administrations. The same could be concluded from the T cell numbers in the spleens of IL-4 treated mice. These were decreased one day after the last IL-4 administration (12), but returned to normal during the three months after the last IL-4 administration (data not shown). The increased IL-4 and IL-6 production found upon *in vitro* stimulation of splenic cells is in accordance with studies that described the induction of Th2 cells as a result of IL-4 (50, 51, 52). To exclude the possibility that the persistent serum IgE level in IL-4 treated mice was the result of an increased endogenous IL-4 production, we examined the mRNA expression for IL-4, IL-10 and IFN-γ in spleen cells. No differences in IL-4 mRNA expression were observed between spleen cells from control and IL-4 treated mice. On the other hand, the mRNA expression of IL-10 and IFN-γ were increased in IL-4 treated mice. No increase in IFN-γ production was observed in the serum of IL-4 treated mice. It is possible that IL-10, for which the mRNA expression was also increased, inhibits the IFN-γ production at a posttranscriptional level. This despite the fact that no IL-10 could be detected in the serum of both

control and IL-4 treated mice. These results indicate that the persistent IgE production is not mediated by an increased endogenous IL-4 production.

Using a virus infection model it was shown that mice with persistently elevated total serum IgE levels were not impaired in developing a protective immunity against lethal challenge with a virulent virus strain after previous vaccination with the non-virulent strain. This despite the fact that lower levels of virus-specific serum IgG_{2a} are generated in IL-4 treated mice upon vaccination and challenge with the non-lethal and lethal PRV-stain, respectively. No IFN-γ response could be detected in serum of both control and IL-4 treated mice, neither after vaccination nor after challenge. Notwithstanding the fact that virally induced responses are generally associated with the production of IFN-γ induced IgG_{2a} antibodies (24, 25). Other studies revealed a 3-fold increase in serum IFN-γ at day 6 after immunization of BALB/c mice with 10^8 p.f.u. of the same virus in both hind-paws (Bianchi et al., manuscript in preparation) and PRV-specific IgG_{2a} production is strongly reduced in IFN-γ-receptor deficient mice (Schijns et al., manuscript in preparation). In non-treated mice a small increase of total serum IgG_{2a} was observed at day 21 after vaccination, indicating that probably the previous priming with TNP-KLH adsorbed on alum prevented a measurable total serum IgG_{2a} response in control and IL-4 treated mice. Viral infection did not lead to an increase of the total serum IgG_1 level, not even in non-treated mice that did mount a small IgG_{2a} response. Upon vaccination and challenge with the PRV-strains no virus-specific IgG_{2a} could be detected in mice with persistently elevated serum IgE that had been vaccinated 46 days after the last of 10 IL-4 administrations, suggesting that these mice are completely biased in the Th2 direction with respect to the isotypes produced. However, mice vaccinated and challenged 208 days after the last of 10 IL-4 administrations did respond with an increase in virus-specific serum IgG_{2a}, indicating a re-establishment of Th1 activity at this time point after IL-4 treatment. All our data concerning IgM and IgG_{2a} versus IgG_1 and IgE as well as the data of the virus vaccination experiment are consistent with our notion that IL-4 treatment has profound effects, it increases the Th2 mediated IgG_1 and IgE responses, and at the same time perturbs a potential Th1 mediated immune response, even 208 days after the last of 10 IL-4 administrations.

Neutralization of IL-4 did not result in a decrease of the persistently elevated serum total IgE level substantiating its IL-4 independence. Therefore, it was thought that the persistent IgE serum level originates from B cells already switched to the synthesis of IgE. Three months after the last IL-4 administration the number of such B cells was indeed 2-fold increased as determined by flow cytometric analysis. The spontaneous IgE secretion, in the absence of a mitogenic stimulus, by these B cells was even a 50-fold higher when compared to B cells from control treated mice in similar condition. Using

membrane fragments of activated Th1 cells we showed that spleens from IL-4 treated mice contained a population of resting B cells that did not need IL-4 to produce IgE, even three months after the last IL-4 administration. Such B cell population was not found in the spleen of control treated mice indicating its induction by IL-4. Moreover, we found that part of the B cells that were IL-4 independent with respect to IgE production, were also CD40 ligand independent. The IgG_1 production, however, turned out to be completely gp39 dependent. This is in accordance with *in vivo* studies in which it was found that IgG_1, but not IgE thymus-dependent responses, could be completely inhibited by an antibody directed to gp39 (personal communication Dr. A.J.M. van den Eertwegh). Other studies described the induction of germline $C\gamma_1$ transcripts by membrane fragments of activated Th1 cells independent of IL-4, suggesting gp39 as a "switch factor" for IgG_1 (53).

It has been described that memory IgE responses, both *in vivo* and *in vitro*, were less dependent on IL-4 than primary IgE responses (12, 13, 14, 34, 54). Furthermore, it has been shown that IL-4 is required to sustain established IgE responses (55). In this report we show that prolonged IL-4 treatment induced a persistently elevated serum total IgE level, that can not be inhibited by neutralizing IL-4. Moreover the number of B cells switched to the IgE phenotype was increased. These results suggest that IL-4 treatment induces B cells to switch to IgE that are responsible for an IgE production that does not need to be sustained by IL-4. Indeed it was shown *in vitro* that a population of B cells in the spleen of IL-4 treated mice was present that did not need IL-4 to produce IgE. It could be argued in other studies that small traces of IL-4 were responsible for IgE synthesis, but this option is not likely in our *in vitro* system.

Collectively, it can be concluded that systemic elevation of IL-4 during a restricted period induces long lasting effects with respect to IgE. These effects could even be observed when IL-4 had returned to normal levels and were not influenced by anti-IL-4 treatment, indicating their IL-4 independence.

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Chapter 8

Suppression of polyclonal and antigen-specific murine IgG_1 but not IgE responses by neutralizing IL-6 *in vivo*

R. van Ommen, A.E.C.M. Vredendaal, and H.F.J. Savelkoul

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René van Ommen,
Astrid E. C. M. Vredendaal and
Huub F. J. Savelkoul

Department of Immunolgy,
Erasmus University, Rotterdam

Suppression of polyclonal and antigen-specific murine IgG_1 but not IgE responses by neutralizing interleukin-6 *in vivo**

The crucial role of interleukin (IL)-4 in the induction of murine IgG_1 and IgE responses, which are coupled through the process of sequential isotype switching, has been well documented. Whereas IL-4 is obligatory for the induction of IgE responses, it enhances IgG_1 responses. In this study, using neutralizing antibodies, we provide evidence that, besides IL-4, also IL-6 is required for obtaining peak IgG_1 responses. The mRNA levels of these two cytokines are coordinately expressed in the spleen of mice immunized with trinitrophenol-keyhole limpet hemocyanin (TNP-KLH). No IL-6 requirement was observed for peak IgE responses. The IL-6 dependence of IgG_1 responses was found for both antigen-specific and polyclonal responses. Moreover, it was noted using TNP-KLH and goat anti-mouse (GAM) IgD as antigen that polyclonal IgG_1 responses are more dependent on IL-6 than antigen-specific responses. *In vitro* experiments revealed that exogenous IL-6 neither enhanced nor inhibited the IgG_1 and IgE production by naive B cells, suggesting that IL-6 did not interfere with the IL-4-induced isotype switch potential. Primary and memory IgG_1 responses were both similarly dependent on IL-6. These observations point to a role of IL-6 in the terminal differentation of B cells switched to IgG_1. Neutralization of IL-6 did not inhibit either antigen-specific or polyclonal IgE responses. Therefore, it was concluded that IL-6 is not involved in the terminal differentiation of B cells switched to IgE. These findings thus provide a distinct role for IL-6, besides IL-4, in regulating murine IgG_1 responses. The formation of IgE, however, is completely dependent on IL-4 alone.

1 Introduction

The crucial role of IL-4 in the regulation of IgE synthesis has been well established by studying mice that were made IL-4 deficient by gene targetting. No IgE synthesis was observed in these mice upon nematode infection [1]. Disrupting the IL-4 gene did not completely impair the IgG_1 production [1], indicating that IL-4 is not an absolute requirement for IgG_1. Functional studies have indicated IL-4 as a "switch-inducing factor" for both IgG_1 and IgE. It alters the chromatine structure of the $S\gamma_1$ region [2] and induces accumulation of germ-line γ_1 and ε transcripts [3, 4], events that are associated with isotype class switching [5, 6].

In line with these results is the observation that IL-4 enhances the IgG_1 production, although less IL-4 is required for peak IgG_1 responses than for peak IgE responses after LPS stimulation of B cells *in vitro* [7]. A linkage between IgG_1 and IgE responses has recently been demonstrated, in that γ_1-positive B cells can switch to ε-positive B cells with a $\gamma_1\varepsilon$-double-positive B cell as an intermediate [8, 9]. Moreover, it has been suggested that these $\gamma_1\varepsilon$-double-positive cells co-secrete IgG_1 and IgE [9].

The role of cytokines other than IL-4 in *in vivo* polyclonal and antigen-specific IgG_1 and IgE responses has not been well investigated. In the human system, IL-6 has been described as a cytokine that is involved in the IL-4-dependent IgE synthesis [10, 11]. Similar observations were made in mice, in that neutralization of IL-6 resulted in a inhibition of the number of IgE-secreting cells in the spleen [12]. Surprisingly, in that study an inhibition of the number of IgE-secreting cells was also observed when IL-6 was administered [12].

Because of these contradictory results we decided to study the role of both IL-4 and IL-6 in IgG_1 and IgE responses. In the study of Auci et al. [12] antigen had been given more than once. It is known that memory IgE responses differ from primary IgE responses with respect to their IL-4 requirement, in that memory IgE responses are partially IL-4 independent [13–15]. Therefore, we studied the effect of neutralizing IL-6 *in vivo* on memory IgG_1 and IgE responses as well as the effect of this treatment on polyclonal and antigen-specific primary IgG_1 and IgE responses. We also studied in *in vitro* cultures of splenic B cells whether IL-6 could enhance IgG_1 and/or IgE production.

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Correspondence: René van Ommen, Department of Immunology, Erasmus University, P.O. Box 1738, NL-3000 DR Rotterdam, The Netherlands

Abbreviations: **GAM:** Goat-anti-mouse **Nb:** *Nippostrongylus brasiliensis*

Key words: IgG_1 and IgE responses / Interleukin-4 / Interleukin-6 / B cell memory

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Collectively, our results show that IgG_1 and IgE responses not only differ in IL-4 dependence, but also deviate with respect to IL-6 in attaining peak levels. This finding thus provides a second regulatory mechanism of IgG_1 and IgE responses coupled through sequential isotype switching.

2 Materials and methods

2.1 Mice

Female BALB/c mice were bred and maintained at the Department of Immunology of the Erasmus University. All mice were at an age of 12–16 weeks at the start of the experiments. The experiments were approved by the Animal Experiments Committee of the Erasmus University.

2.2 Immunization and infection

Mice were injected with 0.2 ml containing 10 μg TNP_{25}-KLH adsorbed on 2 mg alum i.p. [15] or 800 μg goat anti-mouse (GAM) IgD (Nordic Immunology, Tilburg, The Netherlands) i.v. or were infected with *Nippostrongylus brasiliensis* (Nb; a kind gift of Dr. R. L. Coffman, DNAX, Palo Alto, CA) by subcuteneously injecting 750 stage III Nb larvae, as indicated in Sect. 3.4

2.3 Isotype-specific ELISA

Total serum IgE and IgG_1 levels were measured by isotype-specific ELISA as described previously [15, 16]. Detection limits for the IgE and IgG_1 ELISA were 0.5 ng/ml and 0.2 ng/ml, respectively. TNP-specific IgG_1 and IgE were determined as previously described [15], with 0.2 ng/ml and 1 ng/ml as detection limit in the ELISA, respectively. Total serum IgG_{2a} and IgM were determined essentially in the same way using either GAM IgG_{2a} (Southern Biotechnology, Birmingham, AL) or GAM IgM (Southern Biotechnology) both at 1 μg/ml as coat and biotinylated GAM IgG_{2a} or biotinylated GAM IgM both at 0.5 μg/ml as second step, with a detection limit of 0.3 ng/ml and 0.2 ng/ml, respectively.

2.4 Anti-cytokine treatment

Mice were treated *in vivo* by i.p. injection of purified neutralizing antibodies directed to IL-4 (11B11, rat IgG_1, 10 mg/mouse) [17], IL-5 (TRFK5, rat IgG_1 2 mg/ml) [18] and IL-6 (20F3, rat IgG_1, 2 mg/ml) [19]. Alternatively, mice were implanted with 2×10^6 alginate-encapsulated 11B11 and/or 20F3 hybridoma cells i.p. [20]. The hybridoma cells encapsulated in alginate were implanted in mice 3 days before immunization, the purified antibodies were given 2 h before immunization. The rat IgG_1 production by these 11B11 and/or 20F3 cells was determined in the serum using a rat IgG_1-specific ELISA as described previously [20]. Purified rat mAb specific for *E. coli* β-galactosidase (GL113) [21] or 2×10^6 alginate-encapsulated GL113 cells were used as an IgG_1 isotype control. The mAb were purified from culture supernatants by protein G-affinity chromatography [22]. The doses used have been widely shown to be sufficient to neutralize the respective cytokine activities in a variety of systems. All the used hybridoma cells were assayed for their rat IL-6 production, and it was found that in all cases the average IL-6 production by 2×10^6 hybridoma cells/day was less than 8 U. This production was determined in a bioassay using B9 indicator cells as described [23], with a detection limit of 0.1 U/ml.

