

THE UNIVERSITY OF MANITOBA

Production of Antibodies to Murine IgE and
Suppression of IgE Responses
by Treatment of Neonatal and Young Mice
with Anti-epsilon Antibodies

by

Glendon M. Lang

A Dissertation

Submitted to the Faculty of Graduate Studies
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PRODUCTION OF ANTIBODIES TO MURINE IgE AND
SUPPRESSION OF IgE RESPONSES
BY TREATMENT OF NEONATAL AND YOUNG MICE
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A thesis submitted to the Faculty of Graduate Studies of
the University of Manitoba in partial fulfillment of the requirements
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DOCTOR OF PHILOSOPHY

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to Mary, Darin and

Diana

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ABSTRACT

In 1972 when this investigation began, there was general agreement that the separate B cell lines, each committed to the synthesis of one of the known immunoglobulin (Ig) isotypes, had a common precursor which expressed on its surface monomeric IgM (mIgM) and was sensitive to exposure to anti- μ antibodies. More controversial was the surface Ig expression of isotypes other than mIgM on these separate lines, in particular the precursors of IgE-secreting cells. It was against this background of uncertainty that the thesis study was initiated. Specifically, the objectives of the study were (1) to establish whether or not administration of anti- ϵ antibodies to neonatal and adult mice would suppress the IgE antibody response, and (2) to compare the results obtained with the available information on the effects of antibodies directed against other isotypes.

At the outset and throughout this study the main obstacle to progress was the lack of a readily available source of murine IgE (IgEm) required for (1) the quantitation of the IgE antibody response (using e.g., plaque forming cell assays and radioimmunoassays), (2) the measurement of total IgEm levels in mouse serum, and most importantly (3) the production of anti- ϵ m antibodies. Since it was generally accepted that total IgE serum levels were at least 1000 times lower than IgG levels, the production of IgEm in quantities sufficient for its isolation and for its use as an antigen required

the sensitization of large numbers of mice of a suitable high responder strain. Moreover, since all known immunization protocols resulted in the formation of tissue sensitizing IgG1 antibodies in addition to IgEm antibodies and since at the time the only assay available for IgEm was passive cutaneous anaphylaxis (PCA), the first series of experiments were conducted with a view to defining the limits of the biological tests for quantitation of these two distinct cytotropic antibodies, e.g., the 4 hour homologous PCA for the detection of IgG1 and the 4 to 72 hour heterologous PCA in rat skin for IgEm. From the results of these experiments it was concluded that (1) the minimal amount of antigen (i.e., ovalbumin) required to elicit a maximal IgE PCA titer was the same as previously established by others for IgG; (2) SW mice were the most suitable strain for homologous PCA; (3) IgEm antibodies were not detected after a 4 hour sensitization of homologous skin; (4) the variation inherent in the mouse homologous PCA assay required the inclusion of at least 5 replicates per determination, whereas the heterologous PCA assay required at least 3 replicates. It was also found that both IgEm and IgG1 antibodies were heat labile indicating that the then generally accepted method for distinguishing between these two classes of antibodies on the basis of differential heat lability was likely not valid. The potential of B6D2F1 mice to form antibodies of the IgEm and IgG1 classes from birth to 8 weeks of age was investigated by determining their ability to mount the corresponding anti-OA antibody responses. It was found that at the antigen dose utilized (1) the IgE response always preceded the IgG1 antibody response, (2) the ability

to induce an IgG1 antibody response in mice immunized before 12 days of age was erratic, (3) the interval between immunization and the appearance of detectable antibody in both classes decreased with the age of the mice, and (4) the levels of both antibody classes increased with the age of the mice at the time of immunization.

