INDUCTION AND MEASUREMENT OF IgE

A STUDY IN MICE, WITH EMPHASIS ON THE REGULATORY ROLE OF LYMPHOKINES

INDUCTIE EN BEPALING VAN IgE

EEN ONDERZOEK IN DE MUIS, MET SPECIALE AANDACHT VOOR DE REGULERENDE INVLOED VAN LYMFOKINEN

PROEFSCHRIFT

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Science is not the place for those who want certainty, who wish the truths they learned in childhood to reassure them in their old age.

Robert Shapiro
Origins, A skeptic guide to the creation of life on earth, 1987.

Ter nagedachtenis aan mijn vader

Aan mijn moeder

Aan Gertie en onze kinderen

CONTENTS

The sections 4.2 - 4.5, 5.2 - 5.4, 6.2 and 6.3 represent the Appendix papers of this thesis.

VOORWOORD

Immuniteit is een vorm van afweer van mensen en dieren tegen binnendringende infectieuze organismen (bacteriën, virussen en parasieten), allerlei verbindingen (natuurlijk voorkomend dan wel chemisch gesynthetiseerd) en getransplanteerde organen en weefsels. Te onderscheiden zijn twee typen immuniteit: aspecifieke en specifieke immuniteit.

De aspecifieke immuniteit wordt o.a. bepaald door allerlei faktoren (een intacte huid, slijmvliezen, trilharen, hoestreflex, niezen), biochemische faktoren (o.a. eiwitten van het complementsysteem in het bloed, zoutzuur in de maag) en cellen (fagocyterende granulocyten en macrofagen/monocyten).

Bij de specifieke immuniteit worden drie fundamentele kenmerken onderscheiden: de herkenning, de specificiteit, en het geheugen.

De herkenning komt tot stand doordat het lichaam in staat is om met name eiwitachtige stoffen, die qua struktuur verschillen van lichaamseigen strukturen, te onderscheiden. Deze lichaamsvreemde stoffen noemt men antigenen. Antigenen zijn in staat om een immunologisch antwoord (een immuunrespons) op te wekken. Aan een antigeen onderscheidt men een drager (carriër) en een hapteen. Het hapteen (de antigene determinant) is dat onderdeel van het antigeen waartegen de afweer reactie is gericht en welke dus de specificiteit van de immuunrespons bepaalt. Het vermogen van een carriër-hapteen complex om een immuunrespons op te wekken wordt bepaald door faktoren als: molekuulgrootte, chemische samenstelling, en optische en ruimtelijke configuratie. Bovendien zijn de plaats van toediening en de afbraaksnelheid in het lichaam van belang. Eiwitten zijn potente immunogenen, koolhydraten zijn zwakke immunogenen en lipiden zijn niet immunogeen, tenzij ze gebonden zijn aan een eiwit of koolhydraat (of beide). De cellen in het lichaam die de antigenen herkennen zijn de kleine lymfocyten. Zij worden immuuncompetente cellen genoemd. Er worden tenminste twee typen onderscheiden: T (thymus-derived) en B (bone marrow derived) lymfocyten.

Het blijkt dat B lymfocyten onder invloed van antigenen kunnen prolifereren en differentiëren tot plasmacellen welke antilichamen secerneren (de humorale immuunrespons). T lymfocyten daarentegen kunnen onder invloed van antigenen overgaan tot blasttransformatie en uitscheiding van lymfokinen (de cellulaire immuunrespons). Een gevolg hiervan is dat processen die via de humorale immuniteit gemedieerd worden, passief overdraagbaar zijn middels serum. Cellulaire immuniteit is een eigenschap van T lymfocyten en kan alleen door deze lymfocyten of (in bepaalde gevallen) een extract ervan worden overgedragen. Wanneer een antigeen het lichaam binnenkomt, vindt in eerste instantie vaak verwerking plaats door macrofagen, waarna het antigeen gepresenteerd wordt aan de kleine lymfocyten. Er vindt dan klonale selectie plaats. Dit houdt in dat slechts die T en B lymfocyten gaan prolifereren die het betreffende antigeen kunnen herkennen. Meestal is dat slechts 1 op de 10^4 tot 10^5 lymfocyten.

Een antigeen gaat dus een interactie aan met die kleine lymfocyten, die min of meer specifiek zijn voor dat antigeen. In het geval van B lymfocyten heeft het produkt, de antilichamen, dezelfde specificiteit. Antilichamen zijn vaak in staat om een kruisreaktie aan te gaan met antigenen die een soortgelijke struktuur hebben.

Een deel van de door het antigeen gestimuleerde cellen neemt niet aktief deel aan de immuunrespons, maar wordt geheugen (memory) cel. Deze langlevende cellen zorgen ervoor, dat bij herhaald kontakt met hetzelfde antigeen de immuunrespons heviger is en sneller op gang komt.

Antilichamen zijn koolhydraat houdende eiwitten (glycoproteinen) die zich bij scheiding in een elektrisch veld (elektroforese) van serum hoofdzakelijk in de gammaglobuline fraktie bevinden. Antilichamen worden daarom immunoglobulinen genoemd. Het immunoglobuline molekuul heeft een antigeenbindend deel dat de antilichaamspecificiteit bepaalt. Door de grote variatie in de aminozuursequentie van het antigeen-bindende deel is een grote diversiteit mogelijk. Het andere deel van het immunoglobuline molekuul bepaalt de biologische aktiviteiten. Dit deel heeft een min of meer constante aminozuursequentie. Onder biologische aktiviteit verstaat men het vermogen van het immunoglobuline molekuul om, onder andere het complementsysteem te aktiveren, de placenta te kunnen passeren en zich aan bepaalde celtypen te kunnen hechten (cytofylie). Op basis van verschillen in de aminozuursamenstelling van het constante deel worden een vijftal verschillende klassen van immunoglobulinen onderscheiden.

T lymfocyten zijn niet alleen verantwoordelijk voor de cellulaire immuunrespons, maar zij spelen eveneens een belangrijke regulerende rol bij de antilichaamvorming tegen de meeste antigenen. Zulke antigenen worden T-cel afhankelijk genoemd. Tijdens de induktie van de immuunrespons ontwikkelen zich zowel antigeen-specifieke helper T lymfocyten, welke in staat zijn de immuunrespons te versterken, als funktionele suppressor T lymfocyten. Deze laatste remmen de immuunrespons en zorgen dat deze uiteindelijk stopt.

Het is gebleken dat een bepaalde klasse immunoglobulinen (IgE) een essentiële rol speelt bij allergische reakties. IgE komt in het algemeen in zeer lage concentraties voor in het serum van gezonde volwassenen, maar in het serum van patiënten met bepaalde overgevoeligheidsreakties (atopici) worden meestal hoge IgE gehalten gevonden. Door IgE-gemediëerde ('acute') overgevoeligheidsreakties kunnen reeds optreden 15-20 minuten na het contact met het antigeen, bij deze reakties allergeen genoemd. De meest bekende allergenen zijn: huisstofmijt, pollen (gras- en boompollen), huidschilfers van huisdieren (kat, hond, cavia), bepaalde voedingsmiddelen (kippeeiwit, melk) en bije- en wespegif. Deze allergenen zijn van een dusdanige struktuur dat zij meerdere antigene determinanten bevatten (multivalentie). Het IgE heeft bovendien homocytotrope eigenschappen, d.w.z. het hecht zich aan bepaalde celoppervlakken (van o.a. basofiele granulocyten en mestcellen). De binding verloopt via het constante deel van het IgE molekuul, hetgeen impliceert dat het allergeen-specifieke deel nog steeds beschikbaar is voor binding. Indien nu de mestcellen beladen zijn met allergeen-specifieke IgE molekulen, zal bij contact met dat multivalente allergeen binding plaatsvinden. Bij deze binding zullen dan meerdere membraan-gebonden IgE molekulen met elkaar verbonden worden. Dit leidt tot aktivatie van de mestcel waarbij de in granulen opgeslagen ontstekingsmediatoren (o.a. histamine) vrijkomen uit de mestcel. Daarnaast worden produkten van het arachidonzuurmetabolisme (prostaglandinen en leukotriënen) aangemaakt die soortgelijke effekten hebben. Deze vrijgekomen mediatoren zetten een groot aantal processen in gang welke uiteindelijk resulteren in urticariële reakties, conjunctivitis, rhinitis en bronchoconstrictie: de klassieke verschijnselen van atopische reakties.

Het is niet duidelijk waarom niet-atopici toch in staat zijn IgE antilichamen te maken. Ook is het onduidelijk waarom atopici meestal meer IgE antilichamen maken dan niet-atopici. Meer kennis van de vorming van IgE antilichamen zal daarom het inzicht in de etiologie van deze ziekten belangrijk vergroten. Dit kan ertoe leiden dat eventuele behandelingsmethoden meer oorzakelijk gericht kunnen worden.

Het is gesuggereerd dat de IgE-synthese met name wordt gereguleerd door helper T cellen, suppressor T cellen en van T lymfocyten afkomstige IgE bindende faktoren. Recent is bekend geworden dat helper T cellen in twee

groepen ingedeeld kunnen worden op grond van de produktie van lymfokinen. De ene subpopulatie, (Th2), produceert interleukine-4 (IL-4) dat selectief in staat is de produktie van IgE, zowel in vitro als in vivo, te verhogen. De andere subpopulatie, (Th1), produceert gamma-interferon dat alle IL-4 geïnduceerde IgE responsen inhibeert, eveneens zowel in vitro als in vivo.

Het doel van het onderzoek dat in dit proefschrift wordt beschreven, was het vergroten van het inzicht in de faktoren welke de vorming van IgE-antilichamen bepalen. Daartoe werd gebruikt gemaakt van de muis als proefdier. Alvorens met dit onderzoek te kunnen beginnen moesten betrouwbare reagentia en testmethoden worden ontwikkeld voor het aantonen van IgE-antilichaam vormende cellen en de concentratie van gevormd IgE. Nadat wij daarin geslaagd waren, werd aandacht besteed aan de kinetiek van de IgE antilichaamvorming, aan de genetische faktoren die een rol spelen, aan de localisatie van de IgE producerende cellen, en aan de relatie tussen de aantallen IgE-producerende cellen en de IgE concentratie in het serum. Tevens werd onderzocht hoe de vorming van IgE gereguleerd kan worden door lymfokinen, met name door IL-4 en gamma-interferon. Ons onderzoek wijst erop dat de verhouding tussen de aantallen Th1 en Th2 cellen of de aktivatiestaat van Th1 en Th2 cellen bepaalt hoeveel IL-4 en gamma-interferon gevormd worden. Dientengevolge bepaalt de balans tussen IL-4 en gamma-interferon hoeveel IgE er na antigene of allergene stimulatie gevormd wordt.

ABBREVIATIONS

AFC	antibody-forming cell
AFU	arbitrary fluorescence unit
APC	antigen-presenting cell
B	bone marrow-derived
BM	bone marrow
BSA	bovine serum albumin
C	constant domain in Ig molecule
CFA	Freund's complete adjuvants
D	diversity region of Ig molecule
DNP	dinitrophenyl
DTH	delayed type hypersensitivity
EBV	Epstein Barr virus
EFA	enhancing factor of allergy
ELISA	enzyme-linked immunosorbent assay
F_c	crystallizable fragment of Ig molecule
F_cR	receptor for constant part of Ig molecule
FPLC	fast protein liquid chromatography
gel	gelatin
HACA	heterologous adoptive cutaneous anaphylaxis
Ia	I-region coded antigen
IFN-γ	gamma-interferon
Ig	immunoglobulin
Ig-BF	immunoglobulin-binding factor
IL	interleukin
i.m.	intramuscular
i.p.	intraperitoneal
Ir	immune response
i.t.	intratracheal
IU	international unit
i.v.	intravenous
J	joining region of Ig molecule
kb	kilobase
LPS	lipopolysaccharide
LT	lymphotoxin
mAb	monoclonal antibody
MHC	major histocompatibility complex
mIg	membrane-bound Ig
MUF	methylumbelliferon
MUF-G	methyl umbelliferyl galactoside
NMS	normal mouse serum
OA	ovalbumin
PBA	polyclonal B cell activator
PCA	passive cutaneous anaphylaxis
PC-KLH	phosphorylcholine conjugated keyhole-limpet hemocyanin
PFC	plaque-forming cell
PPD	purified protein derivative
PWM	pokeweed mitogen
RAST	radio allergen sorbent assay
RIA	radio immunoassay
s.c.	subcutaneous
SFA	suppressive factor of allergy
Sta	Staphylococcus aureus

T	thymus-derived
TD	T cell-dependent
Th	helper T cell
TI	T cell-independent
TNP	trinitrophenyl
Tw	Tween-20
T15	TEPC-15 myeloma protein
V	variable region of Ig molecule

CHAPTER 1

GENERAL ASPECTS OF IMMUNOGLOBULIN-E PRODUCTION AND LYMPHOKINE MEDIATED REGULATION

1.1 Allergy

Through the now classic experiments of Prausnitz and Küstner in 1921 [1], it was recognized that serum from an atopic patient could passively sensitize the skin of a normal, non-allergic recipient. In 1966, Ishizaka et al. [2] identified this skin-sensitizing serum factor (reagin) as a new class of immunoglobulins: they named this class immunoglobulin E (IgE), because of its capacity to produce erythema. At the same time, Bennich et al. [3] found in the serum of a patient with myeloma large amounts of an immunoglobulin that was found to belong to the newly discovered IgE class. Patients with atopic diseases including allergic rhinitis, allergic asthma, atopic dermatitis and urticaria commonly have elevated total as well as specific IgE levels. The degree of elevation correlates roughly with the severity of the disease [4]. Such a correlation provides indirect evidence that the IgE antibodies play a role in the pathogenesis of atopic respiratory disease. It also suggests that quantitation of IgE antibodies may be useful in the assessment of IgE mediated respiratory symptoms [5,6]. This is further illustrated in patients with rhinitis and asthma in which an elevated total serum IgE level is compatible with an atopic etiology [7]. The availability of myeloma IgE stimulated the development of immunochemical methods for the quantitation of IgE. Serum IgE values are generally expressed in international units per ml (IU/ml) based on a WHO-reference standard. One IU of IgE is equivalent to approximately 2.4 ng. Total IgE values over 200 IU/ml serum are considered elevated. In non-atopic donors, the serum IgE concentration varies between less then 100 and 700 ng/ml, while in atopic individuals concentrations of over 3000 ng/ml can be found. This strong elevation may result from non-specific effects on IgE immunoregulation, since in a number of atopic diseases T-cell dysfunction has also been found. The control of IgE synthesis is believed to be an important factor differentiating between allergic and non-allergic individuals.

Allergic inflammation is the consequence of an interplay between various components of the immune system, including IgE antibody formation and regulation thereof, IgG antibodies and complement, cell-mediated immune reactions and lymphokines as well as immune regulation of the proliferation and differentiation of mast cells, basophils and mucous cells. In addition, histamine and other mediators of inflammatory reaction exert a feed-back regulation on the immune system [8,9]. Eosinophils play a yet not fully understood role, possibly as scavengers of mediators that produce tissue lesions [10] or, as indicated by recent findings, as producers of tissue damaging substances [11]. The significance of IgE is restricted to its role as a cytophilic antibody. The role for IgE in host defence is to provide rapidly mobilizing mediators, serum components and effector cells to the site of entry of a foreign antigen. IgE antibodies are able to trigger mast cells which are already present there. The special feature of these antibodies is that they add immunological specificity to the mast cells and increase the sensitivity of the system. It seems sensible to regard IgE antibody as a 'gatekeeper' which can mobilize other defence systems. Its primary role appears to relate to invasive helminths, mites and ticks [12].

Meanwhile, homocytotropic antibodies similar to human IgE were detected in experimental animals. These animal models are used for studying the immune regulation of atopic allergy. Such studies have obvious advantages, including the possibility to compare effects of experimental manipulations of single parameters in genetically similar individuals. Several of these studies corroborate clinical experience that the allergic inflammatory reaction is more complicated from an immunological point of view than previously appreciated.

In this thesis data with regard to the induction and regulation of IgE formation in the mouse will be presented and discussed. The discussion will be limited to immune regulation of conceivable relevance for the development and regulation of human atopic disease, by which is meant a genetically influenced hyperreactivity to minute amounts of antigens most often accompanied by high levels of serum IgE antibodies. The involvement of the autonomous nervous system and the role of mediator substances like prostaglandins and leukotrienes in the regulation of the cellular events are beyond the scope of this thesis.

As outlined above, it is essential to measure murine IgE accurately and precisely when the mouse is employed as a model for human atopic diseases. Accuracy is the conformity of such measurement to an accepted standard value (reference accuracy) or to a true value. Precision reflects either the calculated result with its standard deviation or the number of digits or decimals in which the result is expressed and may be of low accuracy. Particularly in the mouse measurement of IgE concentration has been very difficult for a number of reasons. The low and transient total IgE levels, the difficulty to induce persisting antigen-specific IgE levels, and the short half-life of IgE antibodies in the serum are all problems shared between various species. Furthermore, in mice no IgE-secreting myeloma has been described. For these reasons together with those mentioned above, it was almost impossible to purify sufficient IgE out of the serum to raise heterologous antisera to murine IgE [13,14].

Because of the lack of suitable antisera no techniques could be developed that allowed determination of secreted IgE or enumeration of IgE-secreting cells, since all such methods are based upon the specific complex formation between IgE and an IgE-specific antiserum. As outlined in Chapter 2, until recently, only the passive cutaneous anaphylaxis (PCA) assay has been used for the detection of murine IgE. It has been shown that this PCA test is not always sufficiently reliable and precise to detect IgE antibodies. For that reason some of the results based solely on this assay might have to be reconsidered while using more advanced techniques that were developed recently.

With the development of antigen-specific mouse IgE-secreting hybridomas, a constant source of murine IgE became available. These monoclonal IgE antibodies are currently isolated and purified using routine methods and used for the preparation of heterologous mouse-IgE-specific rabbit and goat antisera. Such antisera are now prepared and used to design methods for the quantitative determination of murine IgE antibodies. The way the IgE antibodies are purified and the way the resulting antisera are tested for monospecificity and reactivity prompted us to develop our own reagents as will be described in Chapter 4. Mainly for similar reasons, still not many reliable methods are available for the detection of murine IgE-secreting cells. As will be outlined in Section 1.3.3, a lot of the IgE is produced locally. This raises questions as to whether it is reasonable to study only serum IgE levels as a representative for the induction and regulation of IgE antibody formation. Such a study was one of the primary objectives for the work described in this thesis. This prompted us again to adapt techniques for the detection of IgE-secreting cells. It is for these reasons that a substantial part of this thesis deals with the development of suitable reagents and assays for the study of induction and regulation of IgE formation in the mouse.

1.2 Properties of IgE

Like the basic structure of the other immunoglobulin classes, IgE is composed of four polypeptide chains that are covalently linked by disulfide

bonds. The molecular weight of the human IgE molecule is approximately 192,000 ± 4,000 daltons. Upon reduction and alkylation, the protein part of IgE yields light chains (22,600 daltons) and epsilon chains (67,300 daltons) in equimolar amounts which is consistent with the four-polypeptide chain structure. The molecular weight of the heavy chain is suggestive for the presence of approximately 550 amino acid residues in this heavy chain. The light chains may either be kappa or lambda. These light chains do not contain carbohydrates and are indistinguishable from light chains associated with other heavy chains.

IgE is a glycoprotein with a carbohydrate content of about 13%. The intact epsilon chain (containing the protein and the carbohydrate parts) has a molecular weight of 81 kDa. The six oligosaccharide units are exclusively associated with the epsilon chain and are all N-linked at asparagine residues. The role of the carbohydrates is uncertain. They do not play a major role in binding to Fc-epsilon receptors on mast cells. The epsilon chain contains five heavy chain domains (one variable and four constant domains). IgE has 20 disulfide bridges that form sixteen intrachain (and intradomain) bonds and four interchain bonds. Each $C_\epsilon 1$ domain contains two intradomain S-S bonds; the other 12 domains each contain 1.

Two of the three interchain disulfide bridges link the light and ϵ-chains. The remaining two disulfide bridges are unique for IgE and are located in the interdomain regions, $C_\epsilon 1$-$C_\epsilon 2$ and $C_\epsilon 2$-$C_\epsilon 3$, respectively.

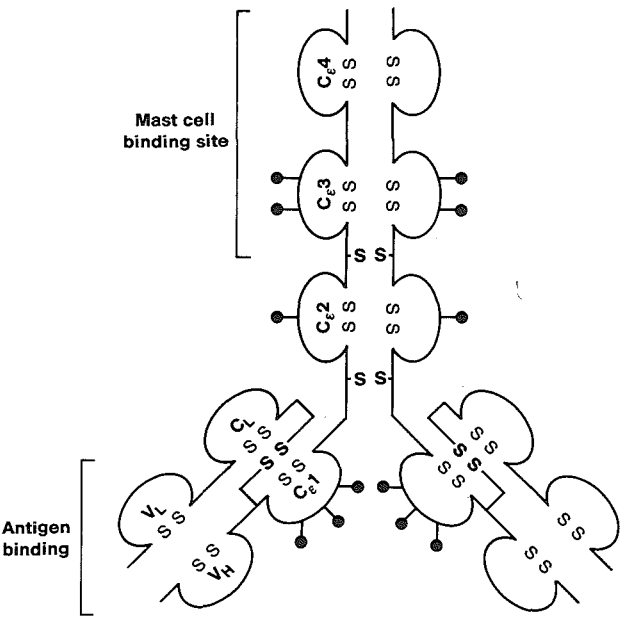

Fig. 1. Diagram of the quarternary structure of the IgE molecule and its Fc and $F(ab')_2$ fragments. The $C_\epsilon 1$ domains contain an additional intradomain bond which is not indicated in this diagram.

These unique interheavy chain disulfides may account for the relative segmental inflexibility of IgE when compared to IgG as measured by rotational correlation times. The $C_\epsilon 3$ and $C_\epsilon 4$ domains show close homology to the $C_\epsilon 2$ and $C_\epsilon 3$ domains of IgG, respectively. With an extra domain ($C_\epsilon 2$) replacing the hinge, the proposed structure of IgE is similar to that for monomer IgM. The IgE molecule, however, lacks tailpieces and shows no tendency to polymerise. The complete amino acid sequence of the epsilon chain has been elucidated. Although this sequence is unique when compared to the sequences of mu, gamma, and alpha chains, the 30% homology with the other known heavy chain constant region sequences is consistent with a common evolutionary origin [13,14].

Membrane-bound and secreted IgE of a murine B cell lymphoma have been reported to consist of structurally different heavy chains. The former is constituted by a 93 kDa molecule, while secretory chains have an apparent molecular weight of 81 kDa. Both forms are heavily glycosylated: in the presence of tunicamycin their apparent molecular weight is reduced by approximately 30% (61 kDa for membrane-bound IgE and 56 kDa for secretory IgE). Glycosylation appears to be necessary for membrane expression and secretion of IgE molecules. Such structural differences between membrane and secretory forms of immunoglobulins have first been demonstrated for IgM and IgG. These differences are limited to the COOH-terminal peptide of the heavy chain. This peptide is highly hydrophobic in the membrane form, which permits anchorage of the receptor molecule to the lipid bilayer. In the secretory form this peptide is more hydrophilic and shorter. The two molecules are encoded by different mRNAs, differing at the 3'-termini. Moreover, the nucleotide sequence of the murine C_ϵ gene is predicting a hydrophobic membrane form being 64 amino acid residues longer than the secreted form [15].

Studies with human IgE molecules have indicated that IgE does not fix complement by the classical pathway. However, fixation by the alternative pathway has been established. The complement-fixing site was found to be in the Fc region. The high amounts of aggregated IgE required suggest that complement fixation by IgE is of little physiological importance.

Similar to human IgE, murine IgE does not cross the placenta, is present in extremely low levels in the serum, has the capacity to passively sensitize mast cells for anaphylactic release of histamine and to bind to mastocytoma cells [14].

The availability of monoclonal IgE from hybridomas made it possible to investigate quantitatively the half-life of IgE in mice. Using radioimmunoassays for the analysis of unlabeled IgE, the half-life of IgE in serum was found to be 5 to 8 hrs [16]. This half-life is somewhat lower than the 10 to 12 hrs obtained by PCA measurement [17]. The difference may be readily explained by the lower precision of the PCA assay, although it can also be envisaged that monoclonal IgE is more easily eliminated from the circulation. No appreciable differences were found in the half-lives of specifically purified IgE antibody, radio-labeled or unlabeled, and IgE antibody present in freshly drawn serum. Although it is well known that murine IgE is easily inactivated (denatured by heating at 56°C for 1 hr), it is quite stable in purified form when kept at lower temperatures.

Enzymatic cleavage of human IgE by papain produces an Fc fragment (MW 98 kDa) which contains the $C_\epsilon 2$, $C_\epsilon 3$, and $C_\epsilon 4$ domains and two antigenic determiants, $\epsilon 1$ and $\epsilon 2$. The loss in binding activity of IgE upon heating at 56°C and upon mild reduction and alkylation (which primarily affects the inter-heavy chain disulfide bonds) has been ascribed to structural changes in the $C_\epsilon 3$ and, to a lesser extent, the $C_\epsilon 4$ domains [18]. Fc_ϵ fragments of mouse IgE have not reproducibly been produced. However, studies on the rate

of sequential digestion of IgE by trypsin in the presence and absence of high affinity receptors for IgE suggest that the $C_\epsilon 3$ domain is binding to the receptor [19]. Resonance energy transfer studies indicate that this $C_\epsilon 3$ domain interacts with the receptor leading to bending of the IgE molecule near its receptor-binding site [20].

High affinity receptors for IgE (K_a approx. 10^{10} M^{-1}) were found on mast cells and basophils (Fig. 2). Such receptors bind the Fc part of the IgE molecule and are therefore called Fc_ϵ receptors ($Fc_\epsilon R$). These receptors appear to be composed of two α components (α_1 of 34 kDa and a more heavily glycosylated 30 kDa α_2) that contain a binding site for IgE. Also a transmembrane β component (33 kDa) and two cytoplasmic disulfide-linked γ components are integrated into the receptor complex [21,22]. The β component is composed of two distinct domains: the larger β_1 domain appears to have a segment within the plasma membrane, while the smaller β_2 domain may be exposed to the cytoplasm. Also macrophages and B and T lymphocytes have been shown to express low affinity IgE receptors (K_a approx. 6×10^7 M^{-1}). These receptors are similar in size (they consist of 47 kDa molecules) and in antigenic determinants. It is presumed that T lymphocytes with such $Fc_\epsilon R$ are involved in regulating IgE synthesis by synthesis of IgE-binding factors (Section 1.4.2) [23,24]. This low affinity $Fc_\epsilon R$ is found on most leukocytes and is recognized by CD23 monoclonal antibodies. This CD23 molecule is able to bind IgE as well as recombinant IgE lacking carbohydrate modification, so it is possible that the CD23 molecule has two binding sites, one for sugars and one for IgE.

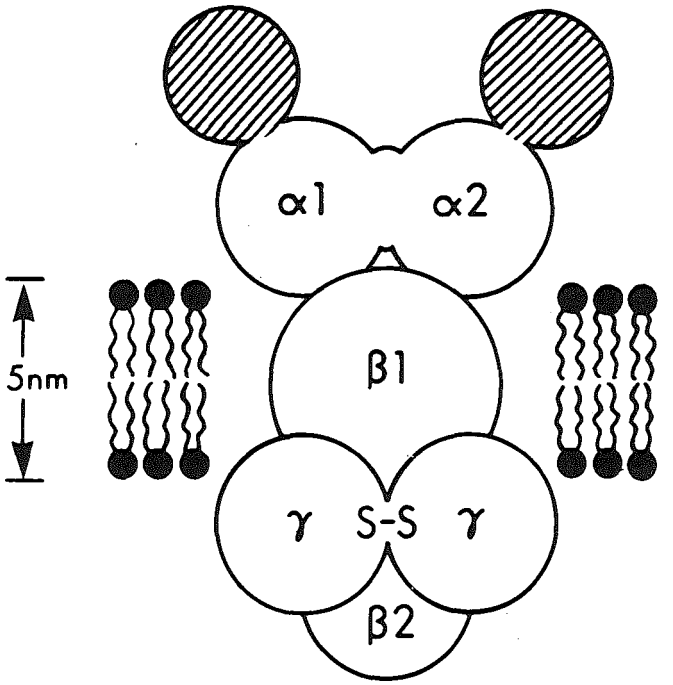

Fig. 2. Schematic diagram of the structure of the IgE receptor. The volumes of the spheres are proportional to the masses of the peptide and carbohydrate (shaded areas) regions. After Metzger et al. [21].

1.3 Formation of IgE

Lymphoid cell interactions resulting in IgE synthesis are basically similar to those involved in the production of other immunoglobulin isotypes. Cooperative interaction between specific T and B lymphocytes is necessary for development of IgE antibody responses in vivo in mice [25]. Furthermore, it was shown that enhanced anti-hapten IgE antibody responses resulted from collaboration of antigen-specific memory B cells and helper T cells specific for the secondary carrier [26,27]. Thymus dependence of IgE antibody formation was demonstrated using thymectomized or congenitally athymic mice [28,29]. Although no specific IgE antibodies could be induced in the serum of these mice, total IgE levels were elevated compared with those of normal mice [29]. It has been suggested that this was caused by the absence of suppressor T cells in these animals. Alternatively, it was suggested that the IgE synthesis could be regulated by distinct isotype-specific helper T cells. Another possibility that has been put forward states that differences in isotype-specific B cells to the same T cell regulatory mechanisms rather than isotype-specific helper T cell control were responsible for immunoglobulin class-specific B cell responses [30].

1.3.1 Ontogeny of IgE-secreting cells

There is little information concerning the ontogenic development of IgE-producing cells in mice. It was shown that 1 to 1.5% of all nucleated cells in spleen and mesenteric lymph nodes are surface IgE positive. These numbers are increased ten-fold upon infection with helminths. In newborn mice, some IgE positive cells were observed within 24 to 48 hours after birth. These values increased to numbers found in adult mice within 4 weeks [31]. More than 95% of all surface IgE-bearing cells coexpress IgM. This suggests that IgE-bearing cells originate from previously IgM positive cells [32]. It was also shown that IgE B cells have a variable (V) region repertoire similar to that of IgM and IgG B cells [33]. Furthermore, the majority of precursor B cells are not committed to a particular isotype, but are multipotential with regard to isotype production [34]. Moreover, normal values of surface IgE positive cells were found in the spleen and lymph nodes of congenitally athymic mice, showing that this development is T cell independent [35].

Ontogenic maturation time of the lymphoid system relative to gestation time differs from one species to another. As measured by the onset of immune responsiveness as well as by the appearance of peripheral T and B cells, mice develop their immune system just before and after birth, whereas humans develop a functional immune system already early in gestation. In man, neonatal B cells express surface IgM, IgG and IgA molecules in adult proportions [36] but they have only a limited capacity to produce Ig which, furthermore, is mainly restricted to IgM [37,38]. It is not clear as yet, whether neonates lack the appropriate helper T cell signals for isotype-switch or have a diminished susceptibility for extracellular differentiation and maturation signals. However, at the DNA level the mechanism for gene rearrangement or heavy chain isotype switching is not defective.

It is well known that the serum IgE level in normal animals and humans is less than 0.01% of that of IgG or IgM. These low serum IgE levels cannot be explained simply on the basis of the precursor frequency of IgE producing cells. Firstly, it was shown by limiting dilution analysis that the precursor frequency is 1 in 2,000 - 5,000 for IgE and 1 in 3 - 10 for IgM or IgG [39]. Secondly, analysis of C_H genes in mice and humans has

revealed no exceptional peculiarities in the localization or the structure of C_ϵ as compared with the C genes of the other isotypes [40-42]. Thirdly, no class-specific recombinase for the preferential expression of the C genes other than the C_μ gene has been demonstrated (Section 1.4.3.4). Fourthly, 1 to 1.5% of the peripheral B lymphocytes have been shown to express surface IgE [43]. These observations suggest the presence of a regulatory mechanism operating specifically on the differentiation and proliferation of B cells able to secrete IgE (precursor IgE-B cells). Studies both in man and rodent systems have demonstrated the presence of IgE isotype-specific subsets of T cells and soluble factors which mediate the enhancement or suppression of induced IgE responses (Section 1.4.2).

1.3.2 B cell activation in the IgE response

An essential aspect in the discussion of activation is the cell cycle. Most (> 95%) of the mature surface IgM and IgD positive ($mIgM^+/IgD^+$) B lymphocytes are in a resting state (G_0-phase), exhibiting a low rate of metabolic activity and no net DNA synthesis [44]. Both IgM and IgD receptors bind antigen. Because of the low frequency of individual antigen-reactive clones anti-receptor antibodies (anti-Ig) have been extensively used to study the consequences of crosslinking mIg. These studies have clearly shown that appropriate anti-Ig reagents, such as $F(ab')_2$ fragments of rabbit anti-μ or anti-idiotypic antibodies (or the intact antibodies on Sepharose beads) will induce B cell activation: low concentrations induce essentially all murine B cells to leave G_0, whilst high concentrations stimulate some 40-50% to synthesize DNA, but not to secrete antibodies. Essentially all B cells bear $Fc_\gamma R$, which bind complexed antigen-IgG antibody complexes. These receptors are implicated in inhibiting B cell activation, and this would be achieved by co-crosslinking mIg and FcR (thus mantaining clonal specificity because of the involvement of mIg). This generates a powerful inhibitory signal via the FcR. Intact (IgG) rabbit anti-Ig provides a polyclonal model to study this effect, since rabbit IgG binds well to mouse FcR. As a result, such anti-Ig does not induce DNA synthesis in B cells, but inhibits the response to the $F(ab')_2$ form. After contact with the stimulating agent, the cell progresses from the G_0-phase into the cell cycle [45,46]. This complex process is called activation. In studying the processes related to activation and subsequent proliferation and differentiation of B lymphocytes in vitro, specific antigen or polyclonal B cell activators (mitogens) are used. The latter stimulate a large proportion of the B cell population and thus evoke responses of sufficient magnitude to permit analysis of membrane related and intracellular changes. Polyclonal B cell activators (PBA) can roughly be divided into two groups. One group of substances can bind to the antigen receptor, i.e. membrane bound Ig, and mitogens of the second group bind to other (mostly unknown) membrane associated structures. Examples of the latter category are pokeweed mitogen (PWM), lipopolysaccharide (LPS), purified protein derivative (PPD), dextran and Epstein-Barr virus (EBV). PBA binding to the antigen receptor are anti-immunoglobulin antibodies (anti-Ig; anti-IgM; anti-IgD; anti-IgG; anti-idiotypic antibodies) and Staphylococcus aureus Cowan I strain (Sta) bacteria [47-50].

Cross-linking of surface Ig (mIg) with mIg-specific antibodies leads to increased cytosolic levels of inositol 1,4,5-triphosphate, a phospholipase C induced breakdown product of phosphatidylinositol 4,5-biphosphate in the B lymphocye membrane. The turnover of the latter product is considered to

be crucial in subsequent B cell responses like Ca^{2+} mobilization, depolarization, increased class II MHC (I-A) message and surface I-A expression [51].

PBA and antigen can be subdivided according to their requirement for T cells to facilitate the B cell response. T cell-dependent (TD)- and T cell-independent (TI)-PBA and antigen can be discerned. TD-PBA and TD antigen can only induce a B cell response in the presence of T cell help (e.g., PWM, PPD and most soluble proteins). In contrast TI-PBA and TI antigen are able to stimulate B cells in the absence of T cells (e.g., LPS, EBV and certain polysaccharides). T cell help in the B cell response against TD-PBA and TD antigen requires physical contact of the helper T cell and the responding B cell. The T cell recognizes the antigen, probably bound to the antigen receptor of the B cell, in the context of class II molecules coded for by the major histocompatibility (MHC) complex [51].

The B lymphocytes probably recognize self and nonself antigens indiscriminately and also recognize a larger spectrum of substances than T cells. B cells normally avoid a response against self by being dependent on signals from activated T cells for stimulation. Even when they bind a substance via their antigen receptors, most of them do not develop into effector cells (antibody-secreting plasma cells) until they have received a further signal from T cells which have encountered the same substance. The details of the transmission of messages between T and B lymphocytes (T-B collaboration) are obscure. The two principal hypotheses are: (a) that the T lymphocytes binds to one region of the antigen (carrier) and the B lymphocyte to another region (hapten) and that the message is transmitted via this 'antigen bridge' between the two cells; and (b) that the B cell binds the antigen first, processes (fragments) it, redisplays the fragments on its cell surface in the context of its MHC molecules, and the T cell then recognizes the complex of the antigen and MHC molecule, thereby stimulating the B cell [39].

The second way in which a B cell can be helped to proliferate and differentiate, is through soluble antigen-specific helper factors and/or non-specific factors - often referred to as lymphokines - produced by activated T cells. Among these are growth factors, regulating the proliferation of activated B cells and differentiation inducing factors (Section 1.4).

The initial activation of helper T cells participating in TD B cell responses is based on the dual recognition of the antigen and class II MHC-molecules, usually present on the surface of antigen-presenting cells (APC) [52]. The factor dependent differentiation of B cells is thought to be a linear sequence of steps leading from the initial activation, subsequent DNA synthesis and cell division to Ig production. In studying these processes two membrane Ig-binding PBA are most frequently used: anti-IgM (mice, humans) and Sta (humans) [53-55].

Resting human B cells (G_0 phase) can be activated by anti-IgM or Sta to express a number of activation markers like the transferrin receptor in the G_1 phase before entering the S phase. The activated cells are then responsive to B cell growth factor [56].

Binding of an antigen to surface Ig can trigger a cascade of events taking place in cytoplasm and nucleus, eventually resulting in the generation of a clone of immunoglobulin-secreting B cells. One of these early changes is the enhanced transcription of the C_δ-gene. This results, by unknown posttranslational events together with an unchanged C_μ-gene transcription, to a decrease in the membrane IgD density. Lamson and Koshland [57] have shown that the increase in the transcription of the C_δ-gene is accompanied by a shift from μm-RNA to μs-RNA. This shift begins shortly

after PBA exposure and within 48 hours μ_s-RNA is the predominant RNA in the activated B cells. Such cells then display two kinds of Ig receptors on the cell surface, IgM and IgD, both carrying identical light chains and identical V-D-J regions, but different C regions. The expression of the δ chain is probably achieved by differential splicing of the same primary transcript used for the production of the μ chains. This event takes place before the activated cells enter the S-phase. Upon activation with a low dose of anti-IgM, resting B cells enter the cell cycle (G_o --> G_1). This is accompanied by cell enlargement and the expression of receptors for B cell growth factors and B cell differentiation factors. Upon binding B cell growth factors, the B cell can enter the S-phase [58]. These factors will be discussed in further detail in Section 1.4.3.

1.3.3 Tissue distribution of IgE-secreting cells

Several lymphoid organs are involved in IgE antibody formation in vivo. When mice are deliberately immunized with a TD antigen employing an adjuvant (Section 1.3.4.4) to induce a primary immune response, antibody-secreting cells were found, dependent on the route of immunization, in the spleen, lymph nodes, gut-associated (GALT) and bronchus-associated lymphoid tissues (BALT), but not in the bone marrow (BM). Upon secondary immunization of mice with TD antigen, the BM becomes the major source of antibody-formation, dependent on migration of reactivated memory B cells from peripheral lymphoid organs into the BM during the secondary type response [59-62].

The 'peripheral' or 'secondary' lymphoid organs like spleen, lymph nodes, the GALT and the BALT provide the architecture and accessory cells (macrophages and dendritic cells) appropriate for the antigen processing and for the presentation of the antigen to the lymphocytes. In these organs, therefore, the antigen-driven differentiation of lymphocytes takes place. There is evidence for a regional production of IgE at sites were also mast cells occur. Several studies have demonstrated regional production of IgE antibodies in lymph nodes draining mucous membranes [63-67]. Furthermore, studies in mice infected with Nippostrongylus brasiliensis show that IgE antibodies can be demonstrated regionally in the draining lymph nodes before a systemic response can be detected [64]. Tracheal sensitivity to worm allergens has been reported to develop prior to intestinal sensitivity. Moreover, this sensitivity correlated with the early local IgE synthesis in the mediastinal lymph nodes, while sensitivity to the allergens in the skin and gastrointestinal tract correlates more closely with serum IgE antibody. Thus local IgE synthesis influences local tissue sensitization and responsiveness to allergens.

The local immune response appears to have some special characteristics. The initial response occurs in the draining lymph nodes and these nodes are the major sites of production of IgG and IgE antibodies found in the serum [68]. The IgG response, on the other hand is also produced in central lymphoid tissues. Facilitation of the IgA transport by the necessary secretory piece (SP) leads to increased levels of specific IgA dimers (IgA_2SP) in the secretions. Presumably, primed or memory B cells committed to the IgE isotype are released from these nodes and localize both at mucosal surfaces and in lymphoid tissue (Fig. 3). However, these cells require restimulation by antigen in order to synthesize specific IgE antibodies. It is also possible that some of the B cells producing local antibody to allergens are primed in the mucosa. It would thus appear that IgE-forming cells predominate in the respiratory and gastrointestinal mucosa and in the regional lymph nodes. Moreover, it appears that distributions of IgE antibody-

forming cells are different depending on the method and the route of immunization. Thus, preferential distribution of IgE-forming cells in the respiratory and gastrointestinal tracts in man might result from frequent exposure of the local lymphoid tissues to small doses of environmental antigens, which may favor IgE synthesis.

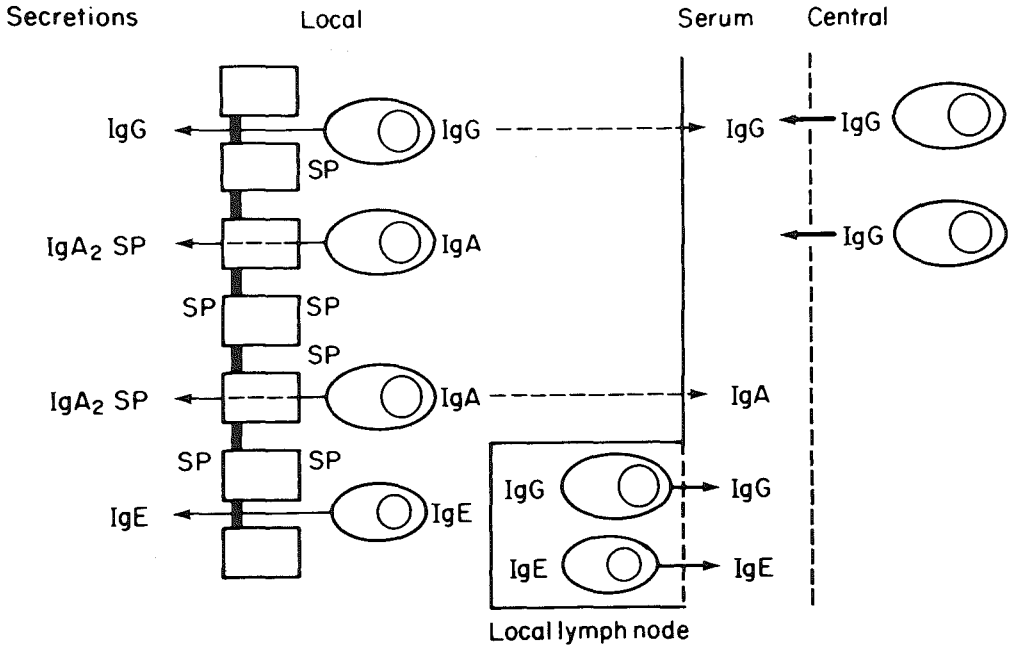

Fig. 3. 'Local' immune response to pollen allergens. Antibodies found in secretions are produced by plasma cells in the mucosa, while the main site for production of IgG and IgE antibodies in the serum appears to be the local lymph nodes. Injections of pollen extract into the stimulate IgG antibody production in central lymphoid tissue and make little effect on antibody production in secretions. SP = secretory piece, IgA_2SP = dimeric IgA containing the secretory component. After Platts-Mills [68].

1.3.4 Factors affecting IgE formation in vivo

The various aspects of IgE antibody formation have been dealt with in several reviews [69-77]. Here only some additional comments will be made.

1.3.4.1 Primary versus secondary-type responses

Upon primary antigen injection small B lymphocytes can be stimulated to transform into B cell blasts in the B cell areas of the lymphoid tissues. These B cell blasts proliferate and differentiate into antibody producing plasma cells. Both the T and the B lymphocyte population can carry immunological memory and such cells are, in contrast to virgin cells, long-lived

small cells with a life-span of several weeks to several months [78,79]. Furthermore, these memory cells are recirculating cells, while virgin lymphocytes are mainly non-recirculating sessile cells [80]. The amounts and the types (affinity, isotypes) of antibody formed vary widely with the conditions of immunization [81-83].

It has become clear that immunization with a relatively low dose of antigen precipitated on alum can give a primary IgE antibody response and that animals immunized by this procedure frequently display secondary IgE antibody responses after booster immunization. It therefore appeared that the requirement of adjuvant for IgE antibody responses is limited to the primary response. Similarly, it was reported in rats that low doses of antigen precipitated on alum are optimal for establishing memory for the IgE antibody response. Rats immunized with a high dose of antigen can also give rise to an IgE antibody response, but secondary IgE antibody responses in these animals are generally lower than those observed in rats primed with a low dose of antigen [69,73].

Recently, several studies have been reported on the induction of secondary type IgE antibody responses in mice without the use of adjuvants. Such immunization protocols invariably are based on multiple injections of low doses of antigen, mostly ovalbumin [84-90].

1.3.4.2 Type of antigen

The induction of maximal antibody production to most antigens requires the participation of antigen-specific T helper cells. In all animal experiments, IgE antibody was formed against TD antigens only [74]. Attemps to produce IgE antibody in mice by immunization with TI antigens were unsuccessful [28,69-77].

The surface of a protein antigen consists of a complex array of overlapping potentially antigenic determinants; in aggregates they approach a continuum. Most determinants depend upon the conformational integrity of the native protein molecule. Those to which an individual responds is dictated by the structural differences between an antigen and the host's self-(glyco)proteins and by host regulatory mechanisms, and is not necessarily an inherent property of the protein molecule reflecting restricted antigenicity or limited number of antigenic sites. As stated above, protein antigens therefore are more immunogenic when administered in aggregated than in soluble form. Thus chemically cross-linked protein molecules (e.g., by formaldehyde [91] or glutaraldehyde [92]) and antigen-antibody complexes, prepared in slight antigen excess are usually highly immunogenic. When, however, the complexes are prepared in antibody excess, their immunogenicity is greatly reduced, probably because the antigenic determinants are blocked [93]. It has been reported that mice immunized with tobacco glycoprotein mount immune responses that are restricted to the IgE class. This is probably due to rutin-like polyphenol groups within the glycoprotein. Therefore, the antigen structure itself can direct the antibody isotype expression [94].

It was suggested that priming the immune system with a carrier protein may activate apart from helper T cells, suppressor T cells capable of specifically suppressing antibody responses. It has been found that a variety of allergenic haptens and allergens could be converted into nonimmunogenic and tolerogenic compounds by conjugation to a variety of synthetic polymers and that these immunosuppressive effects were mediated by activating suppressor T cells [95,96]. Such antigen-specific suppressor T cells could be induced by coupling of ovalbumin to polyethylene glycol [97], polyvinyl alcohol [98] or pollullane [99]. Alternatively, the antigen

could be denatured in urea [100]. Hapten-specific suppressor T cells were induced by coupling the hapten to ovalbumin [95], Mycobacteria [101], muramyl-peptides [102], levan [103] or non-immunogenic carrier proteins like murine-Ig [95].

1.3.4.3 Route of antigen administration

Normally, lymphatic tissues are almost constantly encountered by antigens from transiently invasive or indigenous microbes (normal flora of skin, intestines, etc.) and those that enter the body by inhalation (e.g., plant pollens), by ingestion (e.g., food, drugs) and by penetration of the skin. For deliberate immunization, immunogens are usually injected into skin (intradermally or subcutaneously (s.c.)) or muscle (intramuscular (i.m.), depending on the volume injected and the irritancy of the immunogen. Intraperitoneal (i.p.) and intravenous (i.v.) injections are also used in experimental work, especially with particulate antigens.

Regardless of the route, most antigen eventually becomes distributed throughout the body via lymphatic and vascular channels. Because most food antigens are degraded in the intestines, feeding is effective only under special circumstances. Allergic responses to food are probably due to antigen that resist degradation by intestinal enzymes. Immunization can also be performed by aerosol administration of the antigen. Repeated administration of low dose of antigen in aerosol under relatively physiological conditions was described to be able to induce initially an IgE response. Continued stimulation, however, subsequently led to an antigen-specific and isotype-specific state of tolerance. This was due to suppressor T cells and this tolerance could be transferred with lymphoid cells to naive recipients [104-106].

The way in which an antigen is presented to the immune system is obviously of profound importance in induction of the IgE response. Route of injection as well as type, dose and physical state of the antigen are all extremely important. The kinetics of a localized IgE anti-tetanus antibody response was followed in mice immunized by intratracheal (i.t.), s.c. and i.p. immunization routes. In mice immunized i.t. and i.p. with tetanus toxoid and Bordetella pertussis organisms, the IgE response of bronchial lymph nodes exceeded those of other lymphatic tissues. IgE production by the spleen was second to that of bronchial lymphoid tissue; both responses appeared to correlate with the serum PCA antibody responses. The authors suggested that the serum IgE levels induced by i.p. and i.t. immunization are mediated by IgE-secreting cells present in the draining lymph nodes. In contrast, after s.c. immunization a minimal primary IgE response was detected and only in the draining popliteal lymph node [107].

Initial studies on the induction of IgE formation were largely focussed on hapten-specific IgE antibodies. For the induction of these responses allergens were found to be excellent carriers. It was in these studies that evidence was obtained for the participation of carrier-specific helper cells in secondary IgE antibody responses [25-27].

Secondary immunization with a TD antigen will lead to antibody formation in the BM independent of the route of primary immunization: i.v. and i.p. are equally effective while s.c. priming is effective with a relatively high dose of antigen. Secondary IgE antibody responses localized just like secondary IgM, IgG and IgA responses in the BM as well [108-111].

Every antigen has an optimal immunogenic dose range. Much larger amounts generally elicit high-zone tolerance. With TD antigens, lower amounts can also cause tolerance (low-dose tolerance). Also the physical state of an antigen influences the immunogenicity: aggregated molecules of

bovine gamma globulin, for instance, are immunogenic, while monomers are tolerogenic. It is difficult to induce tolerance to particulate antigens, because such antigens usually are highly immunogenic. The route of administration is another determinant: soluble antigen tends to be immunogenic when injected into tissues, but to be tolerogenic when given i.v.

1.3.4.4 Use of adjuvants

Adjuvants are agents that by non-specific mechanisms can modify the humoral or cellular immune response by simultaneous injection of antigen and adjuvant. Adjuvants not only can stimulate the response to the antigen, but, dependent on the conditions, they can also induce suppression. Furthermore, their effect can be antigen-specific as well as non-specific. The non-specific adjuvant effects are probably mediated by macrophages.

A large variety of substances can act as adjuvants and their modes of action are very heterogeneous [112-114]. Originally, most adjuvants used were of bacterial origin that were themselves good immunogens. Adjuvants like complete Freund adjuvant (CFA) have a strong stimulating effect on most immune responses. However, it also induces excessive granulopoiesis within the BM, which abolishes the ongoing immunoglobulin synthesis in this organ. Other adjuvants which do not induce excessive granulopoiesis, such as alum, do not interfere with antibody formation in the marrow. Many adjuvants probably mediate their effect by decreasing the breakdown of the antigen for prolonged periods of time, while they simultaneously stimulate the mononuclear phagocyte system.

The nature and dose of adjuvant are critical factors in the IgE antibody response to protein antigens. It is well established in mice that some adjuvants, such as alum [115] and Bordetella pertussis bacteria [116] or certain parasites as Ascaris or Nippostrongylus brasiliensis [117,118], enhance IgE antibody responses. Lipopolysaccharides have also been shown to stimulate IgE antibody production, whereas other strong adjuvants, e.g. CFA, are unfavourable to the generation of IgE antibodies [119,120]. The fact that immunizations of experimental animals usually are done parenterally, with relatively high doses of antigen in the presence of adjuvants, is a major source of criticism of the use of rodent models for studies of IgE antibody regulation. In addition, the use of adjuvants may result in antibodies with characteristics partially different from those obtained by immunization without the use of adjuvants, e.g. with different antibody affinity. The use of the B. pertussis as an adjuvant made it possible in rats to use picograms of antigen for the induction of IgE antibody responses. However, in mice, rats and rabbits administration of antigen in the nanogram to microgram range without using any adjuvant can also induce IgE antibody formation (Chapter 5).

Conflicting data exist on the role of complement in the induction of IgE responses [121]. It was observed that during purification of a crude allergen extract, the agent causing skin reactivity in allergic patients copurified with a complement deactivating activity [122]. It was therefore suggested that complement would play a role in allergenicity and adjuvant activity for the induction of IgE responses.

1.3.4.5 Age

The capacity of B cells to proliferate and differentiate is only moderately impaired with age leading to a decrease in the number of effector cells generated [123]. However, the B cell compartment in the BM of aged mice is different from that in young mice since in in vivo transfer systems

it was found that BM from aged mice contained a significantly greater number of differentiated immunocompetent cells than the marrow of young mice [124]. Furthermore, the proportion of long-lived cells in the BM is increased in aged mice [125-128].

The age related changes in the central lymphoid organs are to a large extent based upon deficiencies in T cell function: BM from old mice has a decreased capacity to provide the thymus with T cell progenitors suggestive for a reduced stem cell or prothymocyte population [123]. Also the number of cortisone-resistant mature thymocytes declines with age together with a progressive involution of the thymus after sexual maturation [129].

In mice high-avidity IgG antibody-forming cells preferentially decline during aging [130]. This finding is in contrast to the notion that the secondary immune potential is less severely affected by age than the primary potential [131]. Moreover, the response to TD antigen is more susceptible to age-related decline than the response to the TI antigen LPS [132]. Also, the responsiveness towards low doses of antigen declines faster than towards high doses [123]. No data are available as to such effects on the murine IgE response.

1.3.4.6 Corticosteroid level

The height of the primary immune response to an antigen, especially TD antigen, is influenced by the plasma level of corticosteroids [133]. This influence of corticosteroids on the immune system can be mediated by redistribution of potentially circulating lymphoid cells and mononuclear phagocytes as well as by an effect on the function of these cells. The distribution pattern of these cells is affected because of a decrease of the cellularity of the thymus, spleen and BM, associated with a fall in circulating lymphocytes [134]. The redistribution of several subpopulations of peripheral blood leukocytes to the BM is apparent from the increase of the T cell number and T cell function in this compartment and the prevention of monocyte emigration. Moreover, lymphocyte traffic within the BM, spleen and lymph nodes is almost completely arrested, whereas the influx of lymphocytes into lymph nodes is impaired. Another factor that contributes to corticosteroid mediated redistribution of cells is the lymphoid cell destruction [135].

The effects of corticosteroids on the function of the lymphoid cells, are different for T and B cells, the T cells being more susceptible. It has even been postulated that antigen-reactive T cells are more severely depleted from the peripheral blood than mitogen-reactive T cells [136].

The timing of corticosteroid treatment in relation to the moment of immunization greatly influences the antibody formation during the primary immune response. Administration of high doses of corticosteroids shortly before immunization results in the highest suppressive effect, while administration after antigenic exposure has a smaller effect on serum antibody levels [137]. IgG synthesis appears to be more susceptible to corticosteroid suppression than IgM synthesis [138]. No solid data on the effects of corticosteroid treatment on IgE responses in the mouse were described as yet.

1.3.4.7 Antigen-non-specific IgE response

It was shown that upon immunization with TD as well as TI antigen and independent of the antigen dose, Ig without binding capacity for the eliciting antigen are synthesized [139-141]. This was also found for IgE antibody responses in mice and rats. The ratio of this so-called antigen-non-

specific response to the antigen-specific response is isotype-dependent and is greater during a primary immune response than during a secondary one [90]. Whether this is caused by antigen-non-specific B cell growth and maturation factors or induction of autoantibodies or anti-idiotypic antibodies is still controversial. This non-specific Ig production together with the antigen-specific antibody production upon immunization, is also reflected in the serum Ig fraction [142]. Therefore, measurement of serum Ig levels is not a reliable measure for the capacity to produce antigen-specific antibody upon immunization. This is all the more true since a substantial proportion of the circulating Ig is withdrawn from the circulatory system at secretory sites [143].

Another explanation for the induction of non-specific immune responses has been put forward, relating this phenomenon to the decreased reactivity of non-specific suppressor T cells or the factors they produce. Such non-specific suppressor T cells can be eliminated by low dose X-irradiation, treatment with immuno-suppressive agents (cyclophosphamide, anti-lymphocyte antiserum) or immunization with lipopolysaccharides or Freund complete adjuvant [144-147]. Under such conditions the negative control on antibody production is weakened leading to an enhanced production of both antigen-specific and antigen-non-specific ('bystander') Ig synthesis. Moreover, the activity of non-specific helper T cells and the factors they produce (Section 1.4) may further activate antigen-non-specific B cells [139].

1.3.5 Genetic influences

Many studies have suggested that immune responses are genetically determined in several aspects. A part of the operating genetical control mechanisms are linked to the MHC system. Especially for IgE a double kind of genetic control system is apparent: the first control level being dependent on the immune response (Ir) genes and resulting in a genetically determined ability to respond to minute doses of several types of TD antigens. The second control level is independent of the MHC and determines whether or not a particular strain of mice will respond to the above mentioned conditions by making IgE antibodies. In careful analyses IgE respondership cannot be related to a particular H-2, IgH-C or Mls trait (TABLE I).

Some authors, however, claim that H-2^a and H-2^k mice react poorly and that H-2^s mice react with no or extremely low and transient levels of IgE [148-155]. The same distinction is observed when analyzing the IgE formation in mice infected with helminths. It has been suggested in the IgE non-responder mouse strain SJL, that specific suppressor T cells inhibit IgE formation by B cells. Upon removal of these suppressor T cells, the B cells of this strain were able to produce as much IgE as untreated high responder mouse strains. Breeding experiments with these SJL mice have shown that the suppressor function of their T cells is inherited as a recessive autosomal gene that is not linked to the H-2 haplotype [152,153,155].

It has been reported in humans as well that genetic factors play an important role in determining susceptibility to atopic disease. Several studies have been reported in which significant associations between immune responses to pollen antigens and the possession of certain HLA haplotypes have been described, e.g. HLA-A1, -B8, -DR3/DR3 and HLA-A3, -B7, -DR2/DW2 [5,156-158]. The restricted molecular weight range of atopic antigens (25 - 45 kDa) might suggest that increased mucosal permeability is a causative factor but this now seems unlikely. IgA is the usual protective antibody at mucosal surfaces and several studies have shown an increased prevalence of IgA deficiency in atopic individuals and their relatives [158,159].