2.5 Semiquantitative reverse-transcription (RT)-PCR

RNA was isolated and reverse-transcribed after removing contaminating genomic DNA by DNase I, as previously described [20]. The cDNA was ^{2}log diluted in DEPC-treated H_2O. The separate dilutions were each subjected to PCR as described [24]. For amplification 35 cycles (1 min at 94 °C for denaturation, 2 min at 55 °C for primer annealing and 3 min at 72 °C for primer extension) were performed using a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). Primer sets for IL-4 resulting in a 180-bp product [24], IL-6 resulting in a 348-bp product [24], IFN-γ resulting in a 245-bp product [24] and hypoxanthine phosphatidyl ribosyltransferase (HPRT), a house keeping gene transcript resulting in a 176-bp product, [25] were used. Reverse-transcribed DNase I-treated CDC35 RNA and D1.1 RNA were used as positive control for Th2 and Th1 cytokines, respectively. Both were a kind gift of Dr. D. C. Parker and were maintained as previously described [26]. After amplification the reaction products were electrophorated in 2% SeaKem LE agarose (FMC BioProducts, Rockland, ME). After staining with ethidium bromide the gels were photographed, and the photographs were subsequently scanned with a model 620 Video Densitometer (Bio-Rad Laboratories Inc., Hercules, CA). The reverse-transcribed mRNA amounts in the two cDNA preparations were compared in the linear part of the cDNA dilution curve, and were expressed as dilution of cDNA to obtain a similar optical density.

2.6 Adoptive transfer of spleen cells

Spleens cells (1×10^7) of mice, primed with TNP-KLH 3 months before, were adoptively transferred into naive irradiated (6 Gy) recipients as previously described [15]. All reconstituted mice were i.p. immunized with 10 μg TNP-KLH adsorbed on alum immediately after transfer.

2.7 T cell membrane stimulation

Spleen cells were cytotoxically depleted of T cells using anti-Thy-1.2 (clone F7D5; Serotec, Oxford, GB) and low-tox guinea pig complement (Cederlane, Hornby, Ontario, Canada) as described previously [16]. The percentage of $B220^+$ B cells was routinely > 90%, while the percentage of residual Thy-1^+ cells was < 2%, as determined by flow cytometry. Viable T cell-depleted spleen cells at 2.5×10^5 cells/ml were cultured in flat-bottom microtiter plates together with 100 μg/ml T cell membrane fragments of activated H66 cells (Th1 clone), a kind gift of Dr. P. D. Hodgkin [27, 28] in 0.2 ml complete RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 0.1 M pyruvate, 100 IU/ml penicillin, 50 μg/ml streptomycin, 50 μM 2-mercaptoethanol, 35 ng/ml

purified IL-4, 1.75 ng/ml purified IL-5 and 5 μg/ml anti-IFN-γ (XMG 1.2) [29]. The cultures contained murine recombinant IL-6 (British Bio-technology Limited, Oxford, GB) 100 IU/ml as indicated in the results section. Cultures which did not contain membrane fragments of activated H66 cells served as controls. After 7 days of culture at 5 % CO_2 and 37 °C supernatants were harvested for ELISA.

3 Results

3.1 Cytokine mRNA expression in the spleen during a primary IgE response

In this study we were interested in the involvement of IL-4 and IL-6 in IgG_1 and IgE responses. Therefore, we investigated the cytokine mRNA expression in the spleen during a primary response against TNP-KLH, which predominantly induces IgG_1 and IgE responses in the given dose and the adjuvant employed. At days 0, 2, 4, 6, and 8 after immunization mRNA was isolated from the spleens and after DNase I treatment RT-PCR was performed. Maximum IL-4 and IL-6 mRNA expressions were seen at day 2 after immunization, whereas IFN-γ mRNA expression peaked at day 6 (Fig. 1). Moreover, it was found that the mRNA levels for IL-6 and IFN-γ were significant higher than those for IL-4. For IFN-γ mRNA a higher baseline level was observed when compared to the mRNA baseline levels for IL-4 and IL-6. The largest increase was seen for IL-4 mRNA expression (ninefold), whereas the mRNA levels for both IL-6 and IFN-γ increased twofold (Fig. 1). These results point to an important role of IL-4 and IL-6 in inducing IgG_1 and IgE responses upon TNP-KLH immunization.

3.2 Involvement of IL-4 and IL-6 in TNP-KLH-induced immune responses

Because of these results and former observations [10, 12] we decided to examine the involvement of both IL-4 and IL-6 in primary IgG_1 and IgE responses more closely. To this end, mice were treated with neutralizing antibodies directed to IL-4 and IL-6 prior to immunization with

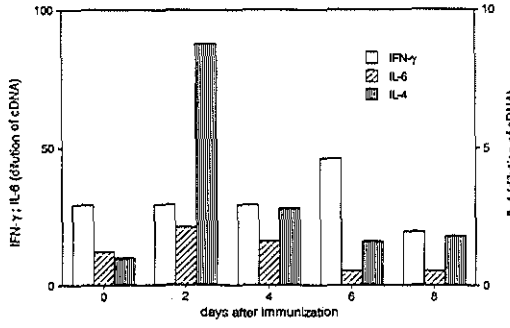

Figure 1. mRNA expression for IFN-γ, IL-6 and IL-4 in reverse-transcribed DNase I-treated total RNA preparations from spleens of mice at day 0, 2, 4, 6, and 8 after primary immunization with 10 μg TNP-KLH adsorbed on alum. Determined as described in Sect. 2.5.

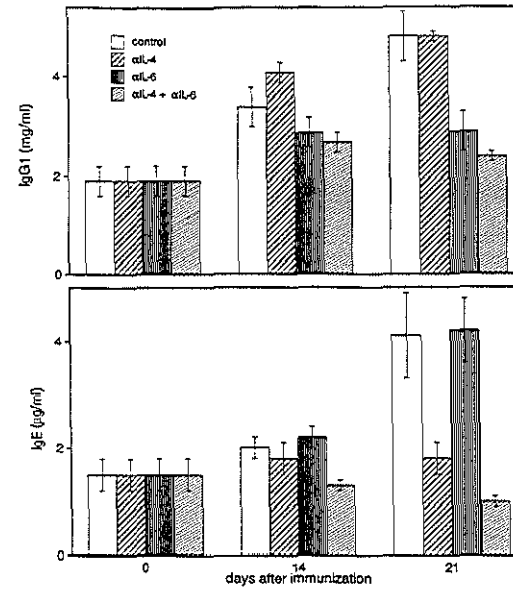

Figure 2. Effect of anti-(α)IL-4, anti-IL-6 or both on the primary total IgG_1 and IgE response. Mice were primed with 10 μg TNP-KLH adsorbed on alum i.p. 3 days after i.p. implantation of alginate-encapsulated anti-cytokine mAb producing hybridoma cells as indicated in Sect. 2.4. Serum levels are expressed as arithmetic mean ± SEM ($n = 5$).

10 μg/ml TNP-KLH, by implanting i.p. alginate-encapsulated 11B11 cells, which produce anti-IL-4 antibodies, and 20F3 cells, which produce anti-IL-6 antibodies. Both 11B11 and 20F3 are of rat origin, and produce antibodies of the IgG_1 isotype. Alginate-encapsulated GL113 cells producing rat IgG_1 antibodies directed to *E. coli* β-galactosidase served as isotype control. At day 21 maximum total IgG_1 and IgE responses were observed in the serum of immunized mice treated with control antibody, 4.8 mg/ml and 4.1 μg/ml, respectively (Fig. 2). Treatment of mice with anti-IL-4 completely abolished the increase in serum total IgE, whereas this treatment did not affect the increase in serum total IgG_1. Treatment with anti-IL-6 had an opposite effect on these two isotypes. It did not disturb the IgE response, whereas it inhibited the increase in serum total IgG_1 by 66 % (Fig. 2). For total serum IgE it was observed that the combination of encapsulated anti-IL-4-producing 11B11 and anti-IL-6-producing 20F3 cells decreased the serum level below the day 0 level, indicating an effect on the background production (Fig. 2).

With respect to the TNP-specific IgG_1 serum levels, other patterns were observed. These responses peaked at day 14. It was observed that neutralization of IL-6 alone did not inhibit the increase of TNP-specific serum IgG_1 (144 μg/ml at day 0 to 455 μg/ml on day 14). The increase in TNP-specific IgG_1 was inhibited by 80 % by treating mice with a combination of encapsulated 11B11 and 20F3 cells. At day 21 no significant inhibition was observed in this situation (data not shown). The production of anti-cytokine mAb by the encapsulated cells was monitored by measuring

rat IgG_1 in the serum of the mice in which they were implanted i.p. At day 7, 87 μg/ml, 108 μg/ml 319 μg/ml and 675 μg/ml rat IgG_1 was observed in animals treated with GL113, 11B11, 20F3 or 11B11 together with 20F3 cells encapsulated in alginate, respectively (data not shown). After day 14 a decline in these rat IgG_1 serum levels was observed. This decline could explain the difference between the TNP-specific IgG_1 serum levels found on day 14 and 21 in mice that were implanted i.p. with both encapsulated 11B11 and 20F3 cells.

3.3 Involvement of IL-4 and IL-6 in GAM IgD-induced immune responses

The results found with respect to the TNP-KLH immunization suggested that IL-6 had a more important role in polyclonal IgG_1 responses than in antigen-specific primary IgG_1 responses. To study this role more precisely we immunized mice with 800 μg GAM IgD i.v. which is known to elicit strong polyclonal IgG_1 and IgE responses [30]. Immunization of mice resulted in an increase in the serum of 24 mg/ml for total IgG_1 and 4 μg/ml for total IgE at day 7 (Fig. 3). As in the TNP-KLH response, treatment of mice with alginate-encapsulated anti-IL-4-producing 11B11 cells completely abolished the IgE response in serum, but in this experiment inhibition (42 %) of the IgG_1 response was also observed (Fig. 3). Neutralization of IL-6 by treating mice with 20F3 cells encapsulated in alginate inhibited the increase in serum total IgG_1 by 80 %, resulting in a serum level of total IgG_1 of 6 mg/ml (Fig. 3). The increase in serum total IgE was not influenced by this treatment. Moreover, no synergistic effect was seen for anti-IL-4 mAb and anti-IL-6 mAb in the GAM IgD-induced IgG_1 response (Fig. 3). GAM IgD injection neither induced an IgM nor an IgG_{2a} response, showing the isotype specificity of the induced response. These responses were not changed by the various anti-cytokine treatments (Fig. 3).

Table 1. Effect of anti-IL-6 treatment on IgE levels during Nb infection[a)]

Antibody injected	Dose	IgE μg/ml Day 0	IgE μg/ml Day 11
None		1.5 ± 9.4	41 ± 9.8
Anti-IL-4	1 mg	1.5 ± 0.4	16.5 ± 1.8
Anti-IL-6	1 mg	1.5 ± 0.4	25.9 ± 3.9
Anti-Il-4 + anti-IL-6	1 mg + 1 mg	1.5 ± 0.4	17.9 ± 2.1
Anti-IL-4	10 mg	1.5 ± 0.4	2.9 ± 0.7
Control	1 mg	1.5 ± 0.4	40.5 ± 5.4

a) Mice were infected with 750 stage III larvae s.c. at day 0. Antibodies were given i.p. in doses of 1 or 10 mg/mouse as indicated. Results are presented as arithmetic mean ± SD ($n = 5$).

3.4 Involvement of IL-6 in an Nb-induced IgE response

We next used a Nb infection model to study the effects of neutralizing IL-4 and/or IL-6 on the induction of a different, but also strong, polyclonal IgE response. Nb infection induced an increase in the total serum IgE level from 1.5 μg/ml on day 0 to 41.9 μg/ml on day 11. Neutralizing IL-4 with 10 mg purified 11B11 inhibited the IgE response by 97 %, whereas neutralizing IL-6 resulted in a 40 %

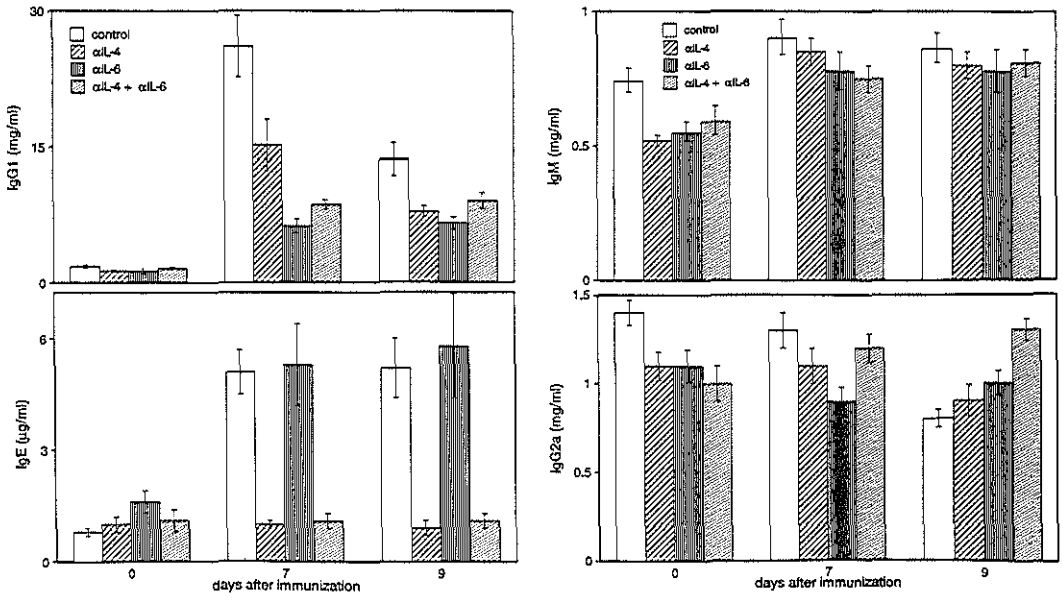

Figure 3. Effect of anti-(α)IL-4, anti-IL-6 or both on GAM IgD induced polyclonal IgG_1, IgE, IgM, and IgG_{2a} responses. Mice were immunized with 800 μg GAM IgD i.v. At day − 3 alginate-encapsulated anti-cytokine mAb producing hybridoma cells were implanted i.p. as indicated in the Sect. 2.4. Serum levels are expressed as arithmetic mean ± SEM ($n = 5$).

inhibition of the IgE increase (Table 1). These results indicate an involvement of IL-6 in obtaining a strong polyclonal increase in IgE. To study whether IL-4 and IL-6 worked synergystically in this response, an experiment was performed in which IL-4 was suboptimally neutralized (1 mg 11B11 i.p.), resulting in a 63 % inhibition. Neutralization of IL-6 at the same time did not increase the percentage of inhibition (Table 1), indicating that IL-4 and IL-6 do not synergize in this polyclonal IgE response.