The low levels of IgEm present in murine reaginic serum (MRS) and the unavailability of monoclonal IgEm until after the completion of this study necessitated expenditure of considerable effort for the isolation of IgEm-enriched fractions from high-titered MRS. For this purpose the most successful procedure proved to require repeated intraperitoneal immunizations with 1.0 μ g OA in 1 to 5 mg Al(OH)₃ followed by precipitation of the IgEm with ammonium sulfate from the corresponding antiserum and its isolation by ion exchange chromatography. Another consequence of not having mg quantities of IgEm was the limitation on the assay procedures available to detect the presence of anti- ξ m antibodies. One technique available at the time of this thesis study adopted for screening and titrating of anti- ξ m activity made use of the capacity of these antibodies to interfere with PCA, hereafter referred to as neutralization of PCA.

In an attempt to produce a potent anti- ξ m antiserum several procedures were examined to reduce the level of contaminants in the IgEm containing fraction and to increase the immunogenicity of the available IgEm. To begin with, rat mast cells were used in the expectation that they would absorb out IgEm, and that the resulting IgEm-mast cell complexes would prove effective to immunize rats; however, this method did not lead to the production of detectable rat

anti-IgEm antibodies. The most successful procedure proved to require the tolerization of the rats with deaggregated normal mouse serum (NMS) prior to their immunization with IgEm-rich fractions. Antisera to IgEm were raised also in guinea pigs and goats and the antibodies produced against the other irrelevant serum antigens were removed with absorbents consisting of insolubilized NMS and mouse myeloma IgG and IgA. These antisera retained their ability to neutralize IgEm mediated PCA and were devoid of detectable antibodies to other NMS constituents.

The eventual availability of pure monoclonal rat IgE (IgEr) facilitated the production of high titered anti- ϵ r. Since it had been known that antibodies to many rat serum proteins crossreacted with mouse serum proteins the possibility existed that the anti- ϵ r may crossreact enough with IgEm to provide an alternate source of antibodies for suppression of the IgEm antibody response. Consequently, an extensive study was made of the degree of antigenic similarity between IgEm and IgEr and the ability of anti- ϵ r antibodies to bind to IgEm. The purified anti- ϵ r antibodies were found to be unable to form a detectable precipitin band against NMS but were able to neutralize IgEm mediated PCA. Although the calculated degree of crossreactivity was less than 4% and the avidity for IgEm was low, nevertheless, these antisera had higher activity than the anti- ϵ m antibody preparations.

The immunosuppressive capacities of all of the anti- ϵ antibody preparations, both anti- ϵ m and anti- ϵ r were tested in adults and neonates. It was found that the IgEm and not the IgG1 anti-OA antibody response could be suppressed by repeated injection of anti- ϵ r

and anti- ϵ m into adult mice prior to immunization. However, it was essential to allow a sufficient time interval (at least 4 days) to elapse between administration of heterologous antibodies and immunization in order to reduce non-specific suppression of the IgE response; the observed non-specific suppression was phenomenologically equivalent to antigenic competition. The most dramatic suppression of IgEm, in the absence of any effect on IgG1, was observed in neonates which received repeated thrice-weekly injections of anti- ϵ r beginning on the day of birth until the 16th day of life and which were immunized 5 days later. Hence, these results were interpreted as indicating that the IgEm-bearing B cells were selectively inactivated or eliminated by treatment with anti- ϵ r antibodies and that the precursor for IgG1 antibody synthesis did not bear the ϵ m isotype on its surface.

Production of IgEm antibodies was also suppressed in mice by administration of rabbit antibodies to murine μ chains prior to immunization. This result was interpreted to mean that the progenitor of the IgEm secreting cell prior to stimulation with antigen expressed both IgM and IgE determinants on its surface. These conclusions are discussed in relation to current models of B cell isotype diversity and to more recent findings in other laboratories. It is worth noting that the results with anti- ϵ were originally obtained in 1977 by the author and have been since confirmed by Bozelka et al in 1982 (395) in mice using hybridoma IgE as a source of anti- ϵ m.