TABLE I

Lack of correlation between some genetic traits and IgE respondership in inbred mouse strains

Strain	H-2	IgH-C	Mls	IgE responder
C57BL/6	b	b	b	low
CBA/J	k	j	d	high
AKR	k	d	a	low
A/J	a	e	c	high
DBA/1	q	c	a	low
DBA/2	d	c	a	high
C3H/He	k	j	c	high
NZB	d	e	a	non
BALB/c	d	a	b	high
SJL	s	b	c	non
SJA/9	s	a	c	non

After Levine and Vaz [150]. H-2 is the MHC complex of the mouse; IgH-C is the allotype of the Ig heavy chain; Mls is allelic type of minor lymphocyte-stimulating antigens.

1.4 Regulation of IgE formation

As outlined in Section 1.3, at the induction level the cellular control of antibody responses of the IgE isotype resemble that of antibody of other classes. However, it has become increasingly apparent that T cells play a decisive role both in induction and regulation of immediate-type hypersensitivity responses. It was shown that athymic nude mice are unable to produce specific IgE upon immunization. Moreover, it was found by adoptive transfer experiments that anti-hapten IgE antibody formation can only be achieved in the presence of sufficient numbers of carrier-specific helper T cells [25-30]. Furthermore, it was shown that the amount of IgE produced by mice could be selectively increased by several treatments, like whole body irradiation, cyclophosphamide treatment, thymectomy or treatment with anti-lymphocyte serum. In view of the sensitivity of suppressor T cells for these treatments it was concluded in these studies that the IgE synthesis is normally down-regulated by suppressor T cells [144-147,160]. IgE antibody formation against priming antigens was also found to be enhanced upon nematode infection [69-76]. This suggested that the IgE antibody formation can be controlled not only by antigen-specific helper and suppressor T cells but also by some additional mechanisms selective for the IgE isotype.

1.4.1 Antigen-specific regulation of the IgE response

T cells may help or suppress immune responses in an antigen-specific or non-specific way, and may regulate idiotype- and isotype-specific responses. This also holds for IgE responses. Regulation of the IgE response by means of antigen-specific mechanisms has been attempted through antigen coupled to natural and synthetic polymers [74,75,95]. Haptenated mycobacteria and muramyldipeptide were shown to induce both IgE-specific and anti-

gen-specific suppressor T cells [161,162]. Antigen-specific IgE responses can be suppressed by injection of antigen or idiotypic antibody coupled to syngeneic cells and by actively produced or passively administered anti-idiotypic antibodies [163,164]. Moreover, feed-back regulation, tolerization of B cells, and manipulation of T cells and the factors they produce can regulate positively and negatively the differentiation of precursor B cells to IgE-forming cells. B cells committed to IgE synthesis can be made tolerant by injection of hapten coupled to a nonimmunogenic carrier. This tolerance induction of B cells is due to active suppression rather than to clonal deletion. The potential importance of suppressor T cells in the regulation of IgE synthesis in both man and rodents was suggested by the following observations: (1) selective depletion of suppressor T cells in low responder mice by either total body irradiation or immunosuppressive agents resulted in marked enhancement of IgE synthesis; (2) serum IgE levels were frequently elevated in patients with primary T cell immunodeficiencies; and (3) certain disorders such as the hyper IgE syndrome, severe atopic dermatitis and acute graft versus host disease were associated with marked elevation in serum IgE and decreased numbers of suppressor T cells [75,161]. It appeared that tolerance induction of B cells is more dependent on a certain affinity treshold (of at least 5×10^5 M^{-1}) than on the actual concentration of an antigen. Moreover, B cells can be made tolerant neonatally only during a narrow time-window.

The effect of such treatments is a persistent suppression of primary and secondary anti-hapten IgE antibody responses. If the anti-hapten antibodies are restricted to a particular idiotype, anti-idiotypic antibodies may be applied for suppression of the IgE antibody response. This type of suppression is much stronger for IgE than for the other isotypes [163,165-167].

1.4.2 Isotype-specific T cells and regulatory IgE-binding factors

Isotype-specific regulation has been described for the synthesis of IgE, IgA and IgG in mice as well as in rats and humans [168-174]. It has been proposed that T cells regulate the key events leading to high or low IgE responsiveness in the models of both Ishizaka and Kishimoto, whereas the Katz group attributes this role to B cells. Some unusual features of the Lyt surface phenotype markers have been noted for T cells providing IgE helper or suppressor signals, but recent data indicate that attemps to make strict correlations of such markers with their cellular function may not be relevant. Certain characteristics appear to be common to such regulation: T cells which regulate the synthesis of a heavy chain isotype are supposed to bear FcR specific for that particular isotype and these FcR-bearing T lymphocytes secrete factors actually accounting for this regulation. In general, the soluble factors bind specifically to the Fc region of the Ig isotype which they regulate, and appear to be related structurally to the lymphocyte FcR. It seems therefore reasonable to hypothesize that the factors are FcR derived from T lymphocytes or include a fragment of the FcR [171,173].

These isotype-specific regulatory factors are referred to as immunoglobulin-binding factors (Ig-BF). Apart from these factors, other isotype-specific factors were described: suppressive factor of allergy (SFA) and en-hancing factor of allergy (EFA) that were able to selectively suppress and enhance the IgE synthesis, respectively [74,75,174]. These factors lack, however, the property to bind IgE. Moreover, the potentiating factor is derived from $Fc_\epsilon R$ positive cells, while the suppressive factor is derived from cells with little $Fc_\epsilon R$ surface expression. In this scheme of IgE regulation, the function of $Fc_\epsilon R$ expressed on the different cells is not

evident. However, earlier studies by Ishizaka's group have shown an increase of $Fc_\epsilon R$ expression when lymphoid cells were exposed to IgE in vitro. Since IgE-BF could comprise fragments of $Fc_\epsilon R$, further characterization of the relevance of $Fc_\epsilon R$ expression to IgE-BF production and biologic activity will be required. Based on these factors a model has been developed. Since in mice the production of IgE antibodies is both T cell dependent and highly susceptible to suppression by both T cells and T cell factors, by analogy in man IgE production could be secondary to abnormally good T cell help or a failure of T cell suppression.

An elaborate theory of 'allergic breakthrough' has been developed, suggesting that periodic fluctuations in T cell suppressor activity control IgE antibody production and thus the symptoms of allergic disease [173,174]. In the animal model, however, the animals receive high-dose immunization in adjuvant and all animals produce high levels of IgG antibody. This model appears to have very little in common with human allergy to inhalants though it might be relevant to the injected allergens.

The IgE-specific immunoregulatory factors have been well characterized in mice and rats. Two factors produced by mesenteric lymph node cells from parasite infected rats have been described [168,171,172]. Both have affinity for the Fc region of IgE, but have different effects on in vitro IgE responses: the potentiating factor selectively enhances the IgE response while the IgE suppressive factor inhibits the IgE response. These two factors appear to share some structural characteristics, including affinity for IgE, molecular size and some antigenic determinants. However, these factors differ in the degree of glycosylation of a common precursor molecule. Although these factors were originally described in the rat, they have recently been found to be produced by human and mouse T cells as well [169,170,173]. Until the biochemical properties of these factors and their congeners have been characterized in molecular terms, these findings will remain difficult to understand.

From studies in the mouse it became clear that the nature of the IgE-binding factors differs depending on the mouse strain. Normal spleen cells of BDF_1 mice incubated with IgE gave rise to IgE-BF that potentiated the IgE response. In contrast, incubation of SJL spleen cells with IgE resulted in the formation of IgE-suppressive factor. The same results were found with antigen primed spleen cells from BDF_1 mice and SJL mice stimulated with the homologous antigen. It appeared that lymphocytes from the two strains differ in the process of glycosylation of IgE-binding factors. The IgE-potentiating factor can be detected whenever the IgE serum level and/or the IgE antibody response is enhanced. On the other hand, IgE-suppressive factor is formed in various conditions in which the IgE response is suppressed. The correlation between enhancement of the IgE response and the formation of IgE-potentiating factor, and that between suppression of the IgE response and the formation of IgE-suppressive factor, strongly suggest that IgE-BF are involved in the regulation of the IgE response in vivo [172]. Considering that human IgE-BF were found to be similar to rodent IgE-BF in their physicochemical properties, and that the biological activities of the human factors are regulated in the same way, it is conceivable that administration of such factors may modulate the IgE synthesis in atopic patients as well [170,171]. Curiously enough, although the events in the cascades in both the Ishizaka and the Katz model were described in great detail, the problem of explaining the mechanism by which an IgE-BF might modulate IgE-bearing B cell responsiveness to antigen has not been tackled.

1.4.3 Isotype regulation by helper T cells and lymphokines

1.4.3.1 Subsets of helper T cells

Cumulative evidence was obtained that helper T (Th) cells are involved in the activation, clonal expansion, differentiation and isotype regulation of B cells which respond to most antigens. The development of clonal Th cell lines and the recent rapid progress in cloning the genes encoding the various lymphokines have made it possible to dissect the regulatory signals involved in the antigen-specific response of B cells. Initially, factors controlling B cell growth and differentiation were identified by the action of crude, ill-defined tissue culture supernatants from mitogen activated T cells. However, the genes for many of these factors have now been cloned (Table II), and the use of defined recombinant gene products has helped to clear up some of the earlier confusions - and perhaps create some new ones. For the most part the following discussion will be confined to what is known of the recombinant factors and has been reviewed before [175-178].

Lymphokines are encoded by genes for which there is only one copy per haploid cell. In common with most eukaryotic genes, the lymphokine genes are segmented, being composed of exons which are complementary to sequences in the mature mRNA and which are separated by introns which are not found in the mRNA. Most lymphokine genes consist of 3-4 introns and 4-5 exons and are located on a number of different chromosomes. It is of interest that in man a number of lymphokine genes are located on chromosome 5 and that tumour necrosis factor (TNF) and lymphotoxin (LT) are closely associated on chromosome 6 within the major histocompatibility complex. The location of genes on the same chromosome raises the possibility that they may be closely linked and under the influence of common regulatory elements.

Lymphokines are usually produced by cells in response to induction signals generated from the cell surface. Many, for example granulocyte-macrophage-colony stimulating factor (GM-CSF), IL-2, IL-3 and interferon-gamma (IFN-γ), have common nucleotide sequences in the 5' flanking region of their genes which may be important in the initiation of transcription. The production of lymphokines seems to be controlled particularly at the level of transcription. Detailed knowledge of the structure of lymphokine genes and their flanking sequences is therefore providing information which will unravel control of their regulation at the DNA level.

cDNAs for lymphokines predict mature proteins of around 100-200 amino acids. Most have a clearly defined hydrophobic signal sequence of around 20 amino acids, which is cleaved to give the mature protein. It is notable that for those factors derived from macrophages (TNF, IL-1α and IL-1β) a pre-sequence is cleaved from the mature protein which is unusually long (70 or more amino acids). Since the signal sequences are associated with transport of the proteins out of the cell, this difference in size may represent an important difference in secretory mechanisms between macrophages and lymphocytes.

Amino acid sequence data show that many lymphokines contain cysteine residues which may be important in the formation of intramolecular disulfide bonds. Using site-directed mutagenesis and chemical reduction the importance of these disulfide bridges for the tertiary structure of the molecule and its biological activity has been determined for several of the lymphokines.

Molecular weight determinations of lymphokines purified form native sources frequently suggest that they are not homogeneous and that they have larger weights than would be predicted from the cloned genes. The discrepancies between predicted and observed molecular weights arise from post-

TABLE II

Cloned lymphokines and their alternative names

	Acronym	Alternative name(s)
Interferon-γ	IFN-γ	
Interleukin 1	IL-1	Lymphocyte activating factor (LAF); mitogenic protein (MP); T cell replacing factor III (TRF-III); B cell activating factor (BAF); B cell differentiation factor (BDF); endogenous pyrogen (EP); leukocyte endogenous mediator (LEM); serum amyloid A (SAA) inducer; proteolysis inducing factor (PIF); catabolin; haematopoietin 1 (HP1); mononuclear cell factor (MCF).
Interleukin 2	IL-2	T cell growth factor (TCGF); thymocyte mitogenic factor (TMF); killer cell helper factor (KHF)
Interleukin 3	IL-3	Multi-potential colony stimulating factor (multi-CSF); burst promoting activity (BP); haemopoietic cell growth factor (HPGF); persisting cell stimulating factor (PSF); mast cell growth factor (MCGF); haematopoietin 2 (HP2)
Interleukin 4	IL-4	B cell stimulation factor 1 (BSF-1); T cell growth factor II (TCGF-II); mast cell growth factor II (MCGF-II)
Interleukin 5	IL-5	T cell replacing factor (TRF), B cell growth factor II (BCGF-II); eosinophil differentiation factor (EDF)
Interleukin 6	IL-6	Interferon β_2 (IFN-β_2); B cell stimulation factor 2 (BSF-2); B cell differentiation factor (BCDF); hybridoma/plasmacytoma growth factor (HPGF); hepatocyte stimulating factor (HSF)
Granulocyte-macrophage-	GM-CSF	Colony stimulating factor (CSF-α); pluripoietin; neutrophil inhibition factor (NIF-T) colony stimulating factor
Macrophage-colony stimulating factor	M-CSF	Colony stimulating factor 1(CSF-1)
Granulocyte-colony stimulating factor	G-CSF	Colony stimulating factor (CSF-β)
Tumour necrosis factor	TNF	Cachetin; tumour necrosis factor (TNF-α)
Lymphotoxin	LT	Tumour necrosis factor (TNF-β)

translational modification, particularly glysosylation. Most lymphokines are variably glycosylated (usually N-glycosylated) and the glycosylated proteins may form oligomers. Size estimations of natural proteins suggest a range of molecular weights which are reduced by separation under reducing conditions on SDS-PAGE. Further reductions in molecular weight heterogeneity may be achieved by incubation of the lymphokine with glycosidases which cleave off glycosylated side chains. The function of the glycosylation is unclear since recombinant products made in Escherichia coli, which are not able to glycosylate proteins, often have the same biological activities and the same half-lives in vivo as their glycosylated equivalents. Furthermore, site-directed mutagenesis of potential glycosylation sites does not alter the biological function.

A careful examination of the lymphokines produced by a panel of activated Th clones has led to the proposition that there are at least two major subsets of Th clones, designated Th1 and Th2 [179-182]. The lymphokine expression patterns and functional distinctions between these two subsets are based in many cases on biological assays and, in all cases, on quantitation of mRNA levels coding for these lymphokines [180,182]. Th1 clones produce interleukin 2 (IL-2), interferon-gamma (IFN-γ) and lymphotoxin (LT) in response to stimulation by antigen or concanavalin A. Th2 clones are unable to do so, but produce interleukin 4 (IL-4) and interleukin 5 (IL-5) in response to the same stimuli (Table III).

To date, no cell-surface antigen differences have been found between murine Th1 and Th2 clones: both are Ly-1$^+$, L3T4$^+$ and Lyt-2$^-$. It is important to point out that many of the described cloned T cell lines fit into

TABLE III

Two subsets of mouse helper T cell clones

	Th1	Th2
Surface markers:		
Ly-1	+	+
L3T4	+	+
Lyt-2	-	-
Lymphokines:		
Interferon-γ	++	-
Interleukin 2	++	-
Lymphotoxin	++	-
GM-CSF	++	+
Tumor necrosis factor	++	+
Interleukin 3	++	++
Interleukin 4	-	++
Interleukin 5	-	++
B cell help:		
IgG2a	++	+
IgE	-	++
Delayed-type hypersensitivity	++	-

After Mosmann and Coffman [185].

TABLE IV

Bioassays for lymphokines

IFN-γ	Virus yield reduction Inhibition of viral RNA and protein synthesis Cell growth inhibition
IL-1	Co-mitogenic stimulation of mouse thymocytes in presence of mitogen Proliferation of D10.G4 T cell line Production of IL-2 by LBRM-33 1A5 line Production of fibroblast prostaglandin Bone resorption Proteoglycan release from cartilage
IL-2	Proliferation of IL-2 dependent T cell lines
IL-3	Haematopoietic colony formation from bone marrow Proliferation of IL-3-dependent lines Induction of 20α-steroid dehydrogenase in nude mouse spleen cells or bone marrow
IL-4	Co-stimulation of mouse splenic B cells in presence of anti-Ig Increase in class II MHC expression by B cells Induction of IgG_1 by T-depleted spleen cells Induction of IgE by T-cepleted spleen cells Proliferation of mast cell lines Proliferation of T cell lines
IL-5	Induction of proliferation and IgM production by B cell lines (mouse)[1] Proliferation or antibody secretion by large B cells (mouse)[1] Induction of secondary anti-DNP IgG antibody by DNP-primed T-depleted B cells (mouse)[1] Differentiation of eosinophils in bone marrow cultures
IL-6	Ig production by normal B cells (human)[1] Ig production by Epstein-Barr virus-transformed cell lines (human)[1]
GM-CSF G-CSF M-CSF	Haematopoietic colony formation of bone marrow cells
TNF LT	Antiproliferative activity on certain tumour cell lines

[1]Indicates that the assay is only available for that species. After [175] and [177].

these two patterns of lymphokine expression, but T-T hybridomas and T lymphoma cell lines often do not. In the rat and in man these two sub-populations of helper T cells can phenotypically be distinguished on the basis of the Ox22 and 2H4 cell-surface marker, respectively [183,184].

Much of the information available about lymphokine biology comes from studies of their generation and assay in vitro. There have been less analyses of the effects of injection of lymphokines into animals, although the recent clinical use of cloned factors is providing a surge of new data (Section 1.4.3.5). There are even fewer descriptions of lymphokine generation and lymphokine receptor distribution in vivo. It follows that our understanding of lymphokine biology is often constrained by the limitations of interpreting in vitro data and these are worth considering before passing to a discussion of general aspects of lymphokine biology.

Detection of lymphokines is usually done by in vitro bioassays although assays based on the antigenicity of molecules e.g. radioimmunoassay (RIA) or enzyme-linked immunosorbent assays (ELISA) are becoming more available. A list of the common bioassays for lymphokines is shown in Table IV. This exemplifies the range of assays which are used. Bioassays for lymphokines have rarely involved the effect of a single factor on a single cell type, although in recent years it has been possible to assay the effect of a recombinant lymphokine on a cell line and use monoclonal antibodies in tests to produce assays with restricted variability [182]. However, where mixtures of lymphokines, as found in cultures of activated lymphocytes, are assayed, or where impure target cells are used, there may be difficulty in interpreting the results. These problems arise because several lymphokines can produce similar biological effects. Thus, IL-4 has been found to cause stimulation of thymocytes or T cell clones used in assays for IL-1 and IL-2, respectively, and TNF and LT are indistinguishable biologically. Furthermore, a lymphokine may induce the production of other lymphokines which may influence different cells in a mixed target cell population, or which may interfere positively or negatively with assays on homogeneous cells. Induction of one lymphokine by another has often been described, for example IL-1 and TNF stimulate fibroblasts to produce IL-6, and IL-1 stimulates T cells to produce a number of lymphokines.

Lymphokine bioassays frequently reflect only one aspect of a particular lymphokine's biological activity. Where the effect of a lymphokine is restricted, such in vitro assays may reflect an important role played by that lymphokine in vivo. Thus, IL-2 is usually bioassayed by its ability to cause proliferation and growth of a T cell line which constitutively carries the IL-2 receptor (e.g. CTLL line) and this mirrors the important role that IL-2 plays in the clonal expansion of T cells in vivo. Other common bioassays reflect only one of many activities which the lymphokine has. For example, IL-1 is assayed for its co-stimulatory effects with mitogen on mouse thymocytes. It can be shown to exert many other biological effects and the relevance of the mouse thymocyte assay to the major functional properties of IL-1 remains to be determined.

Lymphokines are made by different cells (Table V) often in response to their activation. Some, like IL-2, seem to be made only by a restricted type of cell (i.e. T cells) whilst others, like IL-1, can be produced by very different cell types. Whilst there are many cell lines which produce lymphokines in vitro constitutively or after activation, the normal sources of the various lymphokine in vivo is not clear yet. This particularly applies to the colony stimulating factors (CSFs) where the natural source in the bone marrow stroma is not known.

TABLE V

Cellular sources and targets of lymphokines

Lymphokine	Cellular source	Cellular target
IFN-γ	T cells, natural killer (NK) cells	Macrophages, T cells, B cells, NK cells
IL-1 α and β	Macrophages, endothelial cells, large granular lymphocytes, B cells, fibroblasts, epithelial cells, astrocytes, keratinocytes, osteoblasts	Thymocytes, neutrophils, hepatocytes, chondrocytes, muscle cells, endothelial cells, epidermal cells, osteocytes, macrophages, T cells, B cells, fibroblasts
IL-2	T cells	T cells, B cells, macrophages
IL-3	T cells	Multipotential stem cells, mast cells
IL-4	T cells	T cells, mast cells, B cells, macrophages, haematopoietic progenitors
IL-5	T cells (mouse)	Eosinophils, B cells (mouse)
IL-6	Fibroblasts, T cells	B cells, thymocytes
GM-CSF	T cells, endothelial cells, fibroblasts, macrophages	Multipotential stem cells
M-CSF	Fibroblasts, monocytes, endothelial cells	Multipotential stem cells
G-CSF	Macrophages, fibroblasts	Multipotential stem cells
TNF-α	Macrophages, T cells, thymocytes, B cells, NK cells	Tumour cells, transformed cell lines, fibroblasts, macrophages, osteoclasts, neutrophils, adipocytes, eosinophils, endothelial cells, chondrocytes, hepatocytes
LT	T cells	Tumour cells, transformed cell lines, neutrophils, osteoclasts

After [178].

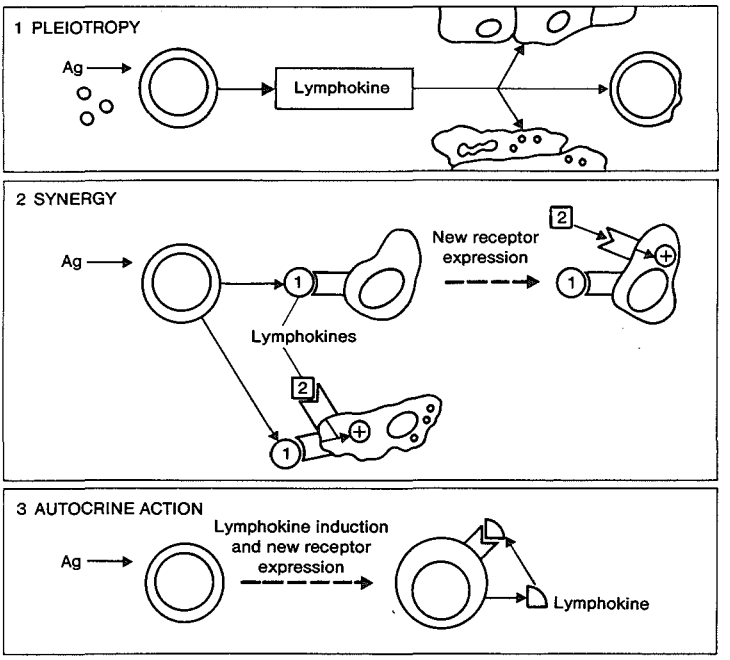

Fig. 4. Models for lymphokine action. Pleiotropy: a single lymphokine produces different effects by acting on different cell types (1). Synergy is seen when one lymphokine induces the receptor for another lymphokine or when both lymphokines are required together to stimulate a cell (2). Some lymphokines act on the cell which produced them in an autocrine fashion (3). After Hamblin [178].

The target cells of lymphokine action may also be restricted or very diverse (Table V). At early stages in lymphokine research it was suspected that lymphokines might have multiple activities on different target cells. The availability of recombinant lymphokines has confirmed that they are often pleiotropic. Thus, IL-1 acts on T cells and is considered important in the immune response to antigens. It also produces inflammatory effects on a variety of target cells. It is a puzzle how one molecule can exert such very different effects on different target cells. It also remains to be seen whether all of these targets are affected in vivo and under what circumstances.

An important aspect of lymphokine activity is that they frequently work together, with each other or with another stimulant, to produce effects. Thus, for example, LT and IFN-γ have a potent synergistic antiproliferative effects in vitro and antitumour effects in vivo, while IL-2 and IL-4 synergize to cause proliferation of T cell clones. Such synergistic action is not understood. However, it may be that the interaction of one lymphokine with its receptor may prime the cell to become responsive to the second

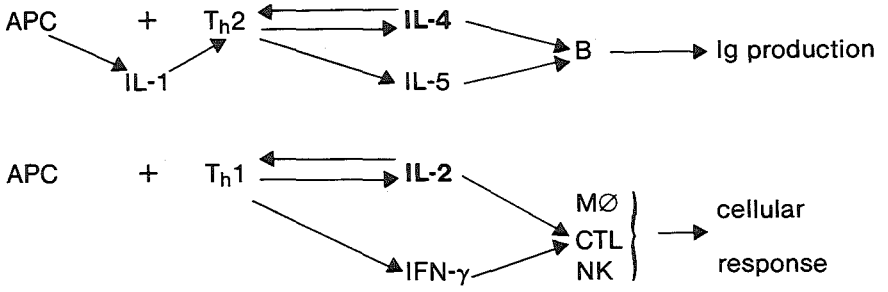

Fig. 5. The concept of two helper T cell subsets Th1 and Th2. Presentation of antigen by antigen-presenting cells to Th cells leads to activation and subsequent lymphokine production. For the activation of Th2 cells an additional signal by IL-1 is neceassary. Th2 cells are primary producers of IL-4 and IL-5 that exert their function mainly as inducing Ig-secretion by B cells. IFN-γ and IL-2 producing Th1 cells are mainly active as mediators of cellular immune responses although they are also able to give help to B cells for Ig-synthesis (see also Section 1.4.3.3). IL-2 and IL-4 are also autocrine growth factors for Th1 and Th2 cells, respectively.

signal. Alternatively, the occupancy of two receptors may deliver the correct signal to allow the cell to respond (Figure 4). A better understanding of lymphokine - receptor interaction and their subsequent intracellular signalling should lead to a further understanding of how these synergistic interactions arise.

The bioassays used to detect most, if not all, lymphokines are now known to be very complex. In addition many lymphokines have effects on more than one cell type. Therefore, cell lines used to assay for lymphokine activities often respond to more than one lymphokine. There are often positive or negative interactions between the activities of different lymphokines. This implies that really monospecific bioassays for lymphokines are difficult to establish. Furthermore, for several T cell products the appropriate information is lacking for the development of monospecific bioassays. Therefore, in various cases mRNA dot hybridization was used to confirm and to further explore the lymphokine synthesis patterns [182]. The principle drawback of the RNA detection procedure, however, is that the presence of mRNA does not prove that the corresponding protein is actually synthesized and secreted as an active lymphokine.

The cloned lines are all IL-2 dependent and nearly all require for periodic stimulation with appropriately presented antigen. Delayed-type hypersensitivity (DTH) responses can be transferred to unimmunized mice by most Th1 clones but not by Th2 clones [186]. Under appropriate conditions, the majority of both Th1 and Th2 clones can provide help to B cells, but there are important differences in the Ig isotypes produced by B cells helped by the different subsets. Moreover, clones of the Th2 subset appear to be significantly better helpers for B cells in vitro, especially if helper function is assessed by the induction of Ig secretion (Fig. 5). It is noteworthy that the lymphokines that have well documented effects on B

cells, i.e. IL-2, IL-4, IL-5 and IFN-γ, are each expressed by only one of the Th subsets. This implies that there are two quite distinct pathways of B cell activation, one that utilizes IL-4 and IL-5 and another in which IL-2 and IFN-γ participate.

1.4.3.2 Lymphokines acting on B cells

The activation, proliferation, and differentiation of B lymphocytes is controlled by a variety of soluble factors, amongst others lymphokines. The latter exert their influence at all stages of the differentiation pathway, from small resting (G_o) B cells right through to the plasma cell, and may act either alone or in concert with other signals (reviewed in [185,187-191]).

Activation

Normal resting (G_o) B cells can be activated without dividing by a variety of different signals including antigen, antibody to surface Ig and certain lymphokines, notably IL-4. The different activation signals do not all act through the same biochemical pathways. Cross-linking of surface Ig with antigen or anti-Ig receptors results in breakdown of phosphatidylinositol bisphosphate into inositol triphosphate and diacylglycerol with subsequent calcium mobilisation and activation of protein kinase C. IL-4 seems not to use this pathway but may involve other receptor linked protein kinases. This is an important point, and may go some way to providing an explanation for the profound synergistic action many B cell growth factors have with activation molecules binding to B cell surface Ig.

The activation of B cells can be measured in a variety of ways. Early events such as calcium flux, protein phosphorylation and changes in intracellular pH are good indicators of transmembrane signalling. Later events such as increases in cell volume and expression of activation antigens (e.g. class II MHC antigens) are also frequently used, but the biochemical link between the membrane signalling event and the measured cellular change is not known. In each of the above, cellular activation can be detected in the absence of cell division.

Proliferation

The most commonly used assay for B cell growth factors depends upon costimulation of B cells with two independent signals, neither of which alone will result in cell division. In most cases, B cells are cultured with growth factor and a suboptimal dose of a polyclonal B cell activator such as anti-IgM, S. aureus Cowan 1 (Sta) in humans, or phorbol esters which directly activate protein kinase C without phosphatidylinositol breakdown and calcium mobilisation. The activation step was thought initially to induce the expression of surface receptors for growth factors but, for IL-4 at least, receptors are now known to be present on resting B cells as well. It seems more likely, therefore, that B cell proliferation depends upon some sort of synergistic interaction between two activation signals which together will drive the cell from G_o/G_1 into cycle. With costimulation assays, IL-2, IL-4, IL-5 (in the mouse), $BCGF_{low}$ and possibly IFN-γ and TNF-α, have all been shown to be B cell growth factors. It is also possible to detect growth factor activity with assays using selected indicator B cell lines. At low cell densities, autocrine factor production by B cell lines may be insufficient for optimal proliferation. Under these conditions, exogenous B cell growth factors will enhance cell division which can be measured easily by ^{3}H-thymidine uptake. B cell growth factors which can be measured in this way are IL-5 (on murine BCL1 cells), and $BCGF_{low}$.

Differentiation

There are essentially three types of assays for B cell differentiation factors. In the first, B cells are costimulated with a B cell differentiation factor and an activation signal, and Ig secretion is measured after a suitable period (usually 5-10 days) as an indicator of B cell maturation. With this kind of assay, IL-2, IL-5 (in the mouse), $BCGF_{low}$ and IL-6 have all been found to be B cell differentiation factors. In the case of IL-6, Ig secretion can occur with no increase in cell dissociated from proliferation. The second type of assay is based on the use of indicator B cell lines for assaying B cell differentiation factors.

The third type of assay is the most physiological one for assaying B cell differentiation factors. This assay is based on the capacity to replace T helper cells in antigen specific antibody responses in vitro. Such factors are called T cell replacing factors (TRF). In humans, only IL-2 and $BCGF_{low}$ have been shown to do this, but in mice, IL-5 has also been reported to have TRF activity. With one or other of the three assays described IL-2, IL-4, IL-5, $BCGF_{low}$, IL-6 and possibly IFN-γ, have all been shown to be B cell differentiation factors.

Most factors which stimulate B cell growth have also been reported to cause B cell differentiation. However, in man, IL-6 has been reported to be a differentiation factor without causing B cell growth. IL-6 is a potent B cell differentiation factor in inducing IgM and IgG secretion by Epstein-Barr-virus-transformed B cell lines and Sta-activated normal human B cells. From the above it is clear that the same lymphokine may act at different stages of B cell development and that there are many different lymphokines involved.

In the above descriptions, B cell growth and differentiation factors are depicted as controlled by antigen-driven T-cell-derived factors augmented by macrophage-derived factors. There is also evidence that B cells have the capacity to control their own growth in an autocrine fashion. Some B cell lines can produce factors such as IL-4 which stimulate their own growth and there is evidence that normal B cells can also respond to factors they make themselves.

B cell growth and differentiation factors have multiple activities

It is quite clear from the above discussion that B cell growth and differentiation factors do not readily fall into separate groups responsible for B cell activation, growth and differentiation. Rather, it seems that each factor may act at more than one stage of normal B cell differentiation. Why there should be so many factors with such similar and overlapping activities is not known, but several possibilities can be put forward.

1. The different B cell growth and differentiation factors may be responsible for regulating responses of B cell subpopulations. Evidence is slowly accumulating for the existence of B cell subpopulations, but it is not yet clear whether these represent cells at different stages of activation, or functionally distinct subsets.

2. Different B cell growth and differentiation factors may regulate antibody responses to different antigens (protein, polysaccharide, particulate, thymus dependent, thymus independent type 1 and type 2, etc.).

3. B cell growth and differentiation factors may regulate the Ig isotype response (IgM, IgG, IgA, IgE, and IgG subclass) to a particular antigenic challenge. It is already known that IL-4 is important for IgG1 and

IgE responses (Section 1.4.3.3 and 1.4.3.5), and there is evidence that the IgA response in mice is enhanced by IL-5. It remains to be seen whether all isotype responses are regulated in this way, and whether this control is mediated by selection of precommitted B cells or by switching (Section 1.4.3.4 and Chapter 6).

4. In vitro artifacts may explain some of these multiple activities. For example a factor may appear to act on B cells by causing the release of another factor from contaminating cells (e.g. T cells or monocytes) present in the B cell preparations. This was thought to be the case for IL-2, but the presence of IL-2 receptors on B cells and the action of IL-2 on homogeneous B cell lines have dispelled this idea. It is likely that similar findings for the other factors will show that these also act directly on B cells. So far, little is known about the micro-environments in vivo where the various lymphokines affecting B cells are generated and exert their action.

The other important attribute of (probably) all the B cell growth and differentiation factors is their lack of specificity. For example, IL-2 is a growth factor for T cells and B cells; IL-4 acts on T cells, B cells, mast cells and monocytes; and IL-5 is an eosinophil differentiation factor as well as a B cell growth and differentiation factor. Moreover, receptors for some of these factors (e.g. IL-4) are found on many diverse cell types. Why these factors should have such a broad spectrum of tissue specificities, and how the various responses are controlled, is not known. If one factor can be used in more than one way the different activities should be partitioned by the local microenvironment. Control could also be maintained if each responding cell type would require synergistic combinations of factors.

IL-4 was first postulated to act as a B-cell growth factor because it could costimulate with anti-Ig and cause proliferation of resting B cells [191]. But IL-4 can also induce the expression of class II MHC molecules [192,193] and low-affinity receptors for the Fc portion of IgE ($Fc_\epsilon R$; CD23) on resting B cells [194,195]. Moreover, cloning of the cDNA encoding murine IgG_1 inducing factor has revealed its identity as IL-4 [196,197]. Mouse IL-4 was originally purified from the supernatants of the thymoma line EL-4 after stimulation with PMA. It is also generated by activated T cell lines. Natural IL-4 has a molecular weight of 20 kDa. The cDNA clones for mouse IL-4 code for 140 amino acids of which the first 20 constitute the signal sequence. The mature 120 amino acid protein has three potential N-glycosylation sites and six cysteines which are all involved in intrachain disulfide bonds. A human clone coding for IL-4 was isolated from a human cDNA library prepared from a concanavalin-A-stimulated human T cells. This clone codes for a protein of 153 amino acids of which the first 22 constitute the signal sequence. It has two potential N-glycosylation sites and six cysteine residues (Fig. 6). It shares 50% homology with murine IL-4. Human IL-4 is inactive on mouse cells as is mouse IL-4 on human. IL-4 shows no homology with other lymphokines. The human IL-4 gene is located on chromosome 5 and consists of four exons spanning 9 kb.

In mice, IL-4 increases IgE and IgG_1 production by activated B cells, both in vitro [189,197-201] (Section 1.4.3.3) and in vivo [202-204] (Section 1.4.3.5). The wide range of both growth and differentiation activities of IL-4 on B cells as well as on T cells [205-208], macrophages [209,210], mast cells [205,211] and hemopoietic progenitors [212] is reflected in the names that were originally used to describe these various activities: B

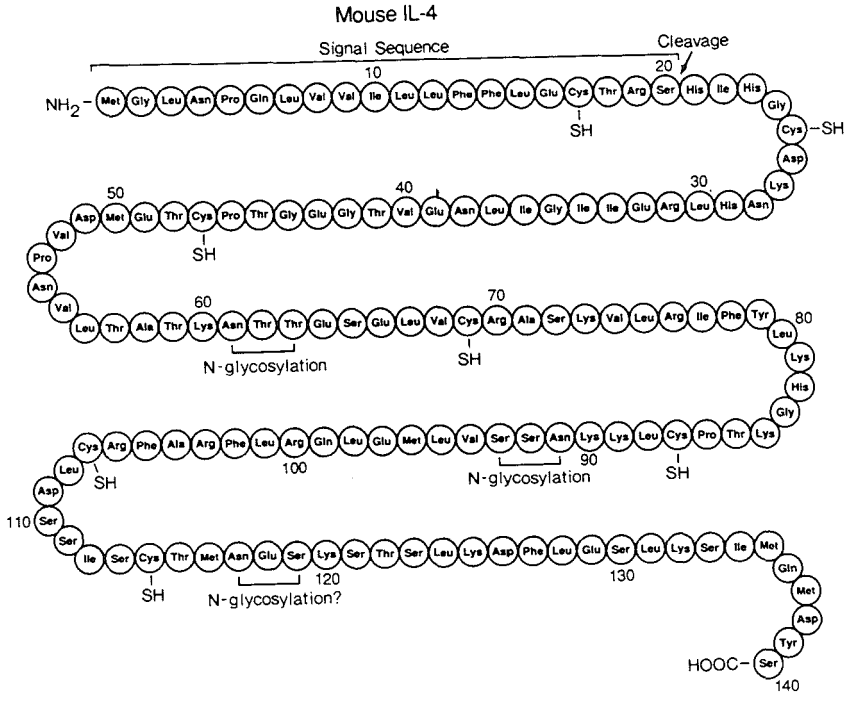

Fig. 6. Schematic representation of IL-4 amino acid sequence. Hydrophobic signal peptides and potential N-glycosylation sites are indicated. The cleavage sites for processing of signal peptides are indicated by arrows.

cell stimulatory factor-1, IgG_1 inducing factor, B cell growth factor, T cell growth factor-2, mast cell growth factor-2 and IgE/IgG_1 enhancing factor. The activities of IL-4 on B cells have many implications: upregulation of MHC class II antigen expression may enhance the ability of B cells to present antigen to T cells, thus making the immune system more sensitive to lower amounts of antigen. This proliferation-enhancing effect of IL-4 accounts for the clonal expansion of antigen-specific B cells. Moreover, the regulation of Ig isotype production may modulate humoral responses to different antigenic stimuli.

IL-5 was initially described as B cell growth factor II (BCGF II) for the murine B cell lymphoma, BCL1 [213]. Subsequently, it was found to cause DNA synthesis and IgM and IgG secretion by preactivated normal B cells. In addition, BCGF II stimulates differentiation of myeloid progenitor cells into eosinophils (eosinophil differentiation factor activity) [214]. T cell replacing factor, which increases antigen-specific antibody responses by B cells from antigen-primed mice, is now known to be identical to BCGF II [215]. All these activities are also mediated by recombinant IL-5 [216,217]. Furthermore, IL-5 can induce the differentiation of thymocytes into cytolytic T cells and enhances IgA formation [218-220].

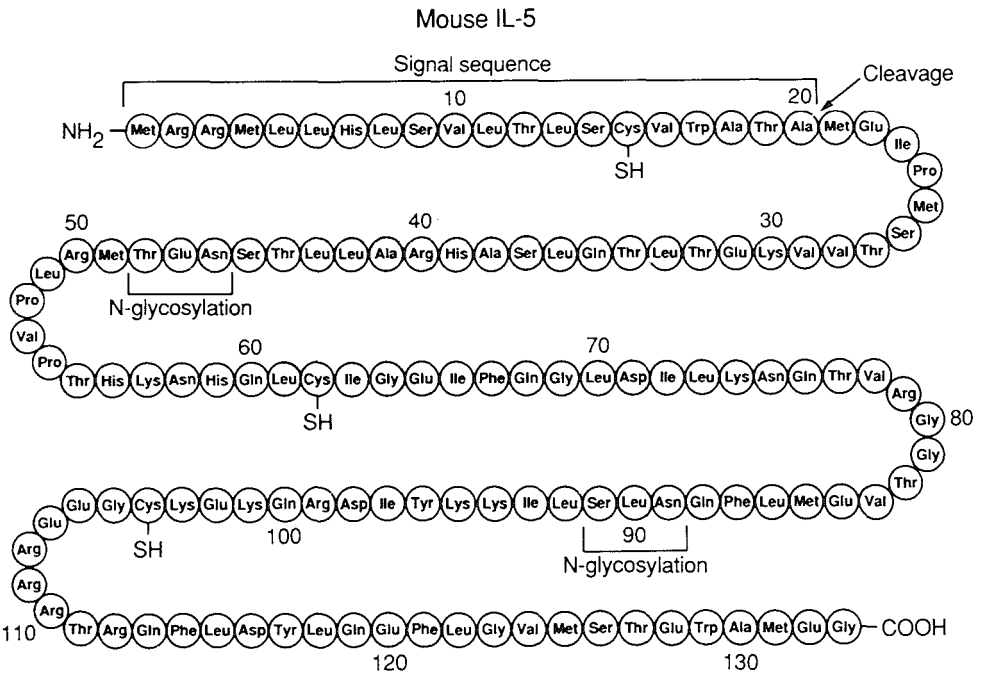

Fig. 7. Schematic representation of IL-5 amino acid sequences. For further details: see legend to Figure 6.

Natural murine IL-5 purified as a protein of molecular weight 42 - 66 kDa, which reduced to 40 kDa on SDS-PAGE. The murine cDNA codes for 133 amino acids with a signal sequence of 18 amino acids. The human cDNA codes for a 134 amino acid protein of which the first 19 are the predicted signal sequence. It has two potential glycosylation sites and two cysteine residues (Fig. 7). The human gene consists of four exons spanning 3.2 kb on chromosome 5. There is 67% homology at the amino acid level between mouse and human IL-5. It is important to note that whilst murine recombinant IL-5 causes both B cell growth and eosinophil differentiation, the human recombinant homologue only causes eosinophil differentiation.

IFN-γ exerts both stimulatory and inhibitory effects on polyclonal and antigen-specific responses by affecting B cell activation and differentiation [221]. Moreover, IFN-γ plays a major role in macrophage activation [222]. It is produced by T lymphocytes from blood or lymphoid tissues upon stimulation with specific antigens, mitogens or alloantigens. Both $CD4^+$ and $CD8^+$ lymphocytes can produce IFN-γ, although the former are considered the major producers in response to antigens. It blocks the IL-4 induced increase in surface Ia expression and cell size as well as DNA synthesis in B cells that have been stimulated by soluble anti-Ig antibodies [223,224]. Also $Fc_\epsilon R$ expression on resting B cells induced by IL-4 is blocked by IFN-γ [194]. On the other hand, IFN-γ is reported to stimulate

polyclonal IgM production by resting B cells in vitro and the combination of IFN-γ and IL-2 has been found to stimulate polyclonal growth and IgM secretion in vitro by activated B cells as well. Furthermore, IFN-γ has been found to be a crucial stimulus for the induction of an in vitro antibody response to sheep erythrocytes in the absence of helper T cells. Recent experiments suggest that IFN-γ also has a role in the control of Ig isotypes produced during an immune response. When recombinant IFN-γ is added to cultures of purified murine B cells, it inhibits both the IgG_1 and the IgE secretion induced by LPS plus IL-4 [200], while it has no effect on the IgM secretion and enhances the secretion of IgG_{2a} [225]. In addition, IFN-γ is a potent inhibitor of most other isotypes in T-cell dependent B-cell responses.

It has been stated that B cells are reactive to **IL-1** during two stages of B cell development, the pre-B cell and the activated mature B cell [226]. In activated mature B cells, IL-1 alone has little effect but appears to potentiate the proliferation and/or differentiation of mitogen- or lymphokine-activated B cells. Because sources of natural IL-1 also produce **IL-6**, many of these activities must be reexamined with recombinant material.

Murine plasmacytoma growth factor (from T cells or the macrophage P388D1 line) or B cell stimulatory factor-2 has recently been cloned by using a human IL-6 probe (Dr. F. Lee, personal communication). This lymphokine is suggested to have growth factor activity for mature activated B cells differentiating into antibody secreting cells. The factor has no activity on small resting B cells.

Recently, a new lymphokine gene was cloned and the amino acid sequence predicted was homologous to lymphopoietin-1. This factor was named **IL-7** and was found to have some stimulating activity on very early pre-B cells (Dr. A. Namen, personal communication). Which of the two helper T cell subsets produces the latter two kinds of lymphokines remains to be established.

1.4.3.3 Effects of lymphokines on B cell isotype expression

After stimulation with a TD antigen, B cells initially bind antigen via antigen-specific Ig receptors, internalize and process the antigen and finally present the antigen in association with Ia molecules to T cells. The expression of putative antigen fragments on the B cell surface in association with Ia molecules results in an immunogenic complex recognized by the antigen-specific T cell receptor. This initial antigen-specific, MHC-restricted T-B cell interaction is an essential step in the eventual secretion of specific antibody [227]. One early consequence of T-B interaction is that IL-4 is released from the T cell. Since IL-4 acts on resting B cells to increase the expression of surface Ia antigens, the release of IL-4 increases the probability of subsequent T-B interactions leading to differentiation into specific-antibody secreting cells [228,229]. Recently, the initial antigen-dependent physical association between helper T cells and B cells have been demonstrated [228-232].

The ability of Th1 and Th2 supernatants to alter the pattern of Ig isotypes produced by B cell cultures, was initially examined in LPS-stimulated B cells in the absence of T cells. The addition of supernatants of activated Th2 clones to LPS-stimulated B cells regularly resulted in a significant enhancement of the production of IgG_1, IgE and IgA [177,193,195,196,213]. Typically, IgG_1 and IgA levels were enhanced 5 to 20-fold, whereas IgE levels were elevated 100 to 1000 times. Using highly

purified IL-4 and a blocking monoclonal antibody to IL-4, both the IgG_1 and IgE enhancing activities have been shown to be mediated by IL-4. These observations have been confirmed with recombinant IL-4 (rIL-4).

The presence of IL-4 in Th2 supernatants did not account for the enhancement of IgA caused by these supernatants, suggesting the presence of a distinct IgA enhancing activity. This activity was found to be mediated by IL-5 [218,219]. It was further demonstrated that IL-4 potentiated the IL-5 induced IgA production, although it cannot enhance IgA production by itself. On the other hand, IL-5 had little effect on the levels of IgE and IgG_1 produced in response to saturating concentrations of IL-4, although IL-5 was able to substantially enhance the IgE and IgG_1 response to suboptimal concentrations of IL-4 (Dr. R.L. Coffman, personal communication).

Supernatants of Th1 clones had relatively little effect on the distribution of Ig isotypes when added to LPS-stimulated cultures, but they contained a potent inhibitor of IL-4 induced IgE and IgG_1 synthesis. This inhibitor has been identified as IFN-γ and, at concentrations of 1-10 antiviral units/ml, it completely suppressed the IL-4 mediated IgE response and reduced the IgG_1 response to the level obtained in the absence of IL-4 [200]. These levels of IFN-γ caused little or no suppression of the production of all other isotypes in response to LPS but did inhibit other responses of B cells to IL-4, such as surface Ia expression and proliferation. The induction of IgE by a Th2 product, IL-4, and the inhibition of this induction by a Th1 product, IFN-γ , offers an attractive and testable explanation for in vivo regulation of IgE synthesis [181,233].

The complex regulatory effects of T cells on B cell antibody production are illustrated in Fig. 6 and involve $BCGF_{low}$ and the CD23 B cell surface antigen. It has been suggested that $BCGF_{low}$ can cause CD23 to be cleaved to release a soluble product which itself has growth factor activity for B cells. In the presence of IL-4 these cells would be induced to produce IgE which is the ligand for the low affinity $Fc_\epsilon R$, CD23. Addition of IgE causes up-regulation of CD23 which, if cleaved from the surface, will further stimulate B cell growth, thereby completing a positive loop. The up-regulation of CD23 (activation) and production of IgE are inhibited by IFN-γ. If IFN-γ is indeed produced by a different helper T cell than IL-4 is, then IgE responses may be controlled at the level of differential stimulation of subsets of T cells.

1.4.3.4 Lymphokine-induced heavy chain isotype switch

It is generally accepted that $mIgM^+$ B cells give rise to cells that can synthesize other Ig isotypes still expressing the same specificity and the same idiotype as their ancestors. By this mechanism, called immunoglobulin class switching or isotype switch, the full range of antibody isotypes can be expressed in molecules having the same specificity and thus the same idiotype [185,234-239].

The C_H gene order in the mouse is: '5-variable region gene-C_μ-C_δ-$C\gamma_3$-$C\gamma_1$-$C\gamma_{2b}$-$C\gamma_{2a}$-C_ϵ-C_α-3'. This locus spans over 200 base pairs. Immunoglobulin gene expression depends upon, among other factors, rearrangement of Ig genes. The switching process is known to occur through a complex set of DNA and RNA splicing and recombination events, involving either deletion of the DNA intervening the rearranged V-sequence and the newly expressed C-region gene, or sister chromatid exchange [238-241]. The deletion model [242] suggests a sequential isotype or subclass expression after switching, corresponding to the sequence of localization of the heavy chain C-genes in the genome (in humans chromosome 14, in mice chromosome 12). This would

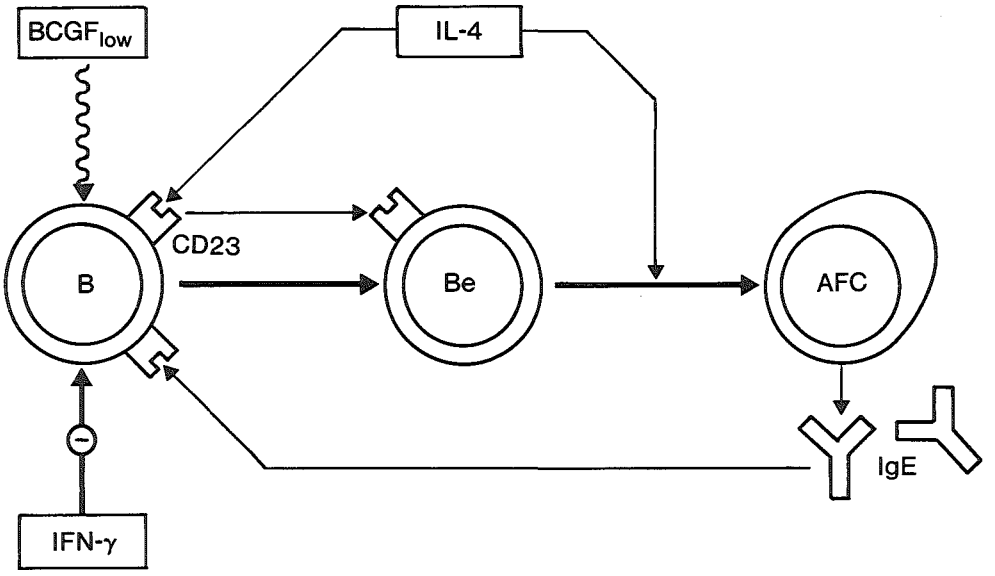

Fig. 8. Control of B cell antibody isotype differentiation by BCGF$_{low}$, IL-4 and IFN-γ. BCGF$_{low}$ acts on B cells to cause the cleavage of CD23, the low affinity Fc R. This fragment promotes differentiation of IgE-producing B cells under stimulation by IL-4. IgE promotes expression of CD23. IFN-γ inhibits IgE production. After Hamblin [178].

indicate a T independent C_H gene expression in Ig-secreting cells that parallels the T independent isotype expression on the surface of B cells and is based upon random switch events [235]. In the case of class switch recombination, differences in the degree of homology and in structure of the various switch regions have led to the proposal of class-specific switch recombination [238]. Indeed, the switch regions of $C\mu$, $C\alpha$, and $C\epsilon$ have some degree of homology. The switch regions of the $C\gamma$ genes, on the other hand, are divergent from the above ones. The fact that the frequency of B cells expressing the various Ig classes can be regulated by T cell derived lymphokines raises the possibility that the switch recombination itself may be induced by certain lymphokines [239,243,244]. This would mean that lymphokines like IL-4 could induce a specific recombinase or a cofactor that confers specificity on a nonspecific recombinase. There are, however, no obvious sequences common to the switch regions of $C\epsilon$ and $C\gamma_1$ genes only, that might provide a substrate for a specific recombinase. Alternatively, lymphokines could alter the 'accessibility' of the $C\epsilon$ and $C\gamma_1$ switch regions, allowing them to become substrates for an isotype nonspecific recombinase. This model is derived from analyses of mouse pre-B cell and B lymphoma cell lines that can be induced to switch only to one or two isotypes [239]. In these cell lines it was found that an identical

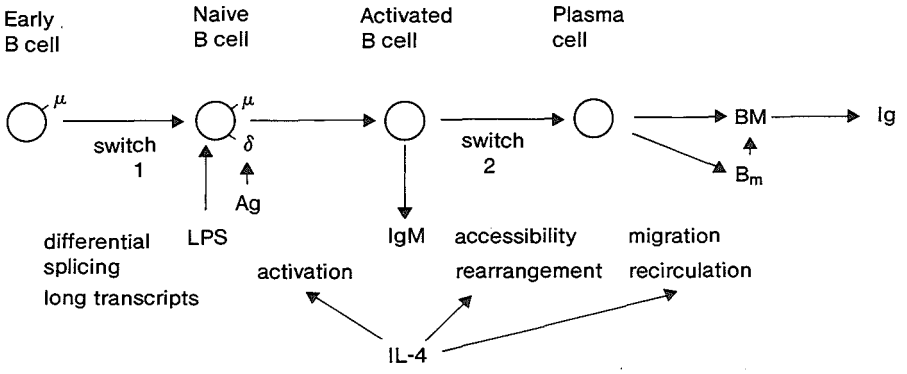

Fig. 9. Hypothetical model of IL-4 induced switching events in B cells after Krawinkel and Radbruch [239,240]. Initial events in class switching involve differential splicing of long transcripts which is controlled at the RNA level. The B cells then can become activated by antigen through their IgD receptor or by LPS through another receptor. The second step of class switching is the recombinatorial joining of two switch regions. Class switch recombination is restricted to switching cells, where it happens on both alleles and involves the same switch regions on both alleles. In this process, the accessibility of the switch regions involved is crucial. After isotype switching, B cells can become recirculating memory B cells (B_m) or differentiate into mature Ig-secreting cells. This second step in class switching and all subsequent events leading to final Ig-secretion are influenced by lymphokines acting as switch factors like IL-4.

switch occurred at both alleles at the same time. In support of this model is the recent demonstration that IL-4, which causes a decrease in the expression of IgG_{2b} in LPS-stimulated B cell cultures, reduces the 'accessibility' of the $C\gamma_{2b}$ locus as evidenced by reduced transcription from unrearranged genes. IL-4, on the other hand, increases the expression of both IgE and IgG_1 in LPS stimulated B cell cultures (Drs. S. Lutzker, P. Rothman and F. Alt, personal communication). This is reflected in an increased transcription from sterile and functional $C\gamma_1$ and C_ϵ genes resulting from an increased 'accessibility' of these C_H loci upon addition of IL-4. The fact that the frequency of B cells expressing the various isotypes can be regulated by lymphokines raises the possibility that this directed switching may indeed be regulated by certain lymphokines, like IL-4 for IgG_1 and IgE, IL-5 for IgA and IFN-γ for IgG_{2b} (Fig. 7).

In the case of IgG_1, it was shown by limiting dilution analysis that IL-4 enhances the frequency of IgG_1 secreting clones resulting from surface IgG negative precursors [198,245]. Recently, it was described that IL-4 is able to enhance polyclonal IgE production in mass cultures of T depleted spleen cells. It was postulated that the IgE response to LPS plus IL-4 results from B cells that undergo an isotype switch in vitro. This was the underlying premise for the studies described in Section 6.2.

1.4.3.5 Effects of lymphokines on in vivo IgE synthesis

The above mentioned model (Section 1.4.3.3) for the regulation of IgE during an in vivo immune response is based on the hypothesis that the relative levels of IL-4 and IFN-γ to which the activated B cells are exposed di-rect the IgE response [179,181]. These IL-4 and IFN-γ levels in turn depend upon the relative numbers or states of activation of Th1 and Th2 cells of the appropriate specificity. This model predicts that Th2 cells will be helpers for an IgE response and that Th1 cells will act as isotype-specific suppressor cells for IgE, at the same time as they may be acting as helper cells for responses of other isotypes.

TABLE VI

Effects of anti-IL-4, IFN-γ and anti-IFN-γ on the Ig isotype pattern

Stimulating agent	Modulating agent	Effect on Ig isotype pattern	Reference
Nippostrongylus	-	IgE ↑ IgG_1 ↑	
	anti-IL-4	IgE ↓ IgG_1 →	202
GAM/IgD	-	IgE ↑ IgG_1 ↑	
	IFN-γ	IgE ↓ IgG_1 ↓	
		IgG_{2a} ↑	204
Brucella	-	IgG_{2a} ↑	
	anti-IFN-γ	IgG_{2a} ↓ IgE ↓	
		IgG_1 ↑	248

The importance of IL-4 and IFN-γ in the regulation of IgE and IgG_1 has recently been evaluated using experimental systems that cause large, polyclonal IgE and IgG_1 responses in vivo (Table VI). Injection of mice with goat anti-mouse-IgD antibodies induced polyclonal B and T cell activation and proliferation and led to substantially increased serum Ig levels within 7 days [204,246]. This increase was predominantly restricted to two isotypes, IgG_1 and IgE, each of which was increased 20 to 200-fold. This isotype pattern suggested greater involvement of IL-4 than of IFN-γ, and thus suggested a response involving predominantly Th2 cells. This interpretation was supported by the observation that T cells from anti-IgD treated mice spontaneously secreted IL-4 in vitro as measured by the Ia induction assay [203], which is quite sensitive to IFN-γ [224]. Most of this anti-IgD induced increase in IgE was blocked by injecting the mice with a monoclonal anti-IL-4 antibody (11B11), although this treatment did not block the IgG_1 response [202]. Striking effects were observed when mice were injected with IFN-γ on day 2, 3 and 4 after anti-IgD treatment. At the highest dose

tested, IFN-γ suppressed IgE and IgG_1 levels by 90% to 95%, but had little effect on the serum levels of IgM and IgG_{2a} [247]. Further evidence that IFN-γ inhibits the IgE and IgG_1 responses in vivo was obtained when mice were injected simultaneously with a monoclonal anti-IFN-γ antibody and the anti-IgD. This caused a 2 to 4-fold isotype-specific enhancement of IgE and IgG_1 as compared to injection of anti-IgD only and suggested that low levels of IFN-γ were, in fact, produced during this response and were inhibitory for the in vivo IgE and IgG_1 response [247].