3.5 IgG_1 production by naive B cells stimulated with T cell membrane fragments is not potentiated by IL-6

To study whether IL-6 can potentiate IgG_1 production by naive B cells we performed *in vitro* experiments in which naive splenic B cells were polyclonally stimulated with membrane fragments of activated Th1 cells in the presence or absence of exogenous IL-6. IL-4, IL-5 and anti-IFN-γ were present in all cultures to facilitate optimal responses [27, 28]. Addition of IL-6 neither enhanced nor inhibited the production of IgG_1 and IgE during the 7 days of culture (Fig. 4). In both stimulation conditions similar amounts of IgG_1 and IgE were produced: 20 μg/ml and 2.8 μg/ml, respectively. This result indicated that enough IL-6 was present in the culture system to facilitate peak IgG_1 and IgE production by naive B cells upon polyclonal stimulation.

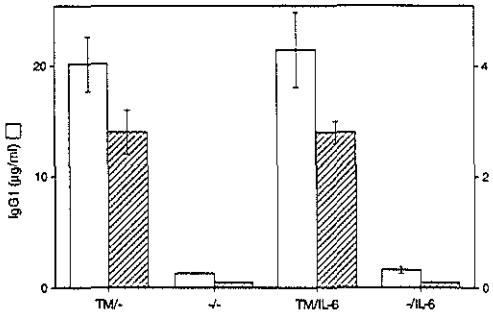

Figure 4. Thy-1-depleted spleen cells of naive mice were stimulated with T cell membrane fragments of activated Th1 cells (TM) in the presence or absence of IL-6. At day 7 the supernatants were harvested and the total IgG_1 and IgE levels determined. Results are expressed as arithmetic mean ± SD (n = 5).

anti-cytokine mAb-producing hybridoma cells. At day 5 the rat IgG_1 serum levels of mice implanted with encapsulated GL113, 11B11, 20F3 or 11B11 combined with 20F3 cells were determined and found to be 185 μg/ml, 149 μg/ml, 320 μg/ml and 397 μg/ml, respectively. On day 7 the 108 μg/ml rat IgG_1 measured in serum of mice implanted with in alginate-encapsulated anti-IL-4-producing 11B11 cells completely abolished the primary IgE response. Anti-IL-4 and anti-IL-6 treatment inhibited the TNP-specific IgG_1 response on day 7 by 34 % and 23 %, respectively. When these two treatments were combined, the TNP-specific IgG_1 response was inhibited by 57 %, indicating that both IL-4 and IL-6 are required for an optimal memory TNP-specific IgG_1 response (Fig. 5).

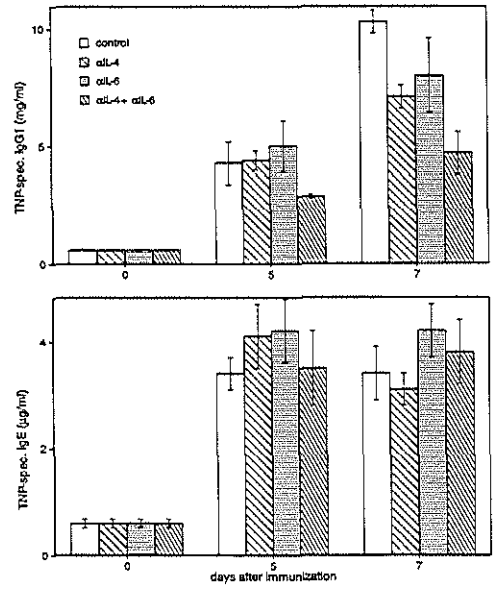

Figure 5. Involvement of IL-4 and IL-6 in memory IgG_1 and IgE responses. Mice that had been primed with 10 μg TNP-KLH were boosted 3 months later with 10 μg TNP-KLH. At day − 3 alginate-encapsulated anti-cytokine mAb producing hybridoma cells were implanted i.p. as indicated in Sect. 2.4. Serum levels are expressed as arithmetic mean ± SEM (n = 5).

3.6 Involvement of IL-4 and IL-6 in memory IgG_1 and IgE responses

We next studied the involvement of IL-4 and IL-6 in the memory TNP-specific IgG_1 and IgE responses. For this, BALB/c mice that had been primed with 10 μg TNP-KLH adsorbed on alum 3 months before were boosted with 10 μg TNP-KLH adsorbed on alum. Maximum TNP-specific IgG_1 responses were seen on day 7, whereas a maximum TNP-specific IgE response was already observed on day 5 (Fig. 5). This TNP-specific IgE response was neither inhibited by either anti-IL-4 or anti-Il-6 alone, nor by the combination of these two anti-cytokine mAb. Treatment was performed by implantation of alginate-encapsulated

3.7 Effect of neutralization of IL-4 and IL-6 on memory B cells to become IgG_1 or IgE-secreting plasma cells

In other studies we have shown that adoptive transfer of primed spleen cells to irradiated control mice leads to enhanced memory IgG_1 and IgE responses after boosting the reconstituted mice [15]. Therefore, we reconstituted 6 Gy-irradiated mice with 1×10^7 TNP-KLH primed spleen cells and studied the effect of neutralizing IL-4 and IL-6 by injecting mice i.p. with 10 mg anti-IL-4, 10 mg anti-IL-4 + 2 mg anti-IL-6. Previously, IL-5 was reported to have an important role in inducing IgG_1-positive B cells to secrete IgG_1 [31]. For this reason we also studied the involvement of both IL-5 and IL-6 in memory IgG_1 and IgE responses in

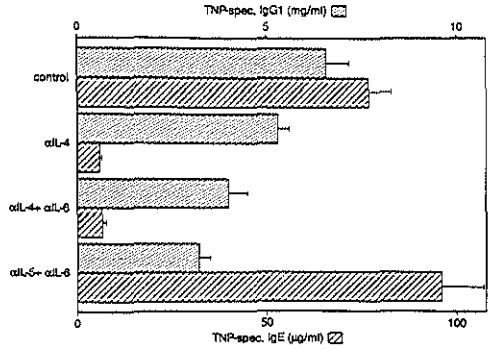

Figure 6. Effect of neutralization of IL-6 and IL-5 on peak levels of memory IgG_1 and IgE responses after adoptive transfer. Irradiated mice (6 Gy) were reconstituted with TNP-KLH primed spleen cells. Mice were injected i.p. with the following antibodies: control (GL113, 4 mg/mouse); anti-(α)IL-4 (11B11, 10 mg/mouse); anti-αIL-5 (TRFK-5, 2 mg/mouse); and anti-αIL-6 (20F3, 2 mg/mouse). All reconstituted mice were boosted with 10 μg TNP-KLH adsorbed on alum i.p. Results represent arithmetic mean ± SEM (n = 5–15).

reconstituted mice. To this end mice were injected i.p. with 2 mg anti-IL-5 + 2 mg anti-IL-6. Control mice received 4 mg rat IgG_1. Subsequently all mice were boosted with 10 μg TNP-KLH i.p., resulting in a peak response for IgE on day 9 and for IgG_1 on day 12. Neutralization of IL-4 inhibited the IgE memory response by 90%, whereas it inhibited the IgG_1 response by 20% (Fig. 6). Neutralization of both IL-5 and IL-6 significantly inhibited the IgG_1 memory response by 52%. This treatment did not influence the memory IgE response (Fig. 6). No synergistic effect was observed for IL-4 and IL-6 with respect to the memory IgE response, suggesting that also in the memory response no IL-6 is required for IgE production, even not in the absence of IL-4.

4 Discussion

This study shows that murine IgG_1 and IgE responses differ in their requirement for IL-6 in reaching peak levels. Neutralization of IL-6 resulted in inhibition of polyclonal IgG_1 responses. Primary and memory antigen-specific IgG_1 responses were slightly inhibited by neutralizing IL-6, but neutralization of both IL-4 and IL-6 resulted in a marked decrease. These results show that these two cytokines act synergystically during a primary and memory antigen-specific IgG_1 responses. However, IL-6 neutralization did not inhibit antigen-specific and polyclonal IgE responses both after primary and secondary immunization. For human B cells a synergistic effect of IL-6 in combination with IL-4 in generating IgE has been described, by showing that anti-IL-6 antibodies strongly inhibited the IL-4-dependent IgE production *in vitro* [10, 11]. In mice we did not observe such synergistic effect. Therefore, we conclude that murine B cells and human B cells differ with respect to the need for IL-6 to give rise to peak levels of IgE.

Neutralization of IL-4 after primary immunization with TNP-KLH resulted in an abrogation of the increase in total serum IgE, whereas it did not result in an inhibition of the increase in total serum IgG_1, an effect that has been well documented by us and other investigators [15, 30, 32]. However, we found that IgG_1 and IgE responses also differ in their need for IL-6 to obtain peak levels after primary immunization with TNP-KLH. Neutralization of IL-6 resulted in an inhibition of total serum IgG_1, whereas it did not have any effect on the induced total serum IgE response. No inhibition of the increase in antigen-specific IgG_1 was observed as result of IL-6 neutralization, but neutralization of both IL-4 and IL-6 resulted in 80% inhibition of the antigen-specific IgG_1 response. This suggests that polyclonal IgG_1 responses are more dependent on IL-6 than antigen-specific IgG_1 responses, most likely as the result of preactivated B cells that only need IL-6 to become IgG_1-secreting plasma cells. Moreover, with respect to IL-4 the opposite can be concluded in that antigen-specific IgG_1 responses are more dependent on IL-4 than polyclonal IgG_1 responses, suggesting that the polyclonal response originates from B cells already switched to IgG_1. The induction of an IgE response, both antigen specific and polyclonal, could be inhibited by neutralizing IL-4, whereas neutralization of IL-6 did not have such effect. Furthermore, no synergistic effects were observed with respect to inhibition of polyclonal and antigen-specific IgE responses, when both IL-4 and IL-6 were neutralized.

It was noted, by studying the mRNA expression for IL-4, IL-6 and IFN-γ in the spleen after TNP-KLH immunization, that IL-4 and IL-6 are expressed simultaneously. After peaking at day 2 the mRNA levels for IL-4 and IL-6 returned to baseline level by day 6. At this time point a peak level for the IFN-γ mRNA expression was found. These results suggest that IL-4 and IL-6 act in concert during a TNP-KLH-induced IgG_1 and IgE response. The up-regulation of IFN-γ mRNA and, at same time, down-regulation of the mRNA levels of IL-4 and IL-6 suggest an active role for IFN-γ in this process. This observation could very well provide the basis for the well-documented inhibition of IL-4-induced IgG_1 and IgE synthesis mediated by IFN-γ [32]. Svetic et al. [33] also found after immunization with GAM IgD a coordinate expression of IL-4 and IL-6 mRNA.

To study the involvement of IL-4 and IL-6 in a strong polyclonal response we did experiments in which we used GAM IgD to evoke large polyclonal IgG_1 and IgE responses *in vivo* [30]. The GAM IgD-induced serum peak IgG_1 levels could be inhibited for 80% by neutralizing IL-6, indicating that IL-6 is important for the induction. As expected, the induced increase in total serum IgE could be completely inhibited by neutralizing IL-4. No inhibition of the IgE response was found upon neutralizing IL-6. Also in the GAM IgD-induced response no synergistic effect was found when both IL-4 and IL-6 were neutralized. Two possibilities can account for this new phenomenon. It has been described that IL-6 is required for terminal differentiation of B cells to Ig-secreting plasma cells [34]. Here, we show that this observation holds for IgG_1- but not IgE-secreting cells. This suggests that B cells switched to IgE are already in a further differentiation stage than B cells switched to IgG_1. This might be a direct consequence of IL-4 which is an absolute requirement for the formation of B cells switched to IgE, but not for the induction of B cells

switched to IgG_1 [1]. More speculative is the possibility that B cells switched to IgE do not express the receptor for IL-6. This could explain the observation that such B cells produce less antibody than B cells switched to IgG_1, as it has been described that IL-6 enhances the murine antibody response [35]. However, the *in vitro* experiments, revealed that both the IgG_1 and IgE production were not influenced by exogenous IL-6. It is possible that no enhancement by IL-6 is observed because enough endogenous IL-6 is produced in the *in vitro* cultures to obtain peak production. Nevertheless, this study makes clear that neither the IgG_1 nor the IgE production is inhibited by exogenous IL-6.

For *Mesocestoides corti*-infected mice it was described that neutralization of IL-6 resulted in a marked inhibition of the IgG_1 serum levels, which are known to increase as result of infection [36]. This is in line with our results. However, *M. corti* infection does not lead to an increase in serum IgE. Therefore, it could not be determined whether IL-6 was necessary for peak IgE responses upon parasite infection. In this study we show that IL-6 in part is necessary for the large increase in polyclonal IgE responses after infection with Nb. However, no synergistic effect of neutralizing IL-4 and IL-6 with respect to the inhibition of the IgE response was seen when IL-4 was suboptimally neutralized.

We also examined the IL-6 dependence of antigen-specific IgG_1 memory responses, because it was shown by Hilbert et al. [37] that primary influenza virus-specific antibody responses were IL-6 dependent, whereas secondary antigen-specific antibody responses were IL-6 independent. In that study, however, no distinction has been made between the individual isotype responses that account for the antigen-specific antibody responses after priming and boosting with the antigen. In our study no difference was seen with respect to IL-6 dependence between the antigen-specific primary and memory IgG_1 response. For the antigen-specific memory IgG_1 response no synergistic effect was observed when both IL-4 and IL-6 were neutralized. As found in former studies, the antigen-specific memory IgE response was not completely inhibited by neutralizing IL-4. However, the amount of anti-IL-4 antibody present in the serum of treated mice was sufficient to completely inhibit the primary IgE response. Hence, it is possible that the amount of anti-IL-4 antibodies present in the serum is not enough to completely neutralize IL-4 after secondary immunization. This in combination with a decreased IL-4 dependence of memory B cells to secrete IgE could account for a normal antigen-specific IgE response after secondary immunization.