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LIST OF ABBREVIATIONS

α	H-chain of IgA
AFC	antibody forming cell
ASC	adult <i>Ascaris suum</i> antigen
BALT	broncho-associated lymphoid tissue
BPO	benzene penicilloyl
BSA	bovine serum albumin
C	constant region of Ig H- and L-chains
CGG	chicken gammaglobulin
CFU-S	colony forming units from the spleen
δ	H-chain of IgD
DTH	delayed type hypersensitivity
ϵ	H-chain of IgE
EFA	enhancing factor of allergy
Fab	antigen binding site of Ig
Fc	isotype specific region of Ig
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
γ	H-chain of IgG
GALT	gut associated lymphoid tissue
H ₂	heavy chain of Ig
HBSS	Hank's balanced salt solution
id	idiotypic
i.d.	intradermal

i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
Ia	I region gene product
Ir-	immune response gene
Ig	immunoglobulin
IEP	immunoelectrophoresis
IgE _m	murine IgE
IgE _r	rat IgE
Igh	H-chain constant region gene
κ	kappa L-chain
KLH	keyhole limpet hemocyanin
λ	lambda L-chain
L, l	light chain of Ig
Ly	T cell marker
LPS	bacterial lipopolysaccharide
μ	H-chain of IgM
2-ME	2-mercaptoethanol
MHC	major histocompatibility complex
MRS	murine reagenic serum
mIgM	monomeric IgM
MØ	macrophage
NMS	normal mouse serum
NQ	neutralization quotient
NIP	4-hydroxy-5-iodo-3-nitrophenyl

OA	ovalbumin
PCA	passive cutaneous anaphylaxis
PC	phosphorylcholine
PBS	phosphate buffered saline
RPMC	rat peritoneal mast cells
RRS	rat reaginic serum
sIg	surface Ig
SFA	suppressing factor of allergy
SRBC	sheep red blood cells
SARb	sheep anti-[rabbit Ig]
SARb-4B	immunosorbent of SARb covalently coupled to Sepharose-4B
s.c.	subcutaneous
Th	helper T cell
Tcyt	cytotoxic T cell
Tdth	inducer T cell of DTH
TI	thymus independent
TD	thymus dependent
TsF	suppressive factor from Ts cell
V	variable region of H- and L-chains
Nb	adult <i>Nippostrongylus</i> <u>brasiliensis</u> antigen

INTRODUCTION

Humoral Immunity

The ability to survive exposure to viruses, toxins, bacteria and eukaryotic parasites relies on the fact that contact with foreign substances (antigens) normally stimulates the animal's lymphoid system to mount an adaptive¹ immune response to the antigens in question. The hallmark of this response is that the animal acquires the ability to "recognize" and react with the antigen more vigorously on subsequent encounters.

For a particular molecule to be antigenic it must be capable of inducing an immune response and of reacting specifically with (i) the "sensitized" cells (lymphocytes or immunocytes) and/or (ii) with antibodies produced as a consequence of stimulation of the immunocytes. Low molecular weight molecules which are able to react with antibodies but are unable to induce an immune response are called haptens. One way to convert a hapten (an antigenic determinant) into an immunogenic moiety is to combine it with a larger molecule (a carrier) which itself is an immunogen. Antigenic recognition and subsequent immune responses to the immunogen involve the interaction

¹ Other forms of protection exist and form the constitutive or non-adaptive immune system (skin, ciliated epithelium, mucous membranes) whose function is to serve as barriers to prevent access of potentially harmful organisms to the host's tissues.

of three different cell populations: macrophages, T lymphocytes and B lymphocytes.