The mechanisms by which normal B cell responses are regulated are far from clear. In vivo, the activation of B cells by TD antigens occurs in the close proximity of T cells and APC. In this environment the availability of antigen will limit the response. Presumably, local lymphokine and lymphokine receptor induction occurs according to the availability of the antigen. Little is known about the structure and function of receptors for B cell factors. Studies of lymphokine receptors and lymphokine expression within the microenvironments of lymph nodes and other lymphoid organs where antibody is produced should yield important information with respect to the regulation of Ig isotype expression and its dynamics in vivo.

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CHAPTER 2

ASSAY SYSTEMS FOR STUDYING IgE FORMATION

The analysis of IgE antibody production can be performed at the cellular level under normal in vivo or controlled in vitro conditions (Section 2.1). To this end, plaque assays and ELISA-plaque assays can be employed (Section 2.2) on the cell suspension itself. Enzyme-linked immunosorbent assays (ELISA) can be employed successfully for the quantitative determination of the concentration of IgE in culture supernatant or in serum (Section 2.3). In this chapter the read-out systems that were actually employed in the work described in this thesis will be discussed briefly. More extensive reviews of these techniques will be published elsewhere [1,2].

2.1 In vitro culture systems

Crude cell suspensions or purified B cells from lymphoid organs have been employed for in vitro analysis of the effects of lymphokines on IgE-antibody formation. This was done in bulk cultures as well as under limiting dilution conditions. With regard to the latter, the in vitro limiting dilution assay according to Lefkovits has been used [3,4]. Initially, in these cultures rat feeder cells necessary for the maturation of the responding B cells were employed. The nature of the feeder activity and the partial substitution of this effect by IL-4 was investigated as described in Section 6.2. In the limiting dilution assays the fraction of non-responding cultures as analyzed by plaque assays, has to be determined. Subsequently, using the zero term of the Poisson distribution, the frequency of precursor cells can be calculated. In this distribution, N cells can be used for culturing, each independently, with probability p of success of containing a precursor B cell. The Poisson distribution gives the relative frequency of wells containing 0, 1, 2,...r cells. The general term of the Poisson formula

$$F_r = \frac{(m^r)\ (e^{-m})}{r!}$$

gives the probability of precisely r cells in a well where m = Np is the mean number of precursors per well. The logarithm of the zero term of the equation is

$$-\ln F_0 = m$$

which means that the negative logarithm of the fraction of nonresponding cultures is linearly proportional to the mean number of precursor cells per well.

Generally, linear regression is used on the data obtained by plotting the fraction of non-responding cultures against the input of cells. As extensively reviewed by Taswell and Fazekas, this method of data analysis is inefficient and misleading [5,6]. Furthermore, linear regression analysis is inferior to the chi-square and the maximum likelihood method with its 95% confidence limit, as shown by an example in Table I. In the latter method it was established that for discrete distributions, the multinomial term consists of the expected frequencies that are raised to the power of the corresponding observed numbers.

For the analysis of the limiting dilution data according to this maximum likelihood method, a BASIC program was employed essentially as described by Fazekas and Porter and Barry [6,7].

Average clone sizes of responding B cell precursors were determined by employing cultures that contain only a single precursor. When 37% of the cultures are negative, 37% contain 1 precursor, 18% have 2 precursors,

TABLE I

Comparison of estimation of precursor frequencies (1/n) as determined by linear regression analysis, chi-square and maximum likelihood

Frequency interval	linear regression	chi-square	maximum likelihood
< 500	65	52	54 (41 - 72)
500 - 5000	2057	2061	1970 (1466 - 2646)
> 5000	28939	44643	46728 (25252 - 87243)

6% have 3 precursors and 2% have 4 or more precursors of Ig-secreting clones. With large numbers of B cells per well a marked quantal distribution of PFC is observed in the individual cultures, as predicted from the Poisson formula, with 'jumps' corresponding to the clonal yield of a B cell. Such jumps are easily recognized if the clone size is large (30 or greater). The clone size is determined by using the estimations of precursor concentration and the average number of precursors per culture (m) for the dilution of B cells under consideration. If the number of cultures assayed is C, the average clone size (c) is

$$c = \frac{\sum PFC}{C \times m}$$

where PFC is the total number of PFC found in C wells.

2.2 Assays for the detection of IgE-secreting cells

Cells secreting IgE antibodies can be revealed in several ways. The first assay employed in this field was the heterologous adoptive cutaneous anaphylaxis (HACA) which lacks enough sensitivity to be of use in the analysis of IgE antibody responses [8]. Plaque assays and ELISA-plaque assays both reveal these cells on the basis of the secreted product [9]. These cells can also be detected by cytoplasmic immunofluorescence although this method does not formally prove the secretion of IgE [10]. Because of a number of reasons plaque assays are considered to be more appropriate for the detection of IgE-secreting cells. In such assays, IgE-plaques develop slower and are smaller than IgM-PFC, apart from the influence of the affinity of the developing antiserum used. Therefore, IgE-antibody-forming cells probably secrete their IgE antibody molecules at a relatively lower rate (number of IgE molecules secreted per cell per unit of time) than IgM antibody molecules are secreted by IgM-antibody-forming cells. Microscopical examination of the plaques is necessary to reveal the antibody-releasing cell in the centre of the plaque and to distinguish in this way true plaques. In the ELISA-plaque assay this is not possible and this is a major drawback in employing this assay for the analysis of IgE-secreting

cells [1,2,11-16]. Moreover, the latter technique is sensitive to the development of false plaques: the addition of proteins like albumin in all washing and incubation steps is necessary to prevent small false spots with a similar size as IgE spots. Also the substrate solution should be filtered in order to prevent the development of false spots that are above the agarose/solid phase interface.

Cells producing Ig can range morphologically from lymphoblast to plasma cell, and many of these cells can be enumerated by means of cytoplasmic immunofluorescence with heterologous antibodies specific for the Ig produced. The availability of fluorescent reagents of high avidity and specificity is a crucial requirement for reliable results. It has been found in cell suspensions of murine lymphoid organs that enumeration of Ig-producing cells by cytoplasmic immunofluorescence and by the protein A plaque assay do not always give similar results [10]. The protein A plaque assay was found to detect as many or several times more Ig producing cells than the fluorescence assay, depending amongst other factors on the Ig class studied. Preliminary studies aimed at enumerating IgE-secreting cells by cytoplasmic immunofluorescence confirms that underestimation of the number of IgE-secreting cells is likely to occur. Moreover, fluorescence based techniques can not reveal the specificity of the immunoglobulin produced.

2.3 Quantitative determination of IgE

In the past, murine IgE antibodies have almost exclusively been detected by using the PCA assay in rats. The results of such tests are mostly calculated as end-point titres, being the reciprocal of the last dilution tested that resulted in a positive reaction, e.g. at least 5 mm staining area. Although these PCA reactions are reliable, sensitive tests, they are at best semi-quantitative, asking considerable time [17,18]. In addition, their use generally requires maintanance of large numbers of recipient animals of defined age and strain. Recently, it was demonstrated that some monoclonal murine IgG_1 antibodies can also sensitize rats for PCA reactions. This was confirmed with some conventional antibodies when the sera were fractionated. However, with monoclonal IgG_1 antibodies about 10^4 times more was needed to sensitize the rat than the amount necessary from IgE [18].

Due to the production of IgE-secreting hybridomas, specific antisera to mouse IgE have become available (Section 4.3). These antisera have been successfully employed as reagents in many assays for the detection of total and specific IgE including radioallergosorbent test (RAST), radioimmunoassay (RIA) and the ELISA [19]. These assays are generally efficient, sensitive and can afford simultaneous assessment of numerous samples, e.g. for screening of hybridoma supernatants. Therefore, they are the assays of choice in studies aimed at unravelling the regulation of IgE production. Furthermore, the precision of the PCA test is questioned because of its dependency on the antigen dose employed. Other complicating factors constitute the large amount of mouse IgE that can bind cytophilically to rat mast cells, the short half-life of IgE in the serum [20,21] and uncertainty whether the serum half-life of IgE antibodies is dependent on the actual serum concentration as shown in man [22]. For these reasons we have adapted an ELISA for the quantitative determination of murine IgE antibodies (Section 4.4), both total and antigen specific. For this ELISA, IgE had to be isolated and purified and used for the production of heterologous anti-IgE antisera (Section 4.2).

For quantitation, a titration of a reference sample is needed, yielding a calibration curve when the signals obtained are plotted against the log

dilution or log concentration. Such curves are S-shaped or sigmoid. When transforming the reading signals into a four-parameter log-logit fit, the sample Ig concentration can be determined. The general form of this logistic equation may be expressed as

$$y = \frac{a - d}{1 + \left(\frac{x}{c}\right)^b} + d$$

Here, y represents the response variable, x is the arithmetic dose (absolute IgE concentration), a is the response at high dose (upper reading plateau), d is the response at zero dose (lower reading plateau), c is the dose resulting in a response halfway between a and d, and b is the slope factor that describes the steepness of the curve. In this equation, y can never exceed a. Therefore, the denominator must be greater than 1, and (x/c) has to be restricted to positive values. The quantity y increases monotonically since the differential equation, of which the logistic function is the general solution,

$$\frac{dy}{dt} = m\, y\, (a - y)$$

implies that dy/dt > 0. For t --> $-\infty$, y tends to zero, and for t --> $+\infty$, y tends a. The growth starts slowly, then becomes faster and finally tapers off. Growth of the signal is fastest in the neighbourhood of the point of inflection. To get its location the second derivative y = y(f) has to be equated to zero.

$$\frac{d^2y}{dt^2} = m\, (a - 2y)\, \frac{dy}{dt}$$

This expression can only vanish if a - 2y = 0 or y = a/2, that is, the point of inflection is halfway between the lines y = 0 and y = a. To get the abscissa, let y = a/2 in the logistic equation and solve it with respect to t:

$$t = \frac{\ln k}{am}$$

This particular abscissa is positive or negative depending on whether k < 1 or k > 1.

It is clear that the curved nature of the sandwich ELISA titration plots is a reproducible effect resulting from Mass Law consideration for the binding of antigen and immuobilized capture antibody when the latter becomes limiting. By converting the data to a log-logit plot these curved titration plots can be linearized to allow further analysis to be performed by linear regression [23]. As described in Section 4.4, the logistic transformation of the data was used to quantitatively determine the absolute concentration of IgE in a Terasaki-ELISA system.

2.4 References

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CHAPTER 3

INTRODUCTION TO THE EXPERIMENTAL WORK

A better understanding of the regulatory mechanisms and cellular interactions of the IgE antibody response is of fundamental interest to allergologists and clinical immunologists because of the role of IgE in the pathogenesis of the immediate type allergic disease. In addition, basic knowledge of the regulation of IgE antibody formation may lead to new forms of treatment including suppression of excessively formed IgE antibody. Beyond the clinical significance, the IgE antibody response provides an excellent model to establish the interdependencies and regulatory events governing the expression of different isotypes at different levels. The formation of IgE antibodies is regulated by T cells and controlled by antigen- and/or isotype-specific interactions. Thus, external modulation of IgE production can be achieved by antigen, idiotype and anti-idiotype, being specific recognition elements in the establishment and control of an immune response. Also IgE specific regulatory factors and receptors on different types of cells exert a regulatory influence. In the case of isotype-specific regulation, total IgE antibody may be affected irrespective of its specificity. This is of relevance for eventual treatment of generalized IgE mediated allergic diseases. On the other hand, an antigen-specific modulation of an immune response will also affect the expression of other isotypes, even in a secondary response.

From the above it is clear that much of the knowledge on the induction of the allergic inflammation gained from animal studies is clinically relevant. Moreover, the basic mechanisms of IgE regulation seem to be similar in man and in mice and rats. This is the underlying premise for these studies.

The purpose of the investigations presented in this thesis was to get more insight into the mechanisms underlying the induction of IgE antibody formation in the mouse and the regulation of this IgE synthesis.

For this study the development of suitable reagents and appropriate assay systems was indicated. Only since the availability of antigen-specific mouse IgE-secreting hybridomas, it became feasible to isolate enough IgE for the induction of heterologous anti-IgE antisera. This purified IgE can also be used as a reference standard in the quantitative determination of IgE. Furthermore, the hybridoma cells can be employed for the standardization of techniques that allow the determination of IgE-secreting cells. It is therefore that in Chapter 4 we focuss on the isolation and purification of monoclonal murine IgE, the generation of heterologous IgE-specific antisera and the development of a Terasaki-ELISA system for the quantitative determination of secreted IgE. Employing one of these antisera, we devised techniques for the determination of both total and antigen-specific IgE-secreting cells. Moreover, in this chapter, some critical factors influencing the applicability of these reagents and techniques are discussed.

In Chapter 5, experiments are described in which the induction of antigen-specific IgE antibodies was analyzed in mice both at the cellular level and the level of the secreted product. At the cellular level, IgE-secreting plaque-forming cell (IgE-PFC) responses were determined in the various lymphoid organs upon immunization. At the serum level, concentrations of antigen-specific IgE antibodies were determined. Moreover, at both levels the antigen-specific IgE response was compared to the non-specific IgE response. In these experiments, several parameters of the immunization were analyzed: type of antigen, dose and route of administration, the use of adjuvant, immunization scheme and the genetic background of the responding mice. Of all these parameters the magnitude and the kinetics of the response was determined.

Immunization protocols were used for the induction of persistent antigen-specific IgE production, employing multiple low dose immunizations without the use of adjuvant. Also the involvement IgE-secreting cells in the peritoneal cavity in serum IgE response was investigated. Evidence is presented suggesting that IgE-secreting cells in the peritoneal cavity are responsible for the long-term antigen-specific IgE production. Treatment of mice during immunization with anti-idiotypic antibodies revealed that the specific IgE response is not inhibitable by such antibody treatment, although the IgE response itself bears the dominant idiotype. The production of other isotypes (like IgM) also bearing the dominant idiotype is strongly inhibited by treatment with anti-idiotypic antibodies. Also the influence of T cells on the IgE synthesis was analyzed in the serum as the read-out system. It is shown that serum IgE levels react in a time-dependent fashion to the absence of mature T cells with increased concentrations.

In Chapter 6, experiments are described on the role of lymphokines on the IgE synthesis, with emphasis on IL-4. In Chapter 6.2, clonal analysis was performed on the role of IL-4 on the induction of IgE synthesis in B cells cultured under in vitro conditions. Strong indications were obtained for the switch enhancing capacity of IL-4, resulting in an increased precursor frequency and clonal size of IgE-secreting cells. In Chapter 6.3, clonal analyses of the B cells from various IgE responder type mouse strains were performed in combination with the analysis of the functional capacities of the T cells from these mice. Evidence is presented that the IgE non-respondership of SJL mice is not caused by an intrinsic B cell defect but rather by T cells defective in the production of IL-4.

Finally, the data obtained are discussed in Chapter 7 in the context of current thinking about the induction of IgE formation and the regulatory role of lymphokines, especially IL-4.

CHAPTER 4

REAGENTS AND ASSAYS FOR THE DETECTION OF IgE AND IgE-SECRETING CELLS

CHAPTER 4.1

INTRODUCTION: ASSAY SYSTEMS FOR STUDYING IgE FORMATION

Antibodies can be used as detecting reagents for a variety of molecules (e.g. Ig) by virtue of their molecular recognition properties. A vast amount of literature exists on different techniques developed to detect Ig in a qualitative, semi-quantitative or quantitative way. Such assays are based on different molecular properties of antibody, antigen and antibody-antigen complexes. As will be discussed below, in this respect it is important to note that different Ig isotypes because of differences in protein (antigenic) structure can behave differently in the various assays. This can result in false conclusions if the data and/or reagents are used for assays different from that used for performance testing.

In all immuno assays detection and, if possible, quantitation is based upon detecting (anti-isotypic) antibodies. The specificity, purity, affinity and titer of these antibodies determines the reliability of the assay. Moreover, these requirements are more stringent for detecting reagents than for capture reagents. Monoclonal antibodies offer tremendous potential as capture reagents in terms of specificity and continuity of supply but because they represent a single antibody lineage within a polyclonal immune response it is sometimes, but not always, necessary to use a mixture of antibodies of differing epitope specificity. Most polyclonal antibodies bind well to plastic with good retention of activity. The formation of antigen-antibody complexes on which all immuno assays are based determine the conditions under which the assays should be performed. Most complex formation occurs optimally at a pH of 6.5 - 7.0 although the reaction is not greatly affected between pH 5.5 - 8.5. Monoclonal antibodies, however, can show drastic changes in specificity with altering pH. The rate of association can be increased by lowering the ionic strength of the buffer, especially when dealing with antibodies of relatively low affinity (Ka $<10^5$ M^{-1}). In many reactions the affinity of antibodies specific for a particular antigen is increased by incubation at lower temperatures (4°C instead of room temperature or 37°C). Changes in reaction temperature do influence the reaction speed but cause minor changes in the affinity constant.

The stability of the antigen-antibody complex both under in vivo and in vitro conditions depends largely upon non-covalent interactions, mainly hydrophobic forces and hydrogen bonds and, to a lesser extent, on electrostatic-charge interactions. These hydrophobic forces contribute to the overall shape of a protein molecule in an aqueous system when the hydrocarbonlike parts of the protein chain are together and the polar parts of the chain are in contact with the solvent. The specificity of an antibody is dependent on the degree of cross reactivity with closely related antigenic determinants. Such interactions mostly occur with a lower affinity.

Several of the biochemical aspects and the nature of the murine IgE molecule have been described already in some detail in Section 1.2. Once an adequate source of IgE was established (antigen-specific IgE hybridomas) and reliable assays developed (ELISA), the purification of IgE antibodies

could proceed [1,2]. Initially, purification procedures relied solely upon gel filtration and ion exchange chromatography resulting in poor recovery of activity (less than 10%) and low degree of purity. Subsequent affinity purification procedures resulted in better purity but only less than 1% of the original material could be recovered. This was probably due to the damage of the IgE upon affinity chromatography employing harsh elution conditions. Until recently, murine IgE molecules were still purified by conventional chromatography only [3]. Therefore, in Section 4.2 a new purification procedure using high performance liquid chromatography (HPLC/FPLC) is presented. A three column FPLC system is employed in which purification of IgE is essentially based on ion exchange chromatography. This procedure unites high resolution and concentration, but selection of the right type of exchanger requires knowledge of the isoelectric point as well as charge distribution and stability of the IgE protein at different pH. This valuable information can only be obtained by running an electrophoretic titration curve prior to designing a purification procedure. In Chapter 4.2 data were obtained explaining the damage of IgE molecules during the isolation procedure. Based on these data a modified procedure was developed in which damage of the IgE molecules was prevented to a large extent.

After the introduction of the radioimmuno-assay (RIA) a variety of immunoassays have been developed in which the detection of Ig is based upon a (ligand) labeled anti-isotypic antibody. Many different labels are available for this purpose: radioisotypes, enzymes, fluorescent dyes, stable free radicals, electron-dense components, etc. Here will only be dealt with enzyme immunoassays (EIA) and especially heterogeneous assays in which the bound and the free fraction of the ligand are physically separated by a washing procedure and in which the antigen or Ig to be determined is either directly or indirectly physically attached to a solid phase (e.g. the well of a microtiter plate or Terasaki tray). Therefore such assays are called enzyme-linked immunosorbent assays (ELISA). For use in sandwich ELISA the quality of the antisera is extremely important for the specificity and the sensitivity of the assay [4]. As mentioned earlier these antibodies are purified out of hyperimmune sera and need to be of a high affinity to be of use as a detecting reagent. Since the specificity of such antibody is most commonly tested in immunodiffusion or immunoelectrophoresis, retesting of the antibody in a new and more sensitive system is necessary [4]. Many times additional affinity purification of the antibody is necessary when employing commercial conjugates.

When using the conjugate two factors have to be taken into consideration: firstly the activity of the enzyme must remain sufficiently high and secondly the affinity of the antibody part of the molecule must remain unaltered. Thus the composition (pH, molarity, presence of activators etc.) of the conjugate buffer plays a major role in the specificity and the sensitivity of the assay. In a sandwich or two-site assay as described in this chapter a much better sensitivity is reached by the double recognition of the Ig to be detected: (a) by the catching antibody or the antigen immobilized on the solid phase and (b) by the detecting antibody.

In Section 4.3 the production, purification and specificity testing of several heterologous murine IgE-specific antisera is described. For the detection of the antigen-specific fraction as well as for the detection of the total amount of a particular Ig-isotype in serum a two-site sandwich system is applied. In both cases, the Ig to be determined is sandwiched between the known antigen or an anti-isotypic catching antibody adsorbed to a solid phase and an enzyme labeled second antibody, respectively. The catching as well as the detecting antibody are heterologous compared to the

Ig to be determined. After adsorbing the antigen or catching antibody to the solid phase, all subsequent incubations are done until equilibrium has been reached and competition can no longer occur between the labeled and unlabeled ligand involved. Moreover, all other reagents, except the sample, are in slight excess so that only the Ig that is bound out of the sample is the limiting step. In this way the signal is directly related to the amount of Ig bound.

The majority of ELISA techniques are performed in microtiter plates because a lot of equipment facilitating easy and automatic handling is commercially available. In a moderately sensitive ELISA system a serum sample of 100 ul is enough to determine the concentration of IgM or IgG antibodies [5-8]. Simultaneous detection of a number of Ig classes especially rare ones like IgD, IgE, autoantibodies or anti-idiotypic antibodies, however, does require a fair amount of serum. In a number of occasions like longitudinal studies of sera of mice it is often difficult to acquire enough serum to perform the various assays. Moreover, in the production of monoclonal antibodies investigators like to test for Ig production by the hybrid cells as soon as possible following fusion, thus at a moment that only very small amounts of medium are available for assaying. The amount of reagents required for such micro-ELISA is also fairly large. These reasons lead to our decision to perform ELISA in 60-well Terasaki trays using 5 ul samples per well [9]. In Section 4.4 this quantitative Terasaki-ELISA for the detection murine IgE antibodies is described.

Determination of antibody production at the cellular level is possible in plaque assays [10]. The plaque-forming cell assay can be adapted to the detection of cells forming antibodies to antigens like proteins, polypeptides, polysaccharides and a variety of haptens. The only requirement is that the red cells have to be coated to exhibit an adequate density of antigenic determinants without causing excessive fragility of the red cells or altering their susceptibility to lysis by complement. Two general approaches to coupling determinants to red cells are used: the chemical approach where the antigens or haptens are directly coupled to the red cells, and the immunological approach where proteins or haptens are conjugated to anti-erythrocyte antibodies which then are bound to red cells by virtue of their antibody activity. This is most effectively accomplished by using monovalent Fab fragments of anti-red cell antibodies. Such conjugates, that should not by themselves lyse the red cells in the presence of complement, have been described [11,12].

In the direct plaque assay, the only antibodies detected are of the IgM class, since IgG, IgA and IgE antibodies are not efficient enough in complement binding to mediate cell lysis under these experimental conditions. To detect these antibodies of non-IgM isotype, the so-called indirect plaque assay has to be applied. In the latter assay, an IgG-, IgA- or IgE-specific rabbit antiserum is added, which will bind to antibody of the relevant Ig class produced by the cell suspension and bound to the indicator erythrocytes. The class specific antibodies crosslink the coating antibodies and generate conditions for efficient complement binding and hence for cell lysis. Since also direct plaques will develop, it is essential to note that the number of such 'facilitated' or 'indirect' plaques can in principle be determined by subtracting from the number of plaques developed with the antiserum, the number of plaques obtained in its absence. Haptenic groups may be linked directly to the erythrocyte surface, or be attached as a hapten-protein conjugate which is especially useful to space out the haptenic group or when the coupling conditions are damaging to the red cell membrane.

For the coupling of large protein antigens to target cells for use in

complement dependent lysis it is essential that indicator cells of optimal sensitivity are produced. The simplest procedure for coating red cells with proteins like ovalbumin and chicken gamma globulin [13] is the one that makes use of $CrCl_3$ as coupling agent according to the method described by Gold and Fudenberg [14]. In Section 4.5 a modified and rapid procedure is described in which different proteins like protein A or ovalbumin can be coupled to SRBC within 4 minutes. This procedure is also based upon $CrCl_3$ as the coupling agent but it can essentially be prepared freshly in order to give more reproducible coupling ratios compared to the aged solutions that are routinely used.

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CHAPTER 4.2

PURIFICATION OF MURINE IgE ANTIBODIES AND THE EFFECT OF THE ISOLATION PROCEDURE ON THE DETERMINATION OF CONCENTRATION IN ELISA

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ABSTRACT

A three-column automated FPLC system was employed to isolate mouse monoclonal-IgE antibodies (mAb) from ascitic fluids and culture supernatants with high yield and recovery. This system allowed large scale purification as well as analytical isolation of IgE antibodies.

This method was used to study the different problems that are often encountered in purification of mAb, especially when dealing with ascitic fluids. One problem is degradation caused by proteolytic and glycolytic enzymes. The activity of proteases and glycosidases in the starting material (culture supernatant of mouse IgE secreting hybridomas) results in loss of binding by monoclonal anti-IgE antibodies as determined in ELISA, leading to an underestimation of absolute IgE concentration and affinity constant. This proteolysis also leads to a loss of charged residues as established by a decreased elution strength from a cationic exchanger. A mixture of protease inhibitors can protect IgE antibodies against this proteolytic breakdown.

Elution of bound IgE from an affinity column with acetic acid results in a greater damage of the Fc portion than competitive hapten elution, leading to a decreased binding in ELISA and on an ion exchange column.

INTRODUCTION

The study of murine IgE synthesis has been greatly hampered by the lack of suitable reagents, mainly due to the difficulties in obtaining enough purified IgE from mouse serum in order to raise heterologous specific antisera. Only very few of such antisera have been described (Lehrer, 1976; Lang et al., 1976; Malley et al., 1977). With the introduction of IgE secreting hydridomas, mouse ϵ-chain specific antisera induced by purified monoclonal IgE antibodies became available. In order to isolate such hybridoma IgE from ascitic fluids, purification methods routinely used for the isolation of monoclonal IgG antibodies have been employed without evaluation of the stability of IgE antibodies under these conditions (Böttcher et al., 1978; Eshhar et al., 1980; Liu et al., 1980; Rudolph et al., 1981; Haba et al., 1985; Coffman and Carty, 1986). For example, affinity chromatography methods are highly specific but harsh conditions are usually necessary to break antigen-antibody complexes, which often result in inactivation of the mAb. Ion exchange chromatography does not distinguish between the specific mAb and non-specific host antibodies or hybrid antibodies carrying inappropriate light or heavy chains (due to the myeloma fusion partner).

It is well known that murine IgE has a high carbohydrate content, 13.3% of the total molecular weight (Liu et al., 1980). Furthermore, the IgE molecule is highly sensitive to proteolytic enzymes (Perez-Montfort and Metzger, 1982; Holowka and Baird, 1983). Murine IgE is especially susceptible to glycolytic and proteolytic cleavage of the six carbohydrate attachment points and the last one or two Fc domains, respectively. This makes purification of IgE an ideal model system to establish routine procedures in sample preparation for using in large scale on-line purification methods of (monoclonal) antibodies. The present study was undertaken to establish the influences of sample pretreatment upon recovery of specific activity and the development of a routine isolation procedure of murine IgE.

New advances in fast protein liquid chromatography (FPLC) and the availability of new column materials that provide efficient extraction of biologically active proteins, prompted us to investigate the usefulness of the FPLC system for the purification of mouse monoclonal IgE antibodies from murine ascites. Moreover, we analyzed the effects of various purification methods on yield and purity of the mAb. This approach allowed us to devise an automatically operated multi-column FPLC system for the purification of murine IgE mAb (Savelkoul et al., 1987).

Recovery of the antibody is usually determined by its binding ability in an enzyme linked immunosorbent assay (ELISA). The extent of binding is proportional to the Ig concentration. Recently, we described a Terasaki ELISA system specific for murine IgE that allows not only concentration determination but determination of the affinity constants of IgE as well (Savelkoul and Pathak, submitted). This allowed us to analyze further the effects of various purification methods on the determination of the absolute concentration of IgE in ELISA.

MATERIALS AND METHODS

Source of IgE

Hybridoma cells (Rudolph et al., 1981) secreting TNP-specific mouse IgE antibodies (IGEL b 4.1, IGEL b4 14.2), were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum. After centrifugation, the supernatant was collected and stored at -20°C. To induce ascites, hybridoma cells

(5×10^6) were injected into female syngeneic (C57BL/6 x DBA/2)F1 mice (OLAC, Bicester, United Kingdom) that had received 0.5 ml Pristane (Janssen Chimica, Beerse, Belgium) 7 days previously. After 10 days ascitic fluids were collected, defibrinized, centrifuged at 500 g and subsequently at 10,000 g and stored at -20°C.

Culture supernatant containing a high IgE concentration was obtained by doubling the medium every 2-3 days without spinning of the culture of the DNP-specific IgE secreting B53/A4 hybridoma cells (Böttcher et al., 1980) in RPMI supplemented with 10% FCS.

Sample pretreatment

Pretreatment of 20 ml of ascitic fluid was done by sodium sulphate precipitation at 20% final concentration followed by stirring for 2 hours at room temperature (RT), centrifugation at 1500 g (20 min, RT), washing with sodium sulphate and resuspending the sample in 5 ml of 10 mM phosphate buffered saline (PBS, pH 7.2). Subsequently, the samples were dialyzed against PBS for two hours at RT, after which the dialysis bag was transferred to a 50 mM sodium acetate buffer (pH 4.9). The precipitated albumin was collected by centrifugation (100,000 g, 30 min, 4°C) and the supernatant was dialyzed and concentrated in an ultrafiltration cell equipped with a Diaflow PM-10 membrane (Amicon, Danvers, MA, USA). Partial proteolytic treatment consisted of incubation of the sample for 1 hour at 37°C with 400 U trypsin/mg IgE. Glycolytic treatment consisted of overnight incubation of the sample at RT with 2 U glycosidase F/mg IgE. The solutions of trypsin and glycosidase F (Boehringer, Mannheim, FRG) were freshly prepared in 0.1 N HCl and PBS containing 1% DMSO, respectively. The enzyme concentrations used were found to be optimally effective in initial titration experiments.

As indicated in the Results section, culture supernatant IgE-samples were either non-treated or heat inactivated for 30 min at 56°C after which the sammples were centrifuged. Bovine serum albumin (BSA, type V; Sigma) was dinitrophenylated to a level of 27 DNP molecules per protein molecule (as determined spectrophotometrically) by using dinitrobenzenesulphonic acid (Eastman Kodak, Rochester, NY) as described (Eisen, 1964). All samples were applied to a 10 ml affinity column of DNP_{27}-BSA coupled to Affigel 10 (Bio Rad, Richmond, CA, USA) and eluted either with 0.1 M acetic acid (Sigma, St. Louis, MA, USA) (pH 2.3) containing 0.15 M NaCl or by 10 mM N-2,4-DNP-ϵ-amino-n-caproic acid in 50 mM TRIS-HCl (pH 8.5), containing 0.1 M NaCl. Protection against proteolytic breakdown was provided by adding a mixture of phenylmethylsulfonyl fluoride (PMSF, Sigma; final concentration: 10 uM), leupeptin (Calbiochem, La Jolla, CA, USA; final concentration; 0.5 ug/ml) and pepstatin A (Sigma; final concentration: 1 ug/ml).

Conventional isolation procedure

Immunoglobulins were precipitated from ascitic fluids with saturated ammonium sulphate (SAS) in sequential steps. The precipitated material from 30 to 55% SAS was dissolved and extensively dialyzed against PBS and loaded on 9 ml bed volume of DNP_{27}-BSA-Sepharose. The bound fraction was eluted with 0.1 M glycine-HCl (pH 2.3) and, after neutralization, dialyzed against 0.01 M TRIS-HCl (pH 8.1). This material was then loaded onto a 17 ml DEAE cellulose anion exchanger (Whatman DE 52, Maidstone, UK), and the bound fraction was eluted with a linear gradient of 0 to 0.2 M NaCl in 0.01 M TRIS-HCl buffer. This fraction was then dialyzed, concentrated and stored at -70°C.

FPLC system

The set up of the multi-column fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden) as well as the columns used in the system and their various characteristics are described elsewhere (Savelkoul and Van Der Linde-Preesman, 1987). All buffers used for FPLC were made up in glass-distilled, de-ionized water and passed over a 0.22 um filter. After the described preparation of the sample, these were passed over 0.22 um Millex GV Durapore filter (Millipore, Milford, MA, USA). As outlined in the Results section, the optimal settings for pretreatment of samples of murine IgE were determined while using a 1 ml MONO S HR 5/5 cationic exchange column with 50 mM Na-acetate (pH 4.9) as running buffer and an elution buffer consisting of the same buffer supplemented with 0.35 M NaCl. When using the multi-column set up, 5 ml of an ascitic fluid either or not precipitated previously was loaded in 20 mM TRIS-HCl (pH 8.2) on a 25 ml Fast Desalting Column HR 8/25. The void peak of this column was then loaded onto the MONO S column. While the effluent was fractionated, the relevant elution peak employing 0.35 M NaCl was stored in a Superloop. This Superloop was emptied on 25 ml bed volume Superose 6 HR 10/30 column, which allowed purity check as well as determination of molecular weigths of the relevant peaks stored in the fraction collector. Recovery of the IgE antibody activity during various treatments was established by the elution strength of the IgE from the sample applied onto a MONO S column on the FPLC system with 20 mM TRIS-HCl (pH 7.0).

Purity and yield of isolated IgE

Purity of isolated material was checked on sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) of the samples performed under reducing conditions (2-mercaptoethanol), by using 8 to 25% slabgels. Gels were run and silver-stained or stained by using the periodic acid-Schiff reaction in the Pharmacia PHAST system. On the same system electrophoretic titration curves were run on pH 3-9 iso-electric focussing gels. Western blotting analysis was performed by running either the purified IgE-antibodies of all the individual hybridoma samples on 8-25% SDS-PAGE. The gels were blotted onto wetted nitrocellulose (Schleicher and Schüll, Dassel, FRG) by diffusion for 60 min at 70°C in the PHAST System. The blots were incubated with PBS containing 0.05% Tween 20 (Merck, Darmstadt, FRG) and 0.02% gelatin (UCB, Brussels, Belgium) for blocking remaining protein binding sites. Subsequently, incubation was performed with optimal concentrations (10 ug/ml) of the various antiserum preparations for 60 min at room temperature. Specific binding was revealed by employing horse radish peroxidase labelled second stage antibodies: horse anti-rabbit antiserum (Amersham, UK) for the rabbit-anti-mouse-IgE antiserum (RAM/IgE) and rabbit anti-goat antiserum (Nordic, Tilburg, The Netherlands) for the goat-anti-mouse-IgE antiserum (GAM/IgE). Substrate reaction was performed by using 0.3 mg/ml 4-chloro-1-naphtol (Sigma) in 50 mM Tris/HCl (pH 7.2) containing 7% (w/v) poly-ethylene glycol 6000 (Baker, Deventer, The Netherlands). Immediately before use, 0.4% cold hydrogen peroxyde was added to the solution. The reaction was stopped by flushing the blot with water.

The yield of isolated IgE was determined by protein determination by optical density at 280 nm or by using fluorescamine (Udenfriend et al., 1972). Briefly, 5 ul samples diluted in 25 ul 2.25 M Na-borate buffer (pH 9.0) were allowed to react with 25 ul 0.7 mM fluorescamine (Serva, Heidelberg, FRG). The reaction mixture was tested by adding 10 ul to standard 60-well Terasaki trays and the fluorescence intensity at an emission wavelength of 475 nm was measured in a microfluorimeter with an excitation wavelength of 390 nm. The results were quantified by an albumin standard curve.

ELISA

Quantitative determination of mouse IgE was performed in an ELISA system employing Terasaki trays. The assay is based upon sandwiching the IgE between two non-crossreacting specific anti-mouse IgE antisera, a conjugate of protein A linked to the enzyme β-galactosidase and the use of a fluorogenic methylumbelliferyl galactoside substrate (Savelkoul et al., 1985). Measurements of the immunofluorescence were performed in an automatically scanning microfluorimeter. In this ELISA, the IgE molecules are bound by immobilized goat anti-mouse IgE polyclonal antibodies. The bound IgE fraction is further revealed by the IgG fraction of a rabbit anti-mouse IgE hyperimmune polyclonal antiserum. This procedure has been described along with a detailed description of the preparation and characterization of the mouse-IgE specific antisera (Savelkoul, Soeting, Radl and Van Der Linde-Preesman, submitted). The RAM/IgE preparation was found to be pure on SDS-PAGE as well as monospecific for murine ϵ-chains in inhibition-ELISA and in immunoblotting. Quantitation was based upon a standard curve of a mixture of highly purified monoclonal IgE of several mouse IgE secreting hybridomas.

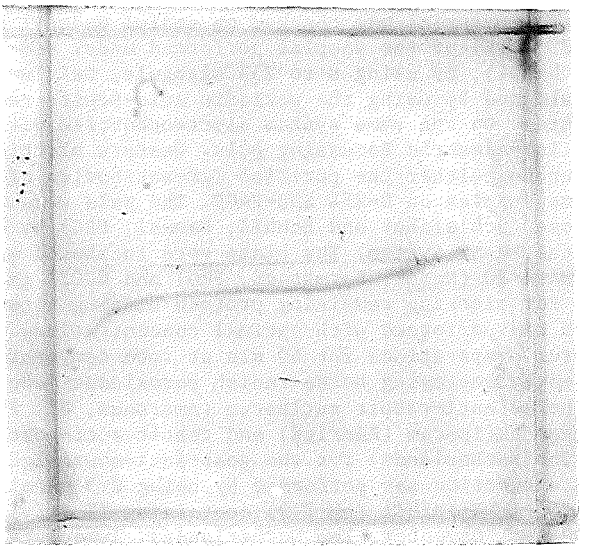

Fig. 1. Electrophoretic titration curve of purified IGEL b4 14.2 IgE on a pH 3-9 isoelectric focussing gel. pH 3 is located to the right while the anode was located at the lower position.

Statistics

Results are expressed as the arithmetic mean of n determinations ± the standard deviation. P-values were obtained by Student t-test to estimate the significance of differences observed. Values of p greater than 0.05 were considered not to be significant.

RESULTS

Before applying a large sample containing IgE to the multi-column FPLC system, a electrophoretic titration curve was produced from a small IgE sample purified on FPLC columns in preliminary experiments without the use of salt precipitation. As a result (Fig.1), a shallow titration curve was found with an iso-electric point of 4.2. Only below this pI, IgE molecules carried enough charge to allow binding to a cationic exchange column (MONO S; see below).

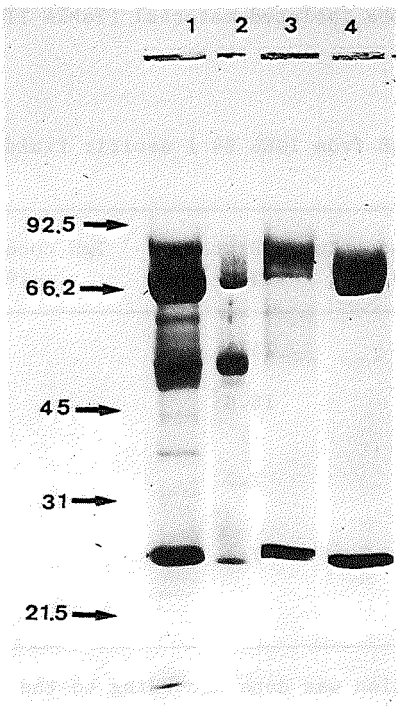

Fig. 2. Isolation and purification of IgE under conventional conditions. Lane 1: IGEL b4 1 ascitic fluid; lane 2: sample after 30 to 55% saturated ammonium sulphate precipitation; lane 3: sample after elution from DNP27-BSA-Affigel; lane 4: sample after elution from DE 52 cellulose ion exchange column. The figures represent molecular weight marker proteins (kDa).

As salt precipitation is routinely used as an initial purification step, the recovery of IgE activity upon salt fractionation was investigated. Precipitation of IgE either by 20% final concentration of sodium sulphate or 30–55% saturated ammonium sulphate resulted in a decreased (10–20%) recovery of IgE as measured by effective binding in ELISA. Moreover, after initial salt precipitation of the sample, a distinct aggregation of IgE molecules was found, which was visible by running such a precipitated sample on the Superose 12 column. In trying to reduce this aggregation, a small amount (2% final concentration) of ethanol was added to the running buffers, which caused a significant increase of monomeric IgE (from 10 to 75%). The addition of ethanol itself to the sample did not cause detectable denaturation and precipitation after incubating the mixture for 30 min at 37°C (data not shown).

Large scale purification of murine IgE out of ascitic fluid employing the multicolumn FPLC system resulted in a high recovery, high yield isolation of IgE. Comparing these results with a conventional purification scheme (Table I and Fig. 2) consisting of sequential salt precipitation, affinity chromatography and ion exchange chromatography, it appeared that the yield of purified material using the FPLC system was much better and so was the recovery of the isolated material (Table II).

TABLE I

Isolation of mouse IgE from IGEL B4 1 ascitic fluid using conventional chromatography

Sample	Volume (ml)	Total Protein[1] (mg/ml)	IgE Concentration[2] (mg/ml)	% Recovery IgE content
ascitic fluid	66	14.5	0.90	100
30–55% SAS[3] precipitate	7	18.8	6.7	79
DNP-Sepharose eluate	23	2.0	1.72	67
DE 52 anion exchange eluate	4.2	7.3	7.3	52

1) Protein determination was done according to the fluorescamine method.
2) The IgE concentration was determined in a quantitative Terasaki ELISA system using absolute standards of pure hybridoma IgE.
3) SAS = saturated ammonium sulphate

TABLE II

Isolation of mouse IgE from IGEL B4 14.2 ascitic fluid using a multi-column FPLC system

Sample	Volume (ml)	Total Protein[1] (mg/ml)	IgE Concentration[2] (mg/ml)	Yield (mg)	% Recovery IgE content
ascitic fluid	50	8.65	0.45	22.5	100
pre FPLC[3]	10	14.42	1.95	19.5	87
post FPLC[4]	10	1.87	1.82	18.2	81

1) Protein determination was done according to the fluorescamine method.
2) The IgE concentration was determined in a quantitative Terasaki ELISA system using absolute standards of pure hybridoma IgE.
3) Sample after precipitation with 20% Na_2SO_4 (3 hours at RT) and overnight dialysis against 50 mM sodium acetate buffer pH 4.9 at 4°C.
4) Sample after passage through Fast Desalting Column, elution from MONO Q at 282 mM NaCl and final cleaning on Superose 12 using 20 mM TRIS-HCl (pH 8.2) as a running buffer throughout the FPLC system.

TABLE III

Molecular weight of the ϵ-chain and complete IgE molecules according to SDS-PAGE analysis

Sample	ϵ-chain	p	IgE	p
IGEL b4 1 ascitic fluid	87.5 $\pm$ 4.6	-	210 $\pm$ 6.8	-
conventional chromatography	68.5 $\pm$ 2.1	< 0.01	183 $\pm$ 5.4	< 0.01
multi-column FPLC	74.2 $\pm$ 2.8	< 0.01	194 $\pm$ 4.3	< 0.01

Results are expressed as arithmetic mean $\pm$ 1 SD (n=3) in kDa molecular weight. P-values were calculated by Student t-test.

However, whether the IgE antibodies were isolated by conventional procedures or by this multicolumn FPLC system, in both cases a decrease of the molecular weight of the heavy chain band was found during purification (Table III and Fig. 3a) according to SDS-PAGE under reducing conditions. This resulted in a significant decrease ($p < 0.05$) of the molecular weight of purified IgE molecules compared to the IgE molecules in the starting

material. A possible explanation for this loss of molecular weight of the ϵ-heavy chain in particular would be the occurrence of proteolytic and glycolytic enzymes that partially copurified with the IgE in the sample.

Therefore, a study was undertaken to determine the susceptibility of the IgE molecules to proteolytic digestion and the possible protection afforded by the addition of a mixture of protease inhibitors. As shown in Table IV, the IgE peak that eluted from the MONO S column showed no significant shift when protease inhibitors were added to the ascitic fluid as compared with the highly purified IgE control. This control IgE sample was obtained by purifying hybridoma IgE from culture supernatant by FPLC as shown in Table II. In the absence of protease inhibitors, however, a distinct peak shift was observed in ascitic fluid. All samples were checked on a 25 ml bed volume Superose 12 HR 10/30 columns for the occurrence of cleaved light chains that could explain the shifted peaks. Such a decrease in molecular weight was not observed.

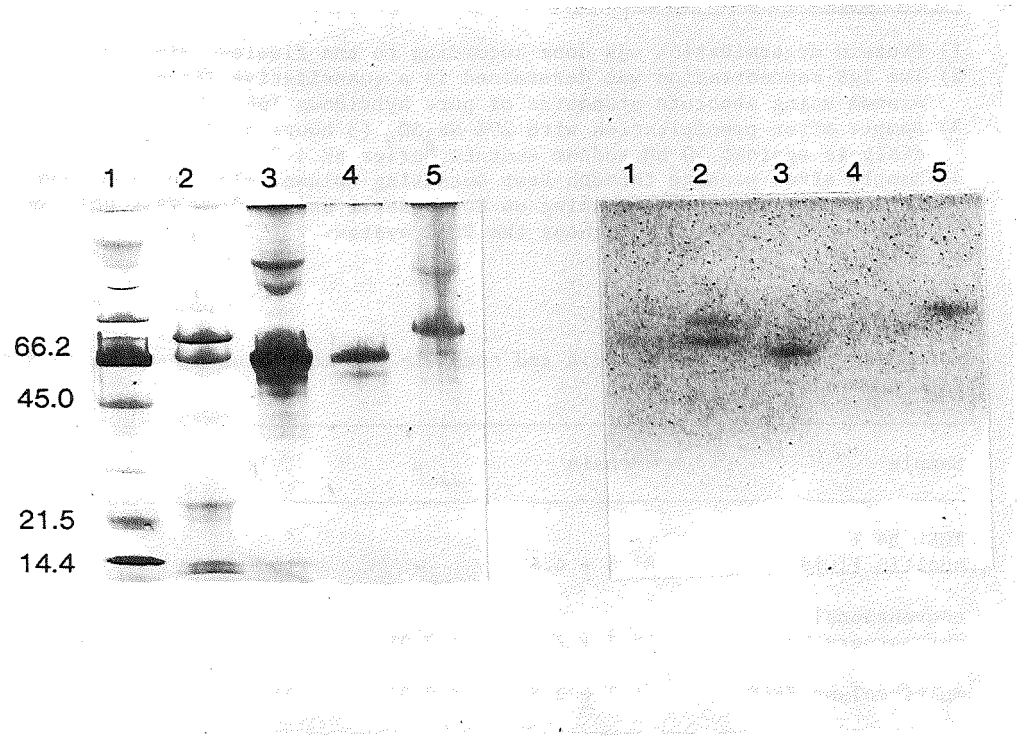

Fig. 3. SDS-polyacrylamide gel electrophoresis of various IgE samples after reduction. The gels were stained with silver (left) or with the periodic acid-Schiff reaction (right). Lane 1: molecular weight marker proteins; lane 2: purified α-glucosidase marker glycoprotein; lane 3: rich culture supernatant containing B53/A4 anti-DNP-IgE hybridoma protein; lane 4: highly purified IgE-sample from the sample in lane 3; lane 5: purified IgE sample from IGEL b4 1 monoclonal antibodies.

TABLE IV

Effects of partial proteolytic and glycolytic treatment on the peak of elution of murine IgE from a MONO S column

Sample	Treatment	Protection	Elution strength (mM NaCl)	Relative peak area (%)
pure IgE[1)]	no	-[2)]	306[3)] ± 15	0[4)]
ascitic IgE	no	-	206 ± 32	100
	no	+	294 ± 17	6 ± 5
	trypsin	-	123 ± 42	86 ± 11
	trypsin	+	287 ± 11	8 ± 7
	glycosidase F	-	341 ± 16	23 ± 6
	glycosidase F	+	294 ± 21	11 ± 7

1) As a sample source ascitic fluid as well as highly purified IGEL b4 14.2 IgE was used.
2) Protection was provided by the mixture of protease inhibitors as described in Materials and Methods.
3) The elution strength is expressed as the amount of NaCl in the lineair gradient of 0 to 0.5 M in the running buffer (20 mM TRIS-HCl, pH 7.0) at which the IgE peak eluted from the MONO S column.
4) Relative peak area is expressed as percentage of the shifted Ig peak from the total accepted peak area during the run while using a integration treshhold level of 5% full scale.
All figures represent arithmetic mean ± 1 SD (n = 4).

Partial proteolytic digestion by trypsin resulted in a substantial shift of the IgE peak (86% of the initial peak was translated from its original elution position) while separate free light chains were not detected on the Superose column. The addition of protease inhibitors (8% peak shift) to the sample could effectively prevent this shift (1-8/86=91%). While proteolytic activity caused a decreased binding of the IgE to the ion exchanger due to a loss of effective charged residues, treatment with glycosidase F increased the binding to the column (23% peak shift), presumably due to the loss of bound sugar residues. The loss of oligosaccharide residues was further confirmed by SDS-PAGE combined with periodic acid-Schiff staining of the gels (Fig. 3b). The addition of protease inhibitors to the sample prevented this glycolytic cleavage to a considerable extent (11 / 23 = 48%).

Alternatively, IgE antibodies were purified by immunoaffinity chromatography on DNP-BSA Affigel. From this column the recovery of monoclonal IgE antibodies was investigated employing different elution conditions. To this end, an IgE rich culture supernatant of the B53/A4 hybridoma was divided into 2 aliquots: an untreated sample and a sample for heat inactivation. All samples were subdivided into two fractions of which one was supplemented with a mixture of protease inhibitors. All samples were applied directly on the DNP affinity column. This column was eluted with either acetic acid or a competitive hapten. From the eluted material, the recovery was determined as binding capacity to a specific antiserum in ELISA as well as binding to an ion exchange column. Therefore, the IgE concentration was

TABLE V

Influence of heat treatment of monoclonal IgE antibodies on the determination of the absolute concentration in ELISA and elution strength from a cationic exchange FPLC column

Sample	Elution	Protection	IgE concentration	Elution strength
Unpurified Supernatant	-	-	2.75 ± 0.37[1)]	305 ± 7[2)]
Untreated Supernatant	HAc	-	0.47 ± 0.38	156 ± 18
	DNP		0.80 ± 0.63	225 ± 23
	HAc	+	2.77 ± 0.12	242 ± 21
	DNP		2.93 ± 0.53	294 ± 18
Inactivated	HAc	-	0.24 ± 0.13	114 ± 39
	DNP		0.67 ± 0.22	207 ± 31
	HAc	+	1.51 ± 0.10	238 ± 26
	DNP		2.47 ± 0.26	257 ± 24

1) Results are expressed as arithmetic mean ± 1 SEM (n = 3) in ng/ml.
2) Results are expressed in mM NaCl at which the peak eluted from the MONO S column in a linear gradient from 0 to 0.5 M NaCl in 20 mM TRIS-HCl, pH 7.0.

determined in the Terasaki-ELISA while the net binding charge was estimated from the elution strength from a cationic exchanger (Table V). Moreover, the molecular weight of the ϵ-heavy chain band was determined by SDS-PAGE.

Western blotting (Table VI) confirmed that the altered ϵ-heavy chains could still be recognized by the polyclonal RAM/IgE antiserum. The same result was obtained when performing Western blotting with goat anti-mouse-IgE antibodies (data not shown). The damage by elution of bound IgE from the affinity column occurred with acetic acid as well as DNP-caproic acid. Moreover, the protection by protease inhibitors against this breakdown prevented the 10% reduction in molecular weight (Table VI).

DISCUSSION

This study was undertaken to establish the influence of sample pre-treatment upon the recovery of specific activity and the development of a routine isolation procedure of murine IgE. In order to select optimal purification conditions, it was considered essential to obtain more information on the charge distribution of murine IgE at various pH values together with the determination of the isoelectric point of the molecule. As shown in the titration curve of IgE, the molecules are only carrying a net charge high enough to allow binding on an ion exchange column at a pH below their pI. The resulting shallow titration curve with a iso-electric point of 4.2 together with the rather broad peaks on the ion exchange columns

TABLE VI

Molecular weight determination of the ε-heavy chain by Western blotting with RAM/IgE antibodies

IgE-sample	Elution[1)]	Protection[2)]	ε-chain[3)]
Culture supernatant	-	-	87,000
Affinity purified	HAc	-	72,000
	DNP	-	77,000
	HAc	+	78,000
	DNP	+	87,500

1)Elution was performed employing acetic acid (HAc) or DNP-caproic acid (DNP).
2)For protection of the samples against proteolytic breakdown, a mixture of protease inhibitors was added as indicated in Materials and Methods.
3)Molecular weights were estimated by using blotted prestained marker proteins.

indicated heterogeneity among IgE molecules. This effect could be due to differences in carbohydrate content, partial proteolytic cleavage and the occurrence of aggregated IgE molecules. Such phenomena have also been described for other Ig-classes like human IgA (Biewenga et al., 1984). Using isoelectric focussing affinity immunoblot analysis, pI micro-heterogeneity has been found in monoclonal murine antibodies (Hamilton et al., 1987). For the these reasons together, we studied the influence of the isolation procedures on the occurrence of heterogeneity in the purified IgE molecules.

During purification of IgE antibodies by salt precipitation, protein denaturation and aggregation of IgE molecules were found in ascitic fluids as well as culture supernatants. Moreover, IgE molecules can be lost during precipitation apart from inactivation as was described in the case of monoclonal IgG antibodies. Furthermore, after salt precipitation the sample may not be sufficiently free of IgM (Philips et al., 1984).

For the isolation of immunoglobulins out of culture supernatants and ascitic fluids high recoveries are common probably because the recovery is calculated from the extent of binding in a ligand based immuno-assay. Any conformational or structural change of the immunoglobulin molecules that does not alter the extent of binding in the ligand based immuno-assay thus does not affect the recovery. Large scale purification of murine IgE out of ascitic fluids employing the multicolumn FPLC system used here, resulted in a high recovery, high yield isolation of murine IgE (Table II). It has been shown that strong ion exchangers (MONO S and MONO Q) have superior resolving properties, greater predictability of retention time and more efficient recoveries than weak ion exchange columns based upon silica supports (Kopaciewiecz and Regnier, 1983; Burchiel et al., 1984). Moreover, when combining cation exchange chromatography with gel filtration, the main contaminants transferrin, alpha acidic proteins and albumin could be re-

moved during the purification of monoclonal antibodies (Carlsson et al., 1985). However, a considerable variation in the molecular weight of the ϵ-heavy chain was found as determined after purification of monoclonal IgE antibodies, ranging from 68.5 to 82.5 kDa. This heterogeneity is typical of heavily glycosylated molecules (Sitia, 1985).

Alternatively affinity chromatography can be employed. During protein A purification it has been found that the amount of copurifying proteins increased when a longer elution protocol was used. Also hydroxylapatite or ion exchange chromatography results in high amounts of copurifying proteins, presumably as a consequence of degradation during purification (Stephenson et al., 1984; Stanker et al., 1985; Manil et al., 1986). For purification of IgE, however, protein A immunoaffinity chromatography cannot be employed because of lack of binding (Johansson and Ingänas, 1978).

In this study the purification of IgE antibodies employing gel permeation chromatography has been omitted because of the low resolving power of the technique unabling the isolation of IgE from IgG antibodies. Also purification by hydrophobic interaction or reverse phase chromatography (Henson, 1985) were excluded from this study.

While collecting culture supernatants or ascitic fluids, lysosomal proteolytic and glycolytic enzymes are released and a general acidification of the medium occurs. At this lowered pH, such proteases become most active and cause break down of the antibodies present. General cocktails of protease inhibitors are considered to be beneficial.

IgE is a glycoprotein with a carbohydrate content of 13% (Liu et al., 1980). The six oligosaccharides are exclusively associated with the ϵ-chain and are all N-linked at asparagine residues (Perez-Montfort and Metzger, 1982; Holowka and Baird, 1983). The carbohydrates can influence the conformation of glycoproteins and also protect them to proteases (Harford et al., 1984; Schauer, 1985). Moreover, oxidizing environments cause rapid denaturation and aggregation. Reducing agents like β-mercaptoethanol, chelating agents like EDTA and avoiding too high protein concentrations are effective in preventing such oxidation (Umezawa, 1972).

As outlined above, isolation of monoclonal antibodies from ascitic fluids bears the risk of glycolytic and proteolytic breakdown of the immunoglobulins to be isolated (Table III). The results as described in Table IV demonstrate that proteases and maybe even glycosidases occur in ascitic fluids and that their activity can be totally or partially blocked by the addition of a mixture of protease inhibitors to the sample (Aoyagi and Umezawa, 1975). Especially PMSF as protease inhibitor of trypsin and chymotrypsin; leupeptin as inhibitor of papain, plasmin, thrombokinase, kallikrein and cathepsin B; and pepstatin A as inhibitor of pepsin, renin and cathepsin D would be most suitable.

The results presented show that the susceptibility of IgE for proteases and glycosidases is different from the other isotypes and that in the search for characteristic chromatographic profiles alterations of the sample can occur due to manipulations during processing (Perez-Montfort and Metzger, 1982; Sitia, 1985). This molecular weight shift could be due to differences in carbohydrate content by removal of neutral sugars, partial proteolytic cleavage and the occurrence of aggregated IgE molecules. Another commonly observed artefact is the charge shift due to partial carbamylation of proteins (Pearson and Anderson, 1983).

In ELISA the determination of the absolute concentration is particularly influenced by the ability of the immobilized catching antibodies to bind the sample IgE. The IgE bound on the hapten affinity column and eluted with acetic acid always resulted in a lower ELISA reading as compared to competitive hapten elution although the yield of eluted material is generally

higher using the former elution method (data not shown). Heat inactivation of the sample results in a considerable loss of IgE recovered from the affinity column (Table V). It is described that decomplementation by heat inactivation (30 min, 56°C) of the sera to be assayed improves the binding of Ig to the coat in ELISA and gives a more consistent determination of IgE concentration. However, heat inactivation results in aggregation of proteins and IgE in particular, which on the other hand may interfere with the determination of the concentration (Parish and Higgins, 1982). Moreover, heating the sample may activate even more the lysosomal proteolytic enzymes from the hybridoma cells, resulting in enhanced breakdown of IgE.

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CHAPTER 4.3

TERASAKI-ELISA FOR MURINE IgE-ANTIBODIES. I. QUALITY OF THE DETECTING ANTIBODY: PRODUCTION AND SPECIFICITY TESTING OF ANTISERA SPECIFIC FOR IgE

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ABSTRACT

In order to develop an ELISA for the quantitative determination of murine IgE, goat and rabbit antisera specific for murine IgE were prepared. As immunogen, monoclonal IgE antibody mixtures of several allotypically different hybridomas were used. Before use, these antibodies were purified employing procedures that allow maximum recovery of binding activity. These goat and rabbit mouse ϵ-chain specific antisera were adsorbed on normal mouse serum. The purified antisera were found to be free of allotypic activity. However, immunoadsorption on NMS could not always remove contaminating anti-idiotypic antibodies. Repeated adsorptions with monoclonal antibodies of different isotypes carrying a similar idiotype were necessary to remove all detectable anti-idiotypic activity. Only after these precautions, the antisera were suitable for detecting IgE molecules on nitrocellulose blots as well as for quantitating circulating IgE-antibodies (ELISA) as well as IgE-secreting cells (plaque assay and reverse ELISA plaque assay).