The adoptive transfer experiments were performed because it is known that in this sort of experiments upon boosting the secondary IgE responses are preferentially enhanced [15, 38]. In these experiments, it was found that only neutralizing IL-4 inhibited the memory IgE response, leaving 10% of the response intact. This part of the response is also independent of IL-6, and is most likely the result of B cells switched to IgE. In addition, neutralization of both IL-5 and IL-6 did not influence the IgE memory response, whereas it did inhibit the IgG_1 memory response, showing that both IL-5 and IL-6 are involved in the IgG_1, but not the IgE memory response.

Collectively, these results show that IgG_1 and IgE responses, both primary and secondary, differ in IL-6 dependence. Whereas IgG_1 responses are inhibited *in vivo* by neutralizing IL-6, IgE responses are not influenced. The difference in IL-6 necessity to obtain peak IgG_1 and IgE responses provides a new regulatory mechanism, besides IL-4, for the murine IgG_1 and IgE responses coupled to each other by sequential isotype switching.

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Chapter 9

General discussion

GENERAL DISCUSSION

Upon antigenic stimulation antigen-specific B cells proliferate and undergo immunoglobulin heavy chain class switch recombination. Activated antigen-specific T cells govern these processes by means of the cytokines they secrete. Subsequently, the activated B cells either differentiate into immunoglobulin secreting plasma cells, or become resting B cells again. These so-called resting memory B cells express immunoglobulin classes other than IgM and IgD due to isotype switching. Moreover, the surface immunoglobulin molecules expressed by these B cells display a higher affinity for the antigen as a result of affinity maturation. This process occurs in the germinal centers and is antigen-driven. The immune system will react upon a subsequent antigenic encounter much more rapidly as a result of these pre-existing antigen-experienced memory B cells. Moreover, it will manifest itself much more strongly with the generation of immunoglobulin molecules other than IgM.

The aim of this study was to improve the insight into the regulatory effects of cytokines on the B cell memory formation. This is essential for the understanding of antigen-specific memory responses that are characterised by the appearance of immunoglobulin classes other than IgM. Since the immunoglobulin class switch to IgG_1 and IgE isotypes is tightly controlled by currently known cytokines as IL-4, we focused our attention on these isotypes.

The following questions were addressed:
- What is the role of IL-4 in the memory formation for IgG_1 and IgE?
- What is the cytokine dependence of memory IgG_1 and IgE responses?
- Are memory IgE responses generated via $\gamma_1\varepsilon$-double-positive B cells?
- Thus, are memory IgE responses a consequence of sequential isotype switching?

In vivo IL-4 treatment

To study the effect of cytokines on the formation of antigen-specific B cells *in vivo* a method was developed permitting a constant systemic level of cytokine for prolonged periods of time (Chapter 2). Adherent growing cells stably transfected with murine genes for either IL-4, IL-5 or IFN-γ were encapsulated in alginate and implanted subcutaneously or intraperitoneally in mice. The use of cytodex beads as a growth substrate provided an excellent means for exact dosage of implanted cells. The encapsulated CV-1 cells stably transfected with the murine IL-4 gene secreted IL-4 for a period of at least 2 weeks after implantation, as was shown by analyzing the IL-4

mRNA expression in CV-1/IL-4 cells recovered out of the alginate capsules in the peritoneal cavity. After these two weeks both the HPRT and IL-4 mRNA expression in the isolated cells slowly declines as the consequence of cell death, resulting in undetectable expression by day 18 after implantation. This limited period of production is mainly caused by breaking or dissolving of the capsules and by diffusion restrictions with respect to nutrients, toxic wastes and oxygen supply [Cheetham et al., 1979; Hashimoto and Shirai, 1990].

The alginate technique could also be used to entrap and implant hybridoma cells producing rat mAb specific for murine cytokines. However, under these conditions no exact dosage of implanted cells could be obtained, as growth will continue until limited by physical parameters, such as charge repulsion between the net negative surface of cells and the negatively charged carbohydrate groups of the alginate [Haug and Smidsrod, 1967]. The mAb production in mice bearing encapsulated hybridoma cells can be easily monitored in the serum using a rat isotype-specific ELISA. Experiments in which *in vivo* IL-4 levels were neutralized by implanting anti-IL-4 producing 11B11 cells every two weeks for a period of three months indicated that no mouse anti-rat-IgG_1 response occurred. These mice still did not mount an IgE response upon boostering with TNP-KLH. In the case of a mouse anti-rat-IgG_1 response no complete neutralization of IL-4 would be expected upon boostering with TNP-KLH, resulting in an elevation of the serum IgE.

The implantation of encapsulated CV-1/IL-4 cells proved to be a powerful technique as it was shown that upon immunization TNP-specific IgE responses could be induced in IgE non-responder SJA/9 mice implanted with encapsulated CV-1/IL-4 cells [Savelkoul et al., 1991]. Under normal conditions these mice do not mount an IgE response upon stimulation, indicating that implantation of CV-1/IL-4 cells yields sufficient functional IL-4 to induce the formation of IgE. This illustrates that implantation of alginate encapsulated cytokine transfected cells is potentially applicable in the modulation of immune responses *in vivo*.

Recently two IL-4 transgenic mouse strains have been developed [Tepper et al., 1990; Burstein et al., 1991; Muller et al., 1991]. These mouse strains express different IL-4 levels *in vivo*. The IL-4 transgenic mouse strain made by Tepper et al. does express elevated serum IgE levels, whereas no elevated IgE levels are expressed by the IL-4 transgenic mice developed by Muller et al. [Burstein et al., 1991; Muller et al., 1991]. A demerit of IL-4 transgenic mice is the disturbed IL-4 level during the ontogeny that could lead to aberrant situations when studying the effect of elevated IL-4 levels on the *in vivo* immune response. This disadvantage does not occur in lethally irradiated mice

infused with bone marrow cells, infected with recombinant retrovirus bearing the IL-4 gene, that expresses high levels of IL-4 [Chambers et al., 1992]. However, it is clear that also irradiated recipients reconstituted with bone marrow cells do not apply to the physiologic situation. We therefore used neither of these models to increase the *in vivo* IL-4 levels. Instead we implanted alginate encapsulated IL-4 producing cells in normally developed mice. Such implantation was carried out every two weeks, because after two weeks the amount of IL-4 produced by the encapsulated cells declined (Chapter 2). The IL-4 production by the encapsulated cells was indirectly examined by means of IL-4 mRNA expression, which does not change during the first two weeks after implantation. Thereafter it slowly declines, and after 18 days IL-4 mRNA is no longer detectable in cells isolated out of the capsules present in the peritoneum (Chapter 2).

Antigen-specific and bystander responses during IL-4 treatment

To study the effect of IL-4 on the antigen-specific memory formation for IgG_1 and IgE, mice were primed with TNP-KLH and subsequently implanted with encapsulated IL-4 producing CV-1/IL-4 cells every two weeks for a period of four months (Chapter 4). IL-4 treatment resulted in elevated serum total IgG_1 and IgE, whereas it did not disturb the serum total IgG_{2a} levels. This illustrates the isotype specificity of the IL-4 treatment. Unexpectedly, the antigen-specific serum IgG_1 and IgE responses decreased as the result of the prolonged IL-4 treatment. This decrease was accompanied by a decrease in the formation of antigen-specific IgG_1 and IgE memory B cells as shown by adoptive transfer of spleen cells from control and IL-4 treated mice in sublethally irradiated non-treated recipient mice. An adoptive transfer system was chosen, because it is known that secondary IgE responses are preferentially enhanced after transferring spleen cells in irradiated control mice [Okudaira and Ishizaka, 1974]. Moreover, adoptive transfer experiments allowed investigation of the TNP-specific memory responses in a more isolated way, without the possible interference of residual systemic effects induced in the treated donor mice by encapsulated IL-4 producing cells that could be still present.

The observed effects of IL-4 with respect to the total and antigen-specific IgG_1 and IgE production could be exerted directly at the B cell level, or indirectly e.g. on APC and/or T cells. For T cells it has been suggested that IL-4, when persistently present, could reduce their IL-4 receptor expression [Renz et al., 1991]. Since IL-4 can act as a proliferation factor of activated T cells [Brown et al., 1988; Mosmann and Zlotnik, 1990] it is tempting to speculate that IL-4 receptor downregulation can lead to decreased T cell proliferation, resulting in a reduced availability of T cells. Indeed, we did find a decrease in the number of T cells in the spleens of IL-4 treated mice (Chapter 4), and we cannot exclude that the observed decrease in $CD4^+$ T cells of IL-4 treated mice is to

some extent due to a (selective) disappearance of antigen-specific Th2 cells. On the other hand, no difference in the IL-4 production by Con A stimulated spleen cells was observed as a result of the IL-4 treatment.

Considering the results of Chapter 4, an indirect effect of IL-4 on B cell memory formation for IgG_1 and IgE seems unlikely, since TNP-specific IgG_1 and IgE responses in the adoptive transfer experiments were not influenced by differences in the percentage of T cells in the spleen cell samples of IL-4 treated mice used to reconstitute the irradiated mice. These results indicated that a more likely explanation for the observed phenomena is a direct negative effect of IL-4 on the antigen-specific B cell. This would be consistent with the observation that IL-4 selectively inhibits antigen-specific B cell activation by antigen-specific T cells [Asano et al., 1988]. Furthermore, the adoptive transfer results show that the observed inhibition of the TNP-specific memory formation for IgG_1 and IgE by IL-4 is a long term effect as the same degree of inhibition was found three months after the last administration of IL-4 (Chapter 4). Adoptive transfer experiments using combinations of B cells and T cells from control and IL-4 treated mice to reconstitute sublethally irradiated mice revealed that the observed IL-4 induced effect on the antigen-specific IgG_1 and IgE memory formation was indeed directly mediated at the B cell level and not indirectly via T cells (Chapter 5).

The markedly elevated bystander responses, that is responses to other antigens than TNP-KLH, in IL-4 treated mice could be the result of enhanced differentiation or prolonged survival of pre-activated B cells [Croft and Swain, 1991; Hodgkin et al., 1991]. We have shown that immunization with TNP-KLH alone or adsorbed on alum induces enough endogenous IL-4 to mount IgE and IgG_1 responses (Chapter 3). The effect of CV-1-secreted IL-4 may be to enhance responses to "e.g. environmental antigens" that do normally not induce levels of IL-4 high enough to result in IgE or IgG_1 responses, possibly by reversing the Fc receptor-mediated inhibition of B cell activation [Sinclair and Panoskaltis, 1988]. In the absence of IL-4, crosslinking of the Fc receptor for IgG to membrane Ig on B cells delivers a negative signal that blocks B cell activation and subsequent differentiation [Amigorena et al., 1992]. In the presence of IL-4 the Fc receptor-mediated inhibition is reversed [O'Garra et al., 1987], allowing B cell activation by "environmental antigens". Alternatively, the IL-4 induced increase in CD23 expression on B cells (Chapter 4) might result in increased antigen focusing and presentation by IgE displaying various specificities [Mudde et al., 1990]. Both proposed mechanisms can lead to activation of B cells that can subsequently switch to IgG_1 and IgE, causing high levels of these isotypes.

These high polyclonal IgG_1 and IgE levels themselves could have a negative effect on

TNP-specific IgG_1 and IgE formation. It is possible that the high level of polyclonal IgG_1 not only inhibited the TNP-specific IgG_1 levels, but also the TNP-specific IgE response, since the IgG_1 response is coupled to the IgE response [Mandler et al., 1993]. Our hypothesis is that follicular dendritic cells present in germinal centers are loaded with irrelevant immune complexes which prevent binding of TNP-KLH immune complexes, resulting in a decreased generation of antigen-specific memory B cells committed to IgG_1 and IgE.

The IL-4 induced increase in serum total IgG_1 and IgE is accompanied by an increase in the number of cells switched to IgG_1 and IgE (Chapters 5, 6 and 7). This was found both at the membrane level and by stimulating B cells *in vitro* in an isotype-specific but antigen non-specific fashion, using rabbit anti-mouse isotype-specific antibodies and a rabbit Ig-specific Th2 cell clone [Tony and Parker, 1985].

Other studies in which the *in vivo* IL-4 levels were modulated systemically by generating IL-4 transgenic mice, that express IL-4 either in B cells [Muller et al., 1991] or in both B and T cells [Tepper et al., 1990], reported different observations with respect to IgE. These differences were most likely due to differences in IL-4 expression [Burstein et al., 1991; Muller et al., 1991]. A study in which long term *in vivo* IL-4 expression was induced by using retroviral-mediated gene transfer showed that the serum IgG_1 levels increased 3-fold, whereas no increase in serum IgE was observed as a result of this treatment [Chambers et al., 1992]. In our model enough IL-4 was present to result in a 7-fold increased serum total IgG_1 level and a 15-fold increased serum total IgE level (Chapter 4). Similar increases in serum total IgG_1 and IgE were found in mice in which IL-4 was expressed under control of the H-2K regulatory sequences [Erb et al., 1994]. In these mice low levels of IL-4 expression was found in most organs. In contrast to our results is the observation of Erb et al. (1994) that besides the increase in serum total IgG_1 and IgE these mice also express much higher numbers of antigen-specific IgG_1 and IgE secreting cells during an immune response against DNP-KLH. However, no data were shown, making it impossible to verify the results. Moreover, from the presented data it does not become clear at what time point after immunization Erb et al. determined the numbers of antigen-specific IgG_1 and IgE secreting cells [Erb et al., 1994]. This is essential, because our results indicate that the antigen-specific serum IgG_1 levels profoundly decrease in IL-4 treated mice, when compared to control treated mice, starting 30 days after immunization with TNP-KLH (Chapter 4).

Taken together, our results provide evidence for the necessity of a balance between the amount of IL-4 and antigen in order to obtain maximum antigen-specific IgG_1 and IgE responses. They extend the *in vitro* findings of Asano et al. [Asano et al., 1988] who

found that IL-4 induced by high doses of antigen inhibited the antigen-specific IgG_1 production. Disturbance of the IL-4/antigen balance either by neutralizing IL-4 or by administration of IL-4 will lead to the inhibition of antigen-specific IgG_1 and IgE formation. In general, antigen-specific and bystander responses are differentially regulated and caution has to be taken when IL-4 administration is considered to increase the induction of IgG_1 or IgE specific for a particular antigen (Chapter 4).