1. Macrophages ($M\phi$): Peripheral blood monocytes and tissue $M\phi$ s are thought to be derived from the most primitive form of host defense system. They do not recognize antigen but in some manner "process" the antigen for presentation to T lymphocytes and/or B lymphocytes (1). Monocytes either circulate, adhere to endothelial surfaces or migrate into the tissues where they develop into histocytes. Monocytes are attracted to sites of inflammation by chemotactic factors and their development into activated $M\phi$ s represents one of the cellular immune responses. Resting $M\phi$ s are heterogeneous in terms of size, morphology, surface antigens and can be found to adhere to both T lymphocytes and B lymphocytes in a reversible manner. However, when the $M\phi$ is bearing antigen to which a T cell is reactive the binding to the T cell is more avid (2). Activated $M\phi$'s differ morphologically from resting $M\phi$ s; they are more granular, larger, more adherent to glass and more motile. One of the major functions of the $M\phi$ is phagocytosis (3). They have intra-cellular structures called lysosomes which contain enzymes able to digest ingested particles by virtue of a high concentration of acid hydrolases. Since the uptake of antigens by the $M\phi$ and expression of antigenic determinants on its surface has been found to be the first step in the antibody response, it is evident that antigens cannot be completely degraded at this stage. The two processes: i) phagocytosis of

antigen and degradation by lysosomal enzymes, and ii)

"processing" of antigen may be the function of different subpopulations of MΦs (4).

2. T Lymphocytes: This group of lymphocytes is referred to as T cells because their development is dependent upon the presence of a functioning thymus. T cells have been found to provide at least 4 distinct types of cells in the immune response:

- i) a helper function to facilitate B cell activation and differentiation during the course of the humoral immune response (antibody formation and B memory cell formation) mediated by a helper T cell (Th);
- ii) a suppressor function mediated by a suppressor T cell (Ts);
- iii) a killer function mediated by a cytotoxic T cell (Tct) to destroy other cells recognized as foreign;
- iv) activation of and mobilization of mononuclear and polymorphonuclear phagocytic cells involved in an inflammatory or delayed type hypersensitivity (DTH) reaction mediated by a Tdth cell.

3. B lymphocytes: The prefix "B" refers to the relationship to avian immune systems in which a central lymphoid organ, the Bursa of Fabricius, exists which appears to direct the development and maturation of the precursors of antibody forming cells (AFC), hence these are referred to as Bursa derived cells or "B" cells. In mammals, the site of B cell maturation is probably multifocal (5); however the gastrointes-

tinal lymphoid tissues (tonsils, appendix and Peyers' patches to name a few) have been suggested.

The necessity for cellular cooperation between these three cell types for antibody production was demonstrated in vivo (6) and in vitro (7). The role of the $M\phi$ was not identified in the in vivo experiments because $M\phi$ s were not removed or inactivated in these early experiments, which involved mixtures of bone marrow cells and thymus cells, followed by antigenic challenge. It was found that antibody production to protein antigens required both B and T cells; and that either alone was ineffective.

The classical demonstration of T-B cell cooperation involves the "carrier effect" as initially described by Mitchison (8). Two different cell populations of primed lymphocytes [i.e., one primed to the carrier, bovine serum albumin (BSA), and one primed to the hapten, 4-hydroxy-5-iodo-3-nitrophenyl (NIP) which had been conjugated to chicken gammaglobulin (CGG)] were transferred into the same syngeneic irradiated recipient. One day after cell transfer the recipient mice were immunized with NIP-BSA and the resultant anti-NIP response was examined. Maximum anti-NIP antibody production occurred when BSA primed splenic T cells (obtained by passaging thymus cells through an irradiated mouse subsequently primed with BSA) were mixed with NIP-primed splenic B cells (obtained by treating the NIP-CGG primed spleen cells with anti- θ (θ is a T cell antigen) and complement. For an antibody response to occur it was essential that a chemical linkage between NIP and BSA existed; mixtures of NIP and BSA or NIP-CGG and BSA did not induce anti-NIP antibody. Hence it was concluded that the

hapten-carrier conjugate stimulated the B cell to produce antibody to the hapten by a reaction of the T cell with the carrier, presumably within close proximity of the B cell. Mechanisms of lymphoid cell interactions will be discussed later.