Moreover, the purity and reactivity of several commercially available anti-IgE preparations were tested in similar types of specificity assays. Since the specificity of antibodies used in ELISA determines the monospecificity of the assay, retesting for contaminating cross-reactivities in commercial preparations was shown to be necessary.

INTRODUCTION

As pointed out by Shields and Turner (1986), it is essential in sensitive immunoassays like enzyme-linked immunosorbent assay (ELISA) for immunoglobulins (Ig), to employ antisera that are of high purity and react with high affinity and specificity with the Ig to be determined. It is therefore prudent, even while using immunoaffinity-isolated antibodies, to control for unwanted cross-reactivity. It has been reported that especially for the quantitation of rare antibodies like IgE, sandwich systems employing polyclonal heterologous antibodies have broad applicability and sufficient sensitivity (Shields and Turner, 1986; Kemeny et al., 1986).

Until recently, quantitation of murine IgE has been hampered by the lack of suitable reagents. The absence of murine IgE-secreting myelomas and the low concentration of IgE in mouse serum made it extremely difficult to prepare anti-IgE antibodies (Lang et al., 1976; Lehrer et al., 1976; Malley et al., 1977). With the successful development of murine antigen-specific IgE-secreting hybridomas, it became feasible to purify IgE antibodies and to generate heterologous anti-IgE antisera. Employing these reagents, detection of IgE antibodies is possible in sensitive radioimmunoassays or ELISA (Böttcher et al., 1978; Böttcher et al., 1980; Hill and Liu, 1981; Eshhar et al., 1980; Barsumian et al., 1981; Rudolph et al., 1981; Bohn and König, 1982; Coffman and Carty, 1986).

The common way to obtain ϵ-chain specific heterologous antisera is to purify hybridoma IgE out of ascitic fluids or culture supernatant. Subsequently, goats and rabbits can be immunized with this purified IgE (Liu et al., 1980; Eshhar et al., 1980; Kings and De Weck, 1981; Bozelka et al., 1982; Uede et al., 1984; Haba et al., 1985; Coffman and Carty, 1986). Such sera might contain allotypic, idiotypic or light chain reactivity along with the desired anti-isotypic activity since intact IgE molecules were used for the induction of the antisera. The removal of such undesired cross-reactivities is routinely done by immunoadsorption on immobilized normal mouse serum (NMS) as well as on other isotypes carrying a similar idiotype.

In the present study we show that this procedure is insufficient. Therefore, several adsorption procedures were employed to remove allotypic, idiotypic and light chain reactivity together with the undesired reactivity against the other heavy chains than the ϵ-chain. Furthermore, the specificity of the produced and purified anti-mouse IgE antisera were tested in a variety of assays like double immunodiffusion and immunoelectrophoresis, Western blotting, inhibition ELISA and plaque assays. The results show that such performance testing is of utmost importance. Furthermore, after rigorous adsorption and specificity testing, an antiserum preparation could be obtained that is applicable in all the above mentioned procedures.

MATERIALS AND METHODS

Animals

Female BALB/c ($H\text{-}2^d$) mice, at least 10 weeks of age, were obtained from Bomholtgard (Ry, Denmark) while (B10 x B10.D2)F_1 ($H\text{-}2^{b/d}$) mice of various ages were purchased from OLAC Ltd (Bicester, UK). Rabbits (New Zealand White) and goats were bred and maintained at the TNO facilities.

Hybridoma IgE

As a source of IgE we used four TNP-specific IgE-secreting hybridomas of the a and b allotypes all bearing the kappa light chain (IGEL a4 6.3,

IGEL a4 6.5; IGEL b4 1; IGEL b4 14.2). These cell lines were generous gifts of Dr. M.R. Wabl (Tübingen, FRG). Ascitic fluids were induced by intraperitoneal (i.p.) injection of 10^6 hybridoma cells into mice primed with pristane (Janssen Chimica, Beerse, Belgium) 10 days earlier. The hybridomas of the a allotype were injected in BALB/c mice whereas those of the b allotype were injected into (B10 x B10.D2)F_1 mice. Apart from these ascitic fluids, we used culture supernatant as well as ascitic fluid of the ovalbumin-specific hybridoma IgE-14-205 (Böttcher et al., 1979; Sera lab, Crawley Down, UK) and purified IgE from the DNP-specific IgE-secreting hybridoma SPE/7 (Eshhar et al., 1980; kind gift of Dr. Z. Eshhar, Rehovot, Israel). The IgE antibodies were isolated and purified out of the above mentioned samples as described (Savelkoul et al., 1985). The isolated material was proved to contain intact IgE molecules still bearing their complete carbohydrate content. This was confirmed by running the reduced sample on sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were either silver-stained or stained by the periodic acid-Schiff reaction.

Preparation of antisera

A mixture was prepared consisting of equal amounts (based upon the protein content) of all the different IgE preparations from the hybridoma lines used. This mixture was referred to as the absolute IgE standard. The isolated IgE-molecules were injected into rabbits and goats according to a protocol that gave rise to high titre antisera (Dresser, 1986). Hyperimmune sera were collected at various time points after the booster immunization. The IgG fraction of these antisera was purified by ammoniumsulphate precipitation, extensively dialysed against phosphate-buffered saline (PBS; 10 mM containing 0.15% NaCl; pH 7.2) and loaded on a DEAE cellulose column (DE 52; Whatman, Maidstone, UK). The bound fraction was eluted with a linear gradient of 0 to 0.2 M NaCl in 0.01 M TRIS-HCl (pH 8.1), dialyzed against PBS, concentrated and stored at -20°C. The purity of the isolated IgG fraction of the different antisera was confirmed by SDS-PAGE. The reactivity of the antisera was tested in double immunodiffusion and immunoelectrophoresis against rabbit anti-goat-IgG (RAG/IgG) for the GAM/IgE and goat anti-rabbit-IgG (GAR/IgG) for the RAM/IgE antiserum, respectively (Nordic, Tilburg, The Netherlands). Similar procedures were used for several commercially available RAM/IgE preparations that were affinity purified and of immunofluorescence quality grade according to the manufacturers' specifications: Nordic, Sera Lab (lot nr. 1040101), and MIAB, Uppsala, Sweden (lot nr. 60223).

Immunoadsorptions

Affinity columns were prepared from purified immunoglobulin fractions from a pool of normal mouse serum (NMS) coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). Such columns were prepared from serum of non-immunized BALB/c, C57BL/6 and DBA/2 mice. Moreover, purified DNP- or TNP-specific myeloma proteins of classes IgG (J509) and IgA (MOPC 315) were immobilized on CNBr-activated Sepharose 4B. The various antiserum preparations of the immunized goats and rabbits were adsorbed twice on these affinity columns in PBS and the effluents were collected. Again the final preparations were tested for reactivity in immunoelectrophoresis against the mixture of purified hybridoma IgE originally used for the immunization.

Western blotting analysis

The purified IgE-antibodies of all the individual hybridoma samples as well as the respective antiserum preparations were run at 8-25% SDS-PAGE on

a Phast System (Pharmacia). The gels were blotted onto wetted nitrocellulose (Schleicher and Schüll, Dassel, FRG) by diffusion for 60 min at 70°C in the Phast System. The blots were incubated with PBS containing 0.05% Tween 20 (Merck, Darmstadt, FRG) and 0.02% gelatin (UCB, Brussels, Belgium) for blocking remaining protein binding sites. Subsequently, incubation was performed with the various antiserum preparations for 60 min at room temperature. Specific binding was revealed by employing horse radish peroxidase labelled second stage antibodies: horse anti-rabbit-Ig antiserum (Amersham, UK) for RAM/IgE and rabbit anti-goat-Ig antiserum (Nordic) for GAM/IgE. Substrate reaction was performed by using 0.3 mg/ml 4-chloro-1-naphtol (Sigma) in 50 mM Tris/HCl (pH 7.2) containing 7% (w/v) poly-ethylene glycol 6000 (Baker, Deventer, The Netherlands). Immediately before use, 0.4% cold hydrogen peroxyde was added to the solution. The reaction was stopped by flushing the blot with water.

Isotype-specificity testing

Apart from Western blotting analysis, both the RAM/IgE and GAM/IgE antisera prepared were tested for their specificity for IgE class antibodies by inhibition studies in the IgE-ELISA as performed in Terasaki trays (Savelkoul et al., accompanying paper). Therefore, wells of Terasaki trays were coated with purified TNP-specific IgE samples from the four individual TNP-specific hybridomas mentioned above. Subsequently, the RAM/IgE detecting antiserum dilution was separately mixed in tubes (blocked for non-specific adsorption with PBS containing gelatin) with increasing amounts of each of the various other non-IgE isotypes or IgE-antibodies of different specificity (ovalbumin (OA)-spcific IgE). The respective isotype preparations consisted of purified Ig-fractions of myeloma proteins of the IgM (MOPC 104E), IgG_1 (MOPC 21), IgG_{2a} (UPC 10), IgG_{2b} (MOPC 11), IgG_3 (J606), IgA (J558) classes as well as of kappa (MOPC 41) and lambda (RPC 20) light chains (Eurogenetics, Diepenbeek, Belgium). On the other hand, the detecting antibody was mixed with increasing concentrations of OA-specific IgE 14-205 (Sera Lab).

Inhibition studies were performed using increasing amounts of $F(ab')_2$ fragments of the purified TNP-specific IgE preparations. These fragments were prepared as described (Parham, 1983) and purified according to their molecular weight (28 kDa) over Fast Protein Liquid Chromatography (FPLC; Pharmacia, Uppsala, Sweden) equipped with a Superose 12 (HR 5/30) column. The purity of these fragments was assessed on a Phast System for electrophoresis.

Moreover, the specificity of the RAM/IgE preparation was confirmed in protein A and antigen-specific plaque assays on IgG- and IgE-secreting cells. The plaque assays were performed on various dilutions of the hybridoma cell suspensions of the four TNP-specific IgE-secreting hybridomas. The number of viable nucleated cells in these experiments was determined by trypan blue exclusion. Protein A coated sheep red blood target cells were prepared as described (Savelkoul et al., 1988a) and the assay was performed as described previously (Savelkoul et al., 1988b). Also the number of anti-TNP IgE-plaque forming cells (IgE-PFC) were determined in these cell suspensions using TNP-coated target cells (Pohlit et al., 1979). The numbers of protein A IgE-PFC and the numbers of anti-TNP IgE-PFC were compared using two-tailed Student t-test ($\alpha = 0.05$).

Avidity determination

Scatchard analysis was performed to determine the affinity (equilibrium constant) of the anti-IgE antisera for monoclonal as well as polyclonal IgE essentially as described by Friguet et al. (1985). Therefore,

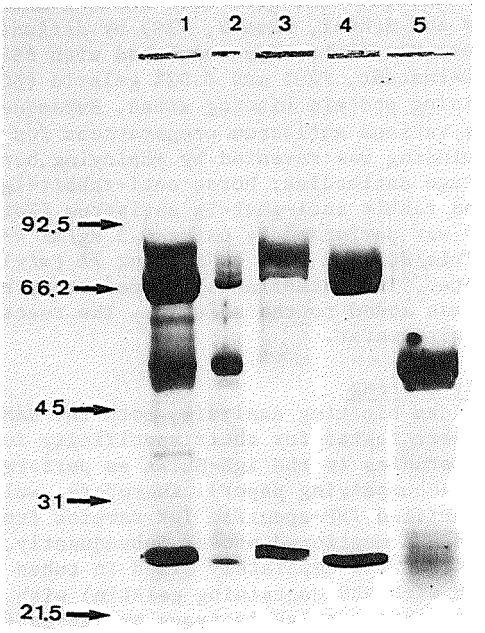

Fig. 1. SDS-PAGE analysis of reduced samples from the subsequent purification steps of IGEL b4 1 ascitic fluid. Starting sample (lane 1), sample after SAS (lane 2), sample after DNP-Sepharose (lane 3), sample after DE 52 cellulose and rabbit anti-mouse-IgE antiserum (lane 5). Figures represent marker molecular weights of marker proteins.

varying concentrations of the RAM/IgE antiserum were mixed with constant concentrations of DNP-specific IgE standards for 30 min at 37'C in wells of microtiter plates that were blocked for non-specific adsorption by overnight incubation with PBS containing 0.02% gelatin. Subsequently, these mixtures were applied on Terasaki trays coated with a constant amount (2 ug/ml in PBS) of $(DNP)_{27}$-BSA. This hapten-carrier complex was prepared according to Eisen (1964) by treating bovine serum albumin (fraction V; Sigma) with dinitrobenzenesulphonic acid (Eastman Kodak, Rochester, NY) after which a coupling level of 27 DNP molecules per protein molecule was assessed spectrophotometrically. From the amount of antibody applied in the well (T) and the amount of antigen-bound antibody (B) after mixing in the microtiter well, the free fraction of antibody (F) could be calculated and a Scatchard plot could be constructed (B/F versus B). From this plot, the inverse of the slope of the curve represented the dissociation constant (K_D).

RESULTS

Purification of the antisera

For immunization, various monoclonal IgE had to be isolated from ascitic fluids and purity had to be assessed by SDS-PAGE. Fig. 1 shows the results of such a typical purification as determined by SDS-PAGE under reducing conditions (lane 1-4). Purified material from all four TNP-specific monoclonal IgE sources was mixed in equal amounts (based upon protein content) and used for immunization. Goats as well as rabbits were immunized

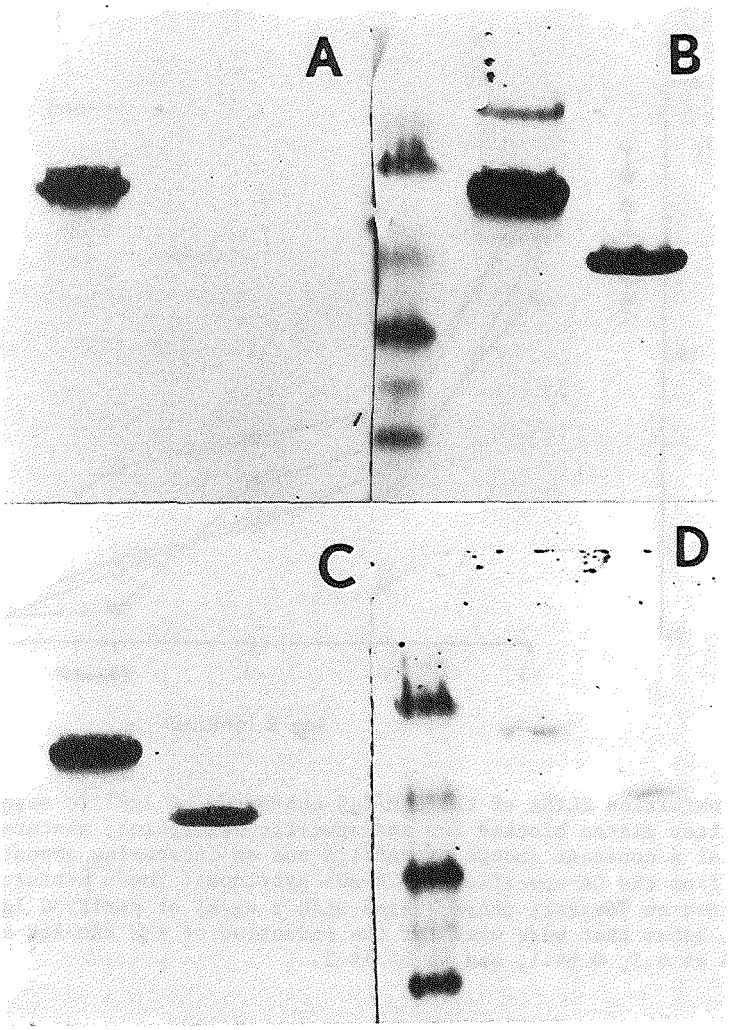

Fig. 2. Immunoblotting analysis of anti-IgE antisera. In panel A and C, purified IgE from IGEL a4 6.3, a4 6.5, b4 1 and b4 14.2 were mixed and blotted onto nitrocellulose in lane 1. In lane 2 purified anti-TNP IgG antibodies were blotted. The same samples were blotted in panel B and D in lanes 2 and 3, respectively. In lane 1 in panel B and D prestained molecular weight marker proteins were blotted. The blots were incubated with the respective rabbit anti-mouse-IgE preparations: the antiserum prepared in this study (panel A) and the commercial antiserum preparations (MIAB, panel B; Nordic, panel C; Sera Lab, panel D).

with this mixture applied in complete Freund adjuvans and boosted every 6 weeks. Sera of the immunized animals were collected 4 days after boosting and stored at -20°C. Subsequently, the IgG-fractions of these sera were isolated and purified as indicated. Purity of these fractions was again confirmed by SDS-PAGE (Fig.1, lane 5).

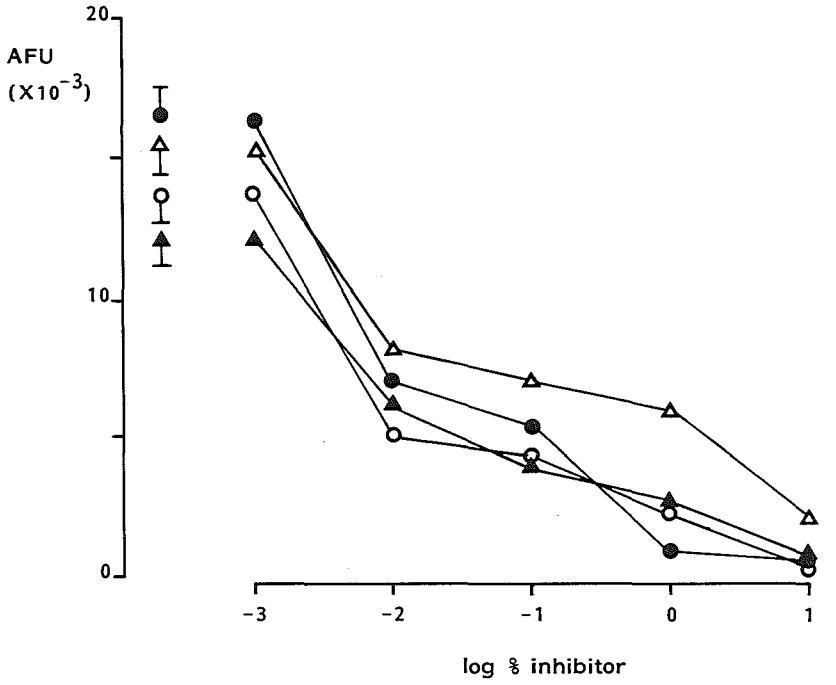

Fig. 3. Inhibition ELISA of the RAM/IgE antiserum by IgE. In separate wells of microtiter plates blocked for non-specific adsorption, mixtures were prepared of a constant amount of RAM/IgE and an increasing amount of purified IgE from the OA-specific IgE 14-205 hybridoma. These mixtures were then applied on Terasaki trays coated with 2 ug/ml of purified IgE from all four IGEL lines that were used for the induction of the RAM/IgE antiserum: ▲ a4 6.3, △ a4 6.5, O b4.1, and ● b4 14.2.

Specificity in blotting analysis

After the immunoadsorptions, the reactivity of the preparations was established initially in double immunoprecipitation and immunoelectrophoresis against different antisera. As a source of antiserum, GAR/Ig was used for the rabbit and RAG/Ig was used for the goat antiserum in these precipitation studies. Only one precipitation line resulted from these incubations, suggesting that pure IgG-fractions were obtained from these antisera. In similar precipitation studies with the IgE-samples used for the initial immunization and the resulting antisera, also single precipitation lines were obtained, suggestive for IgE-specificity of the antisera.

Western blot analysis was performed to confirm the monospecificity of the anti-IgE preparations. The results showed that the RAM/IgE antiserum preparation reacted exclusively with IgE-antibodies blotted onto the nitrocellulose and gave no visible binding with anti-TNP IgG antibodies nor with light chains (Fig. 2, lanes 1 and 2). In the reverse situation, the RAM/IgE blotted on the nitrocellulose membrane did not react with any of the other

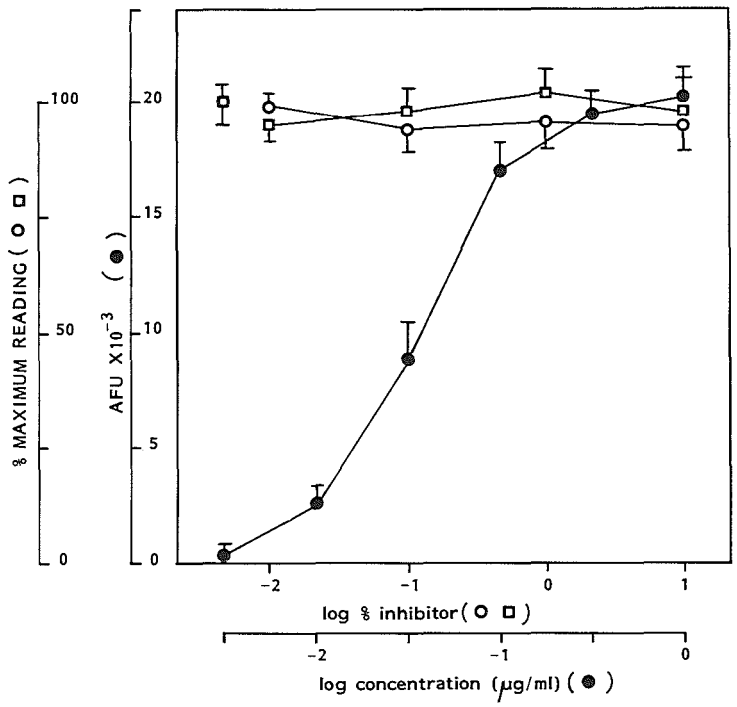

Fig. 4. Inhibition ELISA of the RAM/IgE antiserum. In separate wells of a microtiter plate blocked for non-specific adsorption, mixtures were prepared consisting of a constant amount of RAM/IgE and an increasing concentration of inhibitor. As inhibitors were used a mixture of purified myeloma proteins of all other classes and subclasses containing also light chains (□) or $F(ab')_2$-fragments prepared from purified IGEL b4.1 antibodies (○). These mixtures were then applied on Terasaki plates coated with a constant amount of purified TNP-specific IgE from the IGEL b4.1 hybridoma and further developed as described. Alternatively, in the mixture of all non-IgE isotypes, a constant amount of IgE was added together with an increasing amount of RAM/IgE (●).

isotypes, nor with similar idiotypes and/or allotypes present on IgG antibodies (data not shown).

The commercial antisera were also tested on Western blotting (Figs. 2B-D). All antisera tested reacted with Fc fragments of IgG as well as of IgE although the reactivity was different for the various antiserum preparations. In immunoblotting no light chain reactivity was observed in neither of the antisera tested.

Specificity in ELISA

Since the antisera preparations were intended to be used in an ELISA it was prudent to test the specificity of the antisera in the ELISA as well. This was done in inhibition ELISA in which TNP-specific IgE preparations of

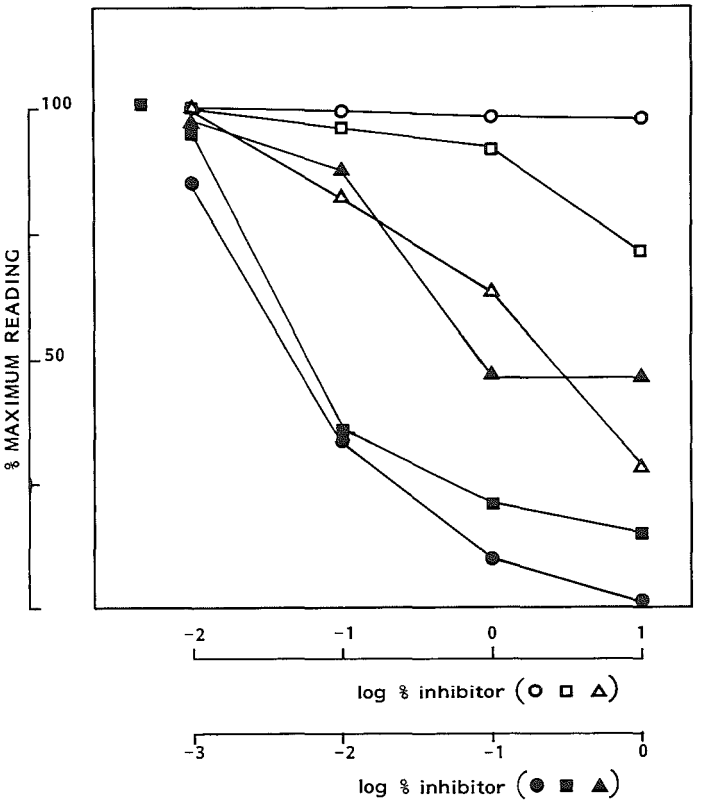

Fig. 5. Inhibition ELISA using commercial RAM/IgE preparations. In separate wells of microtiter plates blocked for non-specific binding, constant amounts of RAM/IgE obtained from Nordic (□, ■), Sera Lab (△, ▲) or MIAB (○, ●) were mixed with increasing amounts of either $F(ab')_2$ IgE-light chains prepared from a mixture of four TNP-specific hybridoma IgE samples (open symbols) or purified IGEL b4 14.2 TNP-specific IgE (closed symbols).

all the four IGEL hybridoma lines used for immunization were coated on wells of Terasaki plates. Subsequently, in separate vials a constant amount of RAM/IgE was mixed with increasing concentrations of OA-specific IgE. When these mixtures were applied to the Terasaki trays coat-ed with a constant amount of TNP-specific IgE, this resulted in a decreased signal (Fig. 3). In a similar type inhibition ELISA, constant concentrations of the detecting RAM/IgE antiserum were mixed with increasing amounts of a mixture of highly purified myeloma proteins of all other non-IgE isotypes (except IgD) as well as purified lambda and kappa light chains. The amount of these proteins added to the RAM/IgE increased up to a 10-fold excess. When these mixtures were applied on plates coated with a constant amount of purified TNP-specific IgE, no decrease of the reading signal was observed

over a wide range of inhibitor concentrations (Fig.4). In the reverse situation, RAM/IgE antibodies titrated in a mixture of all these other proteins on plates coated with a constant concentration of IgE resulted in a linear increase of the reading (Fig.4). In similar type inhibition ELISA, RAM/IgE antibodies were mixed with increasing amounts of TNP and DNP binding IgG antibodies of different allotypes (Fig.4). This indicated that in the assay in which the antiserum is to be used, the RAM/IgE preparation did not detect allotypic or idiotypic determinants.

In order to exclude anti-light chain activity in the antiserum preparations, inhibition studies were performed using $F(ab')_2$ fragments of the mixture of the four purified monoclonal anti-TNP IgE-antibodies that were proved to be essentially pure on SDS-PAGE. The results of this study with all four TNP-specific IgE fragments showed no detectable anti-light chain activity in this RAM/IgE antiserum (Fig.5). For the GAM/IgE similar assays were designed resulting in essentially identical results (data not shown).

Similar inhibition experiments were performed using commercially available antibody preparations. The results (Fig.5) showed that one RAM/IgE (MIAB) could be inhibited completely on anti-TNP IgE coated plates by competition with anti-OA IgE. The antiserum could not be blocked by $F(ab')_2$ fragments of a mixture of hybridoma IgE. Two other antisera (Nordic and Sera Lab) could not completely be inhibited by IgE and did contain some anti-light chain reactivity.

Specificity in plaque assays

The RAM/IgE antiserum was also intended to be used in plaque assays in order to be able to determine the number of antigen-specific and total-IgE secreting cells in various lymphoid organs upon immunization. Therefore, on hybridoma cultures of the TNP-specific IgE- and IgG-secreting cells of different allotypes, plaque assays were performed using protein A and TNP-coated target cells. The results show (Table I) identical numbers of PFC in various cell densities using both types of target cells in combination with the RAM/IgE antiserum, indicating that in plaque assays also no anti-allotypic activity occurred. Since no PFC could be recovered from the anti-TNP IgG-secreting cells using this antiserum (data not shown), also no

TABLE I

Correlation between the numbers of TNP-specific and total IgE plaque-forming cells using TNP-specific mouse IgE-secreting hybridomas

		PFC per viable nucleated cells (%)		
Hybridoma	N	anti-TNP-PFC	total IgE-PFC	P-value
IGEL a4 6.3	7	27.3 ± 12.8	21.4 ± 9.7	0.18
IGEL a4 6.5	7	25.9 ± 14.1	31.8 ± 12.6	0.21
IGEL b4 1	12	36.7 ± 19.2	44.1 ± 21.7	0.12
IGEL b4 14.2	15	38.5 ± 18.8	43.1 ± 19.3	0.14

PFC were determined using TNP30-SRBC and Protein A-SRBC for anti-TNP-PFC and total IgE-PFC, respectively, with rabbit anti-mouse-IgE as developing antiserum. P values were calculated according to the Student's t-test.

evidence for anti-idiotypic activity was obtained. In the plaque assay various dilutions of the developing RAM/IgE antiserum gave rise to an optimum curve of which the optimal dilution resulted in linearly increasing numbers of PFC when testing different numbers of hybridoma cells (data not shown).

Aviditity of RAM/IgE for IgE

Since the RAM/IgE antiserum preparation is intended to be used in ELISA and in plaque assays, we wanted to establish the functional avidity of this antiserum for various IgE samples. Therefore, we determined the equilibrium binding constant by Scatchard analysis in Terasaki-ELISA. The results (Table II) showed the average affinity constant for various monoclonal and polyclonal IgE samples. The antiserum recognized IgE with a rather high affinity (about 10^9 M^{-1}).

TABLE II

Avidity of RAM/IgE for monoclonal IgE as determined by Scatchard analysis in Terasaki-ELISA

Sample	Avidity (K_D)
B53/A4	7.7×10^{-9}
IGEL b4 14.2	6.3×10^{-9}
IgE 14-205	3.5×10^{-9}

Values were calculated in M as described in the Materials and Methods section.

DISCUSSION

In order to be able to produce ϵ-chain specific antisera, IgE should be isolated and purified with a sufficiently high yield and purity. Most important, however, is that also the recovery of the isolated material is high (as determined by binding in a number of different assay systems). As described (Savelkoul et al., submitted), the structure of IgE-molecules can easily be pertubated upon purification, which will be reflected in the titre as well as the specificity of the produced antisera. Therefore, we employed purification methods taking into account these parameters (Savelkoul et al., 1985; Savelkoul and Van Der Linde, submitted).

Since the GAM/IgE and RAM/IgE antisera produced in this study are to be used in ELISA information about the appropriate specificity can only be ascertained in ELISA when a sufficiently large range of antibody concentrations is tested on an elaborate panel of purified myeloma or hybridoma proteins. The panel should include specimens of each Ig (sub)class. Some of the (sub)classes should be represented by a lambda as well as a kappa containing protein. This is essential since the antisera are induced by immunization with whole intact IgE molecules.

It has been described that use of intact immunoglobulin molecules for immunization often results in antisera that contain a considerable degree of anti-light chain contamination. Furthermore, proteins of different allotypes are needed to determine and eliminate anti-allotypic activities. It

has been found in the case of monoclonal antibodies that some mAb that seemed to react anti-isotypically in an ELISA testpanel, turned out to be anti-allotypic in other tests (Haaijman et al., 1984).

Several examples exist in which it is claimed that contaminating anti-idiotypic activity of rabbit antisera can be removed by passing the antiserum over columns of either ragweed-specific monoclonal IgE (Hill and Liu, 1981) or DNP-specific IgG_1 (Giallongo et al., 1982). In the latter study, specificity of the RAM/IgE was tested only in immunodiffusion while using this antiserum in ELISA. In the present study, the specificity of the produced anti mouse-IgE antisera was tested in a variety of assays like double immunodiffusion and immunoelectrophoresis, Western blotting, inhibition ELISA and plaque assays. However, all of these assays have their own detection limit and sensitivity (slope of the dose-response curve). For detection of IgE molecules, immunodiffusion and immunoelectrophoresis are considered to be less sensitive when compared to inhibition ELISA and immunoblotting. On the other hand, immunoblotting is considered to be more sensitive than ELISA in general. The results presented in this study show that after multiple adsorptions an antiserum preparation was obtained that can be used in all of the above mentioned assays. Moreover, this rabbit antiserum can also be used for the detection of IgE-secreting cells in plaque assays and reverse ELISA-plaque assays. This property makes this antiserum useful in the analysis of ongoing IgE responses both at the cellular (IgE-secreting cells) and the humoral level (serum IgE concentration). The production and specificity testing of other mouse IgE-specific antisera that can be used in plaque assays as well as in ELISA have not been described so far.

Several commercially available affinity purified mouse-IgE specific rabbit antisera have been tested along with our own antiserum preparations in all specificity tests. Prior to testing the specificity, the antisera were adsorbed to NMS to remove any contaminating cross-reactivity. This procedure is most commonly used and sometimes performed without testing the specificity of the eluted material. However, even after these adsorptions, some of the commercial reagents still contained cross-reactivity with highly purified polyclonal IgG antibodies as shown by immunoblotting analysis. The antiserum preparations used were claimed to be applicable in ELISA and it must therefore be considered that these antisera might contain additional cross-reactivity when analyzed in blotting analysis. From the data presented it is clear that all of these antisera recognized IgE, but did also contain anti-light chain reactivity as well as cross-reactivity for IgG when analyzed in inhibition-type ELISA. Such contaminating reactivities in these antiserum preparations could only be detected by using different sensitive techniques for the analysis of the monospecificity of these antisera, e.g. immunoblotting. Therefore, not all commercially available antisera are useful in sensitive assays like ELISA when such preparations have been tested in immuno-electrophoresis only. This is all the more true since these antisera were claimed to be affinity purified or rather adsorbed on affinity columns bearing immobilized potentially cross-reacting substances. In short, commercial antisera should only be used in assays having sensitivities that are comparable to those used for obtaining the manufacturers' specifications on specificity testing. When considering the use of such antisera in more sensitive assays, these antisera should be further adsorbed and more rigorously tested for specificity (Shields and Turner, 1986; Snoijink, 1987).

Another point of consideration is that commercial antisera are often claimed to be of high avidity for the relevant isotype, while details substantiating this are usually lacking. As shown in Table II, the rabbit antiserum that we have prepared and tested has an avidity in the range of

10^{-9} M. This value is considered to be sufficient for its successful use as a detecting reagent in ELISA (Steward and Steensgaard, 1983).

Although the choice of antisera employed in ELISA is generally found to be crucial, not many investigations have been conducted to the level of purity required for successful assays. Conflicting data exist regarding the use of either polyclonal or monoclonal antibodies to be employed as catching antibodies in ELISA for the quantitative measurement of human serum IgE. On one hand, monoclonal anti-IgE preparations are preferred because of their superb specificity, lack of cross-reactivity and unlimited availability (Sancho et al., 1986). On the other hand, polyclonal rabbit anti-IgE antisera were described to be superior in sensitivity for use in ELISA, especially in combination with either monoclonal or polyclonal detecting antibodies in a sandwich system (Hill and Liu, 1981; Shields and Turner, 1986; Kemeny et al., 1986). We selected the antisera produced as described in this paper for developing a Terasaki-ELISA allowing the quantitative determination of murine IgE antibodies in the serum (described in a accompyaning paper).

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CHAPTER 4.4

TERASAKI-ELISA FOR MURINE IgE-ANTIBODIES. II. QUANTITATION OF ABSOLUTE CONCENTRATION OF ANTIGEN-SPECIFIC AND TOTAL IgE

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ABSTRACT

A Terasaki-tray based ELISA system was developed for the quantitative measurement of antigen-specific and total IgE antibodies in 5 ul samples of mouse serum dilutions. The assay was based upon non-competitive binding of mouse IgE antibodies between the immobilized appropriate antigen or catching antibodies and the detecting rabbit antibodies. A conjugate of protein A-labelled β-galactosidase and the fluorogenic substrate methylumbelliferyl-β-D-galactoside were used as a detecting system. The resulted fluorescence could be measured in a fast and automatic way by using an inverted micro-fluorimeter. These measurements were automatically transformed into absolute concentration by a microprocessor based program using a four-parameter logistic function and an absolute IgE standard. The assay has a detection limit of 0.04 ng/ml and a range of linearity of 0.04 ng/ml - 20 ng/ml, which is sufficient to measure IgE concentrations in mouse serum.

INTRODUCTION

In a previous study we have described the production and specificity testing of several mouse IgE-specific antisera that were found applicable in enzyme-linked immunosorbent assays (ELISA) for murine IgE (Savelkoul et al., accompanying paper). Using similar approaches, other investigators have also produced heterologous mouse IgE-specific antisera and used for developing IgE-specific ELISA systems (Hill and Liu, 1981; Bohn and König, 1982; Giallongo et al., 1982; Bozelka et al., 1982; Maekawa and Ovary, 1984; Gavériaux et al., 1986; Coffman and Carty, 1986). Quantitation of IgE in all of these studies was based on semi-logarithmic standard graphs using the untransformed absorbance readings.

Results obtained in ELISA are generally expressed as graphically determined end point titres (Malvano et al., 1982; Caulfield and Shaffer, 1984), titres based upon linear regression analysis (Hill and Liu, 1981; Sancho et al., 1986) or absolute concentrations by using transformed data (Beatty et al., 1987). Probably the most general applicable transformation is based upon a four-parameter logistic equation (Rodbard and McLean, 1977; De Lean et al., 1978; Fernandez et al., 1983).

So far all ELISA systems for detecting murine IgE have been performed in 96-well microtiter plates using at least 100 ul serum dilutions that make it often difficult to measure the very low IgE concentrations since only low dilution factors can be used for which a relatively large amount of serum is required. This problem is even more pronounced in longitudinal studies when only limited amounts of serum are available. Therefore, we adapted an ELISA for use in 60-well Terasaki trays so that 5 ul serum dilutions suffice (Pateraki et al., 1981; Van Soest et al., 1984; Labrousse and Avrameas, 1987). By using fluorimetric analysis of β-galactosidase, the sensitivity of the ELISA became sufficient to allow quantitative determination of both antigen-specific and total IgE in mouse serum.

MATERIALS AND METHODS

Mice

Groups (n=6-10) of female mice, 6 to 12 months of age were used for serum collection. BALB/c, C57BL/6 and SJL mice were purchased from Bomholtgard (Ry, Denmark). A/J and (C57BL/6 x DBA/2)F1) mice were purchased from OLAC Ltd. (Bicester, Oxon, UK). C3H/HeJ and C3HeB/FeJ mice were obtained from Jackson (Bar Harbor, Maine, USA) and AKR mice were purchased from the Radiobiological Institute TNO (Rijswijk, The Netherlands).

Serum samples and standard preparations

Sera from mice were collected and stored at -20°C. Before use, the samples were spun down in a microfuge. The primary IgE standard used consisted of a mixture of highly purified hybridoma IgE prepared as described before (Savelkoul et al., accompanying paper).

Antisera

The rabbit (RAM/IgE) and goat (GAM/IgE) antisera to murine IgE were produced by immunization with mixtures of several highly purified hybridoma IgE preparations. The IgG fraction of the GAM/IgE and RAM/IgE antisera reacting exclusively with the Fc portion of IgE molecules were purified and used as described before (Savelkoul et al., accompanying paper). The purified antiserum preparations were adsorbed repeatedly to the reciprocal affinity columns: GAM/IgE and RAM/IgE linked to CNBr-activated Sepharose 4B

as prescribed by the manufacturer (Pharmacia, Uppsala, Sweden). The columns were loaded in 20 mM TRIS-HCl (pH 8.2) and eluted with 0.1 M glycine-HCl (pH 2.3) and washed with 10 mM phosphate buffered saline (PBS, pH 7.2) containing 0.15 M NaCl. Apart from this adsorption to remove contaminating cross-reacting antibodies, the antisera were adsorbed on a protein A-Sepharose column (Pharmacia). This was prudent since a conjugate of protein A coupled enzyme was used for detection. For the goat catching antibody the effluent of this column was collected while for the rabbit detecting antibody the eluted fraction was retained. Both samples were dialyzed and concentrated in an ultrafiltration cell equipped with a Diaflow YM-10 membrane (Amicon, Danvers, MA), filter-sterilized through a 0.22 um filter (Millex GV, Millipore, Milford, MA) and stored in aliquots at -20°C. In some experiments, a rat monoclonal antibody (mAb) specific for mouse IgE (EM95; Baniyash and Eshhar, 1984) was used. This mAb was purified from hybridoma supernatant on a goat anti-rat Ig affinity column.

ELISA for quantitation of murine IgE

Clear polystyrene 60 well standard Terasaki trays (type 653180; Greiner, Nürtingen, FRG) were coated with 5 ul/well of 0.5-10 ug/ml of the GAM/IgE antiserum as catching antibodies in 0.1 M carbonate-bicarbonate buffer (pH 9.6) or the mAb EM95 in PBS. Alternatively, wells were coated with 0.5-10 ug/ml of the relevant antigen in coating buffer. In this and every other incubation step distilled water was applied to the tray to prevent drying. The lid on each plate was closed and plates were incubated overnight at 4°C or 3h at 37°C. After coating, plates were used immediately. Trays were washed with PBS containing 0.05% (v/v) Tween-20 (Sigma, St. Louis, MO), with PBS containing 0.02% gelatin (UCB, Brussels, Belgium; PBS-Gel) and wells were filled with PBS-Gel and incubated for 30 min at 37°C. After washing with PBS containing Tween-20 and gelatin (PBS-Tw-Gel) the plates were dried. The wells were then filled with dilutions of the serum or the standard preparations. PBS-Gel was added to some wells as a control for non-specific reaction. In this and all subsequent incubation steps plates were incubated for 30 min at 37°C. For sera in routine testing, 2 or 3.3 fold serial dilutions (in four steps) were made while for standard curves 8 serial dilution steps were assayed. After extensive washing and removal of liquid from the wells, a conjugate was applied of the enzyme β-galactosidase (Boehringer, Mannheim, FRG) linked to protein A. This reagent was either prepared ourselves (Van Soest et al., 1984) or obtained commercially (Amersham, UK or Zymed, San Francisco, CA) and was used at an optimal dilution (1/200 to 1/1000) in conjugate buffer (PBS containing 0.05% Tween-20, 10 mM $MgCl_2$ and 50mM 2-mercaptoethanol; Eastman Kodak, Rochester, NY). After extensive washing with PBS-Tw-Gel and sucking the liquid from each well, 5 ul/well (= 3.7 uM) of a solution (0.25 mg/ml) of the highly fluorogenic substrate 4-methylumbelliferyl-β-D-galactopyranoside (MUF-G; Sigma) was applied and plates were incubated precisely 60 min at 37°C. Finally, to each well 5 ul stopping solution (1 M Na_2CO_3, pH 10.4) was added and the resulting fluorescence was determined in an automatic scanning inverted microfluorimeter (Leitz, Wetzlar, FRG). The instrument is equipped with a photomultiplier and an average value current meter (De Josselin de Jong et al., 1980) and reflects the measured fluorescence intensity as an arbitrary fluorescence unit (AFU). As pipetting devices when dealing with these trays, a 60 channel 5 ul/well replicator (Biotec, Basel, Switzerland) and a 6 channel Terasaki dispenser (Hamilton, Bonaduz, Switzerland) were used. For washing the plates a washing device was constructed consisting of an air pressured bowl containing washing buffer and a spraying device that ensures washing of every well individually.

Data analysis and representation

For quantitative determination of the IgE concentration, the measured AFU values were plotted in a semi-logarithmic plot versus log dilution (i.e., absolute concentration) of the standard. Such dose-response curves are smooth, symmetrical, and sigmoidal in appearance. These curves may be described by the four-parameter logistic equation:

$$y = \frac{(a-d)}{1 + (\frac{x}{c})^b} + d$$

Here, y represents the response variable (AFU), x is the arithmetic dose (absolute IgE concentration), a is the response at high dose (upper AFU plateau), d is the response at zero dose (lower AFU plateau), c is the dose resulting in a response halfway between a and d, and b is a slope factor that determines the steepness of the curve. From this standard plot, log-logit transformation is performed:

$$\text{logit } Y = \ln (\frac{y}{1-y}) = k + (b \times \ln x)$$

in which Y is the percentage response at dilution i ($Y = 100(AFU_i/AFU_{max})$, and k is an unknown constant (De Lean et al., 1978; Beatty et al., 1987). The slope of the log-logit plot corresponds to the slope factor b of the sigmoidal dose-response curve. As a function of the AFU response, the logit equation can be rewritten as:

$$\text{logit } Y = \frac{AFU}{(AFU_{max} - AFU_i)}$$

Standard linear regression was performed on the logit values of the AFU versus the log of the standard dilutions. The dilutions of known and unknown samples at which the logit is equal to zero are then compared to determine the concentration of the unknown. This was based on the notion that the logit zero point represents half the maximum binding of the standard (Dst) as well as the sample (Dsa). Given a constant amount of coated protein and constant affinity of the bound IgE antibodies to the coat, the IgE concentration bound to the coat at D_{st} (C_{st}) should be equal to the IgE concentration in the sample (C_{sa} at the dilution D_{sa}). Thus at logit zero $C_{st} = C_{sa}$. By comparison with the known IgE content in the standard, the absolute IgE content can be calculated according to:

$$C_{sa} = (C_{st} \times D_{sa}) / D_{st}.$$

The complete data handling is micro-processor controlled.

RESULTS

Optimal ELISA-conditions

The sample volume (5 ul) used for performing ELISA in wells of a Terasaki tray, is only 5% of that of a well of a regular microtiter plate (100 ul). Other parameters also play an important role in establishing useful concentrations of reagents in a Terasaki-ELISA. When comparing a well of a standard Terasaki tray with a round bottem well of a microtiter plate with respect to the effective surface area available for coating

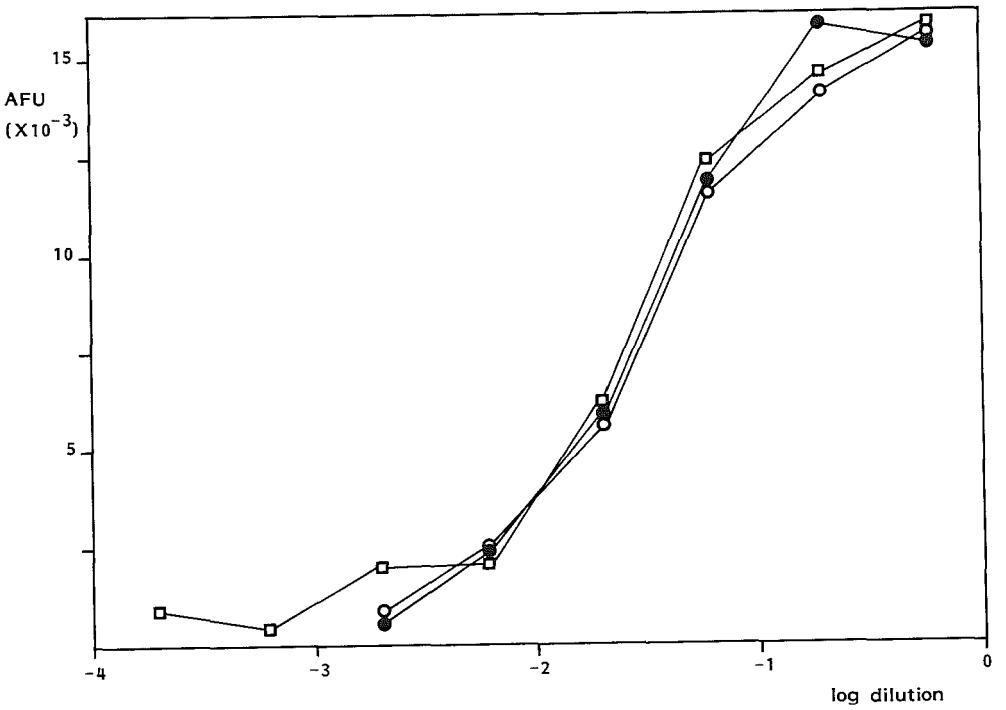

Fig. 1. Determination of the optimal coating concentration of DNP27-BSA in Terasaki-ELISA for IgE. Various concentrations (○,0.5 ug/ml; □ ,2 ug/ml; ● ,8 ug/ml) of DNP-BSA were used for coating. Subsequently, plates were incubated with several dilutions of the IgE standard. After applying the RAM/IgE detecting antiserum, the protein A-β-galactosidase conjugate, and the MUF-G substrate the fluorescence (AFU) was determined.

TABLE I

Comparison of some physical characteristics of round bottom wells of a microtiter plate and wells of a standard Terasaki tray.

	Microtiter well	Terasaki well
Liquid capacity per well (ul)	317	11.4
Available surface area per well (mm^2)	32.25	12.8
Effective coating area per volume (mm^2/ul)	0.11	1.14

Figures were calculated according to the manufacturers specifications.

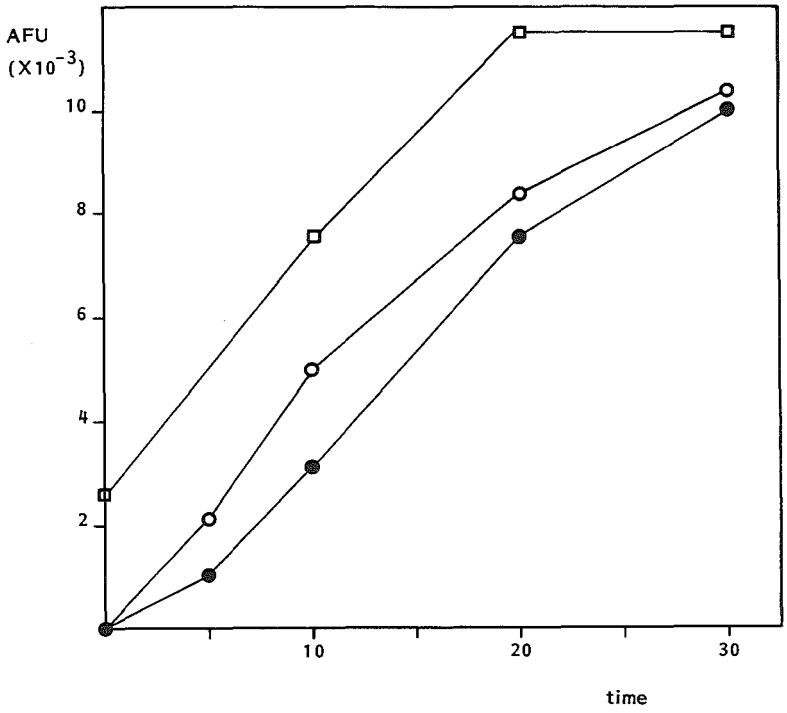

Fig. 2. Determination of the binding kinetics. Terasaki trays were charged with the IgE standard (□) and after various time intervals the amount of bound IgE was determined. Similarly, RAM/IgE was applied for various times on plates coated with either IgE standard (○) or IgE standard bound to the coated GAM/IgE catching antibodies (●). Subsequently, the bound RAM/IgE fraction was determined. The results are expressed as AFU obtained from the bound fractions.

(Table I), it can be concluded that a different geometry exists in a Terasaki well. Because of the ten times larger effective coating area per volume in a Terasaki well, apart from the optimal coating concentration, the concentrations of all subsequent reagents for performing an ELISA will also differ significantly from those used for microtiter plate based ELISA. Therefore, the optimal concentrations of all reagents to be used in a Terasaki-ELISA need to be determined emperically.

The optimal dilutions of all reagents were determined by simple titration and further refined by chequer-board titrations. The effective coating area per unit volume was that large that over a broad range (0.5-8 ug/ml) of coating concentrations of either catching antibodies (GAM/IgE) (Fig. 1) or antigen (DNP-BSA) similar readings were obtained when testing serial dilutions of several DNP-specific monoclonal IgE antibodies. Optimal concentrations of either coat were therefore set to 2 ug/ml. In a similar fashion we established the optimal concentration (12.5 ug/ml) of the detecting antibody (RAM/IgE) as well as the optimal working dilution of the protein A-enzyme conjugate (1 in 1500).

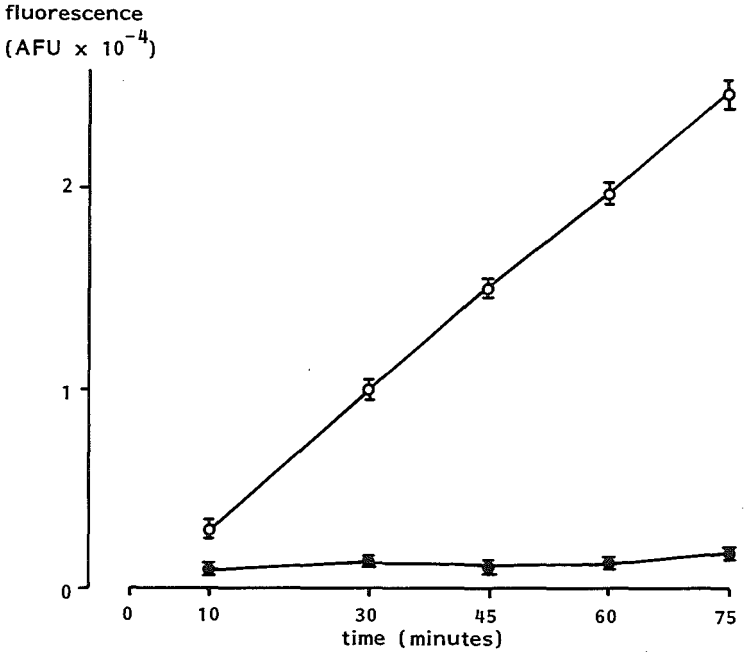

Fig. 3. Kinetics of the product formation (expressed in AFU) by incubating plates charged with a maximum concentration of IgE standard (40 ng/ml) for various periods of time (○). As a negative control plates were charged with PBS-gelatin (●).

Kinetics of the binding

Next, it was established that equilibrium was reached in this Terasaki-ELISA employing incubation times of 30 min at 37°C. To this end, a regular ELISA was performed for total IgE in which the sample incubation times were increased from 1 min up to 60 min. All other incubation steps remained constant at 30 min. The results (Fig. 2) showed a logarithmically increased signal reaching a plateau value of AFU within 10 min reaction time at 37°C.

Kinetic studies of the substrate incubation times showed a linear increase in time of the reading value of a standard IgE sample in a concentration high enough to reach the plateau value (Fig. 3). This increase was not accompanied by an increase in the negative control. From this experiment an optimal substrate incubation of exactly 60 min was selected in order to be able to compare readings from different experiments. The optimal substrate concentration was determined to be 3.7 uM of MUF-G (data not shown).

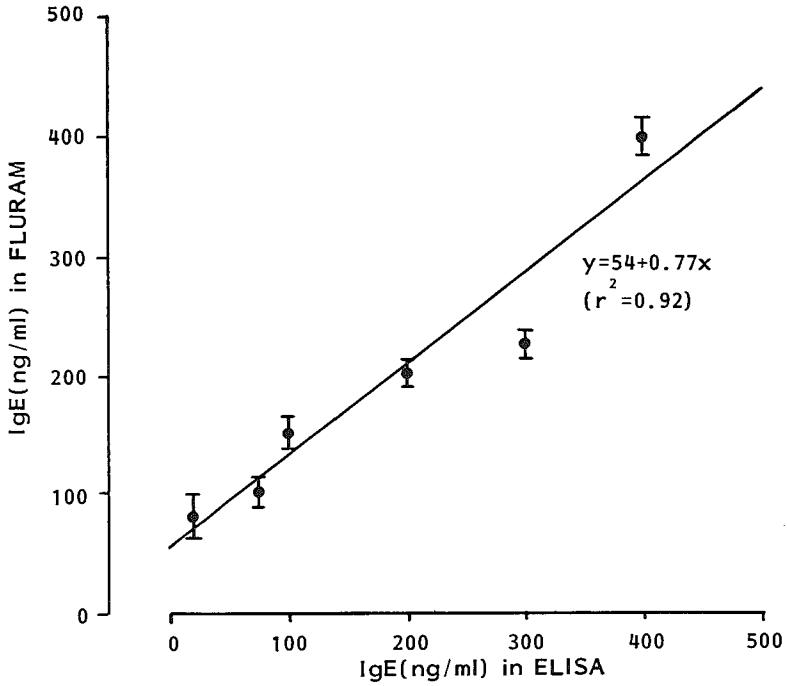

Fig. 4. Standard curve of the absolute IgE standard. The IgE content in various dilutions of the standard was assessed by both the fluorescamin based protein determination and the quantitative Terasaki-ELISA.

Standard curves

An absolute (primary) standard of hybridoma IgE was prepared to enable quantitation of total IgE. This IgE standard contained 40 ug/ml, based upon protein measurement of the purified IgE content (Fig. 4). From the readings of such a standard, a semi-logarithmic plot (Fig. 5) as well as a log-logit plot (Fig. 6) were constructed, showing a straight part over a sufficiently large range of IgE concentrations (0.04 - 20 ng/ml). For routinely performed IgE concentration determinations in mouse sera, in every assay a secondary standard consisting of a mixture of partially purified hybridoma IgE was employed.

In order to test the reproducibility of the Terasaki-ELISA, intra- and interplate variation were repeatedly determined. This was done for the antigen-specific as well as the total IgE standards. It was found that the coef ficients of the calculated concentrations were consistently less than 5%.

Concentration of serum IgE antibodies

Based upon the above mentioned standardizations, the Terasaki-ELISA was applied for measuring the IgE concentration in a number of pooled serum samples from mice of several strains (Table II). The results showed that the IgE levels differed significantly amongst the various strains.

TABLE II

Monoclonal and polyclonal IgE concentration as determined in Terasaki-ELISA

Sample	Origin	IgE content
IGEL a4 6.3	culture supernatant	31 ± 12
IGEL a4 6.5	culture supernatant	55 ± 14
IGEL b4 1	ascites	125 ± 25
IGEL b4 14.2	ascites	8250 ± 167
B53/A4	culture supernatant	40 ± 9
IgE 14-205	ascites	90 ± 16
NMS	BALB/c	328 ± 58
NMS	C3H/HeJ	421 ± 36
NMS	C3HeB/FeJ	4 ± 1
NMS	DBA/2	1250 ± 26
NMS	C57BL/6	124 ± 9

All figures represent the arithmetic mean of the IgE content in ng/ml ± 1 SD (n = 4).