IgG_1 and IgE double-positive cells and sequential isotype switching

The next question we addressed was, whether IgG_1 positive memory B cells could switch to IgE as described for primary responses to *Nippostrongylus brasiliensis in vivo* [Yoshida et al., 1990] and for LPS stimulation *in vitro* [Mandler et al., 1993]. Stimulation of γ_1 positive B cells, both from control and IL-4 treated mice, with a rabbit anti-mouse-IgG_1 antibody and a rabbit-Ig-specific Th2 cell clone *in vitro* resulted in IgE-secreting cells detectable at day 5 of culture. The number of IgE-secreting cells obtained after culturing T cell depleted spleen cells of mice treated with IL-4 for four months is approximately 3-fold higher than when T cell depleted spleen cells from control treated mice are cultured under similar conditions (Chapter 6). Two explanations can be given for the appearance of IgE-secreting cells in these cultures. They can either originate from γ_1-positive cells that have undergone sequential isotype switching [Yoshida et al., 1990; Mandler et al., 1993], or can be the result of pre-existing $\gamma_1\varepsilon$-double positive B cells [Snapper et al., 1988a] that differentiate to IgE-secreting cells after stimulation.

FACScan analysis revealed a profound increase of the pool of $\gamma_1\varepsilon$-double positive B cells in the spleens of mice treated with IL-4 for four months, whereas the number of γ_1-single and ε-single positive B cells did not significantly differ between control and IL-4 treated mice (Chapter 6). It was found that ε-positive B cells could be stimulated with a rabbit anti-mouse-IgE antibody and a rabbit-Ig-specific murine Th2 cell clone to develop in both IgG_1- and IgE-secreting cells during 5 days of culture. This suggests that part of the $\gamma_1\varepsilon$-double positive B cells express IgE on their surface without deleting the γ_1 heavy chain gene. Otherwise no IgG_1 would be expected under the mentioned culture conditions. As described in the General introduction, section 1.10, ε-germline transcripts could act as a substrate for RNA trans-splicing, during which RNA encoding the ε-heavy chain is trans-spliced on a productive γ_1-mRNA [Harriman et al., 1993]. Such process would result in a productive ε-mRNA transcript and as a consequence in B cells expressing membrane bound IgE without deleting their γ_1 heavy chain gene. These $\gamma_1\varepsilon$-double positive cells explain the observed results, because they can be stimulated both via $sIgG_1$ and sIgE to secrete IgG_1.

It is also possible that upon stimulation the $\gamma_1\varepsilon$-double positive B cells develop into B cells that co-secrete IgG_1 and IgE, an option for which evidence has been presented [Mandler et al., 1993]. Such co-secretion of IgG_1 and IgE could be the result of sequential isotype-switching of a γ_1-single positive cell to an ε-single positive cell with a $\gamma_1\varepsilon$-double positive cell as intermediate [Yoshida et al., 1990; Mandler et al. 1993] provided that longlived functional γ_1-mRNA molecules exist.

In all our studies, more IgG_1- and IgE-secreting cells per culture were found upon culturing T cell depleted spleen cells of IL-4 treated mice than upon culturing T cell depleted spleen cells of control treated mice (Chapters 5 and 6). This indicates the pronounced effect of the IL-4 treatment with respect to these isotypes.

The number of $sIgG_1$ and/or sIgE positive B cells were determined to demonstrate the occurence of sequential isotype switching during the *in vitro* cultures. When γ_1-positive splenic B cells were selectively stimulated in an isotype-specific fashion through IgG_1, using a rabbit-Ig-specific murine Th2 clone and RAM/IgG_1 antibody as antigen, the number of γ_1-positive cells decreased while the percentage of $\gamma_1\varepsilon$-double positive B cells increased from 1 to 7% of all B cells (characterised by B220 staining). The 7% $\gamma_1\varepsilon$-double positive B cells correspond with the observation that 9% of all B cells are $\gamma_1\varepsilon$-double positive upon *in vitro* LPS stimulation [Snapper et al. 1988a]. We only observed sequential switching when B cells were obtained from mice that were repeatedly treated with IL-4. Therefore, we conclude that IL-4 commits γ_1-positive B cells to undergo a sequential switching to ε-positive B cells. However, it is also possible that IL-4 preferentially expands B cells that are predisposed to switch sequentially to ε, which could result in an detection of the sequential isotype switching at the membrane level when culturing spleen cells from IL-4 treated mice.

All available data in the literature, supporting the sequential isotype switching model, concerned primary immune responses. In this thesis it is shown that sequential isotype switching is also likely to occur during secondary responses, by showing that γ_1-positive primed B cells upon stimulation develop into $\gamma_1\varepsilon$-double-positive B cells that can differentiate into both IgG_1- and IgE-secreting cells (Chapter 6).

Persistent IgE formation

The alginate technology permits the study of long-term effects of *in vivo* IL-4 treatment. It allows a systemic elevation of the *in vivo* IL-4 concentration during a restricted period of time, depending on the number of injections of alginate encapsulated IL-4 transfected cells. Therefore, it is possible to monitor effects induced by elevated IL-4 levels *in vivo*

at a time point when the alginate encapsulated IL-4 transfected cells do not produce IL-4 anymore. Such studies are not feasible in IL-4 transgenic mice, because of their constitutive IL-4 production. We found that four months after ending the prolonged IL-4 treatment the serum total IgG_1 and IgE levels were still elevated (Chapter 7). In Chapter 2 it was shown that CV-1/IL-4 cells encapsulated in alginate can be recovered out of the peritoneal cavity till day 18, but that IL-4 mRNA can only be detected in the recovered encapsulated cells till day 14. Moreover, 90 days after the last of 10 injections of CV-1/IL-4 cells, we were no longer able to detect intact capsules in peritoneal washes of IL-4 treated mice. Neither could we detect living CV1/IL-4 cells or IL-4 mRNA expression in these washes (Chapter 7). This, and the fact that xenogenic CV-1/IL-4 cells cannot persist out of the alginate capsules, indicates that the elevated IgG_1 and IgE serum levels can not simply be explained by persisting IL-4 producing CV-1/IL-4 cells. Upon Con A stimulation 90 days after the last of 10 control or IL-4 administrations more IL-4 and IL-6 was produced by spleen cells obtained from IL-4 treated mice, than by spleen cells from control treated mice under similar conditions (Chapter 7). In similar cultures, no differences were found with respect to the amount of IFN-γ produced. The increased IL-4 and IL-6 production levels are in accordance with studies describing the induction of Th2 cells by IL-4 [Swain et al., 1990; 1991; Swain, 1991; Abehsira-Amar et al., 1992]. From our studies it can be concluded that the increased IL-4 and IL-6 production are not occurring at the expense of the IFN-γ production.

Con A stimulation of spleen cells, however, does not reveal the actual cytokine production by the spleen cells *in vivo* at a certain time point. Therefore, we examined the IL-4, IL-10 and IFN-γ mRNA expression in spleen cells from mice with persisting IgE, at day 90 after the last of 10 control or IL-4 administrations. No differences with respect to the IL-4 mRNA expression were found between the two groups (Chapter 7). This indicated that the persistently elevated serum IgE levels were not caused by an increased endogenous IL-4 production. Neither were they induced by a decreased IFN-γ production, as it was found that the mRNA expression for this cytokine turned out to be increased in IL-4 treated mice, when compared to control treated mice.

The observation that the persistently elevated serum IgE levels, 90 days after the last of CV-1/IL-4 cell administrations, were neither caused by exogenous IL-4 nor by endogenous IL-4 is corroborated by the finding that the persistently elevated serum IgE levels were neither influenced by neutralizing IL-4. For these reasons we called the persistent serum IgE levels IL-4 independent.

The IL-4 independent serum IgE level suggested the involvement of B cells already

switched to IgE and that do not need IL-4 to sustain the elevated serum IgE level. Such cells could be detected in the spleen three months after the last CV-1/IL-4 cell administration (Chapter 7). Moreover, these B cells were also positive for IgG_1. Three months after the last control or CV-1/IL-4 cell administration, spleens from IL-4 treated mice contained two times more $\gamma_1\varepsilon$-double positive B cells than spleens from control mice. Using membrane fragments of activated Th1 cells we showed that spleens from IL-4 treated mice contained a population of resting B cells that did not need IL-4 to produce IgE, even three months after the last IL-4 administration. Such B cell population was not found in the spleen of control treated mice, suggesting that the induction of this population is dependent on IL-4. Moreover, we found that part of the B cells that were IL-4 independent with respect to IgE production, were also gp39 independent. The IgG_1 production, however, turned out to be completely gp39 dependent. This is in accordance with *in vivo* studies in which it was found that IgG_1, but not IgE responses, could be completely inhibited by an antibody directed to gp39 (A.J.M. van den Eertwegh, to be published). These data are also consistent with studies suggesting that gp39 has a switch inducing activity for IgG_1 [Schultz et al., 1992]. In that study it was shown that gp39 induces germline $C\gamma_1$ transcripts independently of IL-4. Our results do not exclude the possible existence of a ligand for CD40, other than gp39, that is also involved in B cell activation, but which interaction with CD40 can not be blocked by anti-gp39. The existence of such ligand has been suggested by Dr. J.E. de Vries (personal communication).

We and others have described that memory IgE responses, both *in vivo* and *in vitro*, were less dependent on IL-4 than primary IgE responses (Chapters 4, 6 and 7) [Heusser et al, 1989; Finkelman et al., 1990; Katona et al., 1991]. It was shown by Finkelman et al. that IL-4 is required to sustain established IgE responses [Finkelman et al., 1988]. In Chapter 7 we show that prolonged IL-4 treatment induced persistently elevated serum total IgE levels, that can not be inhibited by neutralizing IL-4. In other studies it could be argued that small traces of IL-4 were responsible for IgE synthesis. This option is not likely for the *in vitro* system used in Chapter 7, because activated membrane fragments of Th1 cells were used to stimulate purified B cells and neutralizing antibodies directed to IL-4 were added, to neutralize any possible traces of IL-4.

All the data presented in this thesis concerning the IgG_1 and IgE production by IL-4 treated mice and the IL-4, IL-6 and IFN-γ production upon *in vitro* stimulation of spleen cells from IL-4 treated mice three months after the last CV-1/IL-4 cell administration suggest that treated mice are biased in the Th2 direction, with respect to their immune responses. This notion is supported by the observation that upon vaccination with a non-virulent PRV-strain followed by a challenge with the virulent strain lower levels of

antigen-specific IgG_{2a} are produced by IL-4 treated mice than by control treated mice. This effect lasted even 208 days after the last control or IL-4 treatment. This suggests a perturbed potentially Th1 mediated immune response in IL-4 treated mice, even long after the last IL-4 administration. The lower levels of antigen-specific IgG_{2a}, however, did not result in an impaired protective immunity against a lethal challenge with virulent virus after previous vaccination with the non-virulent strain. This in contrast to all non-vaccinated mice, that did succumb to the infection with the lethal strain (Chapter 6).

Cytokine dependence of memory B cell responses

The crucial role of IL-4 in regulating the primary IgE synthesis has been well established by studying IL-4 knock-out mice, that were made IL-4 deficient by gene targeting. Upon nematode infection no IgE synthesis was observed whereas IgG_1 synthesis could still be detected in these mice, but the level produced was only one-sixth that of control mice [Kuhn et al., 1991]. Moreover, treatment of mice with neutralizing antibodies to IL-4 or IL-4 receptor completely inhibited the production of IgE, but marginally inhibited the IgG_1 response to various antigens [Coffman and Carty, 1986; Finkelman et al., 1986; 1991]. In line with this result is the observation that less IL-4 was required for obtaining peak IgG_1 responses than for peak IgE responses upon LPS stimulation of B cells *in vitro* [Snapper et al., 1988b]. Like IgG_1 responses, secondary IgE responses are partially IL-4 independent [Heusser et al., 1989; Katona et al., 1991]. Finkelman et al. reported that antigen-specific memory IgE responses are like primary IgE responses dependent on IL-4 [Finkelman et al. 1988]. In that study the resting period between priming and boosting with TNP-KLH was rather short (3 weeks). Using sheep red blood cells as thymus dependent antigen it was found that only 12 weeks after priming the maximum memory formation for IgG was achieved [Benner et al., 1974]. We studied the dependence on IL-4 and the susceptibility to IFN-γ, which is known to inhibit the IL-4 induced IgE production, of primary and secondary TNP-specific IgE responses. For IFN-γ no such studies have been described. As expected, the primary TNP-specific IgE response could be completely inhibited by neutralizing IL-4 and extensively by IFN-γ (Chapter 6). Using a resting period of three months between primary and secondary immunization we found the secondary antigen-specific IgE response to be partially IL-4 independent. Moreover, the secondary antigen-specific IgE response was not inhibited by doses of IFN-γ that did inhibit the primary antigen-specific IgE response (Chapter 6). The IL-4 independent part of the memory IgE response is most likely the result of B cells switched to IgE during the primary response. The necessity of IL-4 for this switch was clearly shown in an experiment in which IL-4 was neutralized during the primary response to TNP-KLH, resulting in no detectable IL-4 independent IgE response upon secondary immunization (Chapter 4). It is quite possible that during the three weeks

after primary immunization memory B cells switched to IgE are not formed to an extent that allowed detection of IgE in the absence of IL-4, whereas those cells are formed during the three months after priming. This would explain why Finkelman et al. reports memory responses like primary responses dependent on IL-4, whereas we reported the secondary IgE response to be partially IL-4 independent (Chapters 4, 6 and 7).