Usually the adaptive immune response to environmental antigens is associated with a protective function. However, it is known that certain immunological mechanisms can result in harmful and sometimes fatal reactions. Anaphylaxis,² a term introduced by Hericourt and Richet (9) in 1898 was used to describe an increased susceptibility to a toxic substance rather than an increased resistance. Portier and Richet (10) observed that dogs injected with extracts of sea anemones several weeks after an initial injection of the same extract became ill and died of shock within a few minutes. Anaphylactic reactions were extensively studied in dogs, guinea pigs and rabbits whereby it was shown that these reactions could be transferred from sensitized to normal animals with serum and attributed to precipitating antibody. Von Pirquet (11) coined the term "allergy"³ to include all human disorders in which exposure to a causative agent, the allergen, resulted in a harmful response rather than a protective one. Hay fever was called an allergic disease because the causative agent was believed to be a toxin in pollen to which individuals became sensitive. In 1923 the term "atopy" was advanced by Coca and Cooke (12) as a more restrictive term for hayfever and asthma since both were thought to be distinct from anaphylactic reactions (at that time it

² Greek: ana=against; phylaxis=protection.

³ Greek word meaning altered action.

was controversial whether or not there was any antibody involvement in atopic reactions). Anaphylactic and atopic reactions were further classified by Coka and Cooke (12) as hypersensitivities along with a third category of reactions of which the tuberculin reaction was the most representative. Hypersensitivity is currently classified according to reaction time: In immediate hypersensitivity, a reaction is seen within 30 minutes of exposure, whereas DTH can take as much as 48 hours to manifest itself. For both immediate hypersensitivity and DTH the reaction to antigen is limited to its concurrent presence and is dependent upon a previous exposure.

- i) DTH is a T cell mediated immune response which can also be induced by soluble extracts from stimulated T lymphocytes (13). Four to six hours following intradermal (i.d.) injection of the antigen, M ϕ accumulate at the reaction site. The reaction is elicited by factors released from sensitized T cells i.e., factors which specifically attract and activate M ϕ s, skin reactive factors, and migration inhibition factors. The M ϕ 's destroy the antigen and the reaction subsides, the swelling and induration (an aftermath of the reaction) reaching a maximum 24 to 48 hours after exposure to antigen.
- ii) Immediate hypersensitivity was first shown to be associated with a humoral factor by Ramirez (14). It was not until 1921 that the immunological nature of the reaction was shown by Prausnitz and Küstner (15), who transferred atopic hypersensitivity from an allergic patient to the skin of a non-atopic individual. This passive transfer test (also referred to as a P.K. test) was widely used to titrate skin-sensitizing

antibodies, also called reagins (16) which were, finally, in 1966, identified as belonging to a unique class of immunoglobulin(Ig), referred to as IgE (17). The reaction is triggered when the skin site is challenged with specific antigen, eliciting a wheal and flare. The interaction between skin bound antibody and antigen induces the release of several pharmacologically active substances (primarily histamine and serotonin) from skin mast cells resulting in increased vascular permeability and tissue edema.

In order for B and T cells to recognize and respond to foreign antigens it is evident that they must express on their surface some form of receptor for antigen. It is generally accepted that i) mature resting B cells have on their surface an Ig which functions as a receptor for antigen, and ii) the surface Ig(sIg) expresses an identical antigen binding specificity to the antibody secreted by the activated B cell. As will be discussed later the antigen recognition unit on T cells may be related to Ig, but is clearly different from B cell receptor Ig.

Since the adaptive immune response was shown to involve expansion of relevant antigen reactive cells, Jerne proposed in 1955 (18) that selection and expansion of antigen reactive cells occurred by the combination of antigen with preformed natural antibody. This complex, attached to the appropriate cell, induced division which led to increased synthesis of the appropriate antibody. Specific immune responses were envisaged by Jerne as an increase in the level of natural antibodies, which was normally present in very low amounts.