DISCUSSION

This paper describes a Terasaki-ELISA for the quantitative determination of murine IgE which combines the measurement of absolute concentration with a small sample size of only 5 ul. The ELISA is performed as a sandwich ELISA employing two non-cross-reacting antisera and a conjugate of protein A linked to the enzyme β-galactosidase. Using a fluorogenic substrate and automatic fluorescence measurement and microprocessor-based data acquisition and transformation, the assay became sensitive and reproducible. The quantitative measurement is based on a standard composed of purified monoclonal IgE proteins from six different hybridomas carrying different idiotypes and allotypes.

The results obtained show that Terasaki trays are well suited to act as a matrix for the performance of an ELISA as was shown before (Pateraki et al., 1981; Van Soest et al., 1984; Labrousse and Avrameas, 1987). More important, this study reveals that because of the largely increased effective coating area (Horejsi, and Matousek, 1985), Terasaki-ELISA is especially useful for the determination of antibodies that occur in low concentrations like IgE.

It was described that up to a certain limit, a constant fraction of the proteins is adsorbed to plastic surfaces with a limit of about 1.5 ng/mm^2 for proteins like IgM and BSA (Butler, 1981). As these data were obtained in microtiter plates having an effective coating area of around 0.1 mm^2/ul (Table I), this would imply that at maximum 0.2 ug/ml of protein can be adsorbed before steric hinderance occurs. For Terasaki trays, however, the maximum protein concentration for coating is 10 times larger and it was this value of 2 ug/ml that we selected for routine coating in this Terasaki-ELISA system. Moreover, this would explain the broad range of coating concentrations that can be used. This and the fact that only seldomly

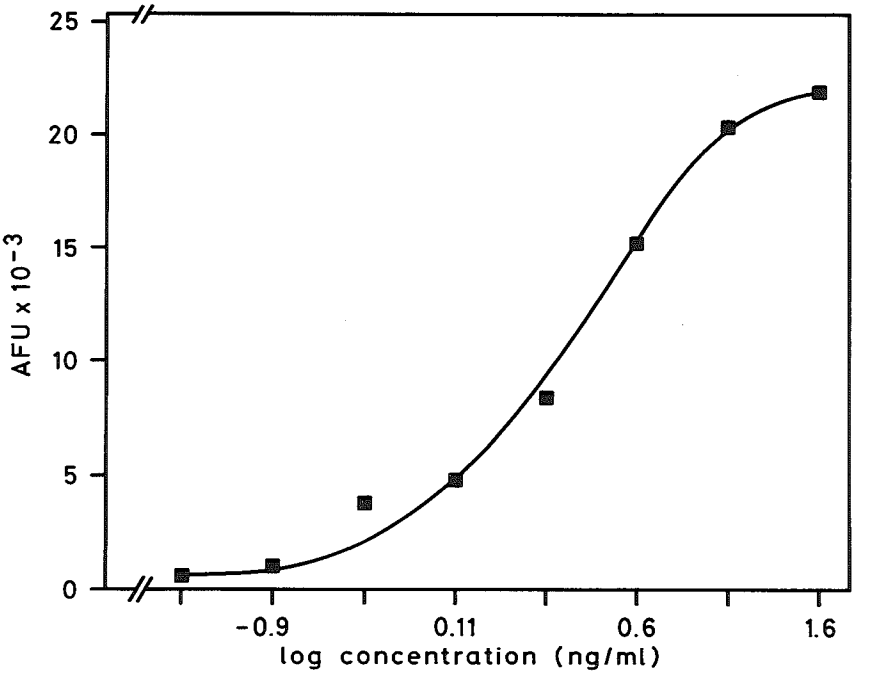

Fig. 5. Standard curve of the absolute IgE standard on plates coated with GAM/IgE catching antibodies. The data represent semi-logarithmic plot of the various concentrations (0.01 - 40 ng/ml) of IgE and the extent of binding (expressed in AFU) as determined by Terasaki-ELISA.

prozone phenomena were observed in Terasaki-ELISA, led us to believe that a largely increased density of antigenic epitopes is available for binding on these trays as compared to microtiter plates. This is in contrast to the prozone phenomenon caused by monovalent binding of antibodies to microtiter plates observed at similar antigen concentrations (Kemp and Morgan, 1986; Vos et al., 1987).

Although we have tested only few different lots of trays from one manufacturer, we never observed a disturbing variation in the binding capacity of different coating proteins to these polystyrene matrices. Such variation has been described as an edge effect for microtiter plates (Murphy et al., 1980; Shekarchi et al., 1984).

Blocking with gelatin was found to be essential after the coating. We have tested several blocking agents similar as described for microtiter plates (Vogt et al., 1987) and found a low concentration of gelatin most efficient.

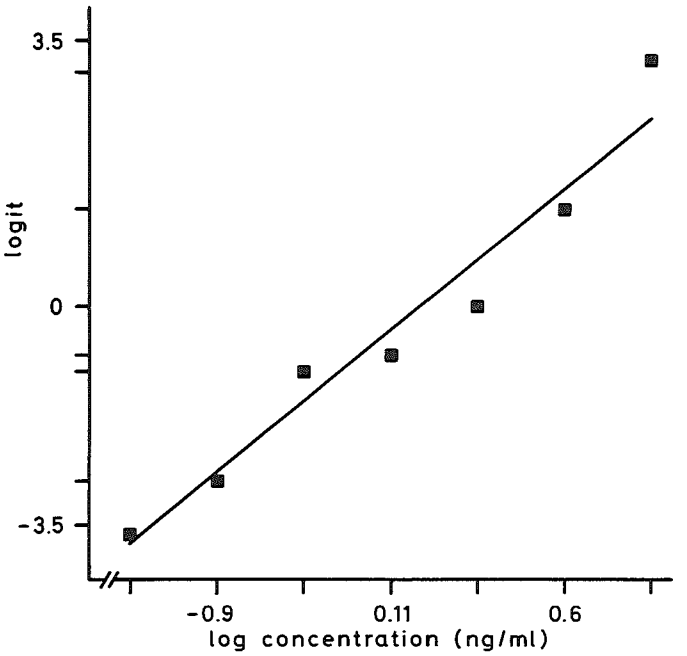

Fig. 6. Log-logit transforms of the data of the standard curves plotted in Fig. 5. Logarithms of the IgE-concentrations at various dilutions of the standard (0.01 - 40 ng/ml) are plotted against the logit of the corresponding AFU readings.

The sensitivity as well as the specificity of the assay are largely dependent upon the detecting antibody employed. As described in detail in a previous study (Savelkoul et al., accompanying paper), the specificity of the RAM/IgE antiserum employed has been tested extensively in inhibition type Terasaki-ELISA. Apart from showing that the antiserum employed is specific for IgE, it was shown that it is also free of any cross-reactivity based on anti-idiotypic, anti-allotypic or anti-light chain contamination.

Detection in this Terasaki-ELISA is based on a conjugate of β-galactosidase labelled protein A. Protein A is especially useful in ELISA as it can behave as a purified anti-antibody of restricted specificity without species specificity (Engvall, 1978; Surolia and Pain, 1981; Langone, 1982). Such an ELISA can be used for assaying Ig without binding affinity for protein A, e.g. rat monoclonal antibodies directed against cell-surface determinants (Van Soest et al., 1984) or murine IgE (this study).

The present study shows that an incubation time of 30 min at 37°C was sufficient for all binding steps to reach binding equilibrium in this system. This, together with the large coating efficiency of Terasaki trays,

allowed determination of the affinity of IgE antibodies in ELISA. This is described in a subsequent paper (Pathak and Savelkoul, accompanying paper).

By employing β-galactosidase labelled conjugates and fluorometry as a measuring device, the ELISA can be made quantitative to the level at which IgE antibodies occur in mouse serum. It has been described that fluorometric methods offer a 1000-fold higher sensitivity than colorimetric methods (Shalev et al., 1980). In choosing a suitable combination of a fluorogenic substrate and one of the enzymes alkaline phosphatase or β-galactosidase as a label, Ishikawa and Kato (1978) and Neurath and Strick (1981) chose the latter although the β-galactosidase activity for 4-methylumbelliferyl-β-D-galactoside (MUF-G) is lower than the alkaline phosphatase activity for 4-methylumbelliferyl phosphate (MUF-P). The reason that they preferred β-galactosidase in combination with MUF-G was that MUF-P has a significantly higher fluorescence background level than MUF-G. This is probably due to nonenzymatic hydrolysis of MUF-P (Neurath and Strick, 1981).

A further necessity for quantitation in ELISA is an absolute standard for calibration of the assay. Such a standard should consist of a known amount of highly purified and intact IgE (Savelkoul et al., 1987).

Moreover, to adapt the method for broad applicability, microprocessor-based analysis is necessary to compare essentially sigmoidal dose-response curves (when the dose is portrayed on a logarithmic scale) of standard and individual test samples. Irrespective of the type of ELISA and probably general for ligand-binding assays, a logistic equation is applicable (Rodbard and McClean, 1977; De Lean et al., 1978; Fernandez et al., 1983). Therefore, this method has become standard for radioimmunoassays and is recently also adopted for the analysis of quantitation in ELISA (Rodbard and McClean, 1977; Fey, 1981; Beatty et al., 1987). This method has the advantage that the absolute concentration of IgE in a sample translates the position of the dose-response curve on the log scale. The method minimizes the influence of the affinity of the sample to the coat and for this reason the shape of the dose-response curve is not significantly different between a sample and the standard or between various samples. This Terasaki-ELISA system has a detection limit that is low enough and a linear measuring scale that is large enough to allow quantitative determination of IgE in mouse serum based upon the log-logit transformation of the data.

It is anticipated that this Terasaki-ELISA system is applicable in various situations in which low concentrations of protein molecules have to be quantitatively determined in small sample volumes in a accurate and reliable way.

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CHAPTER 4.5

RAPID PROCEDURE FOR COUPLING OF PROTEIN ANTIGENS TO RED CELLS TO BE USED IN PLAQUE ASSAYS BY PREWASHING IN CHROMIUM CHLORIDE

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SUMMARY

A rapid and efficient procedure is described for the coupling of proteins (protein A, ovalbumin, albumin and chicken gamma globulin) to sheep red blood cells (SRBC) to be used in antigen-specific or protein A plaque assays. The modification of the original procedure has three distinct features: prewash of the red cells with a low concentration of essentially freshly prepared $CrCl_3$, use of a relatively high concentration of $CrCl_3$ in the reaction mixture and a coupling time of only 4 minutes. Protein A plaque assays performed with such target cells have the same sensitivity as those employing red cells coupled with protein A according to the original procedure. Studies with hybridomas specific for a protein antigen showed that antigen-specific plaque assays employing target red cells coupled with the protein antigen according to the modified procedure have the same sensitivity as the protein A plaque assay. The modified procedure greatly facilitates cellular studies on antibody formation after immunization with protein antigens.

INTRODUCTION

Hemolytic plaque assays are widely used to study antibody synthesis at the single cell level. This holds for the orginal direct type as well as the indirect type plaque assay to study secretion of antibodies of IgM and all other immunoglobulin (Ig)-classes, respectively (Jerne et al., 1974). The protein A plaque assay made it possible to enumerate Ig-secreting cells regardless of the antibody specificity of the Ig (Gronowicz et al., 1976). We have previously shown that plaque formation in this assay is facilitated by using Ig-depleted guinea pig serum as a source of complement (Van Oudenaren et al., 1981). For use as target cells in this indirect plaque assay, protein A is routinely coupled to sheep red blood cells (SRBC) by the chromium chloride ($CrCl_3$) method. To achieve sufficient coupling and to prevent clotting of coupled erythrocytes, incubation times ranging from 4 minutes at room temperature or 1.5 hour at 37°C up to overnight incubation at 4°C are used. All procedures, however, have in common the use of an aged solution of $CrCl_3$.

Studies establishing the magnitude of an immune response to various types of antigens as determined by antibody-secreting plaque-forming cell (PFC) numbers, are mostly restricted to hapten-carrier coupled antigens. This is mainly due to the difficulty to get protein antigens reproducibly coupled to the target SRBC, whereas hapten-coupling of SRBC is sufficiently standardised by a variety of ways (Rittenberg and Pratt, 1969; Inman et al., 1973; Pohlit et al., 1979).

Here we report a modification of the protein coupling method which enables the coupling of a sufficient amount of protein A and other protein antigens like ovalbumin (OA), rat albumin and chicken gamma globulin (CGG). This coupling is achieved within 4 minutes at room temperature using freshly prepared $CrCl_3$ solution.

MATERIALS AND METHODS

Coupling of proteins to SRBC

SRBC kept in Alsever's solution at 4°C for periods from 1 to 3 weeks were washed three times (5 min, 1500 g) in 10 vols of saline (0.15 M NaCl). Concordant with the originally described procedure (Gronowicz et al., 1967), the coupling mixture consisted of 1 ml washed packed SRBC, 1 mg of protein A (Pharmacia, Uppsala, Sweden) in saline (1 mg/ml), 9 ml saline and 50 ul of an aged solution of $CrCl_3.6H20$ (0.05 M in saline). The mixture was incubated for 1.5 h at 37°C, with shaking every 15 min. Thereafter 5 vols of saline were added to the mixture and the cells were washed 3 times with saline and finally resuspended to 17% (volume per volume) in Dulbecco's modified balanced salt solution (BSS). The protein A coated SRBC were used within 3 days of preparation.

In the new procedure described here, SRBC were washed four times (5 min, 1500 g) in 10 vols freshly prepared $CrCl_3$ in saline (12.5 ug/ml). For coupling, 1 ml of washed and packed SRBC, 1 ml of protein A in saline (1 mg/ml) and 1 ml freshly dissolved $CrCl_3$ in saline (1 ug/ml, pH 2.9) were mixed and incubated for 4 min at room temperature. Subsequently, 20 vols 1% fetal calf serum (FCS; Flow, Irvine, Scotland) in saline were added and the cells were washed in this solution 3 times for 5 min at 600 g. Finally the cells were resuspended to 17% (v/v) in BSS and used within 3 days of preparation. For use in the antigen-specific plaque assay, ovalbumin (OA; grade V, Sigma, St. Louis, MA, U.S.A), rat albumin (Calbiochem, San Diego, CA, U.S.A.) and chicken gamma globulin (CGG; Calbiochem) were coupled employing

the same above mentioned new procedure. For inhibition studies, ^{125}I-labelled protein A (specific activity 1.11 GBq/mg; Amersham, Little Chalfont, UK) and ^{125}I-labelled ovalbumin (specific activity 1.11 GBq/mg; New England Nuclear, Dreiech, F.R.G.) were coupled to SRBC using both above described coupling procedures.

Cell suspensions

As a source of antigen-specific Ig secreting cells several murine Ig secreting hybridomas were used: AFLA IV 5-10/4 (gift of Dr. N.A. Bos), M1F5 (gift of Dr. M.P. Mulder) and 408.61 (gift of Dr. F. Lubbe). The specificity of these hybridomas are : ovalbumin, rat albumin and chicken gamma globulin, respectively. These cells were cultured in fresh RPMI 1640 medium supplemented with L-glutamine (4 mM), pyruvate (0.1 M), penicillin (100 IU/ml), streptomycin (50 ug/ml), 2-mercaptoethanol (5×10^{-5} M) and 10% FCS.

Female BALB/c mice, 6 months old, were purchased from OLAC Ltd., Bicester, Oxon, U.K. Upon dissection these mice were free of pathological abnormalities. Cell suspensions from spleen and femoral bone marrow were prepared as described before (Benner et al., 1981). Cells were washed and resuspended in BSS. Total nucleated cells were counted with a Coulter Counter model BZI (Coulter Electronics Ltd., Harpenden, Herts, U.K.). For each assay spleen cells and bone marrow cells from two donor mice were tested separately.

The number of viable nucleated cells was determined in a haemocytometer using 0.2% trypan blue in saline. The viability of the hybridoma cell culture was more than 80%.

Plaque assays

The protein A plaque assay and the antigen-specific plaque assay were performed in BSS using Cunningham type chambers (Jerne, 1974). Cell suspensions were appropriately diluted in BSS. Cell suspensions of spleen and bone marrow were resuspended in 5 ml, and dilutions ranging from 1/5 to 1/80 were assayed. Hybridoma cell suspensions were diluted to 10^3 till 10^4 living cells per ml. 100 ul of the dilution was mixed with 15 ul guinea pig complement (Behringwerke, Marburg/Lahn, F.R.G.) purified (Van Oudenaren et al., 1981) over a protein A-Sepharose column (Pharmacia, Uppsala, Sweden), 15 ul of a 17% suspension of protein A- or ovalbumin-coated SRBC, respectively, and 15 ul of an optimal concentration (around 50 ug/ml of a high titre antibody) of rabbit anti-mouse-Ig antiserum diluted in phosphate buffered saline (PBS). The specific rabbit-anti-mouse IgM serum and rabbit-anti-mouse IgG serum used (kindly prepared and provided by A. van Oudenaren from our laboratory), were purified over a protein A-Sepharose column before use. The plaque assay slides were incubated for 3.5 h at 37°C and from 2 till 16 h at room temperature with protection from direct light. The Ig-secreting plaque-forming cells (PFC) were counted under a dissecting microscope with dark field illumination.

Statistical evaluation

PFC numbers are expressed as the mean number per slide $\pm$ 1 SEM. The mean numbers of PFC are compared using the two tailed Student's t-test for comparison of means with a two-sided 5% confidence limit. Comparisons between groups of differently coupled SRBC were based on the Wilcoxon rank-sum test.

RESULTS

In order to establish optimal concentrations of all reagents to be employed in the new coupling procedure described here, checquer-board titrations were performed for coupling OA to SRBC. Therefore, a freshly prepared $CrCl_3$ solution (0.05 M), OA (1 mg/ml) and a 17% SRBC solution (prewashed in 12.5 ug/ul $CrCl_3$) were mixed in various volume ratios. After the coupling reaction the amount of protein coupled to the SRBC was determined by the fluorescamine assay (Udenfriend et al., 1972). The results indicated that OA can be increasingly coupled to SRBC. The highest coupling efficiency was obtained by mixing the reagents in a 1:1:1 ratio (data not shown). Independent of the protein concentration in the reaction mixture, in the absence of $CrCl_3$, no aspecific protein adsorption to the SRBC occurred. Another controversial parameter in these coupling reactions is the age of the SRBC employed. We found SRBC used within 7 to 14 days after collection and stored at 4'C most suitable. The reproducibility of the coupling reaction resulted in only a 5% SD (n=6) in ug protein bound by 10^8 SRBC (data not shown).

The coupling efficiency of proteins was compared in the two coupling procedures as follows. Reaction mixtures were prepared consisting of a constant number of SRBC (0.5 ml packed cells), varying volumes of a 1 mg/ml solution of protein A or OA , 5 ul 125-iodine labelled protein A (3.7 MBq/ml) or 5 ul 125-iodine labelled OA (3.7 MBq/ml) and depending on the coupling procedure either 500 ul freshly prepared $CrCl_3$ (1 mg/ml) or 50 ul aged $CrCl_3$ (0.05 M). The results (Table I) indicate that when the ratio cold protein : SRBC decreased, both coupling procedures showed a parallel increase in uptake of labelled protein. For OA only the new coupling procedure gave consistent results. The similarity in the slopes of the inhibition curves for the uptake of labelled protein A and OA suggests that different proteins are bound to SRBC with a comparable efficiency.

TABLE I

Coupling efficiency as determined by uptake of radiolabelled protein A or OA to SRBC together with varying volumes of cold protein A or OA using two different coupling procedures.

Protein A or OA (ul)	Original procedure for preparing protein A-SRBC	Rapidly coupled protein A-SRBC	Rapidly coupled OA-SRBC
0	100	100	100
50	100 ± 1	100 ± 1	97 ± 1
100	97 ± 1	100 ± 1	95 ± 4
200	92 ± 3	77 ± 2	76 ± 3
300	73 ± 2	75 ± 3	71 ± 2
400	58 ± 4	57 ± 2	49 ± 6
500	53 ± 6	35 ± 5	31 ± 4

Results are expressed as percentage (± 1 SD; n=4) of a constant amount (5 ul of 3.7 * 10^6 Bq/ml) of ^{125}I-labelled protein A or ^{125}I-labelled OA that could maximally be bound to SRBC in competition with an increasing amount (ul) of cold protein A and cold OA (1 mg/ml), respectively.

TABLE II

Haemolysis of OA-coupled SRBC by OA-specific antibodies and its inhibition

Volume OA added (ul)	Haemolysis (%)	Haemolysis inhibition (%)
0	16 ± 2[1]	62 ± 2[2]
12.5	19 ± 1	41 ± 3
25	29 ± 2	24 ± 2
50	41 ± 2	22 ± 2
75	54 ± 3	N.D.
100	61 ± 2	15 ± 5

[1] Results are expressed as percentage lysis ± 1 SD (n=4) of 10^8 cells in a volume of 2.5 ml.
[2] Results are expressed as percentage lysis ± 1 SD (n=4) of 10^8 cells in a volume of 2.5 ml after addition of increasing amounts of OA (1 mg/ml).
ND = not determined.

TABLE III

Percentage of Ig secreting cells in hybridoma cultures of AFLA IV 5-10/4, M1F5 and 408.61 cells as determined by antigen-specific and protein A plaque assays using rapidly coupled SRBC and SRBC coupled with protein A according to the original procedure.

Hybridoma	Rapidly coupled antigen-SRBC	Rapidly coupled protein A-SRBC	Protein A-SRBC according to the original procedure
AFLA IV 5-10/4	11 ± 3	12 ± 7	6 ± 5
M1F5	28 ± 7	26 ± 3	22 ± 3
408.61	18 ± 6	28 ±12	15 + 7

Results were obtained by analyzing all three hybridoma cultures in antigen-specific and protein A plaque assays and calculating the percentage of secreting cells at various cell densities (10^3 to 10^4 cells/ml). The results were expressed as mean percentage ± 1 SEM (n=6). All antigen-specific plaque assays were compared with both types protein A plaque assays for every hybridoma by the two-tailed Student t-test (α= 0.05). No significant differences were found for each of the hybridomas when tested in the three assays.

TABLE IV

Comparison of the number of protein A PFC in spleen and bone marrow of BALB/c mice using rapidly coupled protein A-SRBC and protein A-SRBC prepared according to the original procedure.

Test sample	Isotype	Rapidly coupled prot. A-SRBC	Protein A-SRBC according to the original procedure	P-value
Spleen	IgM	221 ± 11	252 ± 19	0.19
	IgG	133 ± 20	100 ± 19	0.14
Bone Marrow	IgM	35 ± 2	42 ± 2	0.07
	IgG	69 ± 6	68 ± 3	0.89

Results after using both coupling procedures were compared by the two-tailed Student t-test (= 0.05). Results were expressed as mean number of PFC per slide ± 1 SEM (n=8). The incidence of IgM- and IgG-PFC using routinely coupled target cells was 3.02 and 2.65 * 10^5 for spleen and 4.17 and 6.75 * 10^5 for total bone marrow, respectively. When assayed with rapidly coupled target cells the incidence of IgM- and IgG-PFC was 2.76 and 1.66 * 10^5 for spleen and 3.47 and 6.48 * 10^5 for total bone marrow, respectively.

Most important, the protein coupled SRBC should be lysable by antibodies and complement in order to be of use in plaque assays. Therefore, haemolysis was evaluated after mixing increasing amounts of a hyperimmune OA-specific mouse serum with OA-SRBC and guinea pig complement. To verify whether the haemolysis was caused by antibodies recognizing the OA of the OA-SRBC, the haemolysin reaction was blocked by the addition of free OA. These reactions were performed essentially as described (Koch et al., 1982). The results show that OA coated SRBC can act as target cells for haemolysis that is caused by antigen-specific antibodies (Table II).

To determine the suitability of the protein-coupled SRBC in antigen-specific plaque assays we used cultured antigen-specific Ig secreting hybridoma cells. The hybridomas used were the OA-specific AFLA IV 5-10/4 (IgM,K), the rat albumin specific M1F5 (IgG1,K) and CGG-specific 408.61 (IgG1,K). The same hybridomas were tested in the protein A plaque assay to establish the concordance between the numbers of antigen-specific PFC and protein A PFC. Therefore, various numbers of viable nucleated hybridoma cells, ranging from 200 till 1000 cells/100 ul, were tested using antigen-coated SRBC as well as protein A-SRBC coupled with the two different procedures. As shown in Table III, no significant differences ($p > 0.05$) were found between the numbers of antigen-specific PFC and protein A-PFC using rapidly coupled protein A-SRBC on one hand and protein A-SRBC prepared according to the original procedure on the other hand. This indicates that plaque assays employing the rapidly coupled indicator cells have the same sensitivity as those making use of target cells coupled according to the

original procedure. Moreover, the same coupling procedure could be used to couple protein antigens and protein A. The sensitivity of all three antigen-specific plaque assays as well as both types of protein A plaque assays were not affected by the total number of PFC counted, because of the linear relationship between the PFC numbers on one hand and the input of hybridoma cells on the other hand (data not shown).

The sensitivity of protein A plaque assays employing protein A coated target SRBC prepared according to the two different procedures was also compared by using freshly prepared single cell suspensions of spleen and bone marrow. From each cell suspension a 100 ul appropriately diluted sample was tested in duplicate for IgM and IgG secreting cells. Plaques were developed by incubating the slides for 3.5 hours at 37°C. During this incubation time, examination of the slides every 15 minutes did not reveal any difference in the rate of development of the plaques between both batches of protein A-SRBC. The mean numbers of plaques (Table IV) counted in the slides with the rapidly coupled indicator SRBC cells and in the slides with the indicator SRBC prepared according to the original procedure did not show significant differences ($p>0.05$). This holded for both cell suspensions and for both isotypes. Also no difference was observed in microscopical appearance of the plaques developed by the two batches of protein A-SRBC.

DISCUSSION

Many methods have been tried to couple protein antigens to SRBC for use in plaque assays. Most of the routinely used coupling agents for proteins e.g. glutaraldehyde, maleimide, etc., cannot be used for the coupling of protein antigens as antibody-binding capacity is substantially reduced. Therefore, studies regarding antigen-specific immune responses are often restricted to hapten-carrier complexes merely because of the ease of performing hapten-specific plaque assays. The aim of this study was to make the routine method of protein A coupling to SRBC (Gronowicz et al., 1976) applicable to various proteins, in order to enable plaque assays for protein-specific immune responses in mice.

The simplest procedure for coating red cells with proteins like ovalbumin, albumin, chicken gamma globulin and protein A and the one requiring the lowest amount of protein, is the one that makes use of the chromium chloride coupling procedure of Gold and Fudenberg (1967). $CrCl_3$ is believed to activate carboxyl residues of proteins in the red cell membrane (Faulk and Houba, 1973), thus providing active sites for binding of protein antigens. The coupling conditions that produce indicator red cells of optimal sensivity must be found empirically. We found that all three parameters ($CrCl_3$, OA and SRBC) individually affect the final coupling efficiency according to an optimal titration curve. With the stock solutions used, equal volume ratios turned out to be most effective. For a given number of SRBC, the final concentration of $CrCl_3$ and that of the desired protein to be coupled must be kept constant (Kofler and Wick, 1977). In both procedures described here, 1 mg of protein A or ovalbumine was used per 1 ml of packed SRBC. From $CrCl_3$, however, in the original coupling mixture 0.66 mg is used, whereas in the alternative method presented here 1 mg is used. In the original method the final $CrCl_3$ concentration is 0.063 mg/ml (0.24 mM) whereas in the modified method it is 0.33 mg/ml (1.2 mM). It is also important to note that in the modified method presented here freshly pre-

pared $CrCl_3$ can be successfully used.

In most methods described, the $CrCl_3$ solution to be employed was either buffered (Perucca et al., 1969; Sweet and Welborn, 1971; Truffa-Bachi and Bordenave, 1980) and/or "aged" (Ling et al., 1977; Burns and Pike, 1981; Goding, 1976; Gronowicz et al., 1976). A substantial disagreement exists between the various coupling procedures with regard to the reasons why freshly prepared $CrCl_3$ solutions give lower and more variable coupling efficiencies (Goding, 1976; Kofler and Wick, 1977) and why aging of the $CrCl_3$ solution increases the ability to buffer the solution for a prolonged period. Moreover, in the original procedure for preparation of target red cells for plaque assays, long incubation times of 1 to 1.5 hours at 37°C were prescribed to saturate all protein binding sites on the red cell membrane and to prevent clumping. Sensitization of the erythrocytes by prewashing with a low concentration of freshly prepared unbuffered $CrCl_3$ makes the binding sites more easily available for coupling so that a coupling time of 4 min suffices. Washing the cells immediately after coupling with a 1% FCS solution, effectively prevents clumping of the red cells.

Most important, SRBC that are optimally sensitized for use in haemagglutination are often inappropriate for plaque assays. Such cells have a low density of antigenic determinants so that the rate of attachment of the secreted antibody molecules to the sensitized red cells is too low to cause complement dependent lysis. Necessity for a high degree of coupling may vary with the stage of the immune response (i.e. the affinity of the antibody) and the Ig class being assayed by PFC. Therefore, described rapid coupling procedures for use in antigen-specific haemagglutination tests (Gold and Fudenberg, 1967; Goding, 1976; Kofler and Wick, 1977), need not to be applicable for plaque assays. Moreover, it is essential to verify whether protein-coupled SRBC are specifically recognized by antigen-specific antibodies and whether this complex is able to cause complement-dependent cytolysis of the target cells (Tables I and II). In the experiments described here the specificity of this reaction was shown by the blocking of the hemolysis reaction by competing free antigen.

The data presented in this paper show that the herewith proposed procedure for the coupling of protein antigens to SRBC is appropriate for use in plaque assays (Tables III and IV), so that ongoing protein antigen-specific immune responses can be evaluated. It is expected that by this procedure other proteins can be equally well coupled to red cells for use in antigen-specific plaque assays.

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CHAPTER 5

INDUCTION OF IgE-ANTIBODY FORMATION

CHAPTER 5.1

INTRODUCTION: ANALYSIS OF IgE FORMATION

Investigation for the requirements for induction of IgE antibody formation in vivo was one of the principal aims of the work described in this thesis. Moreover, as reviewed in Section 1.3.4.7, the antigen-specific IgE antibody formation is accompanied by a non-specific IgE response. Such antigen-non-specific responses have been described for the other isotypes as well [1,2]. It was therefore prudent to analyze during ongoing immune responses the ratio of the specific to the non-specific IgE responses. To this end, the techniques described in Chapter 4 had to be developed and tested for their specificity and sensitivity.

Employing the Terasaki-ELISA system for the quantitative determination of IgE concentrations, sera of mice were analyzed that were expected to differ in their total IgE content. To this end, this particular immunization scheme was employed to compare the results of previously determined relationships [3]. It was described that serum Ig levels in the mouse are to a large extent dependent on the antigenic load of the respiratory and digestive tract [4-7]. This is apparent from studies in which serum Ig levels were determined in mice raised under germfree (GF), specific pathogen free (SPF) and conventional conditions. It was found that in SPF and even more in GF animals, the serum levels of IgG_1, IgG_2 and IgA were at least 20-times lower as compared to sera of mice kept under conventional conditions. These serum levels do not fully reflect the Ig-synthesizing activity of the immune system. This is due to the the different half-lives of the various isotypes and the release of a portion of the synthesized Ig in excretions and extravascular fluids. The serum levels of IgM in these mice were not significantly different. No data on serum IgE levels in these mice were available. It is therefore that the studies described in Section 5.2 were undertaken.

Most studies on the induction, formation and regulation of murine IgE antibody responses have relied heavily on the determination of circulating IgE levels using PCA tests [3]. A more direct analysis of IgE synthesis should involve measurement of IgE production at the cellular level, i.e. the stage of the IgE-secreting cell [8]. The sparsely available information on the frequency of IgE-secreting cells and their distribution in lymphoid organs has been obtained in studies using immunofluorescence techniques and heterologous adoptive cutaneous anaphylaxis [9]. These procedures, however, tend to be rather cumbersome and do not lend themselves readily to the enumeration of IgE-secreting cells. The availability of plaque assays for total and antigen-specific IgE-secreting cells (Section 4.5) has made such analysis possible. As outlined in Section 1.3, several parameters relevant to the induction of specific IgE responses in vivo have been analyzed for their effect on the serum IgE levels. Based on these studies several immunization protocols have been formulated for the persistent induction of serum IgE. This situation is regarded as being representative to the human atopic state (Section 1.1). Few studies, however, have been conducted to

the cellular aspects of an ongoing IgE immune response. Those studies have already indicated a lack of correlation between specific and total IgE levels in the serum of mice during an IgE response [3]. Based upon these findings the question arose whether the humoral IgE response is indeed reflected in the response of IgE-secreting cells upon immunization. Therefore, the study described in Section 5.3 was undertaken. Optimal immunization schemes were established with regard to various types of antigens, route of administration and use of adjuvant. Of all parameters the kinetics and the distribution of the IgE-secreting cells over the various lymphoid organs was correlated with the serum levels of both antigen-specific and total IgE. A discrepancy was found between the occurrence of substantial numbers of antigen-specific IgE-secreting cells in various lymphoid organs after immunization and accumulation in the serum of antigen-specific IgE antibodies. At the time when antigen-specific IgE antibodies became detectable in the serum only the peritoneal cavity contained substantial numbers of IgE-secreting cells of which the majority were antigen-specific. It was therefore suggested that the peritoneal cavity contained the antigen-specific IgE-secreting cells that were responsible for the late occurring but persistent antigen-specific IgE antibodies in the serum.

Antigen-specific interactions between cells are mediated by hapten-carrier bridges, and the regulation is conducted through recognition of defined idiotopes by antigen-specific receptors on B and T lymphocytes. The receptors can be defined by their idiotype. Common idiotypic determinants among IgE and other Ig classes were demonstrated in the response to the synthetic polypeptide L-glutamin acid-L-alanin-L-tyrosine and to the p-phenylarsonate hapten [10,11]. In the study described in Section 5.4, an analysis is described of the localization of the sustained IgE antibody response to the PC hapten, and its idiotype expression and susceptibility to anti-idiotype induced suppression.

The response to the PC hapten p-azo-phenyl-phosphorylcholine is characterized by the formation of two distinct antibody populations which express preferred reactivity to PC (group I antibodies) and PC-phenyl (group II antibodies), respectively [11-15]. Expression of the T15 idiotype has been found to be associated with group I but not with group II antibodies. A comparison of amino acid sequences of light chains from serum group I and II antibodies with the sequence of selected monoclonal proteins demonstrated that each antibody population possesses a high degree of homogeneity, being not related to each other [16]. The anti-PC response of mice immunized with Streptococcus pneumoniae R 36A (Pn) consists of group I anti-PC IgM, IgG_3 and IgG_1 antibodies [17]. By immunization with PC conjugates of T dependent antigens, such as KLH and OA, initially group I antibodies were expressed, while later on group II anti-PC IgG_1, IgG_{2a}, IgG_{2b} and IgE antibodies were produced. Thus anti-PC IgE antibodies are found exclusively among the group II population and they do not express the T15 idiotype. However, suppression of the anti-PC IgE response could be achieved by monoclonal anti-T15/HOPC-8 antibodies [18]. It remains to be established whether this is due solely to crossreactivity of T15 anti-idiotypic antibodies.

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CHAPTER 5.2

MODULATION OF TOTAL IgE LEVELS IN THE SERUM OF NORMAL AND ATHYMIC NUDE BALB/c MICE BY T CELLS AND EXOGENOUS ANTIGENIC STIMULATION

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ABSTRACT

Several different grades of T system impairment were studied for their effect on the total serum IgE concentration in BALB/c mice. Homozygous athymic nu/nu mice and their heterozygous nu/+ littermates were compared for serum IgE levels while kept under either barrier-maintained or conventional conditions. The results show a paradox between the T cell dependency of the IgE immune response and the increased levels of serum IgE in the absence of T cells. Both barrier-maintained and conventionalized nu/nu mice have at least twofold increased serum IgE levels as compared to nu/+ mice. With age, IgE levels increased faster and reached higher plateau values in nu/nu than in nu/+ mice. Moreover, after adult thymectomy of BALB/c mice serum IgE levels increased up to 15 fold at 4 months of age, while infusion of immunocompetent T cells in nude mice resulted in a 2 to 5 fold decrease of the IgE level.

INTRODUCTION

Serum immunoglobulin (Ig) concentrations in mice vary widely according to genetic background, age, exogenous antigenic load and the development of the thymus dependent limb of the immune system. Synthesis of the various Ig isotypes requires different degrees of T cell help (Katz et al., 1974; Van Muiswinkel et al., 1976). Thus, genetically athymic nude mice have normal or enhanced levels of IgM, while levels of IgG_1, IgG_2 and IgA are severely reduced in most animals (Wortis, 1974; Holland et al., 1978). However, in BALB/c nude mice a hyperglobulinemic state for IgG_1 can occur especially after helminth infection (Haaijman et al., 1979; Lebrun and Spiegelberg, 1987).

In thymus-bearing animals it was suggested that the regulation of IgE formation is dependent on the presence of helper and suppressor T cells (Ishizaka, 1987; Marcelatti and Katz, 1987). In young BALB/c nude mice, no IgE was detectable in the serum (Ito et al., 1979; Lebrun and Spiegelberg, 1987). No quantitative data on the influence of exogeneous antigenic load on the serum IgE levels are available. Generally, the serum concentration of IgE depends on the rate of synthesis and the rate of catabolism, which both might be affected by the genetic background and the exogenous antigenic load of an individual (Haba, Ovary and Nisonoff, 1985).

Recently, it was shown that helper T cells can be divided in two subsets based on their lymphokine production (Mosmann et al., 1986). One subset of helper T cells (Th2) produces interleukin-4 (IL-4) that selectively enhances the production of IgE both in vivo and in vitro. Interferon-gamma, produced by the other T helper subset (Th1), blocks completely the IL-4 induced IgE synthesis in vitro (Coffman and Carty, 1986). It was suggested that the relative numbers or states of activation of Th1 and Th2 cells determine the regulation of the IgE synthesis (Coffman et al., 1988).

So far measurements of IgE production in mice have been mainly based on the passive cutaneous anaphylaxis (PCA) reaction, which is reliable but only semi-quantitative since it can only establish relative IgE levels depending on a minimal inflammatory skin reaction (Lehrer, 1982). The development of antigen-specific mouse IgE-secreting hybridomas allowed the production of anti-IgE antisera suitable for use in enzyme-linked immunosorbent assays (ELISA) (Lang et al., 1976; Hill and Liu, 1981; Giallongo et al., 1982; Bozelka et al., 1982). With the development of ELISA specific for IgE, quantitation of antigen-specific and total IgE levels in mouse serum became feasible (Maekawa and Ovary, 1984; Savelkoul et al., 1985; Kemeny et al., 1985; Gavériaux et al., 1986).

This study was undertaken to study whether the T-B cell imbalance in nude mice affects the serum IgE level. After having established that the serum IgE level in nude mice is substantially higher than in their heterozygous littermates, we anticipated that thymectomy of normal mice and infusion of immunocompetent T cells into nude mice should have contrasting effects on the total IgE concentration. This study confirms these predictions. The paradox between the T cell dependency of the IgE response and and the increased IgE levels in the absence of T cells on the other hand is discussed.

MATERIALS AND METHODS

Mice

Female nude athymic mice (nu/nu) and their heterozygous littermates of BALB/c mice were bred and maintained in the Radiobiological Institute TNO,

Rijswijk, The Netherlands. All nude and heterozygous mice were raised pathogen free and barrier-maintained. Several cohorts of these barrier maintained mice were transferred to conventional facilities at various ages. The other nude and heterozygous mice were barrier maintained throughout the experiment. All normal BALB/c control mice were bred and kept under conventional conditions. All barrier maintained mice received sterilized pelleted food (Hope Farms, Woerden, The Netherlands) and sterilized water. Conventionally kept mice received similar, but unsterilized food and acidified water (pH 3-4). Food and water were available ad libitum. At dissection, all mice tested were found to be free of symptoms of lymphoreticular malignancies.

Treatment of mice

Thymectomy (Tx) of BALB/c mice was performed at 4-5 weeks of age as described previously (Miller, 1960). For isolation of corticosteroid resistant thymocytes, one intraperitoneal (i.p.) injection of dexamethason sodium phosphate (30 mg/kg) was given to 6-weeks-old female BALB/c mice. These mice were killed two days later and their thymi were removed and prepared for cell suspensions. Thymocytes prepared in this way are enriched for immunocompetent T-cells and are referred to as corticosteroid resistant thymocytes (CRT). Nine-months old BALB/c nude mice received three i.p. infusions of 1 x 10^7 CRT in two-day intervals, whereas control mice received phosphate buffered saline (PBS) at these intervals.

Quantitative determination of serum Ig levels

Small blood samples were taken at varying intervals. The serum samples were investigated within 24 hours or stored frozen at -20'C for later use. The concentration of total IgE in serum was quantified in an isotype-specific enzyme-linked immunosorbent assay (ELISA) specific for IgE as described (Savelkoul et al., 1985). The origin and evidence for the mono-specificity of the anti-IgE antiserum employed have been described (Savelkoul, Soeting, Radl and Van Der Linde-Preesman, submitted). After the appropriate adsorptions, the antisera were titrated and their specificity was assessed in plaque assays using myeloma and hybridoma cells of the appropriate classes. Further confirmation of the specificity was performed in immunoblotting and in inhibition ELISA employing purified hybridoma proteins. The ELISA was performed in standard 60-well Terasaki trays (type 653180; Greiner, Nürtingen, F.R.G.) and employed only 5 ul samples. In this sandwich type ELISA, the IgE was sandwiched between a catching goat antibody coated in the well and a detecting rabbit antibody. After assessment of the specificity, these polyclonal antisera were cross-adsorbed. Furthermore, the antisera were selected on protein A Sepharose (Pharmacia, Uppsala, Sweden) and subsequently the non adsorbing fraction of the goat antiserum and the binding fraction of the rabbit antiserum were used. Next, detection of the bound fraction of the rabbit antibody was based upon binding of a conjugate of the enzyme β-galactosidase linked to protein A and employing a fluorogenic substrate 4-methylumbelliferyl galactoside (Sigma, St. Louis, MO). The resulted fluorescence was measured in an automated microfluorimeter linked to a micro-processor based data handling system.

For standardization, a calibration curve was constructed for a mixture of four purified TNP-specific IgE-secreting hybridomas by transforming the readings of the various standard dilutions with the 4 parameter log-logit transform as described (Savelkoul, Soeting, De Josselin De Jong and Pathak, submitted). Sample readings were transformed and converted to an absolute IgE concentration (ug/ml).

Similar types of ELISA were performed for IgG_1 and total Ig by using rabbit anti-mouse IgG_1 and total Ig antibodies (Nordic, Tilburg, The Netherlands). The standard for total Ig consisted of mouse myeloma proteins and contained 0.385 mg IgM, 2.302 mg IgG_1, 4.328 mg IgG_2 and 1.788 mg IgA per ml (Meloy Lab. Inc., Springfield, Ohio). The anti-Ig rabbit antiserum was able to detect 7.8 $\pm$ 1.6 mg/ml in this total Ig standard which is not significantly different from the protein content (8.8 mg/ml). Similarly, a myeloma standard was employed containing 2.302 mg/ml IgG_1 (Meloy).

Statistical analysis

Of each group of mice, serum Ig levels were expressed as the arithmetic mean $\pm$ 1 SEM. Differences between groups of mice were assayed for significance by two-sided Student t-test. P values greater than 0.05 were not considered significant.

RESULTS

Quantitative determination of total IgE in serum

IgE concentrations were determined in the sera of aging BALB/c mice kept under conventional conditions. The results showed a steady increase in

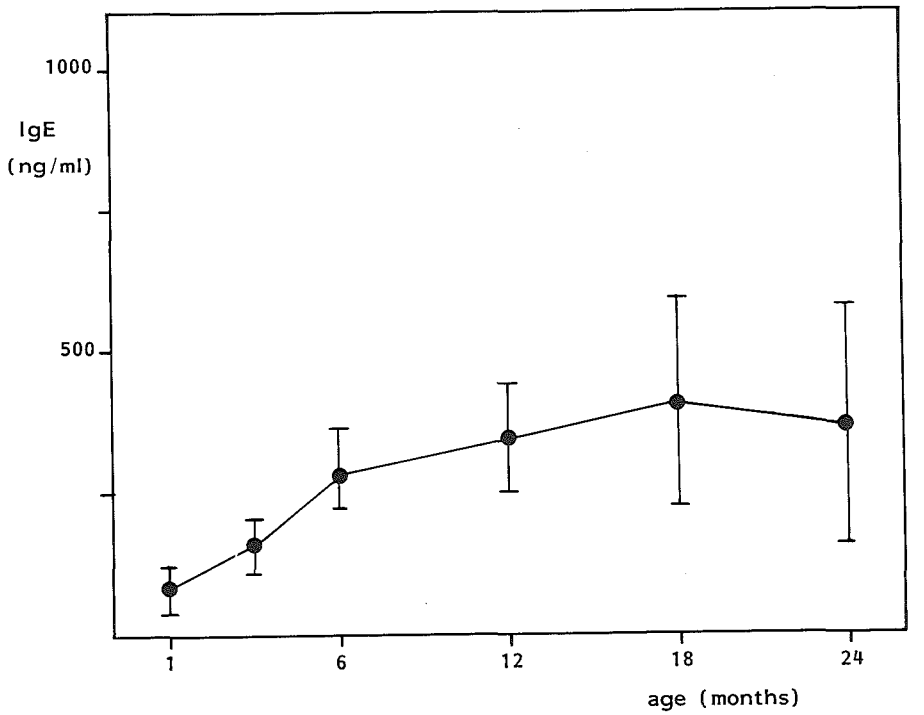

Fig. 1. Age-related increase of the concentration of total IgE in the serum of normal BALB/c mice as determined by ELISA. Figures represent the arithmetic mean $\pm$ 1 SEM (n=12).

serum IgE until a plateau level was reached around 380 ng/ml by the age of 6 months (Fig.1). After 12 months of age, a large degree of variation was found in the serum IgE level of individual mice from the same age. Throughout life in normal BALB/c mice the IgE concentration was consistently less than 0.01% of the total Ig level (data not shown).

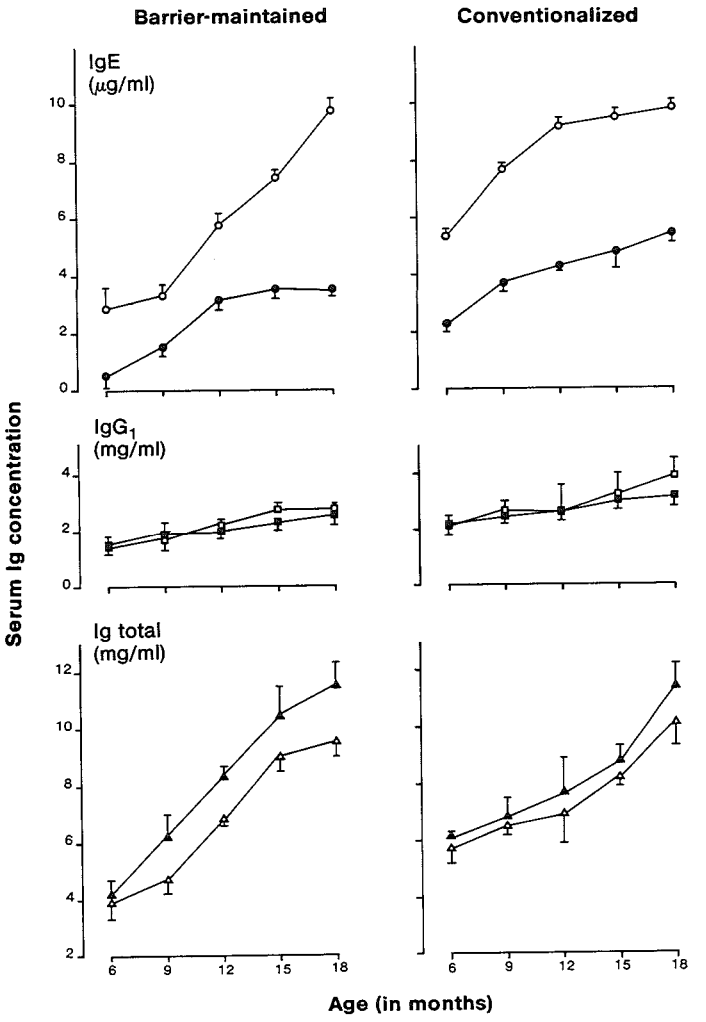

Fig. 2. Serum levels of IgE, IgG1, and total Ig in aging BALB/c nude mice and their heterozygous littermates kept under barrier-maintained (left) or conventional (right) conditions. Figures represent the arithmetic mean ± 1 SEM (n= 12-15) in nude mice of IgE (○), IgG1 (□) or total Ig (△) and in heterozygous littermates of IgE (●), IgG1 (■) or total Ig (▲).

Influence of antigenic load on IgE levels in sera of nude mice

Serum IgE levels were determined in barrier maintained nude mice and their heterozygous littermates at various ages, ranging from 6 to 18 months. At the age of 6 months, IgE levels were found to be significantly increased in nu/nu mice. Further analysis showed (Fig.2) an age-related increase in the serum IgE level of both groupes of mice. The absolute concentration in the serum of nu/nu mice was consistently higher than in nu/+ mice. Even at the age of 15 months, a plateau level was not reached for the IgE level in barrier maintained nude mice while such plateau was reached in heterozygous barrier maintained mice. In the same sera, absolute concentrations for IgG_1 as well as total Ig were determined in similar types of ELISA employing specific antisera. Both the IgG_1 and total Ig levels showed an age-related increase, but at all ages tested the IgG_1 and total Ig levels in nu/nu and nu/+ mice did not differ significantly ($p > 0.05$).

From another cohort of barrier-maintained mice, groups of nu/nu as well as nu/+ mice were transferred to conventional conditions at 4, 6, 10, 12, and 15 months of age. These mice were called conventionalized. Between 6-9 weeks after conventionalizing, sera (n=12-15) were collected for determination of IgE, IgG_1 and total Ig levels. Prolonged follow-up of the conventionalized nu/nu mice was not possible because of their short survival time (2 to 3 months) under conventional conditions. Histological examination was performed on conventionalized nude mice dying between 12 and 18 months of age. Lesions consistent with hepatitis virus infection were found in the majority of these mice while such lesions were almost absent in nu/nu barrier maintained mice. The results (Fig.2) showed that conventionalized nu/nu mice at the age of 6 months had higher levels of IgE than barrier maintained nu/nu mice. Moreover, serum IgE levels of conventionalized nu/nu mice increased faster with age so that the plateau level of IgE (around 10 ug/ml) was reached earlier in these mice: namely, already at the age of 12 months, whereas barrier-maintained mice reached this level at the age of 18 months.

Similar phenomena were observed in sera from conventionalized nu/+ mice, although the IgE content in these sera reached a higher plateau level (5.4 ± 0.3 ug/ml) than in the sera of barrier-maintained mice (3.5 ± 0.2 ug/ml).

Just as in the groups of barrier-maintained nu/nu and nu/+ mice, the concentration of IgG_1 and total Ig in the sera of age-matched conventionalized nu/nu and nu/+ mice did not differ significantly. For both IgG_1 and total Ig an age-related increase of the values was observed with a higher starting concentration at 6 months of age than in the sera of barrier maintained mice. The IgG_1 and total Ig levels in the sera of nude and heterozygous mice was also not significantly different from those of normal age-matched BALB/c mice. The normal IgG_1 and total Ig levels reached at the age of 12 to 15 months 1.95 ± 0.45 and 9.82 ± 1.76 mg/ml, respectively.

Influence of thymectomy on serum IgE levels

At the age of 4 to 5 weeks, normal BALB/c mice, kept under conventional conditions, were thymectomized (Tx). At the ages of 2, 3 and 4 months (1, 2 and 3 months after Tx, respectively) serum of these mice was collected and total IgE levels were determined in ELISA. After the last serum collection, mice were killed and dissected to examine the presence of thymic remnants. No structures resembling thymic remnants were present in any of the nude mice examined.

The serum IgE levels at 1 month after Tx (Table I) showed a significant increase from around 0.2 ± 0.1 ug/ml in the controls to 1.2 ± 0.5 ug/ml in the Tx mice. A plateau level of 5.7 ± 1.3 ug IgE/ml was reached already 2 months after Tx and the IgE concentration continued at this level up to at least 3 months after Tx. This IgE concentration resembled the levels found in heterozygous nu/+ mice (Fig.2).

Influence of T cell supplementation on serum IgE levels

At the age of 9 months, 82 nude mice received CRT infusions and 89 other nude mice were injected with PBS. All mice were conventionalized 6 weeks previously. Up to 15 months of age, little difference in survival could be detected between the two groups. At that time (6 months after infusion), 83% of the CRT versus 79% of the control group had survived. However, at the age of 20 months (11 months after infusion), 70% of the CRT group versus 50% of the control group were alive.

TABLE I

IgE concentration in the sera of adult thymectomized and normal BALB/c mice at various intervals after surgery

Age (months)	Months after surgery	IgE (ug/ml)	
		Tx	+/+
1	-	0.1 ± 0.1	0.1 ± 0.1
2	1	1.2 ± 0.5	0.2 ± 0.1
3	2	5.7 ± 1.3	0.4 ± 0.2
4	3	5.2 ± 1.2	0.4 ± 0.2

Figures represent the arithmetic mean ± 1 SEM of the IgE concentration (n=6) in the serum of thymectomized (Tx) and normal (+/+) mice as determined by ELISA.

At the age of 9 months, PBS control as well as CRT infused groups of mice displayed (Fig.3) high serum IgE levels (8.2 ± 0.4 and 9.3 ± 0.8 ug/ml, respectively). Within 1.5 months after the infusion, however, the CRT infused mice showed already a markedly decreased serum IgE level as compared to the control group. At the age of 13 months the CRT infused mice reached a plateau level of serum IgE around 5 ug/ml. This level of 5 ug/ml was of the same magnitude as the serum IgE level in age-matched conventionalized nu/+ heterozygous littermates (Fig.2).

In the same sera, levels of total IgG_1 were determined in a similar type ELISA. The results showed that, while in the sera of PBS control mice the level of IgG_1 did not differ significantly during the period of observation, CRT infused mice displayed a slow decrease of the total serum IgG_1 level. However, only at the age of 16 months (7 months after after CRT infusion) the decrease of the serum IgG_1 level was significant ($p < 0.05$).

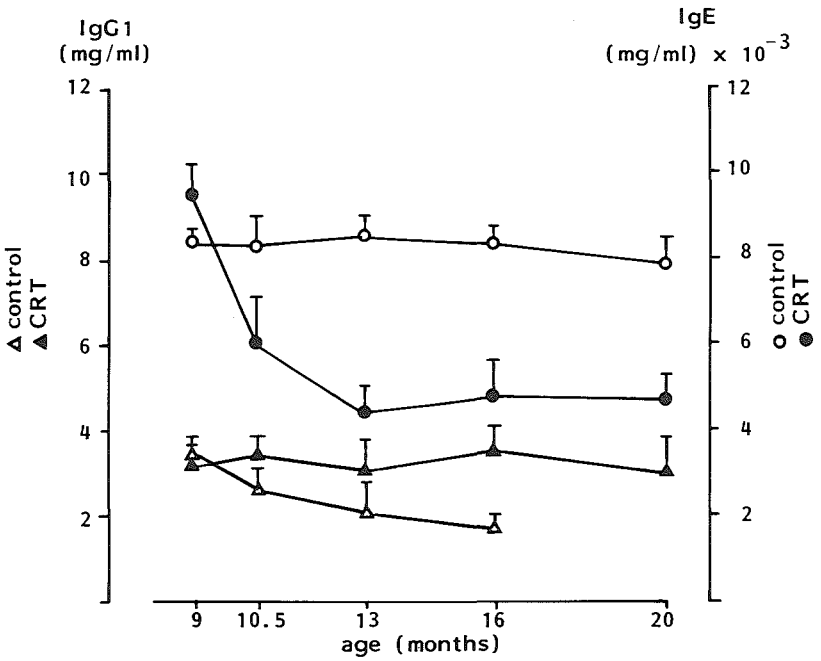

Fig. 3. Serum levels of total IgE (O) and IgG_1 (▲) in BALB/c nude mice infused with 1 x 10^7 T cells at the age of 9 months (n=25). For comparison IgE (●) and IgG_1 levels (▲) were determined in PBS infused control mice (n=25).

DISCUSSION

Several different grades of T system impairment accompanied by relatively unaffected B-cell functions were studied for their effect on the concentration of total IgE within the BALB/c strain. Nude mice displayed markedly increased total serum IgE levels independent of whether they are kept under conventional or barrier-maintained conditions. These results are consistent with the idea that the presence of T cells and/or the factors they produce is crucial in the isotype-specific regulation of IgE synthesis (Ishizaka, 1984; Marcelletti and Katz, 1987; Coffman et al., 1988). Compared with the nude mice, the heterozygous littermates distinctly showed lower serum IgE levels independent of the age and/or antigenic load of the groups of mice investigated. However, in comparison with the mice of the background strain, the serum level of IgE was higher and the age-related onset of increase was much earlier in the heterozygous mice. This may indicate that the T immune system in mice heterozygous for the nu gene is not entirely normal.

There are a few studies on serum levels of IgE in normal mice. No data, however, are available on the IgE serum concentration during aging in specific pathogen free (SPF), barrier maintained nude mice, which have a much longer lifespan than conventionally maintained nude mice (Holland et

al., 1978). Limited data are available on IgE levels in young (8 to 10 weeks old) BALB/c nude mice kept under conventional conditions (Ito et al., 1979; Lebrun and Spiegelberg, 1987). The serum IgE level is to a large extent dependent on the antigenic load of the respiratory and digestive tracts. This is apparent from studies in which mice were raised under germfree, specific-pathogen-free and conventional conditions (Van Snick and Masson, 1980; Durkin et al., 1981). From such studies it became clear that long lasting antigenic stimulation exerts a positive influence on the number of IgE-bearing as well as IgE-secreting cells and the serum IgE level. For example, it was shown that infection with Nippostrongylus brasiliensis induced an increase in serum IgM, IgE and IgG_1 levels in many strains except athymic nude mice (Lehrer and Bozelka, 1982). Especially with IgE, the majority of the induced IgE was not parasite-specific but polyclonal, 'non-specific' IgE (Jarrett and Miller, 1982). Furthermore, it was established that IgG_1 is the major Ig isotype formed in response to helminth infection (Lebrun and Spiegelberg, 1987).

Because of the T cell dependency of the formation of antigen-specific IgE antibodies, nude mice do not respond with specific IgE upon immunization (Lehrer and Bozelka, 1982; Lebrun and Spiegelberg, 1987). It was shown in rats (Jarrett and Miller, 1982) and mice (Wyczolkowska, Brezinska-Blaszcyk and Maslinski, 1983) that the magnitude of specific IgE antibody responses is not reflected in the total serum IgE level. It is this observed paradox that suggests differences in the regulation of specific IgE antibody formation versus total IgE synthesis. Just as in IgE, adult nude mice display significantly higher IgG_1 serum level than heterozygous mice, while the IgG_{2a}, IgG_{2b} and IgA levels are generally decreased (Haaijman et al., 1979; Mink et al., 1980). This observation contrasts with the generally held view that the T cell dependency of the IgG_1 production is reflected in the reduced serum IgG_1 levels in nude mice (Wortis, 1974). The data presented in this paper suggest that the production of IgG_1 and IgE are similarly regulated, thus also revealing a similar paradox with regard to T cell dependency and increased occurrence in T cell deficient mice.