For human B cells a synergistic effect of IL-6 in combination with IL-4 in generating IgE has been described, by showing that anti-IL-6 antibodies strongly inhibited the IL-4 dependent IgE production *in vitro* [Vercelli et al., 1989; Jabara et al., 1991]. Moreover, we had noted that IL-4 and IL-6 mRNA was expressed simultaneously in the spleen of mice upon immunization with TNP-KLH. We addressed the question whether in mice also a synergistic activity of IL-4 and IL-6 occurs. Therefore, we studied the involvement of both IL-4 and IL-6 in primary antigen-specific and polyclonal IgG_1 and IgE responses by neutralizing IL-4 and IL-6 *in vivo* (Chapter 8). For IgE, we did not observe a synergistic effect of IL-4 and IL-6 on both polyclonal and antigen-specific responses. However, we did find that murine IgG_1 and IgE responses differred in their requirement for IL-6 in reaching peak levels. Neutralization of IL-6 resulted in inhibition of polyclonal IgG_1 responses. Primary and memory antigen-specific IgG_1 responses were slightly inhibited by neutralizing IL-6, but neutralization of both IL-4 and IL-6 resulted in a marked decrease. These results show that in mice these two cytokines act synergistically during a primary and secondary antigen-specific IgG_1 responses. The IgE responses, under similar conditions, were only inhibited by neutralizing IL-4. The observation that no synergistic effect for IL-4 and IL-6 was observed with respect to murine IgE indicates that murine and human B cells differ with respect to the need for IL-6 to give rise to peak levels of IgE.

Two possibilities could account for this new phenomenon. It has been described that IL-6 is required for terminal differentiation of B cells to Ig-secreting plasma cells [Muraguchi et al., 1988]. Our observation that this holds for IgG_1- but not IgE-secreting cells suggests that B cells switched to IgE are already in a further differentiation stage than B cells switched to IgG_1. This might be a direct consequence of IL-4 which is an absolute requirement for the formation of B cells switched to IgE, but not for the induction of B cells switched to IgG_1 [Kuhn et al., 1991]. More speculative is the possibility that B cells switched at the DNA level to the expression of IgE do not express the receptor for IL-6. This could explain the observation that such B cells produce less antibody than B cells switched to IgG_1 [Takatsuki et al., 1988]. Our *in vitro* experiments revealed that both the IgG_1 and IgE production were not influenced by exogenous IL-6 (Chapter 8). It is possible that no enhancement by IL-6 was observed because enough endogenous IL-6 was produced to obtain peak production. Nevertheless, in our study neither the IgG_1 nor

the IgE production was inhibited by exogenous IL-6, an inhibition that was described for IgE by Auci et al. [Auci et al., 1993].

Previously, also IL-5 was reported to be important for inducing IgG_1-positive B cells to secrete IgG_1 [Purkerson and Isakson, 1991]. Therefore, to study the cytokine dependence of memory B cells to become plasma cells that secrete antigen-specific IgG_1 and IgE, we examined the role of IL-4, IL-5 and IL-6, in several combinations, on the memory IgG_1 and IgE responses. For this study an adoptive transfer system was used as it is known that upon boosting the memory IgG_1 and IgE responses are enhanced in sublethally irradiated mice reconstituted with primed spleen cells (Chapter 4). As expected, both IL-5 and IL-6 were necessary for peak antigen-specific serum IgG_1, whereas the antigen-specific IgE response was not inhibited by neutralizing IL-5 and IL-6. On the contrary, the antigen-specific IgE response could be inhibited up to 90% by neutralizing IL-4, which did not influence the antigen-specific IgG_1 response in the adoptive transfer system employed. Surprisingly, in the absence of IL-5 and IL-6 no inhibition of the secondary TNP-specific IgE response could be detected by neutralizing IL-4. The fact that γ_1-positive secondary B cells can undergo sequential isotype switching, resulting in IgE-secreting cells (Chapter 6), could explain these observations. From Chapter 8 it is clear that TNP-specific IgG_1 memory B cells need IL-5 and IL-6 for their maturation into IgG_1-secreting plasma cells. Neutralization of IL-5 and IL-6 will therefore prevent γ_1-positive, or $\gamma_1\varepsilon$-double positive B cells to develop into IgG_1-secreting plasma cells. These cells could subsequently switch to become ε-positive B cells for which they need less IL-4 or eventually could switch independently of the presence of IL-4 and develop into IgE-secreting plasma cells for which no IL-5 and IL-6 are required (Chapter 8). Other factors that induce or potentiate secretion of IgE, like IL-13 in the human system [Punnonen et al., 1993] could also be involved in the development of IgE-secreting cells. As yet it has not been ascertained that IL-13 induces IgE secretion by mouse B cells. Still it might be that under certain circumstances IL-13 induces also murine B cells to produce IgE. Recently, it has been described that neutralizing anti-IL-4 antibodies, when complexed with IL-4, can serve as reservoirs for long-term (at least 9 days) delivery of cytokines *in vivo* [Sato et al., 1993]. This indicates that the "double-edged sword" paradigm proposed for soluble cytokine receptors (reviewed in Debets and Savelkoul, 1994) also holds for anti-cytokine antibodies. It is possible that in the absence of IL-5 and IL-6 a persistent low dose of IL-4, as a result of IL-4-anti-IL-4 complexes, is sufficient to allow differentiation of γ_1-positive and/or $\gamma_1\varepsilon$-double positive B cells into IgE-secreting cells.

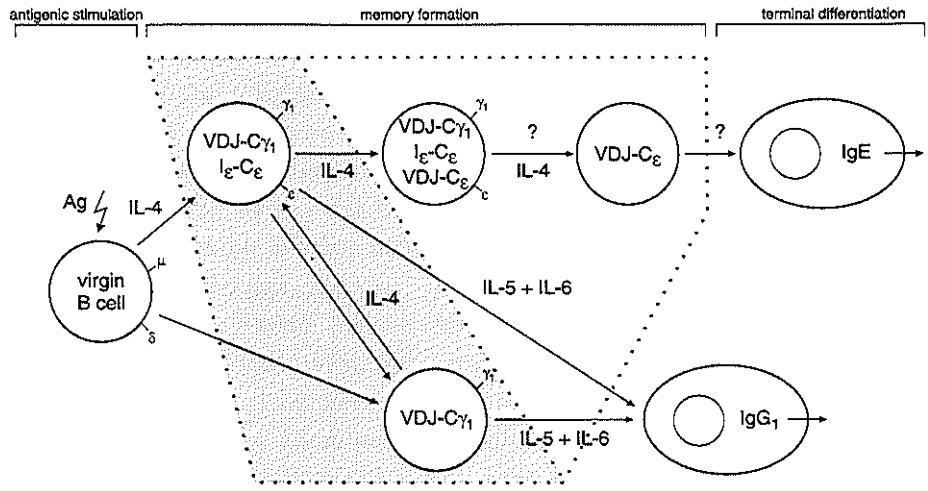

Figure 1. Schematic representation of the B cell memory formation for IgG_1 and IgE. Upon antigenic stimulation virgin B cell develop in γ_1-single positive B cells or, when enough IL-4 is present, in $\gamma_1\varepsilon$-double positive cells exist, namely cells expressing VDJ-Cγ_1 mRNA and ε-germ-line RNA, and cells expressing VDJ-γ_1 and VDJ-ε mRNA. The latter will develop in an ε-single positive B cell, possibly in the absence of IL-4. In the absence of IL-4. In the absence of IL-4 cells that express VDJ-γ_1 mRNA and ε-germ-line RNA will convert into cells expressing only VDJ-γ_1 mRNA. When IL-4 is present the opposite will occur. Whereas IL-5 and IL-6 are required for the differentiation into IgG_1-secreting cells. The balance between IL-4, IL-5, IL-6 and possible other cytokines will determine the route of differentiation. VDJ-Cγ_1 and VDJ-Cε represent mRNA molecules encoding IgG_1 and IgE, respectively. Iε-Cε represents ε-germline transcripts.

Conclusions

The results presented in this thesis are summarized in figure 1. They point to a central role for sequential isotype switching in the development of secondary IgG_1 and IgE responses. Upon antigenic encounter μδ-double positive virgin B cells can develop in the absence of IL-4 into γ_1-single positive memory B cells, whereas in the presence of IL-4 these cells can develop into ε-single positive cells via $\gamma_1\varepsilon$-double positive cells. Upon secondary stimulation, in the presence of IL-5 and IL-6, the γ_1-single positive cells can differentiate into IgG_1-secreting plasma cells.

There appear to be two types of $\gamma_1\varepsilon$-double positive cells. The first type are cells that in the presence of IL-4 can develop in IgE secreting cells, and in the absence of IL-4 but presence of IL-5 and IL-6 can differentiate into IgG_1-secreting cells. In the absence of both IL-5 and IL-6 these cells can develop in IgE-secreting cells in the presence of low amounts of IL-4, or other presently unknown cytokines. The second type are cells that have deleted the γ_1 heavy chain gene segment and can only differentiate into IgE-secreting cells, most likely in the absence of IL-4. This indicates that the memory B cell compartment for IgG_1 and IgE responses is composed of four cell types that under the

influence of cytokines develop into clones of IgG_1- and/or IgE-secreting plasma cells, namely cells expressing VDJ-$C\gamma_1$ or VDJ-Cε mRNA or both and cells expressing VDJ-$C\gamma_1$ mRNA in combination with ε germ-line RNA allowing trans-splicing. The relative concentrations of IL-4, IL-5, IL-6 and possibly other cytokines determine the differentiation of these cells into clones of IgG_1 and IgE secreting cells.

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Summary/Samenvatting

SUMMARY

The aim of the studies presented in this thesis was to investigate the involvement of cytokines, especially IL-4, during the formation of memory B cells switched to IgG_1 and IgE, and the dependence on cytokines of these B cells to develop into IgG_1- and IgE-secreting cells. Special emphasis was put on the recently proposed mechanism of sequential isotype switching which seems to be the major recombination mechanism leading to IgE-secreting B cells after a primary encounter with the antigen (Chapter 1). We studied whether this sequential isotype switching is also the major recombination event in stimulated secondary B cells giving rise to IgE-secreting plasma cells. Furthermore, we questioned whether the sequential isotype switching model could explain the observed IL-4 independence of part of the secondary IgE responses that had been reported in the literature. This despite the fact that IL-4 was reported to be necessary to obtain secondary IgE responses upon secondary exposure to TNP-KLH (Chapter 1). Since the frequency of IgE expressing B cells is normally low, even during secondary responses, we wondered whether it would be possible to increase the number of B cells switched to IgG_1 and IgE by elevating the *in vivo* IL-4 levels, since IL-4 was shown to play an important role in the isotype switch to IgG_1 and IgE (Chapter 1). Studies like this can be performed with the recently described IL-4 transgenic mice. A demerit of such transgenic mice, however, is the already during the ontogeny disturbed IL-4 level that could lead to aberrant situations when studying the effect of elevated IL-4 levels on the *in vivo* immune response. Therefore, we used a new technique, described in Chapter 2, based on encapsulation of cytokine producing cells in alginate, which are implanted in the peritoneal cavity of normally developed mice.

In Chapter 2 it is shown that under these circumstances alginate encapsulated CV/1 cells stably transfected with the murine IL-4 gene produce IL-4 for at least two weeks. Moreover, it is demonstrated that the use of adherent growing transfected cells permits dose-response titrations and provides an easy method for local and systemic cytokine delivery. Also hybridoma cells can be encapsulated and implanted in mice. It is shown that these encapsulated hybridoma cells secrete mAb, that can be monitored in the serum of these mice. No immune response in the host was found upon implantation of encapsulated cells. Implantation of encapsulated mAb producing hybridoma cells proved to be as efficient as the injection of purified mAb.

In Chapter 3 the effect of adjuvant is studied on the isotypes produced upon immunization of mice with TNP-KLH. Adjuvants are considered to play an important role in directing the amount and isotype of antibodies produced upon immunization by conducting

the development of either Th-1 or Th-2 cells. This is based on the different cytokine production patterns that were observed after in vitro restimulation of T cells isolated from mice immunized with antigen either adsorbed on alum or emulsified in complete Freund adjuvant (CFA). However, other studies suggested that primarily the type of antigen determines which isotypes were produced and to what extent. In these studies, however, IgE was not determined. Therefore, we examined whether alum and CFA influenced the amount and/or ratio of IgG_1, IgE and IgG_{2a} produced after TNP-KLH immunization. Similar levels of IgG_1, IgE and IgG_{2a} antibodies were found upon immunization with TNP-KLH either adsorbed on alum or emulsified in CFA. Moreover, administration of IFN-γ in combination with TNP-KLH adsorbed on alum did not increase the amount of IgG_{2a} produced. The presented results indicate that upon immunization with TNP-KLH high IL-4 levels are produced, resulting in an antibody response that is dominated by IgG_1, independent of the adjuvant employed. The IL-4 inducing property of TNP-KLH is substantiated by the finding that repeated immunization of mice with TNP-KLH, without adjuvant, increases the serum total IgE level. This suggests that the carrier part of TNP-KLH preferentially results in Th-2 cell activity and that the adjuvant merely enhances the antibody production.

We also investigated the effect of systemically elevated IL-4 on the antigen-specific IgG_1 and IgE memory formation. In the light of the results presented in Chapter 2, BALB/c mice, after primary immunization with either TNP-KLH (Chapter 4) or TNP-rabbit-IgG (Chapter 5), were implanted with encapsulated CV-1/IL-4 cells every two weeks for a period of four months. The increased IL-4 level resulted in increased serum levels of total IgG_1 and IgE, whereas the concentration of total IgG_{2a} did not change. Unexpectedly, in the same mice the TNP-specific IgG_1 and IgE serum levels were decreased. Similar results were found when the antigen was continuously present during the IL-4 treatment. Furthermore, it was shown that IL-4 decreased the formation of IgG_1 and IgE memory cells. These results pointed to different effects of IL-4 in regulating antigen-specific and bystander responses.

In Chapter 6 it is shown that the effects observed due to systemic elevation of the IL-4 level were mediated at the B cell and not the T cell level. Major changes occurred in the $\gamma_1\varepsilon$-double positive B cell population which increased after IL-4 treatment. Moreover, it is shown that $\gamma_1\varepsilon$-double positive B cells can develop *in vitro* out of γ_1-positive primed B cells and that these double-positive cells can subsequently differentiate into IgG_1- and IgE- secreting cells. The existence of $\gamma_1\varepsilon$-double-positive memory B cells could explain differences in cytokine dependence of TNP-specific memory IgG_1 and IgE responses observed after adoptive transfer of primed spleen cells into irradiated naive recipients. Whereas the IL-4 independent TNP-specific memory IgG_1 response could be blocked

efficiently by neutralizing IL-5 and IL-6, TNP-specific memory IgE responses were virtually not susceptible to such treatment. These IgE responses were also not susceptible to IFN-γ, used in doses that did inhibit the primary IgE response. Inhibition of the TNP-specific memory IgG_1 response by neutralizing IL-5 and IL-6 is accompanied by a 10 fold increase of the IL-4 independent TNP-specific IgE memory response. These data, together with the data concerning γ_1-single, ε-single and $\gamma_1\varepsilon$-double B cells, indicate that secondary IgE responses primarily result from B cells that have either switched to IgG_1, or are double positive for IgG_1 and IgE, and show a minor role for ε-single positive B cells in secondary IgE responses.