Burnet (19) concluded that the antigen-binding moiety existed on the cell surface and proposed the clonal selection theory. In this model antibody was synthesized by specific AFC which developed from a cell expressing surface bound antibody. In studies on B cell development in chickens (20, 21), it was noted that IgM production appeared earlier than IgG production in the developing embryo, and IgA producing cells only appeared after hatching. Initially it was thought, in line with clonal selection, that progenitor cells for IgG and IgA arrived later in the bursa or that these stem cells required a longer residence in the bursa, i.e., a maturation stage. However, it was shown by Cooper et al (20) that treatment of 13 day old chick embryos with antibodies to the IgM H-chain (anti- μ) interfered with the development of not only IgM levels but also IgG and IgA levels in the young chicken. The clonal selection theory still requires a mechanism to account for isotype switching as the clone develops. In addition, the actual role of the surface Ig in providing the signal for proliferation and differentiation has been questioned when it was established that many humoral immune responses required cooperation between T and B cells for induction of antibody synthesis (22-24). Indeed as the immunobiology of the humoral immune response becomes understood, further modifications are being added to the clonal selection theory, nevertheless the major tenets of the theory as proposed by Burnet, such as interaction with antigen, preformed antibody on the surface of a committed B cell and clonal expansion, are still generally accepted. Thus the view is held that one effector cell or the clone resulting from activation of committed B cells

secretes a homogeneous population of molecules each with the same specificity as the preformed antibody on the surface of the original B cell.

Immunoglobulin

The major source of Ig in body fluids and mucosal secretions is a cell derived from the B cell lineage, called the plasma cell. Differentiation from B cell blast to terminally differentiated plasma cell is accompanied at each stage by active secretion of antibody such that the term "Ig-secreting cell" does not apply only to the plasma cell (25). Plasma cells do not contribute significantly to the total white blood cell count and are not regularly found in thoracic duct lymph; being restricted to the lymphoid tissues especially those draining the site of antigen entry. In the lymph nodes, plasma cells tend to be concentrated around the efferent lymphatics and the blood vessels of the hilum. In the spleen they are found within the marginal sinuses and the red pulp.

Description of Ig structural diversity:.

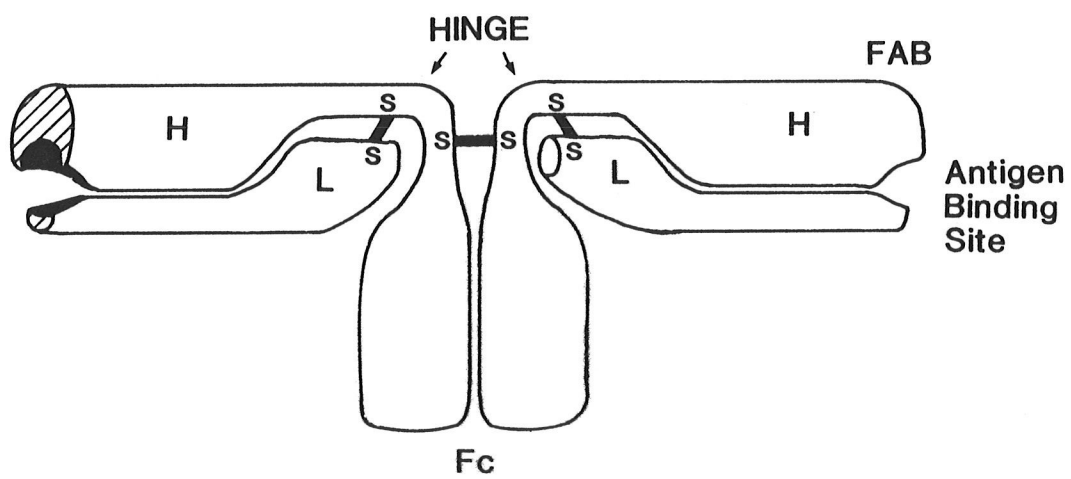
Each antibody molecule is composed of multiple polypeptide chains held together in most cases by disulfide bonds and is able to flex in the region of the hinge (see Figure 1). Functionally, antibody molecules can be divided into at least two regions, one which contains the antigen binding sites (Fab)₂ and one which contains other biological properties (Fc) such as complement activation, binding to a variety of Fc receptors on different cells and passage through the