It has been shown that cytotoxic and helper T lymphocytes can be generated by nude mice, which raises questions about the T cell deficiency of these mice (Hünig, 1983; Ikehara et al., 1984). In view of the existence of some mature T cells in nude mice, the unexpected increase of total IgE and IgG_1 levels in the serum might suggest a specific production of IL-4 by some contaminating T cells on one hand or an absence of the production of gamma-interferon on the other hand. This putative imbalance between Th1 and Th2 subsets in nude mice can thus explain the selective enhancement of the production of polyclonal IgE and IgG_1, a property that has been established in vitro (Coffman et al., 1988). A selective deficiency in especially the production of gamma-interferon linked to the presence of the nude gene, would also explain the increased total IgE levels in heterozygous mice.

The data, however, can not discriminate between which of the various T cell subpopulations are responsible for the T-dependent regulation of the specific versus the total IgE synthesis. Upon depletion of immunocompetent T cells by thymectomy or by using nude mice, the balance between helper and suppressor T cells on one hand and Th1 and Th2 helper T cell subsets on the other hand might be disturbed which apparently results in an overall effect of an increasing IgE level in the serum of these mice. Furthermore, infusion of T cells can, partially, restore the deficiency leading to a markedly decreased IgE level in the serum. It is therefore essential for further studies on T cell regulation of IgE synthesis to study the various T cell subsets separately in order to be able to gain more insight in how the serum IgE level is regulated by T cells and/or the factors they produce.

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CHAPTER 5.3

SPECIFIC IMMUNOGLOBULIN-E ANTIBODY FORMATION IN MICE. I. DELAYED APPEARANCE OF ANTIGEN-SPECIFIC IMMUNOGLOBULIN-E IN THE SERUM COMPARED TO THE ANTIBODY-SECRETING CELL RESPONSE

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ABSTRACT

The antigen-specific and total IgE responses were studied in mice repeatedly immunized with low doses of antigen without the use of adjuvant. Optimal immunization schemes were found to consist of 3 to 7 repeated i.p. or i.v. injections of 10 ug DNP-BSA or OA in saline. After immunization spleen, bone marrow, mesenteric lymph nodes and parathymic lymph nodes were analyzed for IgE-secreting cells by plaque assays. IgE formation was also determined in the serum by a quantitative ELISA. In both assays the ratio of antigen-specific and total IgE responses was determined. Remarkably, while in various lymphoid organs 65% of the IgE-secreting cells were found to be antigen-specific, the maximum value of specific IgE in the serum reached only 35%. Moreover, the occurrence of antigen-specific IgE in the serum was maximal at 30 days after the last injection while the maximum number of antigen-specific IgE-secreting cells was found after 4 days. While BALB/c IgE high responders and to a lesser extent C57BL/6 and AKR IgE intermediate responders showed substantial IgE antibody responses after immunization, no specific IgE formation could be detected in IgE low responder SJL mice or in athymic BALB/c nude mice. The regulatory role of T cells in antigen-specific and non-specific IgE formation and the possibility of differential regulation of these two types of IgE production are discussed.

INTRODUCTION

Due to the importance of immunoglobulin-E (IgE) in the pathogenesis of atopic disease, many studies have focussed on the regulatory mechanisms controlling its production in the mouse (1-3). Generally, immunization of mice with relatively low dose of antigen precipitated on alum or administered with Bordetella pertussis vaccine gave a primary specific IgE antibody response in the serum (4-8). After booster immunization, frequently a secondary IgE response was observed in these animals (9,10). Repeated intraperitoneal (i.p.) immunization of IgE high responder strains with the use of adjuvant resulted in an IgE response that persisted for several months without booster immunization. Such a persistent specific IgE antibody response was considered to be a model for specific IgE formation in humans. However, for simulating IgE production in human allergy, immunizations relying on the use of adjuvants are less relevant. Few immunization schemes not involving adjuvants were used for studying the regulation of antigen-specific IgE responses (10-12).

Quantitation of IgE responses induced after helminth infection or immunization with antigen are almost completely restricted to the determination of specific IgE in the serum employing the passive cutaneous anaphylaxis (PCA) reaction in rats. This test is difficult to quantitate, since it can only establish relative IgE levels depending on a minimal inflammatory skin reaction. The development of antigen-specific IgE-secreting hybridomas, however, allowed the production of anti-IgE antisera suitable for enzyme-linked immunosorbent assays (ELISA) as well as plaque-forming cell (PFC) assays (13-16). With the development of ELISA specific for murine IgE, quantitation of antigen-specific as well as total IgE levels in the sera of mice became feasible (17-20). Few studies deal with the quantitation of the IgE-forming cell response after such imunization (21,22). These studies employed immunofluorescence techniques and heterologous adoptive cutaneous anaphylaxis (23). With the development of plaque assays for the enumeration of IgE-secreting cells, both the antigen-specific and the total cellular IgE-responses can be evaluated (24).

Immunization may lead in addition to the formation of specific antibodies to increased production of immunoglobulins that do not bind with the immunizing antigen, the 'non-specific' response (25). In this study we analyzed the ratio of the antigen-specific to the 'non-specific' IgE-response, both at the cellular and the humoral level.

MATERIALS AND METHODS

Mice

Female BALB/c, athymic BALB/c nude, C57BL/6 and SJL mice, 8 to 12 weeks of age, were purchased from Bomholtgard, Ry, Denmark. Female AKR mice, 8 weeks of age, were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands.

Antigens and immunization

Ovalbumin (type VII; Sigma, St.Louis, MO) was purified by Fast Protein Liquid Chromatography (FPLC; Pharmacia, Uppsala, Sweden) and analyzed for purity by sodium dodecyl sulphate polyacrylamide gelelectrophoresis (SDS-PAGE) on a PHAST system (Pharmacia). For immunization a preparation consisting of 80% monomer and 20% dimer was used. Bovine serum albumin (BSA; type V, Sigma) was dinitrophenylated to a level of 27 DNP molecules per protein molecule (as determined spectrophotometrically) by using dinitrobenzene-

sulphonic acid (Eastman Kodak, Rochester, NY) as described (26). Mice were immunized with 0.5 ml saline containing a varying dose of either antigen as indicated in the RESULTS section. Routes of administration were either i.p., intravenous (i.v.), subcutaneous (s.c.) or oral. As a control mice were injected with saline only. For comparison, mice were also i.p. immunized with antigen precipitated on alum (27).

Plaque assays

Cell suspensions of spleen, bone marrow (BM), mesenteric (MLN) and parathymic lymph nodes (PTLN) were prepared as described (28). Viable nucleated cells were counted in a hemocytometer using 0.2% Trypan Blue in phosphate-buffered saline (PBS) as a diluent. Total IgM-, IgG- and IgE-PFC were determined in a modified protein A plaque assay while antigen-specific PFC were determined in antigen-specific plaque assays. Sheep red blood cells (SRBC) coupled with protein A (Pharmacia) or OA (29) by using chromium chloride were used as indicator cells. SRBC were coupled with trinitrophenyl (TNP) by using 30 mg 2,4,6-trinitrobenzenesulphonic acid (Eastman Kodak) as described (30). Plaques were developed by using Ig-depleted guinea pig complement (Behringwerke, Marburg/Lahn, FRG) by passing the guinea pig serum over a protein A-Sepharose column (Pharmacia) as described (31). The origin, preparation and specificity testing of the anti-IgE (Savelkoul, Soeting, Radl and Van Der Linde-Preesman, submitted) and the anti-IgG developing antisera has been described (32). PFC activity in the BM was determined in the marrow of both femurs and multiplied by a factor of 7.9 to yield the number of PFC in the BM of the whole animal (28).

ELISA for total and antigen-specific IgE

The total as well as the antigen-specific IgM-, IgG- and IgE-concentrations in the serum of mice were quantitated employing a Terasaki-ELISA system (17). For this purpose the same antisera were used as employed in plaque assays. The origin and evidence for the monospecificity of the anti-IgE antiserum in immunoblotting and inhibition ELISA have been described (Savelkoul, Soeting, De Josselin De Jong and Pathak, submitted). For standardization, a calibration curve was constructed from a mixture of purified IgE hybridoma proteins, by transforming the readings of the various standard dilutions with the 4 parameter log-logit transform as described (Savelkoul, Soeting, De Josselin De Jong and Pathak, submitted). Sample readings were transformed and converted to an absolute IgE concentration (ug/ml).

Data analysis

PFC responses of groups of mice (n=3-5), tested individually, were calculated as the arithmetic mean of the numbers of PFC per organ tested $\pm$ 1 standard error of the mean (SEM). This was considered valid since the organs tested did not differ significantly in the numbers of total viable nucleated cells as compared to saline treated controls. Student t-test (two-sided) was used to estimate the significance of differences observed. Differences with p-values greater than 0.05 were not considered to be significant. Serum Ig levels were determined as the arithmetic mean $\pm$ 1 SEM by testing individual mice. The results are expressed in ug/ml for IgE and mg/ml for IgM and IgG.

RESULTS

Effects of antigen dose on the IgE response

BALB/c mice received three i.p. injections of 0.1 to 100 ug OA in 0.5 ml saline on alternate days. On several days after the last injection, cell suspensions of spleen, BM, MLN and PTLN were analyzed in the protein A plaque assay for total IgE-, IgG- and IgM-PFC responses. In all lymphoid organs maximal IgE-PFC responses were found 4 days after the last injection. Fig.1 shows the IgE-PFC responses in the PTLN after 3 and 7 OA injections. As shown in Table I, in all four organs tested IgE-PFC responses reached plateau levels between 1 and 10 ug of OA. Doses between 10 and 100 ug OA resulted in decreasing IgE responses but markedly increasing IgM and IgG responses (data not shown). In control mice receiving injections of saline, only small numbers of background IgE-PFC could be found. Similar findings were obtained when DNP-BSA was used for immunization (data not shown).

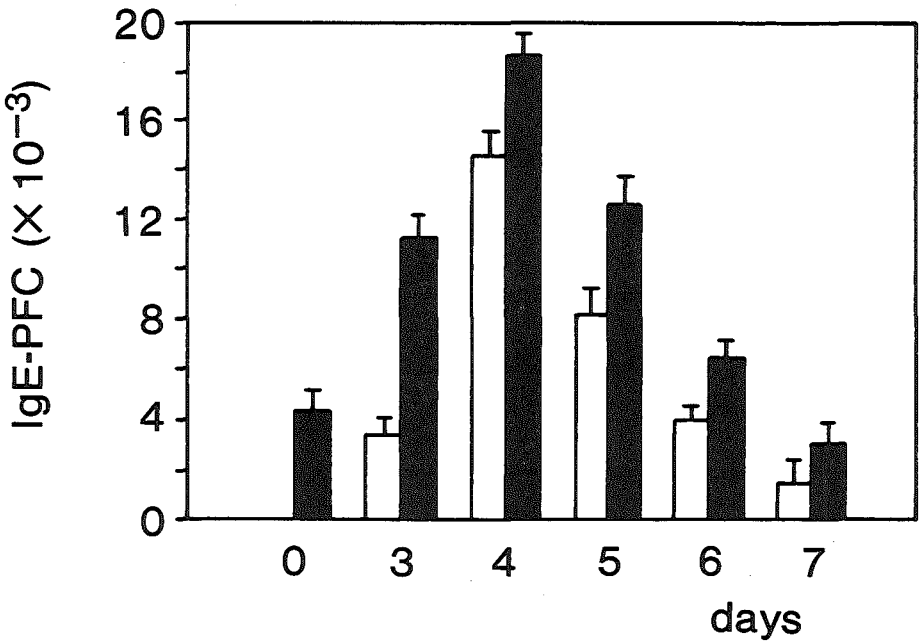

Fig. 1. Kinetics of total IgE-PFC in the parathymic lymph nodes on day 5 after 3 or 7 subsequent immunizations with 10 ug OA in saline i.p. on alternating days. Closed columns represent the results after 3 immunizations while hatched columns represent results after 7 immunizations. Results are expressed as arithmetic mean $\pm$ 1 SD (n = 4).

TABLE I

Total IgE-PFC responses in various lymphoid organs of BALB/c mice immunized with several doses of OA

Organ	Dose of OA (ug/ml)			
	0.1	1	10	100
Spleen	1650 ± 314 [1]	13680 ± 2150	17340 ± 3680	9740 ± 912
BM	1370 ± 490	8850 ± 2645	14290 ± 3190	5127 ± 118
MLN	1240 ± 480	2730 ± 940	7270 ± 1950	9550 ± 238
PTLN	350 ± 163	6033 ± 1470	10680 ± 2750	4360 ± 198

[1] IgE-PFC were determined 4 days after the last of three OA injections. Results are corrected for the numbers of background IgE-PFC in the organs of saline-treated control mice and are expressed as the arithmetic mean ± 1 SEM (n=5).

TABLE II

Total IgE-PFC in spleen and parathymic lymph nodes of BALB/c mice immunized with 10 ug DNP-BSA

Organ	Number of injections			
	1	3	7	10
Spleen	4870 ± 1360	22690 ± 4350	22490 ± 5110	23980 ± 4360
PTLN	390 ± 168	7290 ± 1680	19450 ± 4970	16380 ± 2780

For details: see legend to Table I.

Next, mice were i.p. immunized once, three, seven and ten times with 10 ug of DNP-BSA in saline on alternate days. Cell suspensions of spleen and PTLN were analyzed for total and DNP-specific IgE-PFC at 4 days after the last antigen injection. As shown in Table II, the number of total IgE-PFC reached a plateau after three to seven injections. In the same cell suspensions only a small number of DNP-specific IgE-PFC was found (data not shown).

The effect of the time intervals between the repeated antigen injections was also analyzed for its effect on the total number of IgE-PFC. To this end, mice were immunized i.p. three times with 10 ug/ml of OA in saline while keeping the injections one, four and seven days apart. The results showed that injection on alternate days resulted in maximum numbers of total IgE-PFC (Table III).

TABLE III

Total IgE-PFC responses in the spleen and bone marrow of BALB/c mice immunized with OA employing various time intervals between successive injections

Organ	interval (days)			
	1	4	7	10
Spleen	26980 ± 2145	8453 ± 1324	670 ± 135	567 ± 98
BM	24380 ± 3451	11453 ± 2540	1135 ± 256	687 ± 196

Mice were immunized three times with 10 ug OA in saline i.p.
For other details: see legend to Table I.

Analysis of the serum IgE levels in these mice revealed a slow increase in total IgE concentration from 429 ± 121 ng/ml to 1034 ± 277 ng/ml after immunization (3 injections of 10 ug DNP-BSA in saline i.p. on alternating days) peaking around 14 days after the last immunization. After immunization only little DNP-specific IgE could be detected. Only around day 14 at maximum 56 ± 21 ng/ml DNP-specific IgE was detected (data not shown).

Kinetics of the IgE response

BALB/c mice were immunized by 7 i.p. injections of 10 ug OA in saline on alternate days. Starting on the first day of immunization, total and antigen-specific primary IgE responses were quantitated at the cellular and humoral level. In spleen and BM maximum numbers of IgE-PFC were found at 4 days after the last immunization (day 18). At this day a small antigen-specific response of 5% of the total IgE response was found. By day 32 no longer a significant IgE-PFC response above saline-treated controls was observed (Fig.2a). When such mice were given three 10 ug OA booster injections 3 months later, again at day 4 after the last injection maximum numbers of IgE-PFC were found (Fig. 2b) that were substantially increased as compared to the primary response. Moreover, the portion of antigen-specific IgE-PFC was increased to 65% at 4 days after the last injection (data not shown). The time interval between primary and booster immunization could be increased up to one year without affecting the magnitude of the secondary type response (Fig. 2c).

In the serum, 7 days after the booster injections the total IgE level was found to be increased 12-fold from 356 ± 78 ng/ml to 4320 ± 478 ng/ml. At that time a maximum antigen-specific IgE level of 1510 ± 230 ng/ml (35%) was induced (Fig.3). It was found that while the antigen-specific IgE-PFC response was decreased to control numbers, the total IgE serum concentration was also decreased to the level found before the booster immunizations. The antigen-specific IgE concentration in the serum persisted at the level of 35% of the total IgE concentration for a period up to at least 120 days.

When mice, treated according to the above mentioned primary immunization protocol, received three booster immunizations (each consisting of 3 repeated injections of 10 ug OA in saline i.p. on alternate days), hardly antigen-specific or total IgE-PFC were detected in either lymphoid organ.

In the serum the total IgE concentration and the ratio of antigen-specific versus total IgE remained constant (at 1.57 ± 0.32 ug/ml and 35%, respectively). The numbers of OA-specific IgM- and IgG-PFC, on the other hand, increased substantially. Also in the serum the OA-specific IgM and IgG level increased (results not shown).

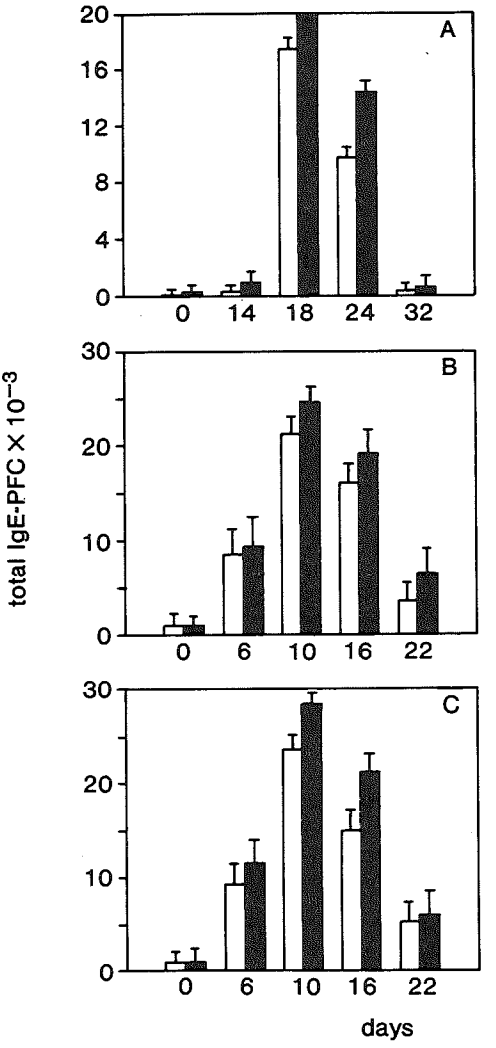

Fig. 2. Kinetics of total IgE-PFC formation in spleen and bone marrow of mice immunized with 10 ug OA in saline i.p. in (A) a primary response (7 subsequent immunzations on alternating days), (B) secondary response (3 additional immunizations after 3 months) and (C) a tertiary response (3 additional immunizations after 1 year). The closed columns represent the arithmetic mean ± 1 SD of the total IgE-PFC in the spleen while the bone marrow results are represented by the hatched columns.

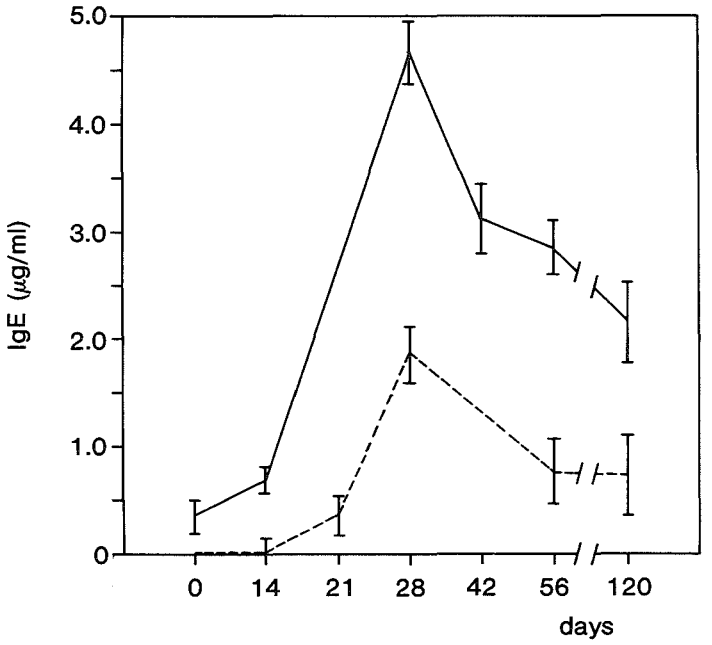

Fig. 3. Kinetics of the serum concentrations of OA-specific and total IgE after 3 booster immunizations of 10 ug OA at 3 months after the priming. Solid lines represent the total IgE concentration while dashed lines represent the OA-specific IgE concentration. Results are expressed as arithmetic mean ± 1 SD (n = 4) in ug/ml.

Effects of route of administration

An immunization protocol consisting of 3 repeated injections of 10 ug OA in saline on alternate days was employed to compare the effect of i.p., i.v. and s.c. routes of immunization in BALB/c mice. The magnitude of the IgE response in the various lymphoid organs was found to be dependent on the route chosen: after i.p. immunization the highest numbers of IgE-PFC were found in PTLN, while after i.v. immunization the highest response was found in the spleen (Fig.4). This response, however, was lower than the splenic IgE-PFC response induced by s.c. immunization. The s.c. immunization also induced the highest IgE-PFC response in the BM. The oral route appeared to induce maximum numbers of IgE-PFC in the MLN. In this case a dose of 1 mg OA was found to be optimal.

For comparison, other groups of BALB/c mice were immunized with a single i.p. injection of 1 ug of OA precipitated on 1 mg of alum. The total and OA-specific IgE-PFC responses were maximal in spleen and BM at 12 days after immunization and steadily decreased to control numbers at 24 days. Around 45% of the total IgE response was found to be OA-specific (Table IV). Also a low transient OA-specific IgG and a gradually increasing specific IgM response were observed. In the serum at day 12 to 21, around 2500

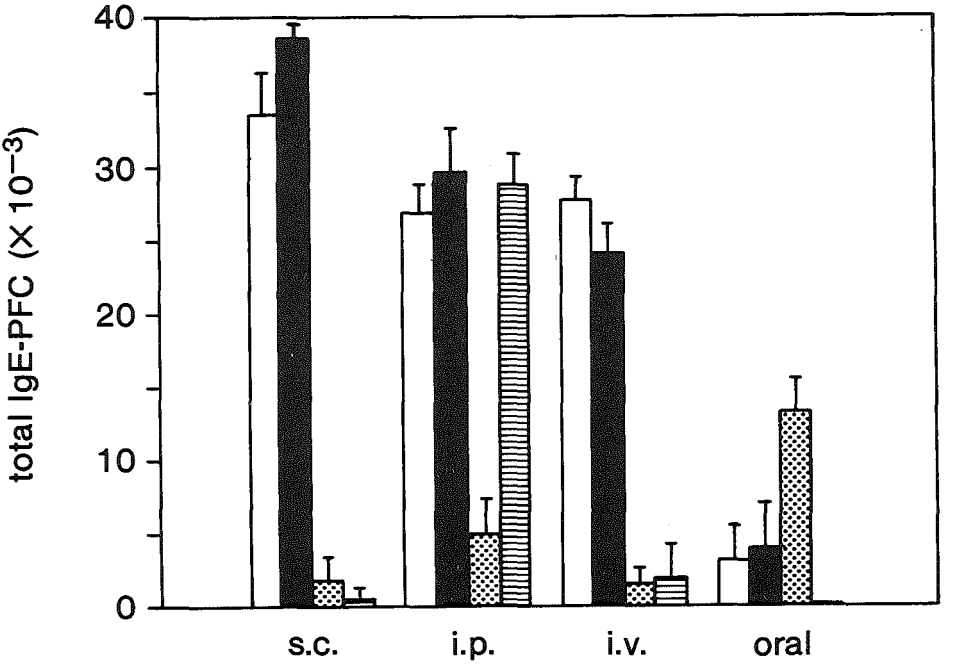

Fig. 4. Peak responses in spleen (open columns), bone marrow (closed columns), mesenteric (dotted columns) and parathymic (dashed columns) lymph nodes 4 days after 3 repeated immunizations with 10 ug OA in saline s.c., i.p., i.v. and oral. Results are expressed as the arithmetic mean $\pm$ 1 SD of the total IgE-PFC.

ng/ml of OA-specific IgE was found, which corresponded to about 30% of the total IgE level. In these sera also a substantial increase of the level of OA-specific IgG was found up to 2.3 $\pm$ 0.7 mg/ml. At 30 days the IgE levels decreased to saline-treated control values (data not shown).

Effect of responder strain

IgE high responder BALB/c mice, intermediate responder C57BL/6 and AKR mice and low responder SJL mice were i.p. immunized with 10 ug OA in saline by 7 repeated injections on alternate days. Table V shows the numbers of total IgE-PFC and OA-specific IgE-PFC found on day 4 after the last OA injection as well as in saline treated control mice. In unimmunized SJL mice no 'background' IgE-secreting cells were found. After immunization of SJL mice with OA, only a very low and rapidly declining total IgE response was observed that did not involve OA-specific IgE-secreting cells. All other strains displayed background, control, total and OA-specific IgE-secreting cells in the decreasing order of BALB/c, C57BL/6 and AKR.

In athymic BALB/c nude mice no total or OA-specific IgE-secreting cells were found independent whether or not they had been immunized with OA (data not shown). In the serum only total IgE could be detected, in values between 5 and 20 ng/ml for both control and OA-immunized mice.

TABLE IV

Kinetics of total and OA-specific IgE secreting cells in the bone marrow and total and OA-specific IgE-concentration in the serum of BALB/c mice i.p. immunized with 1 ug OA precipitated on alum

Day after immunization	IgE-PFC		IgE-concentration	
	Total	OA-specific	Total	OA-specific
0	568 ± 129	<100	316 ± 112	<10
7	4783 ± 1168	2416 ± 368	1433 ± 568	115 ± 35
14	27941 ± 2243	23867 ± 2687	3019 ± 799	674 ± 286
21	24613 ± 1865	18744 ± 1511	5168 ± 1215	1489 ± 377
28	9333 ± 1126	7146 ± 724	5236 ± 1142	1562 ± 743
35	1189 ± 264	<100	2164 ± 1067	489 ± 159

PFC figures represent total numbers of PFC in 2 femurs and are expressed as arithmetic mean ± 1 SD of 4 mice tested individually. IgE concentrations are expressed as arithmetic mean ± 1 SD (n=4) in ng/ml.

TABLE V

Peak response of total and OA-specific IgE-secreting cells in the spleen of various IgE responder strains 4 days after the last immunization

Strain	IgE-responder type	IgE-PFC	
		Total	OA-specific
BALB/c	high	28460 ± 2320	9370 ± 1746
AKR	intermediate	15630 ± 1560	4710 ± 685
C57BL/6	intermediate	3840 ± 971	743 ± 154
SJL	low	1561 ± 153	<100
BALB/c nu/nu	low	269 ± 89	<100

Mice were immunized seven times with 10 ug OA in saline i.p. on alternate days. IgE-responder types according to Levine and Vaz (5). For further details: see legend to Table I.

DISCUSSION

This study shows that antigen-specific IgE-responses can be induced in mice by repeated injection of hapten-carrier complexes or protein antigens without the use of adjuvant. This was also described by others (10-12), but in these studies no data on IgE-secreting cells were provided. In the latter studies the effect of immunization was only analyzed at the level of antigen-specific IgE antibodies in the serum as detected by PCA reactions. Moreover, sofar almost no data were available with regard to the ratio of

antigen-specific versus total IgE responses in the serum (33). With the development of plaque assays for total and antigen-specific IgE-secreting cells and quantitative ELISA for the determination of the concentration of the secreted IgE, it became possible to analyze IgE formation both at the cellular and humoral level.

The conditions for the induction of a maximum antigen-specific IgE response in terms of dose, type of antigens, routes of administration and immunization scheme were found to be similar to those that were described before (10-12). As a primary route of administration i.p. injection was suggested and it was therefore prudent to analyze the PTLN as the major draining lymph nodes of the peritoneum. It was found that the PTLN can contain as many IgE-PFC as the spleen. Previously, similar data have been presented for IgM (34).

A single oral gift of antigen can cause an isotype-specific suppression (35). Oral administration of OA resulted in low numbers of IgE-secreting cells including a small but significant number of OA-specific IgE-secreting cells in the MLN. The i.v. route of immunization resulted in markedly decreased numbers of IgE-PFC in spleen and BM as compared to s.c. and i.p. immunization. It has been described that such i.v. route of immunization may lead to a state of suppression of cellular and humoral responses, by the activation of suppressor T cells (Lagrange 1974; Parish, 1977).

It has been described that specific IgE responses occur only after immunization with thymus-dependent antigens and that this production is dependent on helper T cells and strongly regulated by radiosensitive suppressor T cells and T cells producing IgE binding factors (3,37-39). This was confirmed in the present study since nude mice displayed only low levels of total IgE in their serum (<20 ng/ml) and no specific IgE formation could be induced in nude mice. Also no IgE-secreting cells were detected in the lymphoid organs of these mice.

It has been shown that IgE formation is controlled by genetic factors which involve both major histocompatibility-linked and non-linked loci (1,2). The IgE responder type of several strains of mice was defined by the capacity to produce specific IgE after immunization with hapten-carrier complexes precipitated on alum (4-6). A similar distinction in responders and non-responders could be based on helminth-specific IgE production after infection with Nippostrongylus brasiliensis (36). In all these studies it was found that BALB/c, C3H and DBA/2 mice were IgE high responders, AKR and C57BL/6 mice were IgE intermediate responders and SJL and SJA/9 mice were IgE non-responders. Also after immunization with hapten-carrier complexes or protein antigens, BALB/c mice reacted with higher numbers of specific IgE-PFC than intermediate responders (C57BL/6 or AKR). Low responder SJL mice were almost unable to mount a specific IgE response.These mice also displayed very low total IgE levels in their serum (<20 ng/ml).

With either route of antigen administration, an accompyaning non-specific IgE response was observed along with the antigen-specific IgE formation. A discrepancy was consistently found between the maximum ratio of antigen-specific versus total IgE in serum and the same ratio at the cellular level. In the lymphoid organs a maximum of around 65% antigen-specific IgE antibody formation was observed while in the serum only 35% of the IgE was found to be specific for the immunizing antigen. Moreover, while the cellular response in the various organs tested reached its maximum at 4 days after the last injection, in the serum antigen-specific IgE could not be detected before 24 days after immunization. A possible explanation for the delay in the occurence of specific IgE in the serum could be the time lag in the process of affinity maturation after repeated immunization. By this affinity maturation, antigen-specific IgE antibodies of an

affinity sufficiently high to allow their detection in ELISA might occur in the serum only after some time. Such affinity maturation in the case of antigen-specific IgE antibodies, however, has been found to occur only marginally (Pathak and Savelkoul, in preparation).

This study also shows that already after primary immunization with an antigen in saline high numbers of IgE-PFC can be detected in the BM. On the other hand, when using an antigen precipitated on alum, only after an additional booster immunization specific IgE-PFC could be detected in the BM. Thus, when using alum precipitated antigens for immunization, the kinetics of antigen-specific IgE formation in the bone marrow is comparable to that of other isotypes (28).

Employing low dose of antigen in saline for multiple injections in the primary immunization, the kinetics of the IgE responses in spleen and bone marrow were quite similar. The reaction in the BM raises questions whether in this way only virgin B cells are stimulated by the antigen (28,32). Alternatively, because of the low immunization dose a different regulatory system may operate in which already from the start the BM is involved in the specific IgE formation. Further studies are neceassary to elucidate this apparent controversy.

The observed lack of correlation between total and antigen-specific IgE in the serum was described before (33) and it was suggested that these responses are therefore regulated independently and in different ways. It is not clear whether the non-specific IgE response that was observed before (36) is caused by idiotypic stimulation or by the action of non-specific helper factors from T cells or macrophages (25).

Recently, it was shown that helper T cells can be divided in two subsets based on the lymphokines they produce (40). One subset of helper T cells produces interleukin-4 (IL-4) that is able to selectively enhance the production of IgE both *in vitro* and *in vivo*. Interferon-gamma, produced by the other T helper subset, inhibits this IL-4 induced IgE synthesis. It remains to be established whether the relative numbers or states of activation of these helper T cell subsets differentially regulate the specific and non-specific IgE formation *in vivo* (40).

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CHAPTER 5.4

SPECIFIC IgE-ANTIBODY FORMATION IN MICE

II. PHOSPHORYLCHOLINE-SPECIFIC IgE-SECRETING CELLS IN PERITONEAL CAVITY

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ABSTRACT

This study shows that the peritoneal cavity contains substantial numbers of IgE-secreting cells, especially after repeated intravenous immunization with low doses PC-KLH without adjuvant. The majority (85 ± 10%) of these IgE-secreting cells was PC-specific of which 80% were T15 idiotype-positive. Adult as well as neonatal treatment with anti-idiotypic antibodies did not cause a significant decrease of PC-specific IgE-secreting cells and IgE antibody level in the serum, while the peritoneal and splenic IgE-PFC responses became completely devoid of the T15 idiotype. It was also shown that the peritoneal cavity contained PC-specific IgE-secreting cells for at least 60 days after the last injection and probably account for the major part of the persistent PC-specific IgE in the serum.

INTRODUCTION

After deliberate immunization of experimental animals, antibody forming cells preferentially localize in the lymphoid organs (spleen, bone marrow and lymph nodes). The localization and migration of specific IgM-, IgG- and IgA-secreting cells during primary and secondary immune responses has been described previously (1,2). It was found that the antibody-forming cell response in the bone marrow (BM) starts slowly but is responsible for the long-term specific antibody production both for thymus-dependent as well as several thymus-independent antigens (3,4). The localization and migratory pattern of IgE-secreting cells after immunization is still largely unknown. In a previous study (5), it has been demonstrated that specific IgE-secreting cells are located in spleen, BM and regional lymph nodes. However, because the IgE responses in these organs were transient, they could not account for the long-lasting occurrence of specific IgE antibodies in the serum.

In this study we analyzed the distribution of IgE-secreting cells after repeatedly intravenous (i.v.) immunization with phosphorylcholine conjugated to keyhole limpet hemocyanin (PC-KLH). It appeared that IgE-secreting cells not only occurred in the spleen, BM and lymph nodes, but also in the peritoneal cavity. This was especially true for the antigen-specific IgE-secreting cells. The involvement of these IgE-secreting cells in the long-lasting occurrence of specific IgE antibodies in the serum is discussed.

In BALB/c mice the response to PC is typically restricted in heterogeneity in that the majority of antibodies bear the idiotype (Id) of the TEPC-15 and HOPC-8 tumors (T15-Id) (6,7). It was shown that the elicitation of an anti-PC IgE response is suppressed to a high degree in BALB/c mice which had been injected with the myeloma protein TEPC-15 (T15) and as a consequence actively produced T15 anti-idiotypic antibodies (8). The anti-PC IgE response in these mice, induced with PC-KLH in alum, remained suppressed for several weeks despite further booster injections with antigen (9). In the study presented here we also analysed the expression of the T15-idiotype among the IgE-secreting cells in the various lymphoid organs and the peritoneal cavity.

MATERIALS AND METHODS

Mice

Female BALB/c mice, 8-10 weeks of age, were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. Neonatal BALB/c mice were bred at our own department.

Immunization scheme

Phosphorylcholine-conjugated keyhole limpet hemocyanin (PC-KLH) was prepared as described before (10) and kindly provided by Dr. C. Berek. Adult mice (3 months of age) were immunized i.v. three times on alternate days with 10 ug/ml of the hapten-carrier complex PC-KLH in saline. This protocol is adopted from the one described before (11). On several time points after the last immunization mice were killed, lymphoid organs were removed and cell suspensions were prepared. On the day of the first antigen injection, 0.5 mg purified polyclonal anti-IgE antibodies (12), monoclonal anti-IgM (M41, gift of Drs. M. Leptin and F. Melchers, Basel Institute for Immunology) or anti-T15 (32/65; 13,14) antibodies were i.p. administered.

For neonatal treatment, mice were injected with 0.5 mg of purified

anti-IgM or anti-IgE antibodies on day 3, 10, 17, 24 and 32 after birth (15). These mice were immunized with PC-KLH at the age of 3 months.

Membrane fluorescence

Membrane immunofluorescence was performed essentially as described before (16), employing the above mentioned antibodies. Relevant FITC-conjugated second stage antibodies (Nordic, Tilburg, The Netherlands) were used for detection.

Cell suspensions

Cell suspensions of spleen were prepared as described previously (17). Peritoneal cells were obtained by washing the peritoneal cavity with phosphate buffered saline (PBS): 7 ml PBS was i.p. injected and the peritoneal fluid was collected with a syringe. The cells were spun down and washed twice with Hanks' balanced salt solution before using them for immunofluorescence and plaque assays. About 5 x 10^6 peritoneal cells were recovered from a single adult mouse. Viable cells were counted by trypan blue exclusion and total nucleated cells were counted with a Coulter Counter model BZI (Coulter Electronics Ltd., Harpenden, Herts, UK).

Plaque Assay

IgM- and IgE-secreting cells in each individual well were assayed by a modified protein A plaque assay (18,19). The class of the secreted immunoglobulin (Ig) was determined by using developing rabbit antisera specific for either IgM or IgE in the protein A plaque assay as well as in inhibition type ELISA using excess amounts of all other isotypes. After the appropriate adsorptions, the antisera were titrated and their specificity was confirmed in plaque assays using hybridoma cells of the appropriate classes. It was found that the antiserum preparations employed were monospecific for their respective isotypes. The origin and preparation of the anti-IgM (20) and anti-IgE antisera employed have been described (12).

Antigen-specific antibody-secreting cells were determined by coupling PC to sheep red blood cells (SRBC) as described (13). The number of TEPC-15 idiotype positive (T15 Id^+) anti-PC antibody secreting cells was determined by inhibition of plaque formation with monoclonal anti-T15-Id antibodies.

ELISA

Sera were collected from immunized mice and assayed for total and PC-specific IgE antibodies in a Terasaki-ELISA system (21,22) allowing quantitative determination of the IgE concentration. The anti-IgE antiserum (12) used for detection in this system was verified for monospecificity in inhibition type ELISA as well as immunoblotting. Quantitation was based on a standard consisting of a mixture of several samples of highly purified hybridoma IgE.

RESULTS

Specific IgE-secreting cells in the peritoneal cavity

Peritoneal cells of adult BALB/c mice immunized three times i.v. with 10 ug of PC-KLH on alternating days, were analyzed for the presence of IgE-secreting cells. This was done both by membrane immunofluorescence and plaque assays on peritoneal washings 5 days after the last antigen injection. The results showed a three fold increase in the percentage of IgE-bearing cells upon immunization (Table Ia), that coincided with a large increase in the number of anti-PC IgE-secreting cells (Table Ib). No such

TABLE I

Characterization of peritoneal cells of PC-KLH immunized BALB/c mice

A. Membrane marker expression

Marker	Control	PC-KLH
Thy-1	17 ± 3	16 ± 4
Ra3 6B2	53 ± 4	55 ± 7
MAC-1	23 ± 4	19 ± 7
Ly-1	20 ± 3	40 ± 7
IgE	1.5 ± 0.5	4.4 ± 1.8
IgM	49 ± 8	54 ± 9
lambda	24 ± 2	39 ± 5

Figures represent the arithmetic mean ± 1 SD of the percentage (n=3-6) of positive cells in the total cell suspension obtained 5 days after the last of three i.v. injections of 1 ug PC-KLH.

B. Ig-secretion

	Control	PC-KLH
Viable cells	6.3×10^6 a)	5.8×10^6
Total IgM-PFC	1 ± 1 b)	12 ± 4
Anti-PC IgM-PFC	0	0
Total-IgE-PFC	78 ± 16	1202 ± 146
Anti-PC IgE-PFC	0	899 ± 83
Anti-PC/total IgE-PFC	0 c)	0.75
$Lambda^+$-PFC	9 ± 2	324 ± 54
$Lambda^+$/anti-PC IgE-PFC	0.12 d)	0.27

a) Total number of viable cells as determined by trypan blue exclusion.
b) Arithmetic mean ± 1 SD (n=3).
c) Ratio expressed as percentage of all IgE-secreting cells that are PC-specific.
d) Ratio expressed as percentage of all IgE-PFC cells that secrete the lambda light chain.

changes were observed in the IgM isotype. Moreover, an increase in the number of lambda-bearing (from 24 to 39%) and lambda-secreting cells (from 9 to 324) were found. When calculating ratios it was found that upon immunization, 85 ± 10% of all IgE-secreting cells were PC-specific whereas 27% of these PC-specific IgE-secreting cells secreted the lambda light chain.

In the serum of these mice a 3 to 4 fold increase in the total IgE level from 319 to 1214 ng/ml was observed while PC-specific IgE antibodies constituted 30-35% of the total serum IgE. This response, however, was transient and declined to saline-treated control values within 14 days.

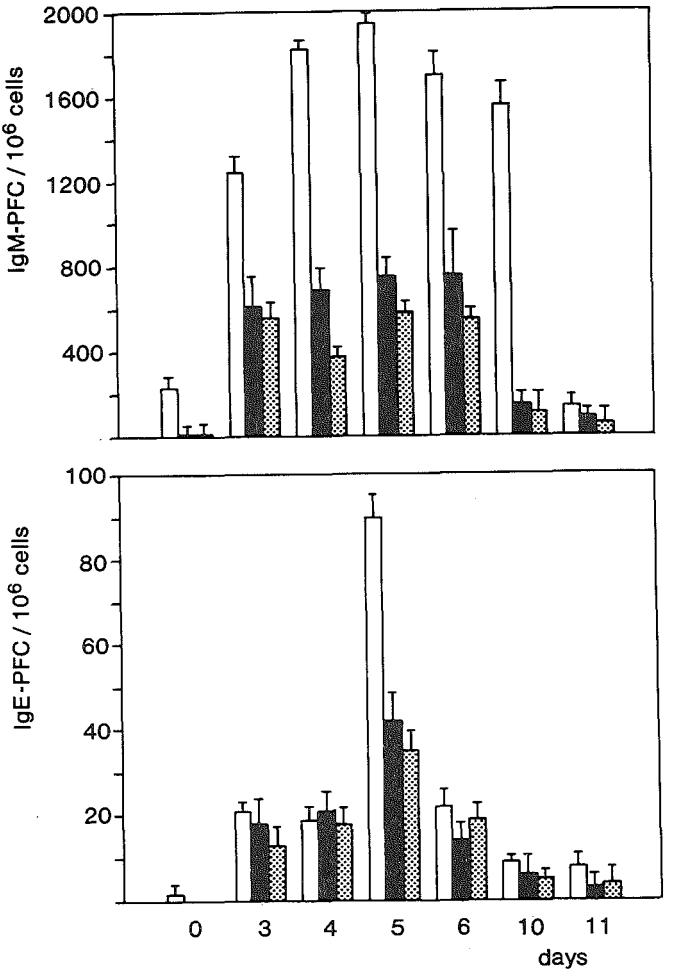

Fig. 1. Kinetics of the IgM (upper part) and IgE (lower part) PFC-response in the spleen after three immunizations with 10 ug PC-KLH in saline i.v. Hatched columns represent total IgM or IgE PFC, solid columns represent PC-specific IgM or IgE PFC while double hatched columns represent T15 Id^+ IgM or IgE PFC. Results are expressed as arithmetic mean $\pm$ 1 SD (n = 4).

Kinetics of IgE-PFC in the peritoneal cavity

Next, the kinetics of the total, the PC-specific and the T15 Id^+ IgM and IgE-secreting cell responses were compared after three i.v. injections with 10 ug of PC-KLH on alternating days. In the spleen a transient response was found, peaking on day 5 for total IgE- as well as PC-specific IgE-secreting cells (Fig. 1). In BM and parathymic and mesenteric lymph nodes similar kinetics of IgE-secreting cell formation was found (data not shown). On the peak day, 38 % of all IgM-PFC were PC-specific while 67% of all IgE-PFC were PC-specific. For either isotype, 78 to 89% of all PC-

specific antibodies were found to bear the T15 idiotype as determined by inhibition of the developing PC-specific IgE-PFC with anti-T15-Id antibodies. In the peritoneal cavity, a specific IgE response was found peaking on day 5 after the last injection and decreasing to a steady-state level of around 30 to 60 specific IgE-PFC per 10^6 cells. This plateau level persisted for at least 60 days after the last injection (Fig. 2).

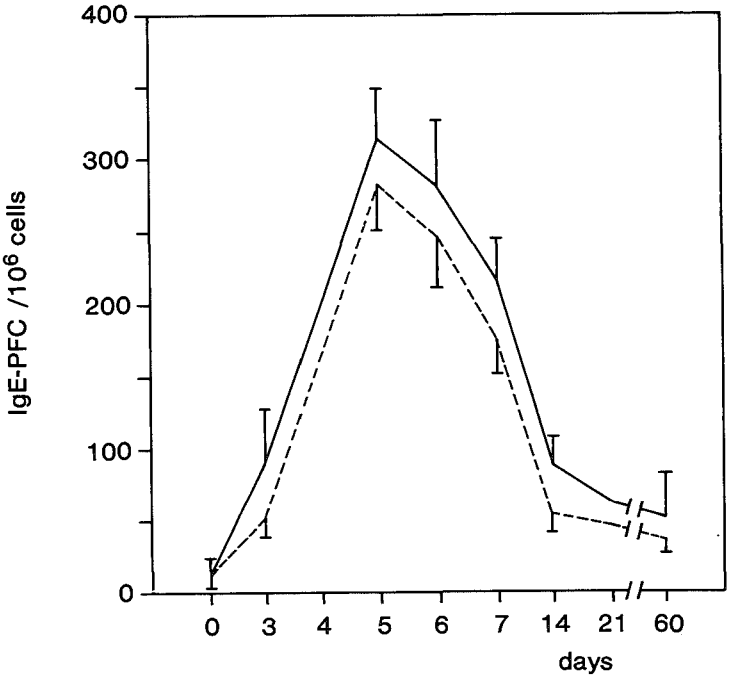

Fig. 2. Kinetics of the total and PC-specific IgE-PFC response in the peritoneal cavity after three immunizations with 10 ug PC-KLH in saline i.v. Solid lines represent total IgE-PFC while dashed lines represent PC-specific IgE-PFC. Results are expressed as arithmetic mean $\pm$ 1 SD of PFC per 10^6 cells.

Kinetics of serum IgE levels

After three injections of 10 ug PC-KLH on alternating days this immunization scheme was repeated three months later. Starting 4 days after the last injection, a three fold increase in the PC-specific IgE level was observed (Fig. 3). A rise in the antigen-specific IgE concentration was found as well. After decreasing to normal concentrations, the specific IgE level persisted to a fraction of around 35% of the total IgE level (320 ng/ml) for a period up to 60 days. Such a long-lasting rise in specific IgE was not noted after a single set of immunization (data not shown).

TABLE II

Effect of treatment with anti-IgM, anti-IgE and anti-T15 Id antibodies on the anti-PC IgE-PFC response in spleen and peritoneal cavity of BALB/c mice

Organ	Treatment	Immunization	Total IgE-PFC		Anti-PC-PFC		T15 Id^+-PFC	
			IgM	IgE	IgM	IgE	IgM	IgE
Spleen	-	saline	210 ± 50	<10	<10	<10	<10	<10
	-	PC-KLH	1980 ± 180	254 ± 42	122 ± 16	216 ± 37	101 ± 19	207 ± 16
	anti-IgM	PC-KLH	156 ± 38	<10	<10	<10	<10	<10
	anti-IgE	PC-KLH	1690 ± 240	<10	139 ± 26	<10	122 ± 14	<10
	anti-T15 Id	PC-KLH	1840 ± 210	191 ± 56	111 ± 54	179 ± 54	<10	<10
Peritoneal cavity	-	saline	<10	<10	<10	<10	<10	<10
	-	PC-KLH	74 ± 9	284 ± 64	25 ± 9	254 ± 33	24 ± 5	236 ± 21
	anti-IgM	PC-KLH	<10	<10	<10	<10	<10	<10
	anti-IgE	PC-KLH	86 ± 18	<10	21 ± 10	<10	<10	<10
	anti-T15 Id	PC-KLH	47 ± 12	268 ± 53	16 ± 8	241 ± 39	<10	<10

Results are expressed as the arithmetic mean ± 1 SD of the data from 4 mice tested in individually.

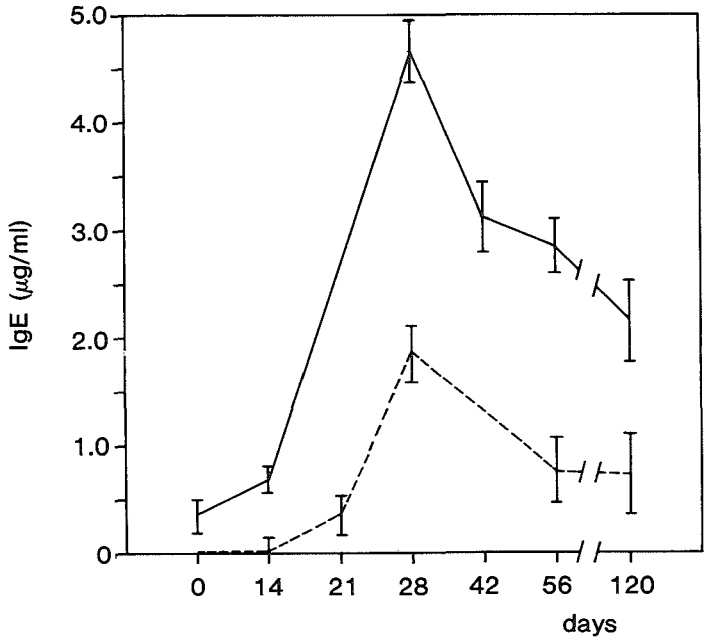

Fig. 3. Kinetics of serum IgE concentrations in ng/ml of total (solid lines) and PC-specific (dashed lines) IgE after two sets of three immunizations of 10 ug PC-KLH in saline i.v 3 months apart. Results are expressed as arithmetic mean $\pm$ 1 SD (n = 4).

Suppression of IgE-PFC responses

Mice received during the standard protocol of three immunizations with PC-KLH inhibiting doses of various purified antibodies. The effects of these suppressive treatments on the total, PC-specific and T15-Id$^+$ IgM- and IgE-secreting cell responses were determined. The results showed that both in spleen and peritoneal cavity the IgE response is largely PC-specific and these PC specific IgE-secreting cells secrete for 80 to 90% T15-Id$^+$ antibodies (Table II). Treatment with anti-idiotypic antibodies, however, did not cause a significant decrease in the number of PC-specific IgE-PFC, although the development T15-Id$^+$ PFC was almost completely inhibited. Moreover, anti-IgM and anti-IgE treatment were equally effective in inhibiting these IgE-PFC responses in both organs tested.

Anti-IgM and anti-IgE treatment also effectively inhibited the increase of IgE levels in the serum. Anti-T15-Id treatment, on the other hand, did not affect the serum level of total or PC-specific IgE (Table III). For comparison, mice were treated neonatally with anti-IgM and anti-IgE antibodies. Such mice did not reveal any detectable serum or cellular IgE response, independent of whether or not they had been immunized. Neonatal treat-

ment with anti-T15-Id antibodies, did not cause a decrease in the anti-PC IgE-PFC response in either spleen or peritoneal cavity as compared to non-treated mice (data not shown). Also, no effect of neonatal anti-T15-Id treatment was found on the serum level of total and PC-specific IgE (Table III).

TABLE III

Serum IgE concentrations in BALB/c mice immunized with PC-KLH that were either neonatally or at adult age treated with anti-IgM, anti-IgE or anti-T15

	Treatment	Immunization	IgE-concentration (ng/ml)	
			Total	PC-specific
Neonatal	-	saline	316 ± 54	<10
	-	PC-KLH	983 ± 162	354 ± 86
	anti-IgM	PC-KLH	<10	<10
	anti-IgE	PC-KLH	<10	<10
	anti-T15	PC-KLH	856 ± 159	276 ± 36
Adult	-	saline	352 ± 126	<10
	-	PC-KLH	1214 ± 238	417 ± 83
	anti-IgM	PC-KLH	<10	<10
	anti-IgE	PC-KLH	<10	<10
	anti-T15	PC-KLH	1063 ± 214	316 ± 26

BALB/c mice were treated with purified antibodies either adult (3 months of age) and immunized with PC-KLH at the same time. Neonatally suppressed mice were at the age of 3 months when immunized with PC-KLH. Serum IgE levels were determined 60 days after immunization. Results are expressed as the arithmetic mean ± 1 SD (n=4).

DISCUSSION

The purpose of this study was to investigate the appearance of IgE-secreting cells in the peritoneal cavity and their involvement in the persistent occurrence of antigen-specific IgE in the serum. This study was indicated by our previous finding of the absence of a clear correlation between the numbers of IgE-antibody secreting cells in the lymphoid organs and IgE-antibodies in the serum as determined by ELISA (5). We therefore immunized mice according to a protocol that usually selectively induces an IgE response without accompanying IgM- and IgG-responses (11). Based upon the results of this study we selected a protocol of immunizing mice three times with 10 ug of antigen i.v. on alternating days without the use of an adjuvant.

The numbers of IgE-secreting cells were dependent on the dose and route of immunization and the use of adjuvants. In this and the previous study (5), it was found that the BM contained specific IgE-secreting cells already during the primary immunization. Interestingly, the BM showed a transient IgE production while in the serum a persistent specific IgE level

was observed. This pointed to a differential regulation of BM IgE production with low dose immunization as compared to the regulation of Ig production in the BM employing high antigen dose on alum. It was described (1-4) the BM accounts for the long-lasting specific Ig formation because of the immigration into the BM of antigen-activated (memory) B cells from the peripheral lymphoid organs. Moreover, within the BM, and a large proliferative activity of the immigrant cells was found (1,2,20). It was suggested in these studies that a lack of feedback suppression in the BM could account for these phenomena.

Just as in the previous study (5), a discrepancy was found between the kinetics of the antigen-specific IgE response in the serum and the IgE-secreting cell responses in various lymphoid organs. The time lag found between the rise and the fall of antigen-specific as well as total IgE-secreting cells in various lymphoid organs on one hand and the appearance of antigen-specific IgE in the serum on the other hand, leads us to suggest that the persistent serumlevels of IgE could be caused by a burst of long-lasting IgE-secreting cells in the peritoneal cavity. These cells may have reached this site by a migratory process from the lymphoid organs like spleen, lymph nodes and BM. A lack of feedback suppression by the antibodies produced may account for the persistent specific IgE formation in this site.

The population of peritoneal cells has also been analyzed for their phenotypic characteristics and potential for IgE secretion in immunized mice (Table I). The values found in saline treated control mice were in agreement with those obtained before (23). The results of immunofluorescence studies indicate that 5 days after ceasing the immunization, there is a two-fold rise in the percentage of surface IgE-bearing cells. These results, suggestive for an IgE-response, were confirmed in plaque assays. It is clearly shown that 5 days after PC-KLH immunization, there is a 15-fold increased number of total IgE-secreting cells in the peritoneal cavity of which 75 % are PC-specific. Moreover, a substantial rise was found in the number of lambda light chain-bearing and -secreting cells. While virtually no IgM synthesis and certainly no IgG synthesis (data not shown) was found in the peritoneal cavity, the results suggest that 27 % of all IgE-secreting cells secrete IgE containing the lambda light chain. These results suggest some involvement of the Ly-1 B cell subset in peritoneal IgE responses. This is all the more apparent from the increased production of lambda light chain secreting cells, that are more abundant in the $Ly\text{-}1^+$ B cell subpopulation (23). Alternatively, a hypothesis has been put forward (24,25) that the omental lymphoid tissue may constitute another primary site of lymphocyte development and may be the source of peritoneal B cells in adult mice. The antigen-specific activation of the relevant peritoneal B cell subpopulations leading to IgE synthesis is currently under examination.

In this study we also established the kinetics of the total, the PC-specific and T15 idiotype positive IgE-secreting cell response in the spleen. For comparison also the IgM response was evaluated. The results (Fig. 1) indicate a transient IgE response peaking at day 5 for total IgE- as well as PC-specific IgE-secreting cells. The number of anti-PC IgM-secreting cells only moderately increased showing the selective effect of this immunization in inducing predominantly IgE responses. Throughout the PC-specific response, the percentage of secreted IgE-antibodies bearing the T15 idiotype was similar (83 % at maximum) as previously has been reported for the IgM- and IgG-responses in BALB/c mice. This is in agreement with other studies describing that the PC-specific IgE-response in BALB/c mice has a similar idiotypic homogeneity as anti-PC antibodies of other isotypes

(8,9,26,27). An analysis of the fine specificity of anti-PC IgE antibodies elicited by immunization with PC-KLH in alum showed that IgE antibodies, express the typical group II characteristics in that the IgE antibodies are T15 Id negative (28-30).

The data presented in this paper show that the peritoneal cavity harbours a substantial part of the total number of PC-specific IgE-secreting cells that are generated after an immunization procedure that almost exclusively induces IgE because of repeated immunization with low antigen doses. Furthermore, these studies show that the fine specificity of these PC-specific IgE-secreting cells is different from those induced by immunization with higher doses of PC containing antigens in combination with an adjuvant.

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CHAPTER 6

LYMPHOKINE-DEPENDENT REGULATION OF IgE-ANTIBODY FORMATION

CHAPTER 6.1

INTRODUCTION: LYMPHOKINE DEPENDENT IgE FORMATION

Cultures of B cells stimulated with bacterial lipopolysaccharide (LPS) in the absence of T cells have proven to be a convenient system for the identification, preliminary characterization and cloning of T cell products that differentially affect the expression of certain Ig isotypes. The addition of supernatants from activated Th2 clones to LPS-stimulated B cells usually resulted in a significant enhancement of the production of IgG_1 [1,2], IgE and IgA [3,4]. In our experiments, IgG_1 and IgA levels were typically enhanced 5 to 20-fold, whereas IgE levels, which were below the threshold of the IgE assay without additions, were elevated to levels which are 100 to 1000 times that threshold by the addition of most Th2 supernatants. Using highly purified IL-4 and a blocking monoclonal antibody to IL-4, both the IgG1 [5] and IgE [6] enhancing activities in Th2 supernatants have been shown to be activities of IL-4. These observations have been confirmed with recombinant IL-4 (rIL-4) [7,8] and indeed, the enhancement of IgG1 was the principal activity used by one of the groups of investigators for isolating their IL-4 cDNA clone [8]. IL-4 need not be present from the start of the cultures but must be added by day 2 for maximum IgE and IgG1 enhancement [3]. If IL-4 was added after day 3, no enhancement of either isotype was observed.