For the induction of the IL-4 independent part of a memory IgE response IL-4 had to be present after primary immunization as was shown in Chapter 3. In Chapter 7 it is described that treatment of mice with IL-4 for a prolonged period led to elevated serum total IgE levels. These elevated IgE levels turned out to be persistent for at least four months after the last IL-4 administration. Subsequent neutralization of IL-4 did not influence this elevated serum IgE level indicating its IL-4 independence. These mice could still develop protective immunity against a viral infection after vaccination, although their virus-specific IgG_{2a} antibody production was profoundly impaired. Cultures in which B cells from mice with persistent IgE were stimulated with membrane fragments of activated Th-1 cells revealed a B cell population that did not need IL-4 to produce IgE. Part of this IgE production turned out to be gp39 independent, whereas the IgG_1 production was completely gp39 dependent. The number of $\gamma_1\varepsilon$-double positive B cells turned out to be increased in the spleen of IL-4 treated mice, whereas almost no ε-single positive B cells could be detected three months after last IL-4 administration. This suggests that the IL-4 independent persistent IgE serum level in long-term IL-4 treated mice is predominantly the result of a $\gamma_1\varepsilon$-double positive B cell population.

Whereas IL-4 is obligatory for the induction of IgE responses, it enhances IgG_1 responses. In Chapter 8 we show that besides IL-4 also IL-6 is required for peak IgG_1 responses. This was done by *in vivo* treatment with neutralizing anti-cytokine mAb. It is shown that the mRNA levels of IL-4 and IL-6 are coordinately expressed in the spleen of mice immunized with TNP-KLH. No IL-6 requirement was observed for peak IgE responses. The IL-6 dependence of IgG_1 responses was found for both antigen-specific and polyclonal responses. Moreover, it was noted by using TNP-KLH and GAM/IgD as antigens that polyclonal IgG_1 responses are more dependent on IL-6 than antigen-specific responses. *In vitro* experiments revealed that exogenous IL-6 neither enhanced nor inhibited the IgG_1 and IgE production by naive B cells, suggesting that IL-6 did not interfere with the IL-4 induced isotype switch potential. Primary and memory IgG_1

responses were both similarly dependent on IL-6. These observations point to a role of IL-6 in the terminal differentiation of B cells switched to IgG_1. On the other hand, neutralization of IL-6 did not inhibit antigen-specific and polyclonal IgE responses. Therefore, we conclude that IL-6 is not involved in the terminal differentiation of B cells switched to IgE. These findings thus provide evidence for a distinct role for IL-6, besides IL-4, in regulating murine IgG_1 responses, that is not required for murine IgE responses.

In Chapter 9 the major points emerging from the experimental studies are discussed in the light of data presented in the literature. A model is proposed that could explain the experimental data described in this thesis with respect to the production of IgG_1 and IgE. A central role is assumed for $\gamma_1\varepsilon$-double positive B cells in IgG_1 and IgE responses. During the course of the studies described in this thesis, evidence for sequential isotype switching during primary IgG_1 and IgE responses was presented in the literature for both mice and humans. We confirmed this and furthermore demonstrate a central role for sequential isotype switching in secondary IgG_1 and IgE responses. Moreover, we show that elevation of IL-4 during a certain period can have profound effects on the IgE synthesis, even when the IL-4 level has returned to normal levels. This is most likely the result of the generation of memory B cells switched to IgE that do not need, or need less IL-4 to give rise to clones of IgE-secreting plasma cells. This finding can have an important implication for the understanding of human asthma, in which disturbed IL-4 levels are considered to be an inducing factor. Observed long lasting IgE serum levels in these individuals might be the result of B cells switched to the expression of IgE early in life, as result of an enhanced IL-4 expression during a critical period of time. Differentiation of such cells into IgE-secreting cells will only partially be prevented by neutralizing IL-4. In this light, immunotherapy based on neutralizing the biologic activity of IL-4 alone might not be the right option for treatment.

SAMENVATTING

Vrijwel iedereen is wel eens gevaccineerd, waardoor hij of zij werd blootgesteld aan gedode of verzwakte ziekteverwekkers. Het feit dat dit proces ook wel actieve immunisatie genoemd wordt, geeft aan dat er in het lichaam een actief proces plaatsvindt, namelijk het ontstaan van geheugen B en T cellen. Dit actieve proces zorgt voor een langdurige bescherming tegen de natuurlijke ziekteverwekker. Het immuunsysteem heeft de ziekteverwekker leren kennen en kan bij hernieuwd contact sneller en doeltreffender reageren. Daardoor kan het immuunsysteem de ziekteverwekker uitschakelen voordat schade ontstaat. De snellere reactie is ondermeer mogelijk, omdat gedurende het eerste contact met de ziekteverwekker (het 'antigeen') B cellen zijn ontstaan die dit antigeen beter herkennen, en bovendien langlevend zijn. Bij hernieuwd contact met het antigeen reageren deze B cellen met de produktie van antistoffen die specifiek binden aan het antigeen. Deze antistoffen zijn eiwitten ('immunoglobuline moleculen'), die samen met fagocyterende cellen ('eetcellen') zorgen voor afbraak van het antigeen.

Een antistof is een molecule dat bestaat uit vier eiwitketens: twee lichte ketens en twee zware ketens. Bij de muis komen acht verschillende klassen van immunoglobuline moleculen voor: IgM, IgD, IgG_3, IgG_1, IgG_{2b}, IgG_{2a}, IgE en IgA. Deze zogenoemde immunoglobuline isotypen worden van elkaar onderscheiden op basis van hun zware keten. Gedurende de immuunreactie op een antigeen, de immuunrespons, produceert een B cel antistoffen van verschillende isotypen, als gevolg van een proces dat isotype switching wordt genoemd. Hierbij verandert de zware keten van isotype zonder dat de herkenning van het antigeen door de antistof verandert. De eiwitten die dit isotype switching's proces sturen worden gemaakt door T cellen en heten cytokinen. Op basis van cytokine productie kunnen T cellen die hulp bieden aan B cellen gedurende een immuunrespons ('Th cellen') onderverdeeld worden in twee subtypen. Namelijk in: Th1 cellen die interleukine-2 (IL-2) en interferon-γ (IFN-γ) produceren en Th2 cellen die IL-4, IL-5, IL-6 en IL-10 produceren.

Een B cel zal, wanneer hij voor het eerst in aanraking komt met een antigeen, reageren met de produktie van IgM. Enkele dagen later ondergaat de B cel onder invloed van cytokinen isotype switching B cel. Als gevolg hiervan gaat hij antistoffen produceren van een ander isotype. Deze zijn echter nog steeds specifiek voor het oorspronkelijke antigeen. Van een aantal cytokinen is het effect op isotype switching bekend. Zo zijn IL-4 en IFN-γ betrokken bij de isotype switch van IgM naar IgG_1, IgE en IgG_{2a}. IL-4 stimuleert de isotype switch naar IgG_1 en IgE, terwijl het de isotype switch naar IgG_{2a} remt. Het omgekeerde geldt voor IFN-γ. Dit cytokine remt de isotype switch naar IgG_1

en IgE, terwijl het de isotype switch naar IgG_{2a} juist positief beïnvloedt. Het is de balans tussen IL-4 en IFN-γ die bepaalt welke isotypen zullen worden geproduceerd door de geactiveerde B cellen.

Geheugen B cellen kunnen op basis van het isotype dat ze tot expressie brengen onderscheiden worden van B cellen die nog niet in aanraking zijn geweest met een antigeen. Deze zogenoemde naïeve B cellen brengen IgM op hun membraan tot expressie, al dan niet in combinatie met IgD, terwijl geheugen B cellen antistoffen tot expressie brengen van een andere immunoglobuline isotype klasse. Het doel van het onderzoek, dat wordt beschreven in dit proefschrift, was meer inzicht krijgen in de rol van cytokinen, met name IL-4, op de vorming van geheugen B cellen die geswitcht zijn naar IgG_1 en IgE. Verder wilden we weten welke cytokinen noodzakelijk zijn voor de uitrijping van deze geheugen B cellen tot IgG_1 en IgE vormende plasmacellen. De aandacht werd tijdens het onderzoek met name gericht op het recent beschreven model van sequentiële isotype switching. Dit lijkt het belangrijkste recombinatie mechanisme te zijn dat leidt tot IgE vormende B cellen na antigeen toediening. De vraag was of sequentiële isotype switching de in de literatuur beschreven IL-4 onafhankelijkheid van een deel van de secundaire IgE respons kon verklaren. Dit in tegenstelling tot de door Finkelman et al. beschreven totale IL-4 afhankelijkheid van secundaire TNP-KLH geïnduceerde IgE responsen (Hoofdstuk 1).

Het aantal B cellen dat IgE tot expressie brengt, is normaal gesproken laag, zelfs gedurende secundaire responsen. Onderzocht werd of het deze celpopulatie toeneemt onder invloed van een verhoogde *in vivo* IL-4 concentratie, aangezien IL-4 een belangrijke rol heeft in de isotype switch naar IgG_1 en IgE (Hoofdstuk 1). IL-4 transgene muizen, die recentelijk zijn beschreven, zouden gebruikt kunnen worden voor de oplossing van deze vragen. Echter dit soort muizen heeft als nadeel dat ze al gedurende hun ontwikkeling zijn blootgesteld aan verhoogde IL-4 concentraties, wat zou kunnen leiden tot een verstoorde ontogenie. Aangezien een verstoorde ontogenie kan leiden tot aberrante situaties wanneer het effect van verhoogd IL-4 wordt bestudeerd, lijkt gebruik van deze muizen niet zinvol. Dit is de reden dat voor de experimenten, die worden beschreven in dit proefschrift, gebruik is gemaakt van een nieuwe techniek gebaseerd op het in alginaat inkapselen van cellen (Hoofdstuk 2). Deze techniek maakt het mogelijk cytokine producerende cellijnen te implanteren in de buikholte van muizen, afgeschermd van het immuunsysteem van de gastheer, maar toegankelijk voor de benodigde voedingsstoffen.

Onder deze condities produceren CV-1 cellen, die stabiel getransfecteerd zijn met het muize IL-4 gen, in ieder geval gedurende twee weken IL-4 (Hoofdstuk 2). Wanneer

gebruik wordt gemaakt van adherent groeiende getransfecteerde cellijnen, is het mogelijk doses respons titraties uit te voeren. Het is een goed bruikbare methode voor lokale en systemische cytokine toediening. Worden antistof producerende hybridoma cellen ingekapseld geimplanteerd, dan is bovendien bepaling van de hoeveelheid geproduceerde antistof in het serum mogelijk. Implantatie van ingekapselde antistofvormende hybridomacellen bleek even effectief als het inspuiten van gezuiverde antistof. Geïmplanteerde, in alginaat ingekapselde cellen zelf veroorzaakten geen immuunrespons in de muizen.

In Hoofdstuk 3 is het effect van adjuvant op de isotype produktie na TNP-KLH immunisatie bestudeerd. Van adjuvantia wordt gedacht dat ze een belangrijk effect hebben op de hoeveelheid en het isotype van de antistoffen die geproduceerd worden na immunisatie. Adjuvatia zouden de ontwikkeling stimuleren van of Th1 of Th2 cellen. Dit werd geconcludeerd op basis van verschillende cytokine produktieprofielen die waargenomen worden na *in vitro* stimulatie van T cellen geïsoleerd uit muizen na immunisatie met antigeen geadsorbeerd aan aluin, of geëmulgeerd in CFA. Uit andere studies blijkt echter dat in de eerste plaats het type antigeen bepaalt welke isotypen er geproduceerd worden, en hoeveel. In deze studies werd echter de IgE produktie niet bepaald. Daarom hebben wij onderzocht wat de invloed is van aluin en CFA op de hoeveelheden IgG_1, IgE en IgG_{2a} antistoffen die na TNP-KLH immunisatie *in vivo* worden geproduceerd. Gevonden werd dat na immunisatie met TNP-KLH geadsorbeerd aan aluin of geëmulgeerd in CFA dezelfde hoeveelheden IgG_1, IgE en IgG_{2a} worden geproduceerd. Zelfs verhoging van de *in vivo* IFN-γ concentratie na TNP-KLH immunisatie, door implantatie van in alginaat ingekapselde IFN-γ producerende cellen, veroorzaakte geen toename van de hoeveelheid IgG_{2a} die werd geproduceerd. Deze resultaten suggereren dat immunisatie met TNP-KLH IL-4 produktie veroorzaakt, wat resulteert in een door IgG_1 gedomineerde antistofrespons. Hierbij maakte het niet uit of aluin, dan wel CFA als adjuvant wordt gebruikt. Dat TNP-KLH alleen resulteert in voldoende IL-4 produktie voor de isotype switch naar IgE, blijkt uit het feit dat herhaalde TNP-KLH immunisatie in afwezigheid van adjuvant een stijging van totale IgE serumconcentratie tot gevolg heeft. Deze resultaten geven aan dat het carrier gedeelte van TNP-KLH preferentiëel een Th2 activiteit tot gevolg heeft, en dat een eventuele aanwezigheid van adjuvant de TNP-KLH geïnduceerde immuunrespons alleen versterkt.