placenta. Figure 1 portrays the basic structure of the monomeric form of all Igs: a Y shape with the Fab regions at the ends of the two arms of the Y. Five major Ig classes have been identified in all mammalian species investigated: IgA, IgD, IgE, IgG and IgM.

- i) IgM. This Ig class is found mainly in the form of a pentamer in which five monomers are joined by disulfide bonds at their Fc regions to a J-chain (small joining peptide). Because of its size, IgM cannot easily pass from intravascular fluids to extravascular fluids and is thereby in highest concentration in serum. It is the most efficient of the Igs in terms of complement fixation and agglutination. IgM is also one of the major B cell surface Igs where it exists as a monomer (mIgM).
- ii) IgE. This Ig class is found predominantly bound by its Fc region to tissue mast cells in mucosal tissues, connective tissues, skin, the peritoneal cavity and near smooth muscle. In the presence of antigen, IgE-sensitized mast cells are induced to release preformed and newly-formed pharmacological mediators causing localized increase in capillary permeability and contraction of smooth muscle. IgE represents only 0.001% of all the serum Ig in normal animals, but during parasitic infection it may increase to 5% of the total circulating Ig. IgE will not fix complement unless it has been aggregated by coupling with a chemical ligand (e.g., bisdiazotized benzidine), then it fixes the third component of complement (27). Even though the IgE molecule in serum

Figure 1.

A model of an IgG molecule to demonstrate the basic unit which contains two identical H-chains and two identical L-chains [adapted from Nisonoff (26)].



has the shortest half-life (12 hours) of all the Ig classes, when it is bound to the Fc_ϵ -receptor on tissue mast cells, it can persist at that site for weeks.

- iii) IgD. This Ig class, like IgE, is found only in trace amounts in serum. Unlike IgE or any other Ig class so far mentioned IgD has not been assigned any significant role in secretions, in circulation or bound to any cell which has not synthesized it. IgD is found predominantly as an integral membrane Ig of mature B cells and often on B cells which also express surface IgM, and therefore may be an important surface receptor for activation of these cells on interaction with antigen.
- iv) IgG. This class is the major Ig class of serum and is equally distributed between intra-and extravascular fluids. IgG can cross the placenta providing the major source of antibody in the developing fetus. Certain subclasses of IgG antibodies can activate a series of serum proteins, called the complement system, to augment agglutination and lysis of large antigens such as bacteria and cells. IgG antibodies bind to Fc receptors on phagocytic cells and killer cells to activate them to effect the destruction of bacteria and cells. In general these two functions, activation of complement and activation of phagocytic cells are the domain of different subclasses of IgG. There are also IgG antibodies which can bind to skin mast cells after interaction with antigen.

- v) IgA. This class of Ig is found predominantly in tears, saliva, colostrum, sweat and secretions of the gastro-intestinal, uro-genital and respiratory tracts. The majority of plasma cells committed to the production of IgA are found bordering the lumens receiving these secretions and only a fraction of the total IgA synthesized is found in the serum. After synthesis in the plasma cell IgA molecules are linked together at the Fc region by disulfide bonds to a small peptide called the J-chain. IgA in secretions are found to contain an additional component, the secretory piece, attached to the Fc region by disulfide bonds, which facilitates transport of locally produced IgA across the epithelial cells bordering all sites of secretion. In addition various cells have receptors for the secretory piece e.g. hepatocytes which pull IgA and complexes of IgA and antigen out of circulation for secretion into bile. The final form of IgA in secretions may be dimeric or trimeric.