The mechanisms by which IL-4 and other lymphokines can enhance or inhibit the production of specific isotypes are not yet understood. IL-4 could enhance the secretion of IgG_1 or IgE either by causing the selective proliferation or maturation of B cells already committed to IgG_1 or IgE production or by specifically increasing the frequency of switching from IgM secretion to the secretion of these other isotypes.

In order to distinguish between these two possibilities, limiting dilution analyses have been performed with LPS-stimulated B cell cultures. Early studies demonstrated that the addition of a T cell line supernatant containing IL-4 caused a substantial increase in the frequency of precursors of IgG_1 secreting cells but only a small increase in the size of the clones originating from these precursors [1,9]. Furthermore, this increase in precursors giving rise to IgG_1 producing clones occurred predominantly in the surface IgG_1 negative B cell population. We took a similar approach to address the mechanism underlying the IL-4 induced enhancement of IgE secretion (Section 6.2).

Th cells of the Th1 and Th2 subsets secrete, when activated, a number of lymphokines that act on a wide variety of cell types and mediate several effector functions. Indeed, some of the regulatory and effector functions are mediated by the same lymphokines that act as B cell proliferation and differentiation factors. The principles that have emerged in the past several years are that most lymphokines or cytokines can act on a wide range of hematopoietic and nonhematopoietic cell types, can have quite different effects (such as growth versus differentiation) on different cells within the same lineage, and can often act differently in combination

with other lymphokines than acting alone. These principles suggest ways in which Th cells could initiate and control a coordinated set of immune and inflammatory responses, perhaps selected for optimum effect against a specific class of pathogens. Since the two Th subsets express quite distinct sets of lymphokines, it seems reasonable to suggest that they regulate very different sets of immune and inflammatory responses.

The dramatic and opposing effects on IgE production of two lymphokines, IL-4 and IFN-γ, made by the two different subsets of Th cells, suggest a model for the regulation of IgE production in vivo. We have proposed that the level of IgE produced during an immune response reflects the relative levels of IL-4 and IFN-γ to which the activated B cells are exposed [10,11]. This, in turn, depends upon the relative numbers or states of activation of Th1 and Th2 cells of the appropriate specificity. This model predicts that Th2 cells will be helpers for an IgE response and that Th1 cells will act as isotype-specific suppressor cells for IgE, at the same time they may be acting as helper cells for responses of other isotypes. On the basis of these assumptions and together with the notion that IgE antibody formation is controlled by genetic factors (Section 1.3.5), a study was conducted on the possible involvement of the IL-4 productive capacity in the differential ability of various mouse strains to produce IgE upon immunization (Section 6.3).

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CHAPTER 6.2

INCREASE OF PRECURSOR FREQUENCY AND CLONAL SIZE OF MURINE IMMUNOGLOBULIN E-SECRETING CELLS BY INTERLEUKIN-4

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ABSTRACT

Interleukin-4 is able to preferentially enhance murine IgE levels in the supernatant of LPS-stimulated T-cell depleted splenic B cell cultures. Clonal and quantitative analysis of this response revealed that this is due partly to a 14-fold increased IgE precursor frequency and partly to a 3-fold increased clone size of IgE-secreting cells. IL-4 increased the precursor frequency and the clone size of IgM-secreting cells not more than two-fold. Both the IgM and IgE response in LPS-stimulated B cells were completely inhibited by the addition of monoclonal anti-IgM antibodies (M41) to the cultures, indicating that the IgE-secreting clones developed as subclones from precursors that express IgM. These cells lacked expression of membrane-bound IgE upto day 5 of the culture. Application of feeder cells in these cultures resulted in an increased precursor frequency of IgE-secreting clones among LPS-reactive B cells that is due, partially, to IL-4 produced by the feeder cells.

INTRODUCTION

Recent studies have indicated the existence of several activities in the supernatant of murine helper T cell hybridomas, clones and lines activated by concanavalin A or the relevant antigen. These factors amplify responses of B cells activated by anti-immunoglobulin M (IgM) in vitro, induce Ia antigens on resting B cells, prepare small resting B and T cells for entry into the cell cycle and enhance IgE and IgG_1 production upon stimulation with lipopolysaccharide (LPS) in vitro. These activities together with the induced proliferation of several IL-2 and IL-3 dependent cell lines, were ascribed to B cell stimulatory factor-1 or interleukin-4 (IL-4) (1-14). Recently, a cDNA coding for IL-4 has been isolated (15,16) and receptors for IL-4 have been identified on most haemopoietic cells (17, 18). It is suggested that IL-4 is a growth and differentiation factor and that it affects all hemopoietic cell lineages apart from the B cell compartment.

It has been shown that the production of IL-4 is exclusively linked to helper T cells ($Lyt\text{-}1^+L3T4^+Lyt\text{-}2^-$) of the Th2 type (19,20). One of the most striking effects of IL-4 in vitro is the 10^2-10^3-fold enhancement of the IgE-production by LPS-stimulated B cells (12, 13, 21). Also in in vivo experiments the relevance of IL-4 and Th2 cells for the induction of significant IgE-responses has been described (22, 23).

It is reported in limiting dilution cultures that the enhanced secretion of IgG_1 in LPS-stimulated B cell cultures by B cell differentiation factor for IgG (now known to be IL-4), was caused by increased isotype switching by activated, surface IgG_1^- B cells to IgG_1 secretion (8, 10, 11). Similarly, it was suggested that the enhancing activity of IL-4 on murine IgE production was also due to an increased switch frequency from IgM to IgE-secretion (13). It is still unclear, however, whether IL-4 instructs B cells to switch to the production of IgE, or whether IL-4 preferentially induces high-rate IgE-secretion in B cells that had already 'switched' to IgE isotype expression at the membrane level. In this report we show that addition of IL-4 causes an increased frequency of IgE-secreting clones. Furthermore we show that the average clone size of IgE-secreting cells increases in the presence of enhanced IL-4 levels.

MATERIALS AND METHODS

Mice

Female BALB/c mice, 6-20 weeks of age, were used throughout this study and were obtained from either Bomholtgard, Ry, Denmark or from the Institute for Medical Research, San Jose, CA, USA. As a source of thymus cells 3-6 weeks old syngeneic BALB/c mice were used or heterologous Lewis rats (Central Institute for Laboratory Animals, Hannover, FRG).

Cell suspensions

Whole intact cell suspensions of murine spleen (24) were prepared by passing the cells through a nylon gauze filter with 100 um openings, as described (25). T-cell depleted spleen cell suspensions were prepared by gently squeezing spleen fragments through a 200-mesh wire screen. The cell suspensions were washed and incubated with anti-Thy-1.2 (30-H12; No.TIB107; American Type Culture Collection ATTC, Rockville, MD, USA) for 15 min on ice. The cells were then pelleted and resuspended in 10% rabbit complement (Lo-Tox M; Cedarlane Lab., Hornby, Ontario, Canada) diluted in RPMI 1640 containing 25 mM HEPES, pH 7.2, and 0.3 % bovine serum albumin (BSA, grade V; Sigma Chemical Co., St. Louis, MO, USA). Cells were incubated with

complement for 45 min at 37°C, and the dead cells were removed by centrifugation over Histopaque (density 1.119 g/ml; Sigma). All steps except the complement treatment were performed in Hanks' balanced salt solution plus 0.3% BSA. Using this procedure, the B cells ($B220^+$) prepared were consistently more than 95% pure, while the contamination of $Thy\text{-}1^+$ cells was less than 1 to 2%. Thymus cell suspensions were prepared by dissecting the thymus avoiding blood contamination and carefully removing the parathymic lymph nodes. The thymus cell preparation was subjected to low dose X-irradiation (0.1 Gy) as described (26) and after washing these cells were used as filler cells in the limiting dilution cultures where indicated. Viable cells were counted by the trypan blue exclusion method. Total nucleated cells were counted with a Coulter Counter model BZI (Coulter Electronics Ltd., Harpenden, Herts, UK).

In vitro cultures

In cultures of whole cell suspensions of spleen and bone marrow (BM), varying numbers of cells were cultured with 3.6×10^6/ml filler cells and 50 ug/ml E.coli lipopolysaccharide (LPS 026:B6; Difco, Detroit, MI) in 0.2 ml of RPMI 1640 medium supplemented with L-glutamin (4mM), penicillin (100 IU/ml), streptomycin (50 ug/ml), 2-mercaptoethanol (50 uM) and 20 % fetal bovine serum, batch 29101086(FCS; Flow, Irvine, Scotland), specifically selected for growth-supporting properties and low endogenous mitogenic activity. Routinely, 32 replicate cultures were set up for each cell concentration and, as controls, for cultures containing no mouse lymphoid cells. The cells were cultured in flat bottom microtitre plates (Costar 3596; Costar, Cambridge, MA). The cultures were assayed on day 5 for IgM- and on day 7 for IgE-secreting plaque-forming cells (PFC).

In cultures using feeder cells, also replicates were studied in which 10 ug/ml purified 11B11 (anti-IL-4; 27) was added to the cultures. In limiting dilution cultures of T-cell depleted spleen cells, known numbers of B cells were incubated in twice the final cell concentration in a similar medium without the addition of filler cells and using 8 ug/ml of LPS from Salmonella typhosa (Sigma). Cells were cultured in round bottom, 96-well plates (Flow Laboratories, McLean, VA). Highly purified recombinant IL-4 (100 U/ml) together with IL-5 (8 U/ml; 28,29) or plain culture medium were added after 1 day of culture. IL-5 was added to these cultures to permit the use of lower concentrations of IL-4. We have shown (Coffman and Shrader, in preparation) that maximum IgE production requires 10-30 fold less IL-4 in the presence of IL-5 then in the absence of IL-5. IL-5 by itself causes no stimulation of IgE production either in LPS-stimulated cultures (28) or in Th-stimulated cultures (30). To evaluate the effect of anti-IgM on the in vitro IgM and IgE responses, B cells were stimulated with LPS in the presence or absence of highly purified monoclonal anti-IgM antibodies (clone M41; kind gift of Drs. M. Leptin and F. Melchers from the Basel Institute for Immunology). As determined by titration studies the plateau of inhibition of the IgM precursor frequency in in vitro cultures was reached at 4 ug/ml. In some experiments, some cultures were supplemented with anti-IgM while 1 day later IL-4 was added. All culture supernatants were harvested 7 days after initiation of the cultures and frozen until assayed.

ELISA and Plaque Assay

The supernatant of every individual culture was assayed in isotype-specific enzyme-linked immunosorbent assays (ELISA) for IgM and IgE as described (12, 31). IgM- and IgE-secreting cells were assayed by a modified hemolytic plaque assay employing Staphylococcus aureus protein A (Pharmacia, Uppsala, Sweden)-coated sheep red blood cells (SRBC) and Ig-class

specific rabbit anti-mouse-Ig antibodies as developing antisera, in the presence of guinea pig complement (Behringwerke, Marburg/Lahn, FRG). We employed the protein A plaque assay with some modifications (32). The origin and specificity of the anti-IgM and anti-IgG_1 antisera (33) as well as of the anti-IgE antiserum employed have been described. The same class-specific antisera were employed in the ELISA. After the appropriate adsorptions, the antisera were titrated and their specificity was assessed in plaque assays using myeloma or hybridoma cells of the appropriate classes. Moreover, purified IgE hybridoma proteins of several antigenic specificities and hybridoma proteins of all other isotypes secreting both lambda and kappa light chains, were used to confirm the specificity of the anti-IgE antiserum in immunoblotting and in inhibition ELISA. Typically, 50 ug/ml of all other isotypes read less than 1 ng/ml in the IgE-ELISA. As a result, the antiserum was found to be monospecific for the F_c portion of murine IgE.

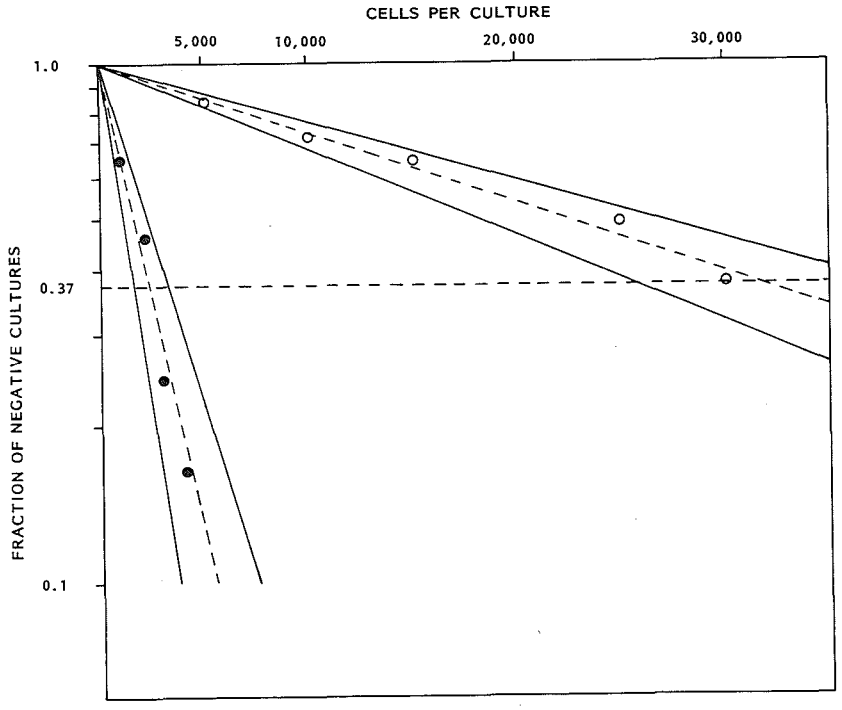

Fig. 1. The enhancing effect of IL-4 on the frequency of IgE-secreting clones. Thirty-two replicate cultures of T-depleted spleen cells were set up for each cell concentration either with LPS (O) or with LPS plus IL-4 (250 U/ml) (●). Cultures were tested at day 7 for IgE-secreting cells with the reverse plaque assay. For each frequency determination, the lower line represents the upper 95% confidence limit, the middle line represents the frequency estimate and the upper line represents the lower 95% confidence limit of the frequency. Precursor frequencies (1/n) (range) of IgE-secreting B cells were: in the presence of LPS 31,800 (25,700-37,900) or of LPS plus IL-4 2,300 (1,400-3,200).

Data analysis

Culture conditions (34) were employed that allow every growth-inducible B cell to grow and differentiate into a clone of Ig-secreting cells upon stimulation by LPS. By reducing the number of lymphoid cells added to each culture, reactive B cells become limiting and fluctuating conditions are reached in which a fraction of all cultures is negative. The results of a limiting dilution analysis of splenic B cells with or without the addition of IL-4 for the frequencies of precursor B cells yielding IgE-secreting clones is shown in Fig. 1. The linear regression of the curves, plotted as numbers of cultured cells against the logarithm of the fraction of nonresponding cultures indicates that precursor B cells reactive to LPS yielding IgE-secreting cells were limiting in these cultures. This fraction of negative cultures for the various cell concentrations was confirmed to the Poisson distribution. Therefore, frequencies of reactive cells could be determined at cell concentrations yielding, on an average, one clone/culture. Evaluation of such quantal dilution assays is performed by the method of maximum likelihood and frequencies are estimated according to the program as described by Fazekas de St.Groth (35). Results are expressed as precursor frequency with the 95% confidence limits and p-value. In some cultures, thymocyte feeder cells themselves gave rise to small numbers of PFC and this was taken into account when setting the threshold for positive cultures. In these cultures a maximum of less then 10 PFC were found per well and cultures were scored positive when they yielded at least 20 PFC (33). For the screening of T-depleted spleen cell cultures in which no feeder cells were employed, the negative cultures without addition of spleen cells did not yield any PFC. Threshhold values for allowing positive-negative screening in ELISA were determined in quantitative ELISA testing control medium only. These levels were set to 1 ng/ml, the detection limit of the ELISA.

The switch frequencies were calculated as the ratio of the frequency of IgE-secreting clones as determined at day 7 to the frequency of IgM-secreting clones as determined at day 5 of the culture. This procedure is based upon the finding that in LPS-stimulated B cell cultures, cells secreting a particular non-IgM isotype developed from clones which contained previously IgM-secreting cells (21,34).

The average clone size, of IgM- or IgE-secreting B cells was calculated according to Layton et al. (8). This analysis is performed in microtitre plates employing an input of B cells in which around 37% of the cultures are negative and, therefore, the positive cultures contain an average of one mitogen-reactive precursor per culture. Clone sizes are expressed as the average number of Ig-secreting cells in a clone resulting from 1 precursor or by the average Ig level (ng/ml) in day 7 supernatant of these cultures.

Immunofluorescence studies

Cell suspensions after T-cell depletion were stained with anti-Thy-1.2 to determine residual contaminating Thy-1^+ cells. In whole cell suspensions of spleen and BM the percentage of surface Ig-bearing as well as B220-positive cells was determined. Therefore, polyclonal rabbit anti-IgM and anti-IgG_1 (Nordic, Tilburg, The Netherlands) and monoclonal rat anti-B220 B-lineage marker RA3-6B2 (12) were employed, respectively. For IgE, the above mentioned polyclonal rabbit anti-IgE antiserum was used. Immunofluorescence was performed as described (36) with goat-anti-rabbit and rabbit-anti-rat fluorescein isothiocyanate labelled second stage antibodies (Nordic, Tilburg, The Netherlands). The results of cytoplasmic IgM and IgE positive cells were corrected for the percentage surface Ig positive cells.

RESULTS

Kinetics of IgE precursor frequencies in LPS activated B cells

Whole cell suspensions of BALB/c spleen were stimulated with LPS and assayed for IgM- and IgE-precursor frequencies on days 3 to 8 of culture. Secreting cells were tested in isotype-specific plaque assays using the appropriate developing antisera. For comparison, B cells were prepared from BALB/c mice and stimulated with LPS. The results (Table I) confirm previous data that the optimal culture period for determining IgM and IgE precursor frequencies is day 5 and 7 in cultures of intact spleen as well as of splenic B cels, respectively. Moreover, these results showed that the frequency of IgM- and IgE-secreting clones in whole spleen cell cultures were not significantly different from those frequencies among purified splenic B cells assuming that BALB/c spleen contains 45% $sIgM^+$ B cells. This was supported by the finding that both the IgM and the IgE response in LPS-stimulated B cells was completely inhibited by the addition of monoclonal anti-IgM antibodies to the cultures (cf. Table IV). This indicates that the IgE-secreting clones developed from precursors that express surface IgM. Based on this assumption one can calculate a switch frequency by dividing the frequency of IgE secreting clones by the frequency of IgM secreting clones. The maximum switch frequency from IgM to IgE among splenic B cells from BALB/c mice was about 1.5%.

TABLE I

Kinetics of precursor frequencies for IgM and IgE in BALB/c spleen cell cultures

	IgM		IgE	
Day	Absolute[a] frequency	Frequency[b] among B cells	Absolute frequency	Frequency among B cells
3	<1 in 200	<1 in 200	<1 in 4,000	<1 in 4,000
4	1 in 169	1 in 84	<1 in 4,000	<1 in 4,000
5	1 in 35	1 in 12	<1 in 4,000	<1 in 4,000
6	1 in 40	1 in 28	1 in 3,300	1 in 2,100
7	1 in 39	1 in 27	1 in 2,300	1 in 1,600
8	<1 in 200	<1 in 200	<1 in 4,000	<1 in 4,000

[a] Whole spleen cell cultures were stimulated with LPS in the presence of rat thymocytes at 3.6×10^6/ml. Absolute precursor frequencies were determined by plaque assays of the individual cultures and represent the arithmetic mean of 4 independent experiments.

[b] Precursor frequencies among LPS-reactive B cells are based on cultures containing purified B cells.

Enhancement of IgE precursor frequency and clonal burst size by IL-4

Before analyzing the effects of IL-4 on the IgE response at the clonal level, we wanted to confirm that IL-4 stimulated IgE production in bulk cultures. IgE levels were determined in supernatants of T-depleted spleen cells stimulated either with LPS alone or with LPS plus IL-4. The typical

results of such experiment (Table II) showed a more than 900-fold increase in the IgE level in cultures stimulated with IL-4. The other isotypes tested did not differ more than 3-fold, with the exception of IgG_1 which increased 21-fold. Simultaneously, a 5.4-fold decreased IgG_3 level was observed in IL-4 stimulated cultures. Addition of anti-IL-4 antibodies to the cultures completely inhibited the effect of IL-4 on the IgE, IgG_1 and IgG_3 isotypes.

TABLE II

Effect of IL-4 on the production of the various isotypes by LPS-stimulated cultures of T-depleted spleen cells as determined by ELISA

Addition to culture medium	supernatant Ig level (ng/ml)						
	IgM	IgG1	IgG2a	IgG2b	IgG3	IgA	IgE
Medium	56,800	1,900	25	499	5,500	89	<1.5
IL-4[a]	69,300	39,500	19	526	1,000	120	920
IL-4 + anti-IL-4[b]	49,700	1,000	31	643	6,300	113	<1.5

[a] Recombinant IL-4 was added at day 1 in 250 U/ml final concentration.
[b] Anti-IL-4 antibodies were added at day 0 in 10 ug/ml final concentration to some cultures that contained IL-4.
Figures represent the arithmetic mean of 4 independent experiments.

Next, we examined whether IL-4 increased the frequency of IgE precursors relative to the frequency of IgM precursors. This was done in a limiting dilution culture system. These cultures were analyzed on days 5 and 7 by performing IgM- and IgE-plaque assays, respectively. The supernatants of these cultures were also tested for IgM and IgE levels by isotype-specific ELISA. We also examined whether IL-4 influenced the clone size of the IgE-secreting clones. To this end T-cell depleted spleen cells were cultured with and without the addition of highly purified recombinant IL-4. As a control, some of the cultures with IL-4 were supplemented with anti-IL-4 in order to inhibit the enhancing activities of IL-4.

The results of the limiting dilution analysis of splenic B cells in the presence or absence of exogenous IL-4 were plotted in Fig. 1. These results together with those of Table III, demonstrate that, according to plaque assays, IL-4 induced on the average a 14-fold increase in IgE precursor frequency, while for IgM less than a 2-fold increase was observed. Precursor frequencies as determined by analysis of the supernatant levels showed a 7.9-fold increase for IgE and a 2-fold increase for IgM in the presence of IL-4. The clone size of IgE-secreting clones displayed a 3-fold increase as revealed by plaque assays. Anti-IL-4 antibodies added to the IL-4 stimulated cultures effectively blocked the enhancing activities of IL-4. This suggests that part of the enhancing effects of IL-4 are due to an increase in frequency of cells that give rise to an IgE producing clone and part are due to an increase in numbers of IgE-producing cells made by each clone.

TABLE III

Effect of IL-4 on IgE precursor frequency, switch frequency from IgM to IgE and clonal burst size in limiting dilution cultures of LPS-reactive T-depleted splenic B cells

Assay	Addition	Precursor frequency (1/n)		Switching frequency (%)	Clone size	
		IgM	IgE		IgM	IgE
					PFC	
Plaque assay	Medium	352 ± 116[a]	31,800 ± 6,100	1.0	24 ± 4	7 ± 3
	IL-4[b]	226 ± 48	2,300 ± 900	9.8	36 ± 7	17 ± 4
	anti-IL-4	298 ± 73	24,700 ± 4,200	1.0	13 ± 3	5 ± 1
					ng/ml	
ELISA	Medium	359 ± 166	20,000 ± 3,600	1.8	14 ± 4	2 ± 1
	IL-4	165 ± 17	2,500 ± 587	6.5	17 ± 6	7 ± 4
	anti-IL-4	384 ± 77	26,200 ± 8,600	1.5	2 ± 1	1 ± 1

[a] Precursor frequencies are expressed as the number of B cells representing 1 IgE precursor as determined either by plaque assay or by ELISA on the supernatant of the culture. In ELISA day 7 supernatants were tested for IgM and IgE. Threshhold values for allowing positive-negative screening were set at 1 ng/ml of IgM or IgE produced in the supernatant when analyzed by ELISA. Switch frequencies were calculated as the ratio of the frequency of IgM- secreting precursors to the frequency of IgE-secreting precursors. These results are expressed as the precursor frequency with its 95% confidence limits ($p < 0.01$). These experiments were repeated 6 times and all precursor frequencies determined fell within these 95% confidence limits shown for this typical experiment. Clone sizes are expressed either as the average number of Ig-secreting cells or by the average Ig level (ng/ml) in day 7 supernatant of these cultures. These results are expressed as the arithmetic mean ± 1 SD (n=6).

[b] At day 1, IL-4 (100 U/ml) and IL-5 (8 U/ml) were added to the cultures while anti-IL-4 antibodies were added at day 0 in a final concentration of 10 ug/ml.

Blocking of the IL-4 induced enhancement of IgE production by anti-IgM antibodies

The most likely explanation for this increase in precursor frequency is that IL-4 enhances the frequency of isotype switching that occurs during proliferation and differentiation of clones of IgM^+IgD^+ B cells although it is formally possible that B cells that have switched to IgE and therefore bear sIgE require IL-4 in order to grow or differentiate into a detectable clone. In order to test these possibilities, limiting dilution assays, both with and without IL-4, were performed in the presence of a monoclonal IgM antibody which has been shown to inhibit the responses of IgM-bearing cells. At days 5 and 7 precursor frequencies as well as clone sizes were determined for IgM and IgE, respectively. Anti-IgM antibodies (Table IV) were able to decrease the frequency of LPS stimulated Ig-secreting clones

TABLE IV

Effect of anti-IgM antibodies on the precursor frequency, switch frequency and clonal burst size of IgE as determined by plaque assay

T-depleted spleen cells cultured with		precursor frequency (1/n)		Switching frequency	Clone Size	
IL-4	anti-IgM	IgM	IgE	(%)	IgM	IgE
-	-	332 ± 92	29,400 ± 7,800	1.1	26 ± 22	8 ± 3
+	-	263 ∓ 77	3,600 ∓ 1,400	7.3	37 ∓ 10	23 ∓ 5
-	+	1,217 ∓ 214	<32,000	-	6 ∓ 4	2 ∓ 1
+	+	910 ∓ 246	<32,000	-	10 ∓ 7	2 ∓ 1

T-depleted spleen cells were stimulated with LPS and cultured with or without an optimal inhibitory dose of anti-IgM antibodies (4 ug/ml). IL-4 (100 U/ml) and IL-5 (8 U/ml) were added to the cultures at day 1. Results are expressed as arithmetic mean ± 1 SD (n=3).
For details: see the legend to Table III.

TABLE V

Kinetics of surface- and cytoplasmic-Ig positive cells of various isotypes in LPS-stimulated T-depleted spleen cell cultures supplemented with IL-4 as determined by immunofluorescence

Marker	Day 0	Day 3		Day 5		Day 7	
		+IL-4	-IL-4	+IL-4	-IL-4	+IL-4	-IL-4
sIgM	93	74	52	39	22	10	2
sIgG1	1	0.8	<0.5	2.5	<0.5	17	2
sIgE	<0.5	<0.5	<0.5	2.0	<0.5	20	1
cIgM	<0.5	6.5	2.3	23.5	5.5	3	1
cIgG1	<0.5	2.5	<0.5	5.5	<0.5	15.5	<0.5
cIgE	<0.5	<0.5	<0.5	<0.5	<0.5	5	<0.5

Bulk cultures containing 5 x 10^4 T-depleted spleen cells per well were stimulated by LPS in the presence or absence of IL-4 (100 U/ml) and incubated for 7 days. On alternate days, cells were stained with polyclonal antisera and the percentage of positive cells was determined by counting 300 viable nucleated cells. The percentages cytoplasmic positive cells were corrected for the percentage surface positive cells. The detection limit of this procedure is 0.5%. Results are expressed as percentage positive cells.

TABLE VI

Effects of mouse and rat feeder cells and the inhibition by anti-IL-4 antibodies on the development of clones of IgM and IgE secreting cells

T-depleted cells cultured with			Precursor freq. (1/n)		Switch frequency	Clone size (PFC or ng/ml)	
Feeders	IL-4	anti-IL-4	IgM	IgE	(%)	IgM	IgE
A. ELISA							
-	-	-	525	27,500	1.9	ND	0.74
-	+	-	270	5,300	5.1	ND	4.05
-	-	+	483	22,500	2.1	ND	1.04
mouse	-	-	223	4,700	4.7	ND	1.20
mouse	+	-	187	2,100	8.9	ND	7.05
mouse	-	+	157	11,200	1.4	ND	1.16
rat	-	-	291	7,500	3.9	ND	1.52
rat	+	-	221	3,100	7.1	ND	6.51
rat	-	+	351	12,500	2.8	ND	1.64
B. Plaque-forming cell assay							
-	-	-	345	42,600	0.8	14.4 $\pm$ 3.8	2 $\pm$ 1
-	+	-	82	2,100	3.9	36.7 $\pm$ 6.9	17 $\pm$ 4
-	-	+	416	34,700	1.2	9.6 $\pm$ 3.4	3 $\pm$ 2
mouse	-	-	325	21,000	1.5	64.7 $\pm$ 9.8	21 $\pm$ 5
mouse	+	-	45	1,000	4.5	34.4 $\pm$ 12.3	41 $\pm$ 7
mouse	-	+	226	38,400	1.2	78.9 $\pm$ 9.4	15 $\pm$ 7
rat	-	-	230	26,000	0.9	66.6 $\pm$ 8.2	10 $\pm$ 3
rat	+	-	30	871	3.4	129.5 $\pm$ 12.8	32 $\pm$ 6
rat	-	+	126	34,000	0.9	89.7 $\pm$ 11.4	17 $\pm$ 4

For details: see the legend to Table III.
ND means not determined.

as well as the growth of the IgM- and IgE-secreting clones in these cultures. This was found both in the presence and in the absence of IL-4. Thus IL-4 cannot overcome the anti-IgM induced inhibition of the IgE precursor frequency and this inhibition is caused by a severely decreased activation and/or clonal growth of IgM expressing B cells. Although the IgE precursor frequency in the anti-IgM treated cultures was too low to be determined, plaques could still be found, suggesting that the decrease was not due to toxic effects of the anti-IgM antibodies present. IL-4 is thus more likely to stimulate isotype switch recombination than to result in a selective growth advantage of cells that were already switched to IgE at the onset of the culture.

To further support this conclusion, we analyzed during culture the frequency of IgM and IgE bearing cells at various time points. At day 0, 3, 5 and 7 cells were harvested and stained for cytoplasmic and membrane-bound IgM and IgE. The results (Table V) show that by day 3 detectable IgE bearing cells were not found yet. On day 7, especially in the LPS plus IL-4 stimulated cultures, detectable numbers of cytoplasmic as well as surface IgE positive cells were identified. No significant changes in the numbers

of IgM positive cells were obtained, while IgG_1 showed a similar trend as IgE. These results are therefore suggestive that IgE-secreting cells as detected by day 7 arise during culture from newly-formed LPS-reactive B cells that express IgM. The absolute number of those cells appears to be increased in cultures that contain IL-4.

Effect of feeder cells on development of Ig-secreting clones

In the original culture system for mitogen-activated murine B cells (28), rat thymocytes were used as a source of growth promoting feeder cells. The frequency of IgE precursors in cultures of intact spleen cells with thymocyte feeder cells (Table I) was significantly higher than the frequency of IgE precursors in cultures of T-depleted spleen cells in the absence of feeder cells (Tables III, IV). In order to investigate whether the feeder cells employed facilitated the development of IgE secreting clones by producing IL-4, T-depleted spleen cells were stimulated by LPS with or without IL-4 and rat or syngeneic mouse thymocytes. A monoclonal antibody to mouse IL-4 was added to some cultures with mouse thymocyte feeder cells to inhibit the enhancement of the IgE precursor frequency caused by IL-4 produced by feeder cells. The individual cultures were analyzed both by plaque assay and ELISA. From the data of the plaque assay the clone sizes were calculated (Table VI).

Addition of mouse feeder cells resulted in a 2 fold increase in frequency of IgE-secreting clones (1 in 21,000 vs. 1 in 42,600) as determined by plaque assay. Addition of IL-4 resulted in a further increase of the frequency of IgE-secreting clones (1 in 1,000 vs. 1 in 21,000). The majority of this increase was inhibitable by anti-IL-4 antibodies (1 in 38,400 vs. 1 in 1,000). These results suggested that addition of feeder cells resulted in an increase of precursor frequency of IgE-secreting clones that was largely due to production of IL-4 and for the remaining to other growth promoting capacities of feeder cells.

DISCUSSION

Clonal analysis of T-depleted spleen cells stimulated with LPS plus IL-4 consistently showed an increase in the frequency of IgE secreting clones as determined by plaque assay on the cultured cells and by isotype-specific ELISA on the culture supernatant. The average 14-fold increase of the frequency of IgE secreting clones and the 3-fold increased average clone size of the IgE-secreting clones induced by IL-4 are similar to the 16-fold increase of the IgG_1 precursor frequency and the 2.8-fold increase in clonal burst size of IgG_1-secreting cells induced by addition of IL-4 containing supernatant in B cell populations depleted of surface IgG bearing B cells (8). However, in the latter study LPS-stimulated B cells were cultured in the presence of rat thymocyte feeder cells. Moreover, since in that study no IgM precursor frequencies were determined, the effect of the lymphokine on the on the process of isotype switching could not be evaluated. More recently we have shown that IL-4 enhances polyclonal IgE production in cultures of T-depleted spleen cells (12, 13). This enhanced IgE production by IL-4 could be caused by an increase in the frequency of IgE-secreting clones, an decreased cell cycle time (decreased doubling time) in the population of daughter cells or an increased rate of synthesis of IgE molecules by IgE-secreting cells. In order to discriminate between these three possibilities we have performed limiting dilution analysis on purified B cells.

In these experiments, we analyzed the frequency of IgM as well as of IgE secreting clones in the presence or absence of IL-4. This enabled us to calculate a 'switch frequency' which is a ratio between the frequencies of IgM to IgE precursors, each assayed at the optimum times (day 5 for IgM, day 7 for IgE). No such frequency at the molecular level can be determined in these cultures. However, we found this calculated ratio to be useful for distinguishing isotype specific from non-specific changes in precursor frequencies. Additions that cause comparable changes in both IgM and IgE precursor frequencies do not change this ratio and thus should not involve changes in the frequency of isotype switching. Additions that differentially change the precursor frequency of IgE relative to IgM will alter this ratio and could be explained by changes in the frequency of switching to IgE. Since direct precursor-product relationships between IgM and IgE clones cannot be established in these cultures, the IL-4 induced increases of 'switching frequency' do not by themselves prove that IgE precursors have undergone a switch. Based on the data presented, the process of isotype switching itself cannot be demonstrated. When combined with the evidence that nearly all of the IgE-producing clones arise from IgM-bearing precursors, however, this suggests that IL-4 does, in fact, enhance the frequency with which IgM^+ cells can switch to IgE production. Formally it is possible that all IgE precursors have switched at the molecular level to IgE but recently enough to still retain IgM on their surface, but no evidence has been presented that such cells in fact exist (8,21,34). Furthermore, immunofluorescence analysis of the cultured cells at various time points during culture revealed that by day 3 IgE-bearing cells were not yet detectable. If IL-4 would alter the amount of IgM bearing cells that had switched at the molecular level to IgE, such cells had been detected early in culture. By day 7 membrane IgE as well as cytoplasmic IgE-positive cells could be detected and these numbers were three to five fold increased in the IL-4 stimulated cultures.

On the average, IL-4 induced a 3-fold increased clone size of IgE-secreting clones. This suggests that IL-4 induced IgE-secreting cells have undergone an enhanced differentiation or have a slight selective growth advantage in that their doubling time is decreased, probably by the reported property of IL-4 to prepare B cells for entry into the cell cycle (2). Alternatively, in the presence of IL-4, an increased frequency of IgE secreting clones could occur within the daughter cells of a single clone. This would thus reflect the pleiotropic effects of this lymphokine.

Assuming an average IgE precursor frequency of 1 in 1250 LPS-reactive B cells cultured in bulk cultures of 5×10^4 B cells per well, this would equal 40 IgE precursors that give rise to clones of 20 cells each. In total some 800 IgE-secreting cells should thus be found in such cultures resulting in supernatant levels of around 200 ng IgE/ml. Supernatant levels of 100 to 1000 ng/ml for IgE were found in such IL-4 containing mass cultures (Table II; 12, 13). The correlation of the clonal burst sizes of IgM- and IgE-secreting clones as determined in ELISA as well as by plaque assays allows on a statistical basis the estimation of the amount of Ig produced after 7 days of culture of 1 Ig-secreting cell. It can be calculated that in these experiments, on the average, 0.98 ng IgM/ml was produced by a single IgM-secreting cell while 0.25 ng IgE/ml was produced by a single IgE-secreting cell, neglecting the possible influence of differences in the half-life of the 2 isotypes.

In order to evaluate possible effects of thymocyte feeder cells in the limiting dilution culture system (34), B cells stimulated with LPS plus IL-4 or anti-IL-4 were cultured with rat as well as mouse thymocyte feeder cells. The results demonstrated that some, but not all of the growth promo-

ting capacity of feeder cells was contributed by the active production of IL-4. The frequency of IgM-secreting clones was higher in cultures with feeder cells than in cultures with IL-4. Therefore, a part of the feeder effect on the development of Ig-secreting clones is not due to IL-4 production but to other growth supporting activities. These activities facilitate the develop-ment of IgE-secreting clones by an increased switch frequency. Syngeneic mouse thymocytes were found to be more inhibitable by anti-IL-4 antibodies, that are specific for mouse IL-4, than rat feeder cells. This reflects a certain degree of species specificity between mouse and rat IL-4 (37). For the evaluation of the IL-4 effects on IgE responses under limiting dilution culture conditions it is therefore more appropriate to use culture systems without the addition of feeder cells.

These experiments, together with those of Layton et al. (8) strongly suggest, but do not prove, that a substantial part of the IL-4 mediated enhancement of IgG_1 and IgE formation is due to an increased frequency of switching to those isotypes. In LPS-stimulated total spleen cell cultures, we found a maximum IgE switch frequency of 1.5%. This frequency would fit in the sequence of switch frequencies of the various isotypes that parallels the order of the C_H genes in the genome (33,38). This would indicate a T independent C_H gene expression in Ig-secreting cells that parallels the T independent isotype expression on the surface of B cells. This model is based upon random switch events determined solely by the distance of the various isotype switch regions to the $C\mu$ switch region (39,40). However, lymphokine mediated differential expression of the genes depending on the distance from the $C\mu$ gene could provide a second molecular mechanism superimposed on this model (8, 10, 11, 14, 41). Alternatively, such differential changes in the distance of an isotype switch region to $C\mu$ could be based upon the certain degree of homology between the switch regions of $C\mu$, $C\alpha$, and $C\epsilon$ genes. The switch regions of C genes on the other hand, are divergent from the above ones (42-44). The changes in switch frequency due to the addition of IL-4, could involve the lymphokine mediated induction of a specific recombinase or of a cofactor that confers specificity on a nonspecific recombinase (45). There are, however, no obvious sequences common only to the switch regions of the $C\gamma_1$ and $C\epsilon$ genes that might provide a substrate for a specific recombinase (44). Alternatively IL-4 could alter the 'accessibility' of the $C\gamma_1$ and $C\epsilon$ switch regions, allowing them to become substrates for an isotype-nonspecific recombinase. This model is derived from analyses of mouse pre-B and B lymphoma lines that can be induced to switch to only one or two isotypes (46,47). In support of the latter mechanism is the recent demonstration that IL-4, which causes a decrease in the expression of IgG_{2b} in LPS-stimulated B cell cultures (48), reduces the 'accessibility' of the γ_{2b} locus as evidenced by reduced transcription from unrearranged genes. The fact that the frequency of B cells expressing the various isotypes can be regulated by T cell-derived lymphokines (8,10,11,14,39,41) raises the possibility that this directed switching may be regulated by certain lymphokines (49).

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CHAPTER 6.3

FREQUENCY ANALYSIS OF FUNCTIONAL IMMUNOGLOBULIN C_E GENE EXPRESSION IN THE PRESENCE AND ABSENCE OF INTERLEUKIN-4 IN LPS REACTIVE MURINE B CELLS FROM HIGH AND LOW IMMUNOGLOBULIN-E RESPONDER STRAINS

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ABSTRACT

Non-responder SJL mice produce low levels of antigen-specific IgE after immunization, compared to responder strains. Young athymic BALB/c nude mice are unable to produce antigen-specific or total IgE in their serum. These mice also have very low numbers of background IgE-secreting cells in their lymphoid organs. High-responder BALB/c mice do have substantial numbers of background IgE-secreting cells while low responder AKR mice show intermediate numbers. Similar differences were found when analyzing lipopolysaccharide (LPS) reactive B cells in cell suspensions of spleen and bone marrow in limiting dilution cultures. Limiting dilution analysis of T-cell depleted splenic B cell cultures revealed that the defective IgE production in SJL mice is not due to an intrinsic B cell defect. This defect can be substantially overcome by addition of exogenous interleukin-4 (IL-4) to these cultures. Furthermore, it was shown in limiting dilution cultures that SJL thymocyte feeder cells were able to suppress IgE production by LPS-activated high responder BALB/c B cells. The addition of IL-4 or neutralizing antibodies against IL-4 or IFN-γ to these cultures were able to overcome this suppressive effect to a large extent. We conclude that different IgE responder types are caused, at least in part, by a defective IL-4 production or by a defect in the Th2 system that is functionally detectable at the level of thymocytes.

INTRODUCTION

It has been shown that immunoglobulin-E (IgE) antibody formation is controlled by genetic factors which involve both major histocompatibility linked and non-linked loci (1,2). Various mouse strains have been classified into different IgE responder types on the basis of their capacity to produce a persistent level of antigen-specific IgE in the serum after antigenic stimulation under conditions that are optimal to induce an IgE response. This IgE formation was usually measured by the semiquantitative method of passive cutaneous skin reaction in rats. Commonly, a distinction is made in 'high-responder' (BALB/c, C3H, CBA), 'low-responder' (AKR, A/He) and 'non-responder' (SJL, SJA/9) mice (3-7). Although the different antigen-specific and polyclonal IgE responses in these mouse strains are well documented, little data is available about the total IgE level in their serum (8,9). Nor have studies dealing with 'spontaneously' occurring ('background') IgE-secreting cells in the various lymphoid organs of these different responder strains been described. With the development of highly sensitive quantitative IgE enzyme-linked immunosorbent assays (ELISA) (10) and adapting the reverse type hemolytic plaque assay for the enumeration of IgE-secreting cells (11) such studies of background IgE production have become feasible.

It has been established that the initiation of IgE formation is highly dependent on T helper cells (2,12,13). Recently, it was shown that T helper cells can be divided in two subsets on the basis of their lymphokine production (14,15). One subset of helper T cell (Th2) produces interleukin-4 (IL-4) that is able to selectively enhance the concentration of IgE in the supernatant of T-cell depleted splenic B cell cultures stimulated by lipopolysaccharide (LPS) (16-18). Interferon-gamma, produced by the other T helper subset (Th1), reduces the IL-4 induced IgE-synthesis *in vitro* (17). We reinvestigated the IgE responder types of several mouse strains by determining the serum levels of total IgE in ELISA and the numbers of background IgE secreting cells in various lymphoid organs by plaque assays. Moreover, B cell populations from spleen and bone marrow (BM) of mouse strains of both types, were stimulated to proliferate and to mature into clones of IgE secreting cells by polyclonal activation with LPS under limiting dilution conditions. Clonal and quantitative analysis of the response by purified B cells in the absence or presence of IL-4 enabled us to study IgE production at the B cell level (19). Using the protein A plaque assay, the frequency as well as clone size of IgE secreting clones were determined in individual cultures. The results suggest that the IgE respondership is not an intrinsic B cell property, but is determined by the T cell compartment. The addition of exogenous IL-4 to the cultures could compensate for the apparent defect in IL-4 production by feeder cells from IgE non-responder mouse strains.

It has been suggested that the magnitude of *in vivo* IgE responses is dependent upon the relative levels of IL-4 and IFN-γ encountered by antigen-stimulated B cells, and, therefore is dependent upon the relative numbers of Th1 and Th2 subsets (15,20,21).

MATERIALS AND METHODS

Mice

Female BALB/c and AKR mice, 8-12 weeks of age, were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. Female SJL mice, 8-12 weeks of age, were obtained from GL. Bomholtgärd, Ry, Denmark. Female

Lewis rats, 3-6 weeks of age, were obtained from OLAC 1976, Bicester, Oxon, England.

Cell suspensions

Cell suspensions of spleen, bone marrow (BM) and mesenteric and parathymic lymph nodes were prepared as described (22). T-cell depleted spleen cell suspensions were prepared by treatment with anti-Thy-1.2 antibodies and complement (Low Tox M; Cedarlane Lab., Hornby, Ontario, Canada) as described (17,18). Thymuses from rat, BALB/c and SJL were dissected with special care to avoid blood contamination and removing the parathymic lymph nodes. The thymus cell suspension was subjected to low dose X-irradiation (0.1 Gy) as described (23) for use as feeder cells. Viable cells were counted by trypan blue exclusion method and total nucleated cells were counted with a Coulter Counter model BZI (Coulter Electronics Ltd., Harpenden, Herts, UK).

In vitro cultures

Spleen and BM cell suspensions were cultured under limiting dilution conditions according to Andersson et al. (24). Briefly, varying numbers of cells were cultured with 3.6 x 10^6 rat thymocyte feeder cells/ml and 50 ug/ml E.coli lipopolysaccharide (LPS 026:B6; Difco, Detroit, MI, USA) in 0.2 ml of RPMI 1640 medium supplemented with L-glutamine (4mM), penicillin (100 IU/ml), streptomycin (50 ug/ml), 2-mercaptoethanol (50 uM) and 20% fetal bovine serum (FCS; Flow, Irvine, Scotland), specifically selected for growth-supporting properties and low endogenous mitogenic activity. Routinely, 32 replicate cultures were set up for each cell concentration. Cultures containing no mouse lymphoid cells were used as controls. The cells were cultured in flat bottom microtitre plates (Costar 3596; Costar, Cambridge, MA, USA). The cultures were assayed on day 5 for IgM- and on day 7 for IgE-secreting plaque-forming cells (PFC). In the absence of added spleen cells, a maximum of 10 PFC were found per well. Cultures were scored positive when they yielded at least 10 PFC.

In limiting dilution cultures of T-cell depleted spleen cells, B cells were incubated in twice the final cell concentration in a volume of 100 ul per well. Again 32 replicate cultures were put up for every cell dose. For the determination of the precursor frequencies of IgM secreting clones, 3 or 6 different B cell inputs varying between 20 and 500 B cells per well were tested. For the analysis of IgE precursor frequencies, input B cell numbers between 1,000 and 32,000 were tested. After 1 day of culture, 100 ul of highly purified recombinant IL-4 (250 U/ml) or 100 ul of culture medium was added. The dose of IL-4 employed gave rise to optimal precursor frequencies of IgE secreting clones in initial titration experiments. When feeder cells were applied in cultures of T-depleted spleen cells, 3.6 x 10^6 irradiated rat, BALB/c or SJL thymocytes per ml were used. Where indicated, purified monoclonal anti-IL-4 antibody (clone 11B11; Ref. 25) was added to the cultures at 10 ug/ml being an effective dose. Similarly, anti-IFN-γ antibodies (clone F_1; Ref. 26) was added to the cultures in an optimum dose of 5 ug/ml.

ELISA and Plaque Assay

Supernatants were harvested on day 5 (for measuring IgM) and on day 7 (for measuring IgE) in order to compare the numbers of recovered plaques with the Ig level in the supernatant. The supernatant of each individual culture was assayed in isotype-specific enzyme-linked immunosorbent assay (ELISA) for IgM and IgE as described (10). Day 5 supernatants were always found to be negative for IgE (< 1 ng/ml) while day 7 supernatants contained

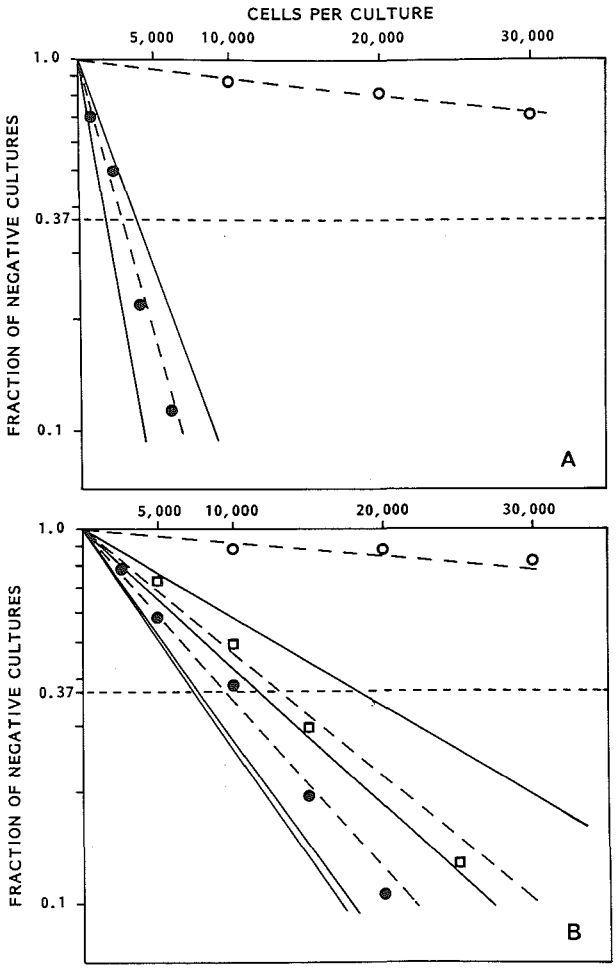

Fig. 1. **A.** The inhibitory effect of SJL thymocyte feeder cells on the frequency of IgE-secreting clones of BALB/c B cells. Thirty-two replicate cultures of T-depleted spleen cells of BALB/c mice were set up for each cell concentration either with SJL (O) or with BALB/c thymocyte feeder cells (●). Cultures were tested at day 7 for IgE-secreting cells with the reverse plaque assay. For each frequency determination, the lower line represents the upper 95% confidence limit, the middle line represents the frequency estimate and the upper line represents the lower 95% confidence limit. Precursor frequencies (1/n) (range) of IgE-secreting B cells were: in the presence of SJL feeder cells <25,000 or of BALB/c feeder cells 2,954 (1974-3934). **B.** The enhancing effect of IL-4 on the frequency of IgE-secreting clones of SJL B cells cultured in the presence of SJL feeder cells. T-depleted SJL spleen cells were cultured with SJL thymocyte feeder cells and either with medium (O) or with the addition of IL-4 (250 U/ml) (●). SJL B cells were also cultured in the presence of BALB/c thymocyte feeder cells (□). Precursor frequencies (1/n) (range) of IgE-secreting SJL B cells were: <25,000 in the presence of SJL feeder cells without the addition of IL-4; 9,632 (7462-11,802) for SJL feeder cells in the presence of IL-4; and 13,084 (7874-18294) for BALB/c feeder cells.

large amounts of IgM (> 10 ug/ml). This was due to the high B cell input numbers for analyzing IgE precursor frequencies and these inputs will always contain multiple IgM precursors.

IgM- and IgE-secreting cells in each individual well were assayed by a modified protein A plaque assay (27). The class of the secreted Ig was determined by using developing antisera specific for either IgM or IgE in the protein A plaque assay as well as in inhibition type ELISA using excess amounts of all other isotypes. After the appropriate adsorptions, the antisera were titrated and their specificity was confirmed in plaque assays using hybridoma cells of the appropriate classes. It was found that the antiserum preparations employed were monospecific for their respective isotypes. The origin and preparation of the anti-IgM (28) and anti-IgE antisera employed have been described (Savelkoul, Soeting, Radl and Van Der Linde-Preesman, submitted).

Data analysis

Culture conditions (24) were employed that allow every growth-inducible B cell to grow and differentiate into a clone of Ig-secreting cells upon stimulation by LPS. By reducing the number of lymphoid cells added to each culture, reacting B cells become limiting. The results of a limiting dilution analysis of splenic B cells of SJL mice cultured in the presence of SJL thymocytes with or without the addition of exogenous IL-4, for the frequency of precursor B cells yielding IgE-secreting clones is shown in Fig. 1. The linear disregression of the curves, plotted as numbers of cultured cells against the logarithm of the fraction of nonresponding cultures indicates that precursor B cells reactive to LPS and yielding IgE-secreting cells were limiting in these cultures. Therefore, frequencies of reactive cells could be determined at cell concentrations yielding, on an average, one clone per culture. Evaluation of such quantal dilution assays is performed by the method of maximum likelihood and frequencies are estimated according to the program described by Fazekas de St.Groth (29). For the screening of T-depleted spleen cell cultures in which no feeder cells were employed, the negative cultures without addition of spleen cells did not yield any PFC. Treshhold values for allowing positive-negative screening in ELISA were determined in quantitative ELISA testing control medium only. These levels were set to 1 ng/ml, the detection limit of the ELISA.

The switch frequencies were calculated as the ratio of the frequency of IgE-secreting clones as determined on day 7 to the frequency of IgM-secreting clones as determined on day 5 of the culture. This procedure is based upon the finding that in LPS-stimulated B cell cultures, cells secreting another isotype than IgM developed from clones which initially consisted of IgM-secreting cells (24).

The average clone size was calculated as described (19). The analysis is performed on microtitre plates employing 96 wells of a cell dose at which around 37% of the cultures are negative and therefore the positive cultures contain on the average one LPS-reactive precursor per culture. Clone sizes are expressed as the average number of Ig-secreting cells in a clone resulting from 1 precursor or by the average Ig level (ng/ml) in day 7 supernatant of these cultures.

For the statistical analysis of the significance of differences observed, p-values were calculated by Student t-test. Values of p greater than 0.05 were considered not to be significant.

Immunofluorescence studies

Cell suspensions were suspended in phosphate-buffered saline containing 2% bovine serum albumin at 1 x 10^7 per ml and were stained with either polyclonal rabbit anti-IgM (Nordic, Tilburg, The Netherlands) or monoclonal anti-Thy-1.2 (30-H12; American Type Culture Collection; ATCC, Rockville, MD, USA) for 30 min on ice. A purified rat monoclonal antibody to the B220 B-lineage surface marker, RA3-6B2 (17,18) was also employed. Positive cells were scored after incubation with goat-anti-rabbit and rabbit-anti-rat fluorescein isothiocyanate labelled second stage antibodies (Nordic). Cells stained with the latter antibodies only were used as controls.

RESULTS

Background IgE-secreting cells in lymphoid organs and IgE levels in serum

'Background' IgE-secreting cells in spleen, BM and lymph nodes of different responder strains were enumerated in the protein A plaque assay. The results expressed as total number of IgE-PFC per organ showed that most background IgE-secreting cells reside in the spleen (Table I). High-responder BALB/c mice had higher numbers of IgE-secreting cells in all lymphoid organs tested as compared to low-responder AKR mice. Non-responder SJL mice had no detectable IgE-secreting cells in their lymphoid organs. Also athymic BALB/c nude mice did not have detectable numbers of IgE secreting cells in the various lymhpoid organs tested.

In the serum, on the other hand, old BALB/c, AKR and SJL mice showed comparable levels of total IgE as detected by isotype-specific ELISA (Table I). Young adult SJL mice (12 weeks of age) as well as BALB/c nude mice displayed no detectable IgE levels in their serum. Young BALB/c and AKR

TABLE I

IgE levels in the serum and background numbers of IgE-PFC in lymphoid organs of unimmunized mice of various responder strains

Strain	Total IgE (ng/ml)	Total IgE-PFC per organ		
		Spleen	BM	Lymph nodes
BALB/c	383 ± 196	4,654 ± 1,648	3,688 ± 1,471	794 ± 261
BALB/c nu/nu	<10	<100	<100	<100
AKR	451 ± 243	1,250 ± 754	1,191 ± 473	<100
SJL*	<10	<100	<100	<100
SJL	539 ± 256	<100	<100	<100

Of at least 5 mice of every strain, 12 weeks (*) or 6-10 months of age, sera were tested individually in ELISA. Protein A plaque assays for IgE-PFC were performed on cell suspensions of spleen, BM and lymph nodes (mesenteric plus parathymic lymph nodes) of the same mice. All results are expressed as arithmetic means ± 1 SD.

TABLE II

Frequencies of IgE secreting clones in cultures of whole spleen or BM cells from various responder strains

Strain	Organ	$sIgM^+$ B cells (%)	Isotype	Absolute frequency (1/n)	B cells activated by LPS (1/n)
BALB/c	Spleen	41	IgM	32 ± 2[1]	13[2]
			IgE	2,456 ± 246	1,007
	BM	8	IgM	200 ± 26	16
			IgE	27,728 ± 12,682	2,218
BALB/c nu/nu	Spleen	81	IgM	28 ± 6	23
			IgE	<34,000	-
	BM	12	IgM	214 ± 37	26
			IgE	<32,000	-
AKR	Spleen	39	IgM	92 ± 4	36
			IgE	3,709 ± 679	1,447
	BM	9	IgM	530 ± 69	48
			IgE	16,804 ± 4,932	1,512
SJL	Spleen	34	IgM	83 ± 18	28
			IgE	<32,000	-
	BM	9	IgM	518 ± 80	47
			IgE	<32,000	-

1 Calculated as fraction of all viable nucleated cells and representing arithmetic mean ± 1 SD (n=4).

mice showed total IgE serum levels that were not significantly different from those in 6 month old individuals (data not shown). In the ELISA system employed, detection was based upon a purified anti-ϵ chain specific rabbit antibody that was also used in the protein A plaque assay. This antiserum reacted equally well with different monoclonal IgE antibodies of unrelated specificities and was free of antibodies to idiotypic determinants of the IgE antibody mixtures used for immunization. The slopes of serial dilutions of the various sera were identical to those of isolated monoclonal IgE antibody preparations. The lowest IgE concentration that could be measured reproducibly was 0.1 ng IgE/ml.

In similar type ELISA, levels of total IgM and total IgG_1 were determined in the serum of the same mice. The results did not show significant differences between the sera of the various mouse strains tested (data not shown).

Clonal analysis of LPS reactive B cells in intact cell suspensions

Precursor frequencies were determined in spleen and BM of mouse strains of different IgE responder types as well as in BALB/c nude mice. This was done by employing a culture system that allows every LPS-reactive B cell to proliferate and mature into a clone of IgM secreting cells. The results of the limiting dilution analyses of cultures of cell suspensions of spleen

TABLE III

Precursor frequencies and clonal burst sizes of IgE-secreting clones in T-depleted spleen cell cultures

Strain	T-depleted spleen cells cultured with[1]		Precursor frequency[2] (1/n)		Clone size[3] (PFC)	
	Rat feeders	IL-4	IgM	IgE	IgM	IgE
BALB/c	-	-	247	17,600	8.9	2.3
	-	+	198	2,800	21.8	35.5
AKR	-	-	352	28,500	9.7	1.8
	-	+	212	3,000	24.2	33.4
SJL	-	-	401	<32,000	7.1	1.3
	-	+	337	19,100	20.4	18.0
	+	-	344	13,600	21.6	8.9
	+	+	231	3,400	59.2	25.8
BALBC/c	-	-	218	<32,000	6.8	0.8
nu/nu	-	+	183	15,700	9.8	4.9
	+	-	188	12,800	14.8	9.4
	+	+	198	2,700	34.2	18.3

[1] LPS-stimulated T-depleted spleen cells of various mouse strains were cultured with or without 3.6 x 10^6/ml rat thymocyte feeder cells and/or 250 U/ml of recombinant IL-4.