Om het effect van IL-4 op de vorming van antigeen-specifieke IgG_1 en IgE geheugen B cellen nader te bestuderen, hebben wij gekeken naar de invloed van een systemische verhoging van de *in vivo* IL-4 concentratie op deze geheugenvorming. Op basis van de resultaten beschreven in Hoofdstuk 2 werden muizen geïmmuniseerd met TNP-KLH (Hoofdstuk 4) of TNP-konijn-IgG (Hoofdstuk 5), beide geadsorbeerd aan aluin. Vervol-

gens werden in alginaat ingekapselde CV-1/IL-4 cellen geimplanteerd in de buikholte van de muizen. Dit gebeurde om de twee weken gedurende een periode van vier maanden. De op deze manier verhoogde *in vivo* IL-4 concentratie resulteerde in een toegenomen serumspiegel voor totaal IgG_1 en IgE, terwijl de serumspiegels voor totaal IgG_{2a} niet veranderden. Onverwacht correspondeerde de toegenomen totale IgG_1 en IgE serumspiegels met verlaagde antigeen-specifieke serumconcentraties voor deze isotypen. Vergelijkbare resultaten werden gevonden wanneer niet alleen IL-4, maar ook het antigeen continu aanwezig was. De verlaagde serum concentraties voor antigeen-specifiek IgG_1 en IgE correspondeerden met een afname van het aantal antigeen-specifieke geheugen B cellen voor IgG_1 en IgE. De in Hoofdstuk 5 en 6 gepresenteerde gegevens maken duidelijk dat antigeen-specifieke en totale IgG_1 en IgE responsen verschillend worden gereguleerd door IL-4.

Dat de door systemisch verhoogd IL-4 veroorzaakte effecten worden geïnduceerd op B cel en niet op T cel niveau, wordt beschreven in Hoofdstuk 6. Het is voornamelijk de $\gamma_1\varepsilon$-dubbel positieve B cel populatie die is toegenomen onder invloed van IL-4 behandeling. Deze dubbel positieve B cel populatie ontstaat *in vitro* uit een γ_1-enkel positieve B cel populatie, en kan *in vitro* tot zowel IgG_1 als IgE secernerende plasmacellen differentieren (Hoofdstuk 6). De aanwezigheid van een populatie $\gamma_1\varepsilon$-dubbel positieve geheugen B cellen kan de cytokine afhankelijkheid verklaren van TNP-specifieke secundaire IgG_1 en IgE responsen die na immunisatie optreden in bestraalde naïeve ontvangers die gereconstitueerd zijn met geprimede miltcellen. Neutralisatie van IL-5 en IL-6 blokkeerde de IL-4 onafhankelijke TNP-specifieke geheugen IgG_1 respons. De TNP-specifieke geheugen IgE respons, daarentegen, werd niet geremd door neutralisatie van IL-5 en IL-6. Deze respons bleek ook ongevoelig voor IFN-γ, toegediend in doses die de primaire IgE respons verlagen. De door anti-IL-5 en anti-IL-6 geïnduceerde remming van de TNP-specifieke geheugen IgG_1 respons gaat gepaard met een tienvoudige toename van de IL-4 onafhankelijke geheugen IgE respons. Deze resultaten, samen met de data van de $sIgG_1$ en sIgE membraan kleuringen, duiden erop dat secundaire IgE responsen voortkomen uit B cellen die of geswitcht zijn naar IgG_1, of dubbel positief zijn voor IgG_1 en IgE. Ze geven aan dat ε-enkel positieve B cellen geen belangrijke rol spelen in secundaire IgE responsen.

Hoofdstuk 3 laat zien dat de aanwezigheid van IL-4 gedurende de primaire respons noodzakelijk is voor de inductie van geheugen B cellen die geen IL-4 nodig hebben om uit te rijpen tot IgE secernerende cellen. In Hoofdstuk 7 beschrijven we dat langdurige behandeling met IL-4 bij muizen leidt tot de inductie van sterk verhoogde IgE serum spiegels, die aanhouden tot tenminste vier maanden na het stoppen van de IL-4 behandeling. Deze verhoogde IgE niveaus konden niet verlaagd worden door neutralisa-

tie van IL-4, wat suggereert dat ze IL-4 onafhankelijk zijn. Hoewel met virus gevaccineerde muizen met verhoogd serum IgE op een virale infectie reageren met een verlaagde virus-specifieke IgG_{2a} respons, blijken ze toch beschermd te zijn tegen een letale dosis van dit virus. *In vitro* B cel kweken, waarin B cellen van IL-4 behandelde TNP-KLH geprimede muizen met persisterend IgE worden gestimuleerd met geactiveerde T celmembraanfragmenten van Th1 cellen, toonden een B cel populatie aan die inderdaad geen IL-4 nodig heeft om IgE te produceren. Een deel van deze IL-4 onafhankelijke IgE produktie bleek bovendien gp39 onafhankelijk, terwijl de IgG_1 produktie volledig afhankelijk bleek van gp39. Het aantal $\gamma_1\varepsilon$-dubbel positieve B cellen in de milt van IL-4 behandelde muizen met persisterend IgE was drie maanden na laatste IL-4 toediening nog steeds verhoogd, terwijl nauwelijks ε-enkel positieve B cellen gedetecteerd konden worden. Dit geeft aan dat de IL-4 onafhankelijke persisterende IgE respons gegenereerd wordt via een $\gamma_1\varepsilon$-dubbel positieve B cel populatie in muizen die lang met IL-4 zijn behandeld.

IL-4 is noodzakelijk voor de vorming van IgE, terwijl het de produktie van IgG_1 verhoogt. In Hoofdstuk 8 beschrijven we dat naast IL-4 ook IL-6 noodzakelijk is voor maximale IgG_1 responsen *in vivo*. Dit werd duidelijk na *in vivo* behandeling met anti-cytokine monoclonale antilichamen. Na TNP-KLH immunisatie komen IL-4 en IL-6 mRNA gelijktijdig tot expressie in de muizemilt. In tegenstelling tot IgG_1 is IL-6 niet noodzakelijk voor maximale IgE responsen. De IL-6 afhankelijkheid van IgG_1 responsen werd gevonden voor zowel antigeen-specifieke als polyklonale responsen. De GAM/IgD geïnduceerde polyclonale IgG_1 respons bleek echter meer afhankelijk van IL-6 dan de TNP-KLH geïnduceerde antigeen-specifieke IgG_1 respons. Toevoeging van exogeen IL-6 aan *in vitro* B cel kweken beïnvloedde noch de IgG_1 noch de IgE produktie door naïeve B cellen. Dit geeft aan dat IL-6 niet de IL-4 geïnduceerde switch verstoort. De resultaten suggereren dat IL-6 noodzakelijk is voor de terminale differentiatie van naar IgG_1 geswitchte B cellen tot IgG_1 secernerende plasmacellen, terwijl IL-6 niet nodig is voor de terminale differentiatie van naar IgE geswitchte B cellen. Dit betekent dat IgG_1 en IgE responsen niet alleen verschillend worden gereguleerd door IL-4, maar ook door IL-6.

In Hoofdstuk 9 worden de belangrijkste punten die naar voren zijn gekomen in de eerdere hoofdstukken bediscussieerd in het licht van de resultaten die door andere onderzoekers gepubliceerd zijn. Een model wordt voorgesteld dat de resultaten van het onderzoek, beschreven in dit proefschrift, naar de regulatie van de IgG_1 en IgE produktie samenvat. In dit model wordt een centrale rol toegedacht aan $\gamma_1\varepsilon$-dubbel positieve cellen. Gedurende de uitvoering van het in dit proefschrift beschreven onderzoek werd in de literatuur het bewijs geleverd voor sequentiële isotype switching, waarbij B cellen geswitcht naar IgG_1 verder kunnen switchen naar IgE. Deze vorm van isotype switching

vindt zowel plaats in de muis als in de mens. Voor de muis hebben wij deze manier van isotype switching bevestigd. Bovendien laten wij zien dat sequentiële isotype switching ook een centrale rol speelt gedurende secundaire IgG_1 en IgE responsen. De $\gamma_1\varepsilon$-dubbel positieve B cel, die ontstaat tijdens sequentiële isotype switching, is het celtype dat preferentieel verhoogd aanwezig is in de milt van langdurig met IL-4 behandelde muizen. Dit celtype lijkt verantwoordelijk voor de in deze muizen gevonden verhoogde, IL-4 onafhankelijke totaal IgE serumconcentraties. Het is mogelijk dat tijdens de langdurige IL-4 behandeling cellen ontstaan die geen IL-4, of minder IL-4 nodig hebben voor de uitrijping tot IgE secernerende plasmacellen. Dit gegeven kan belangrijke implicaties hebben voor humaan astma, waarbij een verstoorde IL-4 spiegel wordt gezien als directe oorzaak. De lang aanhoudende IgE serum spiegels in deze patiënten kunnen het gevolg zijn van B cellen die al op jonge leeftijd van het individu geswitcht zijn naar IgE. Dit zou het gevolg kunnen zijn van een tijdelijke verhoging van de IL-4 produktie tijdens een kritische periode van de ontwikkeling. Deze geswitchte cellen zullen minder afhankelijk zijn van IL-4 voor het uitrijpen tot IgE secernerende plasmacellen, dan cellen die nog niet geswitcht zijn naar IgE. Dit zou inhouden dat immunotherapie gebaseerd op neutralisatie van IL-4 geen goede optie is voor behandeling van deze patiënten.

ABBREVIATIONS

AP	alkaline phosphatase
APC	antigen presenting cell
bp	base pair
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
BSAP	B cell specific activator protein
C	constant region
CD	cluster of differentiation
cDNA	complementary DNA
CFA	complete Freund adjuvant
Con A	concanavalin A
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunosorbent assay
$F(ab')_2$	divalent antigen-binding fragment
FcR	receptor for the Fc portion of immunoglobulins
FCS	fetal calf serum
FDC	follicular dendritic cell
FITC	fluorescein isothiocyanate
GAM	goat-anti-mouse
Gy	Gray
h	hour
HPRT	hypoxanthine phosphatidyl ribosyltransferase
HRP	horse radish peroxidase
HSA	heat stable antigen
IFN	interferon
IL	interleukin
Ig	immunoglobulin
i.p.	intraperitoneal
i.v.	intravenous
KLH	keyhole limpet hemocyanin
LD	lethal dose
LPS	lipopolysaccharide
mAb	monoclonal antibody
MHC	major histocompatibility complex
min	minutes
mRNA	messenger RNA
PALS	periarteriolar lymphocytic sheath
PBS	phosphate buffered saline

PCR	polymerase chain reaction
pfu	plaque forming unit
PNA	peanut-agglutinin
PRV	pseudo rabies virus
Ra	rat
RaAM	rat-anti-mouse
RAM	rabbit-anti-mouse
RARa	rabbit-anti-rat
RNA	ribonucleic acid
S	switch region
s	surface
SA	streptavidin
SCID	severe combined immuno deficiency
SD	standard deviation
SEM	standard error of the mean
SNAP	switch nuclear A protein
SNIP	switch nuclear protein
TcR	T cell receptor
TD	thymus dependent
Th	T helper
TI	thymus independent
TK	thymidine kinase
TNP	trinitrophenylated
U	unit

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René

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 19 juni 1965 in Rheden geboren. In 1984 behaalde hij het diploma V.W.O. aan het Christelijk Lyceum te Arnhem. In datzelfde jaar begon hij met zijn studie Biologie aan de Katholieke Universiteit te Nijmegen. In 1985 werd het propaedeuse examen en in 1989 het doctoraal examen met goed gevolg afgelegd. De doctoraalfase bestond uit een hoofdvak Immunologie, met als onderwerp "Interrelatie, functionele betekenis en signaaltransductie van twee typen IgG Fc receptoren op humane monocyten", onder verantwoordelijkheid van dr. J.G.J. van de Winkel en een hoofdvak Biochemie met als onderwerp "De genomische organisatie van het A641 gen", onder verantwoordelijkheid van prof. dr. W.J.M. van de Ven.

Van 1989 tot en met 1993 werkte hij op de afdeling Immunologie, hoofd: prof. dr. R. Benner, van de Erasmus Universiteit te Rotterdam. Gedurende deze periode werd het in dit proefschrift beschreven onderzoek verricht in het kader van een door N.W.O. gesubsidieerd project onder supervisie van dr. ir. H.F.J. Savelkoul. Tijdens deze tijd heeft hij de opleiding tot immunoloog, volgens de SMBWO, gevolgd. Tevens werd een bijdrage geleverd aan het onderwijs in de Histologie voor studenten in de Geneeskunde, en begeleide hij een afstudeerproject van een student van het Hogere Laboratorium Onderwijs. In 1992 werd het certificaat onderzoeker ex art. 9, Wet op de Dierproeven verkregen.

PUBLICATIONS

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3 J.G.J. van de Winkel, R. van Ommen, T.W.J. Huizinga, M.A.H.V.M. de Raad, W.B. Tuijnman, P.J.T.A. Groenen, P.J.A. Capel, R.A.P. Koene and W.J.M. Tax. 1989. Proteolysis induces increased binding affinity of the monocyte type II FcR for human IgG. *J. Immunol. 143:571-578.*

4 H.F.J. Savelkoul, R. van Ommen, A.C.T.M. Vossen, E.G. Breedland, R.L. Coffman and A. van Oudenaren. 1994. Modulation of systemic cytokine levels by implantation of alginate encapsulated cells. *J. Immunol. Methods. 170:185-196.*

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6 R. van Ommen, A.E.C.M. Vredendaal and H.F.J. Savelkoul. 1994. Prolonged *in vivo* IL-4 treatment inhibits antigen-specific IgG_1 and IgE formation. *Scand. J. Immunol. 40:1-9.*

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9 R. van Ommen, A.T.J. Bianchi, V.E.C.J. Schijns and H.F.J. Savelkoul.

1994. Prolonged in vivo IL-4 treatment leads to persistent IgE production that is IL-4 independent. *submitted for publication.*

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11 A.J.M. van den Eertwegh, R. van Ommen, H.F.J. Savelkoul, W.J.A. Boersma and E. Claassen. 1994. *In vivo* localization, gp39 expression, cytokine production and antibody formation are sequential immunological events after injection of anti-mouse-IgD antibodies. *submitted for publication.*

12 A.J.M. van den Eertwegh, M. van Meurs, R. van Ommen, H.F.J. Savelkoul, W.J.A. Boersma and E. Claassen. 1994. Differential *in vivo* appearence of activated T cells, cytokine producing cells and antibody-forming cells in peripheral lymph nodes and spleens after immunization with TI-2 antigens. *submitted for publication.*

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