[2] Precursor frequencies are expressed as the reciprocal number of B cells that result on the average in one IgM- or IgE- secreting clone analyzing 3 inputs at 32 wells per input of B cells.

[3] Clonal burst sizes are expressed as the average number of IgM or IgE secreting cells in 96 wells that contained statistically one precursor.

and BM (Table II), were not only expressed as precursor frequencies calculated as fraction of all nucleated cells but also as the fraction of all B cells in that organ. The fraction of B cells was determined in cell suspensions of spleen and BM by counting the number of viable nucleated cells in each cell suspension and staining these cell suspensions with antibodies specific for B220 and membrane-bound IgM. No consistent differences were found between the various strains tested when analyzed by Student t-test (data not shown).

By determining the precursor frequencies of IgM as well as IgE secreting clones, switch frequencies could be calculated as the fraction of IgM secreting cells that had switched to the secretion of IgE. The data of Table II show that the precursor frequency of IgM secreting cells was higher in BALB/c than in AKR and SJL mice. This holds true when calculated as absolute frequencies or frequency of LPS-reactive B cells. The latter frequencies in spleen and BM were approximately the same (within a three-fold difference) for IgM as well as IgE in all three strains tested. The precursor frequencies of IgE-secreting cells were comparable in BALB/c and AKR mice, both for spleen and BM when calculated as fraction of the B cells. For spleen and BM of BALB/c mice switch frequencies around 1 percent were calculated (1.3% for spleen and 0.7% for BM). For AKR mice, substantially higher switch frequencies were found (2.5 and 3.2%, respectively).

Since IgE precursor frequencies in SJL as well as in BALB/c nude mice were too low to measure in this limiting dilution culture system, no switch frequencies could be calculated. The frequency, however, must be less than 0.2%.

Clonal analysis of the effect of IL-4 on LPS activated T-depleted spleen cells

In order to determine possible differences in precursor frequencies for IgM and IgE secreting cells between B cells from different type responder strains, T-depleted cell suspension were analyzed. To this end, spleen cell suspensions of BALB/c, BALB/c nu/nu, AKR and SJL mice were stimulated by LPS and cultured under limiting dilution conditions. In the case of SJL mice, B cells were also cultured in the presence of rat thymocyte feeder cells. Precursor frequencies and average clone sizes were determined both for IgM and IgE, with or without the addition of highly purified recombinant IL-4. The typical result of such experiment (Table III) showed for BALB/c and AKR mice that addition of IL-4 increased the frequency of IgM-secreting clones only slightly (within two-fold) while the frequency of IgE-secreting clones increased dramatically (5 to 10-fold). This led to a marked increase in the calculated switch frequency (7-fold). The average clone size of IgM- and IgE-secreting clones was also affected by IL-4 addition. Essentially the same results were obtained when the supernatants of these cultures were analyzed in isotype-specific ELISA (data not shown).

SJL B cells cultured with or without feeder cells also showed not more than a two-fold increase in the frequency of IgM secreting clones upon addition of IL-4 to the cultures. The frequency of IgE-secreting clones in SJL spleen was undetectable in the absence of exogenous IL-4 or feeder thymocytes. The addition of IL-4 to the cultures increased the frequency of IgE-secreting clones to a level that was approximately equal to the frequency in BALB/c B cell cultures (1 in 19,100) without additions. SJL B cells cultured in the presence of both IL-4 and feeder cells had a frequency of IgE-secreting clones (1 in 3,400) that was comparable to that of BALB/c or AKR with only IL-4 added, and this was reflected in the switch frequency (6.8). Also the clone size of the IgE-secreting clones in cultures of SJL B cells with IL-4 and feeder cells was comparable to the clone sizes of BALB/c or AKR B cells cultured with IL-4 only.

Clonal analysis of the effect of SJL thymocytes on cultures of LPS-reactive B cells

Next, the possibility was studied that the low IgE precursor frequency in intact cell suspensions of SJL mice is not an intrinsic B cell defect but is possibly a difference in the T cell compartment. To test whether this defect is functionally present at the level of thymocytes, splenic B cells of BALB/c, BALB/c nude and SJL mice were cultured at limiting dilutions with irradiated thymocyte feeder cells of either BALB/c or SJL origin. Exogenous IL-4 as well as blocking antibodies to IL-4 and IFN-γ were added to some cultures to establish whether the possible defect was due to a defective IL-4 production.

As shown in Table IV, BALB/c, BALB/c nude and SJL B cells gave equal frequencies of IgM secreting clones (1 in 153, 1 in 286 and 1 in 229, respectively) and IgE secreting clones (1 in 8,800, 1 in 12,500 and 1 in 13,100, respectively) upon addition of BALB/c thymocytes. This was reflected in the switch frequencies calculated (1.7, 1.7 and 1.8 %, respectively). The addition of exogenous IL-4 to these cultures increased the frequency of IgE-secreting clones to around 1 in 3,000 as had been observed in previous experiments. The addition of anti-IL-4 antibodies to cultures that did not contain exogeneous IL-4 inhibited the precursor frequency of IgE-

TABLE IV

Clonal analysis of T-depleted spleen cells cultures with mouse thymocyte feeder cells

B-cell source	Thymocyte source	T-depleted spleen cells cultured with			Precursor frequency (1/n)	
		IL-4	anti-IL-4	anti-IFN-γ	IgM	IgE
BALB/c	BALB/c	-	-	-	152	8,800
		-	+	-	314	<32,000
		+	-	-	187	2,900
		-	-	+	167	2,300
	SJL	-	-	-	496	<32,000
		+	-	-	259	6,400
		-	+	-	306	<32,000
		-	-	+	256	<32,000
SJL	SJL	-	-	-	513	<32,000
		+	-	-	315	4,600
		-	-	+	376	<32,000
	BALB/c	-	-	-	229	13,100
		-	+	-	327	<32,000
		+	-	-	172	3,300
		-	+	-	243	<32,000
		-	-	+	215	2,800
BALB/c nu/nu	BALB/c	-	-	-	286	17,800
		-	+	-	436	<32,000
		+	-	-	182	2,800
		-	-	+	253	18,400

T-depleted spleen cells were cultured with or without the addition of IL-4 (final concentration 250 U/ml), anti-IL-4 antibodies (final concentration 10 ug/ml) or anti-IFN-γantibodies (final concentration 5 ug/ml). For further details: see the legend to Tables 2 and 3.

secreting clones to below the detection limit of the reading system in all combinations tested.

Thymocyte feeder cells of SJL origin consistently failed to support detectable frequencies of IgE-secreting clones from B cells of all three strains tested. The addition of IL-4 or neutralizing antibodies to IFN-γ to cultures with SJL thymocyte feeder cells, however, supported approximately the same frequencies of IgE-secreting clones as BALB/c thymocytes. The addition of anti-IL-4 antibodies to these cultures with either strain of thymocytes resulted in a marked inhibition of the frequency of IgE-secreting clones. The frequencies of IgM-secreting clones did not change more than two-fold under these various conditions so that the calculated switch frequencies changed in parallel with the frequencies of IgE-secreting clones. IL-4 dependent increase and decrease in the clone sizes of the IgE-secreting clones were found similar to those in previous experiments (data not shown).

The results of a typical limiting dilution analysis of splenic B cells

of SJL or BALB/c mice cultured in the presence of either strain thymocytes with or without the addition of exogenous IL-4 is shown in Fig. 1. In Fig. 1A the inhibitory effect of SJL thymocytes on the precursor frequency of IgE-secreting clones as compared to cultures containing BALB/c thymocytes is shown. SJL thymocytes caused at least 8-fold decrease in IgE precursor frequency. In Fig. 1B the enhancing effect of IL-4 on the frequency of IgE-secreting clones of SJL B cells is shown when these cells were cultured in the presence of SJL feeder cells. SJL feeder cells caused in the presence of IL-4 at least 7-fold increase in precursor frequency of IgE-secreting clones comparable to the frequency determined when SJL B cells were cultutred in the presence of BALB/c thymocytes.

DISCUSSION

Clonal and quantitative analysis of LPS-reactive B cells revealed that B cells from SJL mice displayed lower precursor frequencies of IgE secreting clones than those from AKR or BALB/c mice. When IL-4 was added to the cultures, however, BALB/c and AKR splenic B cells gave rise to comparable frequencies of IgE-secreting clones. In the case of SJL B cells a partial restoration could be achieved by adding rat thymocyte feeder cells to the cultures. Subsequent addition of exogenous IL-4 to the rat thymocyte feeder cell containing cultures resulted in a similar frequency of IgE-secreting clones as compared to the other strains tested (Table III). The beneficial role of the addition of feeder cells to these B cell cultures is partly due to IL-4 production (19).

Addition of SJL thymocyte feeder cells to LPS-activated B cell cultures of BALB/c or SJL mice inhibited the frequency of IgE-secreting clones as well as their clonal size (Table IV). BALB/c feeder cells did not have this inhibitory effect on the precursor frequency of either BALB/c or SJL B cells unless anti-IL-4 antibodies were added to the cultures. Therefore, the B cells of SJL mice do not carry an intrinsic defect resulting in a severely decreased IgE formation. No distinction can be made at present whether this inhibition of IgE formation is the result of active suppression by an overproduction of IFN-γ or the absence of help leading to insufficient IL-4 production. Analysis of the effect of IL-4 in bulk culture systems of LPS activated B cells from SJL and SJA/9 mice suggested that in the presence of IL-4 these B cells were capable of producing as much IgE as in BALB/c control B cells. Since addition of anti-IFN-γ antibodies to SJL B cell cultures in the presence of SJL thymocytes did not result in an significant increase of the precursor frequency of IgE secreting clones, active suppression does not seem to be of relevance. Also, immunized SJA/9 mice that were simultaneously treated with anti-IFN-γ antibodies were still unable to produce IgE in detectable amounts (R.C., unpublished observation). It is therefore likely that insufficient IL-4 production would cause, at least in part, the defective IgE formation in SJA/9 and SJL mice (30, this study).

The haplotype of H-2 as well as non-H-2 linked loci were suggested to influence the IgE respondership of various mouse strains as classified according to the inducible specific IgE formation (1,3-5). Both the SJL and BALB/c mouse strains are highly susceptible to isotype-specific and allotype-specific suppression by T cells (8,13,31-34) or by IgE-binding factors secreted by T cells (6,35,36). Alternatively, this down regulation of IgE formation could be caused by Th1 cells (15,17). On the other hand, a clear helper T cell effect in primary and secondary IgE responses was described (2,36-38). The IgE responder type might thus be determined by the helper T

cell compartment only.

In the past, IgE formation in mice was usually measured by the semi-quantitative method of passive cutaneous skin reaction in rats (1-6). This method did not allow to study the increase of polyclonal IgE compared to specific IgE after parasitic infection or specific immunization. Available data on total IgE levels in mouse sera showed a deficient IgE production in the serum of SJL and BALB/c nude mice (9,30,39). It was also shown that IgE production is highly influenced by environmental factors like bacterial products and parasites (6,31,32,35,40).

In young SJL mice up to 12 weeks of age, IgE levels of less than 10 ng/ml were found. A marked age-related increase in serum IgE level in SJL mice was found since at the age of 6 to 10 months of age, these mice displayed no significantly different total IgE level when compared to other strains. The numbers of background IgE-secreting cells in various lymphoid organs of SJL mice, however, were lower than those of BALB/c mice at both ages analyzed. This difference could be due to the fact that the serum level of IgE is caused by IgE-secreting cells that are located outside the spleen, BM, or mesenteric and parathymic lymph nodes. Alternatively, the half-life time of IgE in the serum of SJL mice could be substantially increased as compared to that in BALB/c mice because of certain strain-dependent differences in mechanisms for clearing of IgE molecules from the serum. Others described that strains differences and exogenous antigenic load influenced the half-life time of IgE in the serum (41).

Most of the data presented in this study were obtained by analyzing the IgM and IgE production in cultures of LPS-activated B cells in reverse plaque assays. Essentially the same phenomena, however, were found when analyzing the culture supernatants in isotype-specific ELISA for IgM and IgE. We have shown before that limiting dilution analysis by plaque assays on cultured B cells and by ELISA on culture supernatants result in consistently similar frequencies of IgM- and IgE-secreting clones (19). We have also previously shown by clonal analysis of LPS-stimulated splenic B cells that IL-4 results in an increased IgE production partly because of an increased precursor frequency and partly because of an increased clone size of the IgE-secreting clones. In the same study evidence was presented that strongly suggested that the observed increase in IgE-secreting clones is due to an increased switch frequency from IgM to IgE upon addition of IL-4 to LPS-activated B cell cultures, although the switch process was not studied at the molecular level (19).

Putting these findings together, we conclude that in IgE non-responder mouse strains that are defective in the production of IgE, the defect is not due to an intrinsic B cell defect but is most likely caused by a partially defective T cell compartment. Based on the evidence presented, it is tempting to conclude that this effect is found selectively in the Th2 type T cell compartment because the defect in IgE production can be partially overcome in vitro by the addition of IL-4 to the B cell cultures. So far, the data do not allow to conclude whether T-cells defective in IL-4 production only or a totally defective Th2 system account for the observed lack in IgE production in SJL mice.

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

7.1 Reagents and assyas for studying IgE formation

The aim of the study described in this thesis was to gain more insight in the IgE antibody formation in vivo and more precisely, the influence of the various parameters that play an essential role in the induction of an antigen-specific IgE response. Therefore, the ratio of the specific to the accompanying non-specific IgE response were investigated after appropriate immunization, both at the humoral level and the cellular level. Also the isotype-specific suppression of the IgE response was investigated. On one hand, a study was undertaken aimed at the suppression of an ongoing specific IgE immune response by injection of anti-idiotypic antibodies. On the other hand, the role of IL-4 in the isotype-specific regulation of the IgE response was further elucidated. However, before starting these investigations, suitable reagents had to be prepared and assays had to be set up since they were not available at the start of this study.

Because of the relative inability to isolate and purify the low and transiently occurring amounts of IgE in the serum of mice, it has been virtually impossible to produce heterologous murine IgE-specific antisera [1]. For this reason, techniques necessary for the analysis of the induction of antibody formation and the regulation thereof were not available for the IgE isotype. Such techniques dependent on specific antisera are: Jerne-type plaque assays for the detection of antigen-specific Ig-secreting cells, the protein A plaque assay for the detection of total Ig-secreting cells of a given isotype, ELISA-plaque assays, cytoplasmic and membrane immunofluorescence and RIA or ELISA for the detection of the Ig produced [2]. Only recently a number of ELISA systems have been described for the determination of secreted IgE molecules, while assays for the analysis of murine IgE-secreting cells are still scarce [3-7].

The availability of antigen-specific murine IgE-secreting hybridomas has enabled the isolation and purification of large amounts of IgE [8-13]. As described in Sections 1.2 and 4.2, several special characteristics of the IgE molecule need to be considered carefully before selecting a purification procedure. Analysis on gel electrophoresis, immunoblotting and in ELISA confirmed that the isolation procedure presented in Section 4.2 results in monoclonal IgE molecules that are intact with regard to their protein as well as carbohydrate content. A mixture of purified IgE samples from four different hybridomas was subsequently employed for the immunization of rabbits and goats for the induction of high titre specific antisera. The IgG fraction of these antisera was purified and analyzed for monoreactivity with intact IgE molecules (Section 4.3). This specificity testing was performed both in inhibition type ELISA as well as in immunoblotting using various purified Ig fractions of all the other isotypes including both types of light chains. This was found all the more necessary in order to avoid contaminating anti-light chain, anti-idiotypic and anti-allotypic activity in the antiserum preparation. These types of activities have to be removed when for example in ELISA the IgE detected has to be quantitated on the basis of a reference sample of the identical purified hybridoma IgE mixture [14,15]. It was shown that further adsorptions of these antisera have made the contaminating activities undetectable by the most sensitive techniques available. For comparison, several commercial preparations were included in this study which showed the necessity of this specificity testing procedure prior to the use of the antisera.

Having both the specific heterologous antisera and a reference IgE sample available, a ELISA method suitable for the quantitative determination of murine IgE antibodies was designed employing Terasaki trays (Section 4.4). This Terasaki-ELISA method has the advantage of employing only

5 ul samples per well [16-18]. Therefore, this method is especially useful for measuring IgE levels in the serum of mice during follow-up studies.

ELISA can be used to: (a) accurately measure polyclonal and monoclonal antibody to the same antigen; (b) measure antibodies to both the whole protein molecule and its components; (c) distinguish between strongly and weakly reacting determinants of the same antigens; (d) discriminate between determinants on native and denatured structures; (e) detect the effects of charge on antigen-antibody reactions; and (f) rapidly evaluate idiotypic anti-idiotypic interactions. Thus, the ELISA technique is useful in studying immunochemical properties of antigens and antibodies. However, ELISA exhibits certain characteristics whicg distinguish it from other procedures. These include: (a) steric limitations of antibody-antigen reactivity; (b) affinity differences in inhibition assays; (c) potential for denaturation of the antigen on plastic surfaces; and (d) difficulties in utilization of the charged antigen [19,20]. The ability of such ELISA to measure absolute antibody concentration has thus been questioned. It has become apparent that antibody affinity may significantly influence the results of solid phase assays. In addition, although antibody responses are often considered solely in terms of concentration, it is important to realize that antibody affinity may play a critical role in determining biological activities of antibodies. Therefore, it is necessary not only to be able to characterize an antibody response in terms of affinity as well as magnitude but also to understand how antibody affinity may affect a particular assay [21].

The Terasaki IgE-ELISA set up allows the quantitation of both antigen-specific and total IgE antibodies. Furthermore, it has become clear that the logistic equation is a useful method for data reduction in quantitative ELISA [22]. Microprocessor-based automatic data handling results directly in absolute concentrations based upon the calibrated IgE content in the standard. However, similar as to many other ELISA systems for the quantitative determination of Ig, the reference standard curve for antigen-specific IgE antibodies appeared to be linear over a smaller concentration range than the standard curve for the total IgE determination. A general explanation for this phenomenon is assumed to be a function of: available epitopes on the immobilized antigen, affinity for the various epitopes presented on the coat, crossreactivity between the various antibody populations present in the sample, etc. The Terasaki-ELISA presented functions essentially similar as described for microtiter plate-based ELISA methods and these characteristics have been further elucidated elsewhere [19,20,23,24].

Using the rabbit mouse-IgE-specific antiserum produced, the antigen-specific and the protein A plaque assay have both been adapted for the detection of IgE-secreting cells. Furthermore, a rapid procedure has been developed for the production of SRBC coupled with protein antigens for use as indicator cells in specific plaque assays (Section 4.5). This was necessary because of the inconsistency in the coupling reaction for protein antigens to SRBC employing routine procedures [2].

7.2 Induction of IgE antibody formation

With the Terasaki IgE-ELISA method developed studies were performed for the analysis of total IgE levels in the serum of aging mice while kept under conditions of a different antigenic load and with differential defects in the T cell system. The results (Section 5.2) confirm previous data of other studies in mice, rats and humans in which the total serum IgE level was found to increase progressively with age and to decline slowly at the end of the life-span [25-28]. Simultaneously, the spreading of the

individual serum levels was found to increase progressively with age.

It was also shown that the total IgE level in the serum is dependent on the housing conditions of the mice and more precisely their antigenic load. It has become clear that the serum IgE level is relatively more dependent on this antigenic load than the total Ig or the IgG_1 level.

Large differences in serum levels of various Ig classes and subclasses can be found among different mouse strains, even when factors such as antigenic stimulation and age are similar [27,29,30]. In a number of cases it was found that BALB/c mice react with the highest serum levels of especially IgM and IgG_1. Also for total IgE, BALB/c mice show high levels in their serum. Within 6 months of age these mice reach a plateau of around 300 ng/ml after which the individual variability in total IgE serum level increased markedly. It is therefore unpredictable whether at old age the serum IgE increases further.

Even more profound than the effect of age on the serum IgE level was the effect of the presence or absence of functional T cells. The athymic nude mice displayed markedly increased total IgE levels as compared to their heterozygous littermates and certainly as compared to normal euthymic mice. It was also shown that nude mice displayed significantly increased serum levels of IgG_1 [31]. These observations contrast with the T cell dependency of antibody formation of particularly these two isotypes [32,33]. The observed paradox was interpreted as a difference in the regulation of specific versus total IgE and IgG_1 responses. The mere fact that these two isotypes are regulated strongly by the balance between IL-4 and IFN-γ (cf. Section 1.4), suggests a putative imbalance within the residual Th cell population in nude mice, especially between the Th1 and Th2 subsets. This could explain the selective enhancement of polyclonal IgG_1 and IgE responses in nude mice. Reconstitution experiments of nude mice with either suppressor T cells or helper T cells from either the Th1 or Th2 subset should reveal the regulation of the enhanced background serum level of these two isotypes.

A clear discordancy has been established between the total serum IgE level and the induced specific IgE concentration upon immunization (Section 5.3). It was found that only late (around 30 days) after the induction of a specific IgE immune response (based upon multiple low dose antigen injections without adjuvant) substantial serum IgE concentrations were found. Furthermore, at maximum only 30% of the total serum IgE level was antigen-specific. Several authors reported similar data (obtained with different techniques and different experimental set up) before and interpreted these as a reflection of a differential regulation of the specific and the total IgE response. So far, however, no data were available on the cellular level. The results of the study described in Section 5.3 suggest that, employing cellular assays, the antigen-specific response is transient in all the lymphoid organs tested, even under optimal conditions that result in a persistent serum level of specific IgE. Maximum total IgE-PFC responses were mostly obtained at 4 days after the last immunization. Up to 65% of the total number of IgE-secreting cells found on the peak day appeared to be antigen-specific. The observed lack of correlation between the antigen-specific and the total IgE response implies that ongoing IgE production at the cellular level cannot be predicted from IgE levels in the serum.

The observed time-lag between the cellular response in the lymphoid organs and the appearance of specific IgE antibodies in the serum, have prompted further investigations. Because of the short half-life of serum IgE antibodies (total as well as antigen-specific) [34], and the inability to detect IgE antibody-secreting cells in peripheral lymphoid organs at time points at which specific IgE antibodies occur in the serum, have

indicated a special site of longlasting antigen-specific IgE formation. The nature of this site can either be another organ or local production in lymphoid tissue along the respiratory or digestive tract. The route of immunization in our experiments, however, points to the peritoneal cavity as a possible site of IgE synthesis in contact with the circulation. The peritoneal cavity is selectively drained by the parathymic lymph nodes. In the latter organs IgE synthesis was always detected, independent of the immunization type or route. In the omentum lymphoid like structures were found consisting of lymphocytes, macrophages, mast cells and antigen-presenting cells [35,36]. These particular structures were already marked as a potential site of lymphocyte development and may be the source of Ly-1 positive B cells in the adult mouse [37]. We found high numbers of IgE-secreting cells in the peritoneal cavity after immunization. Remarkable was that this response involved a relatively high percentage of lambda bearing IgE-secreting cells (26%) and that this response almost exclusively consisted of antigen-specific IgE-secreting cells. Studies in progress will reveal whether this response involves $Ly\text{-}1^+$ B cells. Other investigators have presented evidence for a special function of this $Ly\text{-}1^+$ B cell subset with regard to development of the antibody specificity repertoire in fetal and neonatal live and the development of autoantibodies that progressively increase with age [37-42].

As described in Section 5.3, from these results the following migratory model can be postulated. Antigen-primed IgE-B cells in peripheral lymphoid organs will react with a short burst of proliferative activity in which a number of cells differentiate all the way to IgE-secreting cells that can be detected optimally at day 4 after the last immunization independent of the type of antigen and the dose. The simultaneously produced IgE memory cells migrate through the circulation and the bone marrow as well and finally home at the site of antigen deposition, i.e. the peritoneal cavity (Section 5.4). Upon a subsequent booster immunization these IgE memory cells react vigorously by proliferation and differentiation to IgE-secreting cells. The IgE produced will be almost exclusively antigen-specific and, after a certain lag time, will accumulate in the serum. By that time, part of the IgE will be cytophilically bound to Fc R on mast cells and be released into the serum with an additional time lag of around 12 days, the half-life of IgE bound to mast cell $Fc_\epsilon R$ [34]. These mechanisms together with the short half-life of IgE antibodies in the serum, might account for the low specific IgE level in the serum of maximally 30% of the total IgE level. This was shown before employing injections with antigen on alum [43-46].

In the study described in Section 5.4 it was shown that the particular immunization scheme employed with PC-KLH gives rise to a preferential IgE response. This IgE anti-PC response was largely $T15^+$. Moreover, it was demonstrated that the anti-PC IgE response cannot be suppressed by injection of anti-T15 anti-idiotypic antibodies, while the expression of the T15 idiotype among IgE anti-PC-PFC did no longer occur. The mechanism underlying the apparent association of a given variable region with a particular isotype is not clear. It is, however, of interest that IgG_3 and IgM antibodies, being group I antibodies and bearing the T15 idiotope are most protective against pneumococcus R 36A (Pn) [47-51]. Since the immunological memory to Pn is established early in life, protective T15 idiotope bearing antibodies may be formed after challenge with PC-KLH. However, the formation of anti-PC antibody isotypes, which are elicited by T-dependent mechanisms may develop later and only after appropriate immunization with PC in a T-dependent antigenic form. These newly elicited antibodies recognize an antigenic determinant, the PC structure including the aromatic spacer,

which differ from PC presented by Pn [52]. The IgE antibodies clearly display these characteristics. The question arises why the anti-PC IgE response can be suppressed by anti-T15 anti-idiotypic antibodies, in spite of the fact that IgE are group II antibodies and of different clonal origin than the T15 positive group I antibodies [45,53]. From these studies [54] it was concluded that group II anti-PC antibodies are genetically expressed independent from group I antibodies and do not represent somatic variants of group I. This was apparent from the finding in a panel of group II anti-PC hybridomas utilizing a H_H gene different from the T15 family. Although it has been shown before that the anti-PC IgE antibody response differs from IgM and some IgG classes in its clonal origin, it remains to be solved whether this is an exclusive characteristic of the PC system or a general phenomenon [52,54]. The basis for this observation is not yet clearly understood and may be related to the priming by previously induced cross-reactive responses and/or distinct affinity of certain clones. Further studies of epitope relationships to different isotypes may provide more insight into the molecular requirements for IgE antibody formation and its regulation.

7.3 Regulation of the IgE synthesis by lymphokines

A few models of the mechanism controlling IgE synthesis have been described [55-63]. These models provide a general concept of how IgE synthesis might be controlled in mice, rats and humans. However, further dissection of these regulatory mechanisms has led to divergent and sometimes contradictory interpretations by the various groups as to how genetic, antigenic and environmental factors could affect the outcome of animal exposure to antigen. Since the same variables have no influence on the IgG response, the concept of isotype-specific regulation in addition to the antigen-specific T helper and T suppressor regulatory mechanisms was proposed.

The IgE isotype response may be selectively enhanced under the following conditions: (a) in humans with the hyper-IgE syndrome; (b) in rodents exposed to low dose of X-rays or a low dose of cyclophosphamide injected prior to the antigen; (c) in rodents and humans suffering from an intestinal nematode infestation; (d) in humans or rodents immunized with antigens in adjuvants such as aluminium hydroxide and Bordetella pertussis; and (e) in rodents immunized with antigens chemically coupled to rutin-like polyphenolic compounds. By contrast, the IgE response can be selectively depressed: (a) in selected strains of mice, and is even absent in strains such as SJL; (b) in rodents immunized with antigens in certain adjuvants such as complete Freund's adjuvant (CFA); (c) in rodents repeatedly exposed to CFA components prior to antigen injection; and (d) in rodents immunized with antigens chemically coupled to the active component of CFA, i.e. the Mycobacterium, and possibly even with antigen-conjugated muramyl peptides [56-58].

It has been proposed that T lymphocytes regulate the key events leading to high or low IgE responsiveness in the models of both Ishizaka [55,61] and Kishimoto [59,62], whereas the Katz group [60] gives this role to B cells. Some unusual features of the lyt surface phenotype markers have been noted for T cells providing IgE helper or suppressor signals, but recent data indicate that attemps to make strict correlations of such markers with their cellular function may not be relevant.

Apart from the above mentioned regulatory systems controlling IgE synthesis, another isotype-specific regulatory mechanism involving lymphokines has been described recently (Section 1.4.3).

The study described in Section 6.2 revealed that IgE production in vitro is strongly enhanced by IL-4. This was shown in a model system employing polyclonal activation of the B cells by LPS. The addition of IL-4 to LPS-stimulated limiting dilution cultures caused an approximately tenfold increase in the frequency of clones that give rise to IgE-secreting cells without causing any substantial increase in the frequency of IgM secreting clones. IL-4 also increased the size of the IgE-producing clones 3 to 4-fold. Similar data have been described by others with regard to the effects of IL-4 on the production of IgG_1 by LPS-activated B cells [64]. A monoclonal antibody directed against IgM inhibited both the IgM and the IgE response in these cultures, demonstrating that the precursors of the IgE-secreting cells express mIgM. Taken together these studies suggest that IL-4 enhances both IgG1 and IgE secretion by LPS-stimulated B cells by specifically facilitating heavy chain class switching. This conclusion is in concordance with the results of Layton et al. [64].

Although LPS is a convenient and reproducible stimulus for B cells, it is unclear how faithfully it mimics activation by Th cells and lymphokines. Certainly LPS, in the absence of Th cells, efficiently induces growth, differentiation and isotype switching of B cells. It seems likely that only those lymphokines that cause effects different from those of LPS will appear active in LPS-stimulated cultures. This would give an underestimate, and perhaps a distorted view of the activities contained in a Th supernatant that is added to such cultures.

Although these and other results strongly suggest that IL-4 is inducing surface IgM-bearing B cells to switch to IgE and IgG_1, it does not prove it at the clonal level. They leave open the possibility that the additional IgE and IgG1 producing clones came from cells that had already switched prior to stimulation with LPS, but required IL-4 (together with LPS) to develop into detectable clones. Definitive proof that IL-4 directly regulates the frequency of isotype switching requires analysis of the products of individual B cell clones either with or without IL-4. To this end, the ability of IL-4 to alter the pattern of isotypes expressed during the clonal growth and differentiation of B cells has to be examined in single cell cultures.

Furthermore, the results presented lend support to the proposed mechanism of isotype switching and the involvement of lymphokines like IL-4 in this process [65]. It has been proposed that a necessary prerequisite for switch recombination is the 'accessibility' of a constant region (C_H) locus to the enzymes responsible for the recombination. Accessibility of specific C_H genes, as evidenced by 'sterile' transcription, DNAse hypersensitivity, and changes in methylation of nonrearranged C_H genes, correlates well with the subsequent switch to those C_H genes in several mouse B and pre-B cell tumor lines [66,67]. Preliminary evidence now suggests that IL-4 can induce changes in the accessibility of several C_H genes that correlate with the frequency of subsequent switching to these genes. Normal B cells cultured with LPS and IL-4 show substantially reduced levels of both sterile and productive $\gamma 2b$ mRNA transcripts as compared to B cells cultured only with LPS, and this correlates well with the approximately 5-fold reduction of secreted IgG2b caused by IL-4 (Lutzker et al. submitted). In addition, both sterile and productive ϵ-transcripts are made by LPS-stimulated B cells only in the presence of IL-4 (P. Rothman, R. Coffman and F. Alt, unpublished). These data support the idea that IL-4 regulates the frequency of switching by altering the accessibility of specific C_H loci to the switch recombination mechanism, rather than by altering the specificity of the recombination mechanism itself. The role of lymphokines in directing the isotype switching process was further discussed in Section 1.4.3.4. The

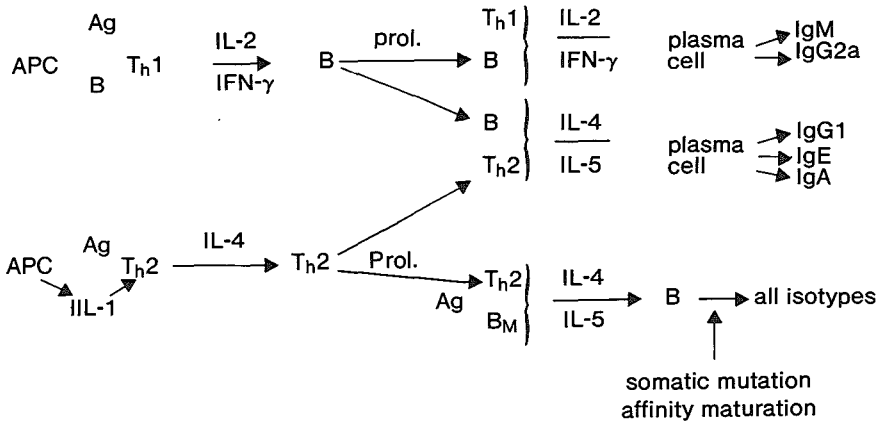

Fig. 1. Schematic representation of the mechanisms underlying the production of the different lymphokines that are able to regulate the production of the various Ig isotype.

combination of the various lymphokines present locally probably regulates the production of the various isotypes (Fig. 1).

The study described in Section 6.3 was undertaken to investigate whether IL-4 may be involved in the IgE production ability of different mouse strains. The results show that spleen cells from low responder mouse strains have a defective IgE production by LPS stimulated B cells in vitro. Moreover, the results show that IgE low responder strains do not have an intrinsic B cell defect. Instead, the results are suggestive for a defective IL-4 production or a defective state of activation of Th2 type cells in IgE low responder strains. These data are in agreement with the model put forward by Mosmann and Coffman and explained in Sections 1.4 and 6.1. Some additional comments with regard to this model will be made here.

The lymphokines produced by Th2 clones stimulate several key steps in the development of allergic or immediate hypersensitivity responses. As described above, only Th2 clones can stimulate a primary IgE response and IL-4 is (one of) the key factors responsible for this process. In addition, the combination of IL-4 and IL-3 leads to the optimum growth of IL-3 dependent mucosal mast cells in vitro [68,69]. IL-5, another Th2 derived product, is a potent stimulus for the growth and differentiation of eosinophils in vitro [70,71]. Th1 clones do not appear to make a comparable activity (D. Rennick, personal communication). Both mast cells and eosinophils bind IgE via IgE-specific $Fc_\epsilon R$ and, when they encounter antigens recognized by the bound IgE, can release an assortment of mediators of inflammatory reactions and, in the case of eosinophils, can also directly kill microorganisms [72]. Thus, the Th2 cell can, in principle, stimulate the production of the major cellular and humoral components of the immediate hypersensitivity system (Fig. 2). The recent demonstration that some

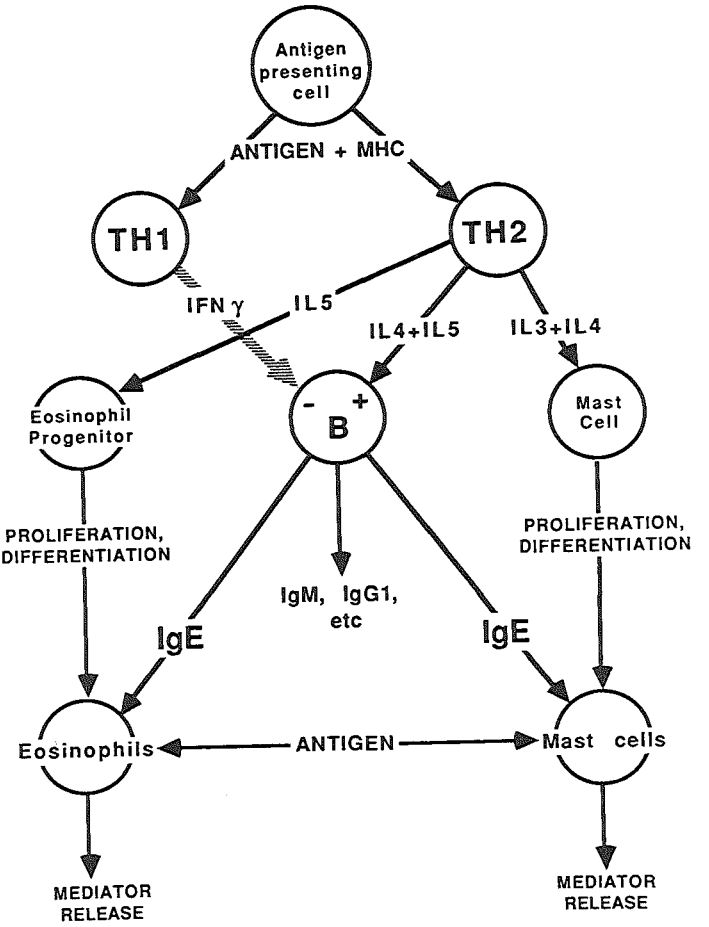

Fig. 2. Schematic representation of the regulatory role of Th2 type cells in immediate type hypersensitivity responses. After Coffman et al. [77].

IL-3 dependent mast cell lines secrete IL-4 [73] could add yet another arrow to this diagram.

There are many conditions, both in man and in experimental animals, in which IgE, eosinophil and mast cell levels are all significantly elevated. One well-studied example is the helminth parasite infection of mice and rats. Acute infection with a variety of parasitic worms, such as Nippostrongylus brasiliensis, results in substantial increases in serum IgE levels, eosinophil production [74], and mucosal mast cells in the intestines [75], all within two to three weeks. None of these responses occur in nude or neonatally thymectomized animals; they all require the presence of

T cells. It has been demonstrated that the *in vivo* IgE response to *Nippostrongylus* does, as expected, require IL-4 [76] and experiments are in progress to determine whether the inhibition of IL-3 and IL-5 function by antibodies has the effects predicted in Figure 2. If this scheme is correct, it offers some insight into the coordinated regulation of a complex response to an invading pathogen. It may be that the Th2 system stimulates the optimum combination of responses for dealing with certain classes of pathogens, such as multicellular parasites.

The production of IFN-γ by Th1 clones is expected to be inhibitory for IgE production but, based on *in vitro* assays, should not inhibit increases in either mast cells or eosinophils. The scheme in Figure 2 therefore represents only part of the responses expected to be stimulated by Th2 products.

7.4 Conclusions

In conclusion, this thesis describes in detail the development of reliable and suitable reagents and techniques for the investigation of IgE antibody formation in mice. Subsequently, immunization protocols were designed for the induction of persistent levels of specific IgE antibodies in the serum. This is regarded at least in part a reflection of the situation in human atopic disease. Various parameters that are essential for the understanding of the induced IgE response were analyzed. During the course of these studies a time lag was noted between the response of IgE-secreting cells in the various lymphoid organs and the appearance of specific IgE antibodies in the serum. Whereas the cellular IgE response could reach a high degree of antigen specificity (up to 80% of the total IgE response), the maximum degree of specificity in the serum was 30%. The peritoneal cavity was found to be a major site of localization of long term IgE antibody producing cells after repeated i.p. immunization. The regulation of IgE formation by T cell derived lymphokines was investigated especially in the light of the proposed balance between IL-4 and IFN-γ, the ratio of which would determine the net IgE synthesis. It was shown in clonal assays that IL-4 acts on B cells most probably by inducing a selective switch from IgM to the synthesis of IgE. This resulted in an increase of precursor frequency as well as clone size of IgE-secreting clones among LPS activated B cells. Moreover, the difference in IgE responder type of several mouse strains was investigated at the clonal level. It was shown that the observed lack of IgE production in IgE low responder mouse strains was not due to an intrinsic B cell defect. On the other hand, T cells defective in IL-4 production or a defective state of activation of Th2 cells probably account for this defective IgE production *in vivo*.

The combination of reagents and functional assays developed and the data from the studies on the induction and regulation of IgE synthesis in the mouse provides a fruitful basis for the further unravelling of the IgE antibody formation and the regulation thereof in the mouse as a animal model system for human atopic disease.

7.5 References

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SUMMARY

IgE antibodies play a crucial role in immediate type hypersensitivity reactions in atopic patients. The serum levels of total IgE are in general higher in atopics than in non-atopic individuals. The reasons for the overproduction of IgE, which contribute to the characteristic symptoms of allergic disease in atopic patients, are largely unknown. By studying in the mouse model system the mechanism of IgE antibody formation under defined conditions, more insight can be obtained into the cause underlying the overproduction of IgE in allergic patients. This may lead to a better understanding of the etiology of allergy-associated disease.

As reviewed in Chapter 1, antigen-specific IgE formation can be regulated by antigen, negative feed-back by the antibodies produced, by idiotypic-anti-idiotypic interaction and by antigen-specific helper and suppressor T cells. Alternatively, independent of the antigenic specificity of the IgE antibodies produced, isotype-specific regulation can be exerted by IgE binding potentiating and suppressive factors produced by T cells. Recent studies suggest that such isotype-specific regulation of IgE antibody formation can be mediated by lymphokines.

The purpose of the studies described in this thesis was to obtain more insight into the mechanism of induction of IgE formation and the regulatory role of lymphokines in this IgE synthesis.

The plan of investigation necessitated the availability of reliable reagents and suitable and sensitive assay systems for the detection and quantitation of total as well as antigen-specific IgE-secreting cells and IgE antibodies. The production of such reagents and the development of the required methods is described in Chapter 4.

To this end, purification procedures were developed for the isolation of IgE antibodies with a high yield but, even more important, with a high recovery of biological activity (Chapter 4.2). This recovery of activity of the purified IgE antibodies was evaluated by determining the ability to precipitate in double immunodiffusion and immunoelectrophoresis, the salt concentration necessary for the elution from an ion exchange matrix and the ability to bind in immunoblotting as well as in ELISA. Almost all of these detection methods were based on the use of specific anti-IgE antiseram.

For the production of such heterologous antisera (Chapter 4.3) purified IgE molecules were necessary for the immunization. Repeated immunoabsorptions were imperative for the removal of contaminating anti-light chain, anti-allotypic and anti-idiotypic activities, while performing elaborate specificity testing in inhibition type-ELISA. Moreover, from these studies it appeared that IgE molecules are sensitive to the purification procedure employed and this plays a crucial role on the binding in, for example, an ELISA. This problem was worth noting since the purified IgE samples were intended for use as an absolute standard in an ELISA for the quantitation of murine IgE (Chapter 4.4). This quantitation is based on a four-parameter logistic transformation of the data obtained from the standard as well as the sample readings.

For the enumeration of antigen-specific IgE-secreting cells by plaque assays it is prudent to couple macromolecular proteins to indicator SRBC in such a way that these SRBC can still be lysed by activated enzymes of the complement system. To this end, a method was developed that allowed covalent coupling of antigens by prewashing the SRBC in a low concentration of chromium chloride and blocking of the coupling reaction by irrelevant protein (Chapter 4.5).

By using the methods developed, the induction of IgE antibody formation was studied (Chapter 5). Total IgE levels were determined quantitatively in the serum of athymic BALB/c nude mice as well as in serum of heterozygous and normal BALB/c mice. Special attention was given to the effect of the housing conditions (more precisely: the environmental antigenic load) on the serum IgE level. It appeared that high antigenic load correlated with increased IgE levels in the serum although roughly a 6 weeks delay was apparent in increase of serum IgE level after increasing the environmental antigenic load (Chapter 5.2). A similar time delay was found while studying the effect of functional T cells on the serum IgE level. Injection of T cells in athymic nude mice decreased the serum IgE level while thymectomy of normal mice resulted in increased serum IgE levels.

Active immunization of BALB/c mice by repeated low dose i.p. injection of certain antigens without the use of adjuvant resulted in detectable IgE synthesis in lymphoid organs (Chapter 5.3). Interestingly, in these induced IgE responses, a short term transient response of antigen-specific IgE-secreting cells as well as a time lag in the accumulation of specific IgE antibodies in the serum were apparent. Moreover, the bone marrow contained specific IgE-secreting cells already after the first immunization. This suggested obvious differences in the regulation of these specific IgE responses as compared with immunization schemes employing adjuvant and high doses of antigen. It was also found that in cellular responses up to 75% of the responses were antigen-specific whereas in the serum at maximum only 35% of the IgE concentration was antigen-specific. This shows that the specific IgE concentration in the serum is only a weak representative of the production at the cellular level.

In a subsequent study it appeared that the peritoneal cavity contained many specific IgE-secreting cells after immunization with PC-KLH even after the short term IgE synthesis in the lymphoid organs had been disappeared (Chapter 5.4). Treatment by injection of monoclonal anti-T15 anti-idiotypic antibodies was able to inhibit selectively the T15 idiotype-positive IgE response without inhibiting the antigen-specific IgE response. This is in contrast to the IgM and IgG responses in BALB/c mice, where both the $T15^+$ and the anti-PC response are inhibited by treatment with anti-T15. These results suggest that various groups of antigen-specific antibody responses exist which differ in the expression of the fine specificity of a certain dominant idiotype. The IgE response apparently belongs to a group which expresses of a dominant idiotype and differed with IgM and IgG antibody responses in this respect of fine specificity.

Subsequently we analyzed the regulation of the IgE antibody formation by lymphokines as helper T cell products (Chapter 6). This study was focussed on the potentiating role of IL-4. Clonal analysis of the effect of IL-4 on purified B cells revealed that IL-4 most probably acts as a 'switching' factor that is able to activate IgM-bearing B cells to synthesize IgE (Chapter 6.2). IL-4 increased the precursor frequency of IgE-secreting clones of LPS-activated B cells 14-fold, while IL-4 caused a 3-fold increase in the clone size of IgE-secreting clones. IL-4 (produced by Th2 type cells) is inhibited in its potentiating activities completely by IFN-γ (produced by Th1 type cells). It is therefore possible that the ratio between the numbers of Th1 and Th2 type cells or their respective state of activation determines how much IgE is synthesized under *in vitro* as well as *in vivo* conditions.

Clonal analysis of B cells from SJL mice, which have a genetically determined, markedly depressed IgE productive capacity, revealed that this low IgE production both *in vitro* and *in vivo* was probably due to an absolute defect or a functional defect in the IL-4 producing Th2 cell compart-

ment (Chapter 6.3). It appeared that LPS-activated B cells from these mice were able to mount normal production of IgE if cultured in the presence of sufficient amounts of IL-4 or when cultured with T cells from IgE high responder BALB/c mice. However, culturing BALB/c B cells in the presence of SJL T cells resulted in a severely depressed IgE production.

In summary, this study has expanded the understanding of the mechanism and the regulation of murine IgE antibody formation. The conclusions from this study may be important for understanding the etiology of immediate type inflammatory diseases mediated by IgE antibodies, like atopic rhinitis and allergic asthma. The data presented will certainly facilitate and stimulate further investigations in IgE antibody formation and hopefully opens avenues to specifically limit overproduction of this intriguing class of immunoglobulins.

SAMENVATTING

IgE antilichamen spelen een cruciale rol bij acute overgevoeligheids reakties in atopische patiënten. De serum spiegels van totaal IgE zijn bij atopici in het algemeen hoger dan in niet-atopici. De achterliggende oorzaak van deze overproduktie van IgE antilichamen welke in belangrijke mate bijdraagt aan de karakteristieke verschijnselen van allergie bij atopici zijn nog onvoldoende bekend. Door in het proefdiermodel van de muis het mechanisme van de IgE antilichaamvorming onder gedefiniëerde omstandigheden te analyseren, kan meer inzicht worden verkregen in de oorzaak van het ontstaan van de verhoogde IgE antilichaam produktie bij atopici en dus in de etiologie van deze met allergie en astma geassocieerde ziekten.

Zoals beschreven in Hoofdstuk 1, kan de vorming van antigeen-specifieke IgE antilichamen worden gereguleerd via een negatieve terugkoppeling door de gevormde antilichamen, door idiotype-anti-idiotype interacties en door antigeen-specifieke helper en suppressor T cellen. Daarnaast kan onafhankelijk van de antigene specificiteit van de gevormde IgE antilichamen een isotype-specifieke regulatie plaatsvinden door IgE bindende potentiërende en suppressieve T cel faktoren. Recentelijk is beschreven dat door helper T cellen geproduceerde lymfokinen een regulerende funktie kunnen vervullen in de vorming van IgE antilichamen.

Het doel van het onderzoek was het vergroten van het inzicht in het mechanisme van de inductie van de IgE antilichaamvorming en de regulatie van deze IgE synthese door met name lymfokinen.

Voor het uitvoeren van dit onderzoek waren betrouwbare reagentia en eenduidige en zeer gevoelige detectie methoden nodig voor het aantonen en kwantificeren van zowel het totale aantal als het aantal antigeen-specifieke IgE producerende cellen en de geproduceerde IgE antilichamen. De produktie van dergelijke reagentia en de ontwikkeling van de benodigde methodieken wordt beschreven in Hoofdstuk 4.

Allereerst werden zuiveringsmethoden ontwikkeld waarmee monoklonale IgE antilichamen geïsoleerd konden worden met een hoge opbrengst maar vooral met behoud van hun biologische activiteit (Hoofdstuk 4.2). Dat wil zeggen dat het intact zijn van de gezuiverde IgE moleculen gecontroleerd werd door middel van bepaling van de precipitatie in dubbele immunodiffusie en immuno-electroforese, de benodigde zoutsterkte voor elutie van een ionenwisselaar en mate van binding in immunoblotting en in ELISA. Al deze detectiemethoden zijn gebaseerd op het gebruik van een specifiek anti-IgE antiserum.

Voor het opwekken van een dergelijk heteroloog antiserum (Hoofdstuk 4.3) waren gezuiverde IgE molekulen nodig als immunisatiemateriaal. Bij het uitvoerig testen van de monospecificiteit van de verkregen antisera in o.a. inhibitie ELISA bleken herhaalde immunoadsorpties noodzakelijk voor de verwijdering van contaminerende anti-lichte-keten, anti-allotypische en anti-idiotypische activiteit. Bovendien bleek bij deze studies de gevoeligheid van IgE moleculen voor de gevolgde zuiveringsprocedure en de invloed hiervan op de binding in bijvoorbeeld ELISA een cruciale rol te spelen.

Aan probleem werd aandacht besteed omdat de gezuiverde IgE monsters als absolute standaard dienden te fungeren bij het opzetten van een ELISA voor de kwantificering van IgE (Hoofdstuk 4.4). Deze kwantificering is gebaseerd op transformatie van de resultaten volgens een vier-parameter logistische functie van een standaard monster.

Voor het bepalen van het totale aantal en het aantal antigeen-specifieke IgE-secernerende cellen met behulp van de plaque test was het nodig

macromoleculaire eiwitten te koppelen aan indicator SRBC op een zodanige wijze dat deze SRBC konden worden gelyseerd door geaktiveerde complement enzymen. Daartoe werd een methode ontwikkeld waarbij door voorwassen van de SRBC in een lage concentratie chroomchloride gevolgd door blokkering van de opengebleven reactieve plaatsen door irrelevant eiwit, de antigenen covalent gekoppeld werden (Hoofdstuk 4.5).

Met behulp van de aldus ontwikkelde methoden werd de inductie van de IgE antilichaamvorming bestudeerd (Hoofdstuk 5). Totale IgE serum spiegels van naakte thymusloze BALB/c muizen alsmede serum spiegels van heterozygote en van normale BALB/c muizen werden bepaald. Hierbij werd speciale aandacht gegeven aan het effekt van huisvestingsomstandigheden (meer precies: de antigene belasting door de omgeving) op de serum IgE spiegel. Hogere antigene belasting bleek samen te gaan met verhoogde IgE concentraties in het serum alhoewel er een tijd van één tot anderhalve maand nodig was na het blootstellen aan die verhoogde antigene belasting alvorens de verandering aantoonbaar was (Hoofdstuk 5.2). Eenzelfde trage verandering van de serum IgE concentratie werd gevonden na injectie van T cellen bij thymusloze muizen. Het bleek dat injectie van T cellen in thymusloze muizen de IgE spiegel na enkele weken doet afnemen terwijl thymectomie van normale muizen na enige tijd leidt tot verhoging van de IgE spiegel.

Herhaalde i.p. immunisatie van BALB/c muizen met een herhaalde lage dosis antigeen zonder gebruikmaking van adjuvants leidde tot aantoonbare IgE synthese in de lymfoïde organen (Hoofdstuk 5.3). Interessant bij deze aldus opgewekte IgE responsen was de kortdurende respons van antigeen-specifieke IgE-secernerende cellen en de vertraging waarmee specifieke IgE antilichamen in het serum accumuleerden. Tevens bleek niet alleen de milt, maar ook het beenmerg reeds enkele dagen na immunisatie IgE-secernerende cellen te bevatten. Dit duidt op een ander regulatie-mechanisme van de op deze wijze geïnduceerde IgE responsen dan bij immunisatie met een hoge dosis antigeen in combinatie met een adjuvans, waarbij uitsluitend tijdens de secundaire respons antilichamen in het beenmerg worden gevormd. Van het totale aantal IgE secernerende cellen bleek tot 75% antigeen-specifieke antilichamen te secerneren, terwijl in het serum maximaal slechts 35% van het IgE antigeen-specifiek was. Dit toont aan dat de serum concentratie van specifiek IgE slechts een zwakke afspiegeling is van de produktie op cellulair niveau.

In een vervolgstudie bleek de peritoneaal holte na immunisatie veel specifieke IgE-secernerende cellen te bevatten, ook na de uitdoving van de kortdurende IgE synthese in de overige lymfoïde organen (Hoofdstuk 5.4). Deze IgE-secernerende cellen in de peritoneaalholte lijken verantwoordelijk te zijn voor het handhaven van de relatief hoge concentratie van antigeen-specifieke IgE antilichamen in het serum. In deze laatste studie werd gebruikt gemaakt van het antigeen PC-KLH, waarvan bekend is dat de anti-PC antilichaam respons in BALB/c muizen het dominante T15 idiotype draagt. Behandeling van muizen met monoklonale anti-T15 anti-idiotype antilichamen bleek in staat selectief de T15-idiotype positieve IgE respons te remmen zonder de antigeen-specifieke IgE respons significant te beïnvloeden. Dit is in tegenstelling tot de IgM en de IgG respons in BALB/c muizen, waarbij zowel de $T15^+$ als de PC-specifieke respons geremd wordt door behandeling met anti-T15 antilichamen. Deze resultaten suggereren dat de PC-specifieke antilichaamresponsen verschillen in fijnspecificiteit binnen het in de gebruikte BALB/c muizestam dominante T15 idiotype.

Tenslotte werd geanalyseerd op welke wijze de IgE antilichaamvorming op cellulair niveau gereguleerd kan worden door lymfokinen die door helper T cellen worden geproduceerd (Hoofdstuk 6). Hierbij werd met name aandacht besteed aan IL-4 als potentiërende regulator. Klonale analyse van het

effekt van IL-4 op geïsoleerde B cellen toonde aan dat IL-4 hoogst waarschijnlijk als een 'switch' faktor fungeert die IgM-dragende cellen aanzet tot vorming van IgE antilichamen (hoofdstuk 6.2). IL-4 vergroot de precursor frequentie van IgE-secernerende klonen die ontstaan uit door LPS-geaktiveerde B cellen 14-voudig, terwijl IL-4 de kloongrootte van deze IgE-secernerende klonen 3-voudig doet toenemen. IL-4 (geproduceerd door type 2 helper T cellen: Th2) wordt in zijn potentiërende effekten op de vorming van IgE volledig geremd door gamma-interferon (afkomstig van Th1 cellen). Derhalve is het mogelijk dat de verhouding tussen de aantallen Th1 en Th2 cellen of de mate van hun aktivatie de IgE produktie reguleert.

Onderzoek van SJL muizen, die een genetisch bepaalde sterk verminderde IgE produktie hebben, toonde aan dat deze geringe IgE produktie waarschijnlijk een gevolg is van een absoluut dan wel een funktioneel defekt in de IL-4 producerende Th2 cellen (hoofdstuk 6.3). Het bleek dat door LPS-geaktiveerde B cellen afkomstig van deze muizen in vitro wel degelijk in staat waren tot normale produktie van IgE antilichamen, mits zij werden gekweekt in aanwezigheid van voldoende IL-4 of wanneer zij tezamen werden gekweekt met T cellen van BALB/c muizen (die in staat zijn tot een sterke IgE produktie). Indien BALB/c B cellen gekweekt werden met SJL T cellen produceerden ook deze B cellen nauwelijks of geen IgE. Dit vermogen kon echter hersteld worden door toediening van IL-4. Dit toont eens te meer aan dat IL-4 een centrale rol heeft in de regulatie van de IgE antilichaamvorming.

Samenvattend kan gesteld worden dat ons onderzoek het inzicht in het mechanisme en de regulatie van de IgE-antilichaamvorming belangrijk heeft vergroot. De verkregen inzichten kunnen van belang zijn voor het inzicht in de etiologie van acute inflammatoire IgE gemediëerde ziekten zoals atopische rhinitis en allergisch astma. De beschreven resultaten kunnen onderzoek naar de IgE antilichaamvorming mogelijk maken en verder stimuleren. Daardoor kunnen nieuwe mogelijkheden gevonden worden om selectief de overproduktie van deze intrigerende klasse van antilichamen te remmen.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd op 11 juli 1956 te Grevenbicht (L.) geboren. In 1974 werd het diploma Atheneum B behaald aan het Bisschoppelijk College St. Jozef te Sittard. In datzelfde jaar werd begonnen met de studie Biologie aan de toenmalige Landbouw Hogeschool te Wageningen. In 1982 slaagde hij voor het doctoraalexamen met de drie hoofdvakken Celbiologie, Biochemie en Genetica. De stage vervulde hij aan het Department of Chemical Immunology van het Weizmann Institute of Science te Rehovot (Israël).

Vanaf 1 februari 1982 was hij als wetenschappelijk assistent verbonden aan de afdeling Celbiologie, Immunologie en Genetica van de Erasmus Universiteit te Rotterdam, aanvankelijk ten laste van de Nederlandse Organisatie voor Zuiver-Wetenschappelijk Onderzoek (Z.W.O.), en vanaf 1 januari 1985 ten laste van het Nederlands Astma Fonds. Binnen deze vakgroep werd het in dit proefschrift beschreven onderzoek verricht onder leiding van Prof. Dr. R. Benner.

In 1984 werd het C-diploma Stralingsbescherming behaald aan het J.A. Cohen Instituut voor Radiopathologie en Stralenbescherming (I.R.S.) te Leiden. In 1986 werd in het kader van post-academisch onderwijs in de geneeskunde een nascholingscursus Biostatistiek gevolgd. In 1987 werd als mede-initiatiefnemer een landelijke FPLC-discussiegroep opgezet. Gedurende de onderzoeksperiode werd praktikum onderwijs in de Immunologie en Celbiologie gegeven aan eerstejaars studenten Geneeskunde. Tevens werd als gastdocent onderwijs binnen het Hoger Laboratorium Onderwijs gegeven en werd een post-HLO cursus 'ELISA' opgezet en verzorgd.

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