All Ig isotypes when considered in the monomeric form are composed of 4 polypeptide chains joined together by disulfide bonds: 2 identical H-chains (the largest) whose N-terminal regions contain the antigen binding site (28) and 2 identical L-chains, which are associated with the N-terminal regions of the H-chains and potentiate the antigen binding capacity of H-chains while possessing little intrinsic affinity for antigen on their own. Each polypeptide chain contains an N-terminal variable (V) region and a C-terminal constant (C) region. The N-terminal region of both H- and L-chains resides in

the Fab region. X-ray crystallographic studies (29) indicate that the L-chain V (Vl) region and the H-chain V (Vh) region interact to form a cleft associated with the antigen binding site. Light and H-chains are folded and held together by weak non-covalent chemical bonds at this site. Most of the amino acid differences between antibody molecules have been attributed to three small areas called hypervariable regions located in the primary sequence of the V-regions of both H and L chains.

With respect to the H-chain C (Ch) region the primary amino acid sequence has been subdivided into domains based on the polypeptide loops established by intrachain disulfide bonds which occur along the chain. The domains are numbered from N-terminal to C-terminal; Ch1 is located within the Fab region; all others reside within the Fc region. The L-chain C (Cl) region is also composed of a polypeptide loop and is situated adjacent to the first domain of the H-chain. While it is known that the Fc region is responsible for the biologic activity of each Ig class, the domains involved have not been clearly identified in all cases. L-chains are generally composed of 212 amino acids (23,000 daltons) which display two sets of mutually exclusive antigenic determinants to form the two subclasses kappa (κ) and lambda (λ). Either light chain class has been found in all isotypes at the same frequency, namely, 90% κ and 10% λ . While light chains are quite uniform in size, H-chain molecular weight is dependent upon isotype. Shown below are the average molecular weights of H-chains ranked from largest to shortest.

<u>CLASS</u>	<u>H-CHAIN</u>	<u>MOLECULAR WEIGHTS</u>
IgE	epsilon (ϵ)	72,500 daltons
IgM	mu (μ)	70,000 daltons
IgD	delta (δ)	67,000 daltons
IgA	alpha (α)	64,000 daltons
IgG	gamma (γ)	50,000 daltons

Antigenic determinants on antibody molecules.

Antisera raised against antibody molecules or immunoglobulin fractions have been used to study the genetics of Ig synthesis. The various antigenic determinants as defined have been used as markers to obtain chromosome maps for the genes coding for the V and C regions of both H- and L-chains. Some interesting features have been revealed not the least of which is that the genetic information required to synthesize one antibody molecule exists in three different chromosomal regions: one carrying the H-chains loci; one the k chain loci; and, one the λ chain loci. Within each region the variable and constant loci are linked.

- i) isotypic determinants: H-chain determinants shared by all antibodies of a given class or subclass within a species.
- ii) allotypic determinants: H- and L-chain determinants shared by all antibodies of the same isotype within a given strain or haplotype. Genetic polymorphism has been found to exist

for seven of the eight known H-chain constant regions (Igh) genes in the mouse (30). All of the Igh loci are located on chromosome 12 and since no genetic recombination has ever been recorded to take place within this region the allele assigned to the γ_2a isotype becomes the designated allele for all of the genes at that locus. For example, the BALB/c allele for γ_2a is Igh-1a, therefore, all of the other BALB/c H-chain alleles are designated "a". The genes are numbered according to the order of their discovery:

gene:	Igh-1	Igh-2	Igh-3	Igh-4	Igh-5	Igh-6	Igh-7	Igh8
isotype:	γ_2a	α	γ_2b	γ_1	δ	μ	ϵ	γ_3

- iii) idiotypic determinants: Antisera may also recognize the antigen binding site itself or other sites within the variable region. Those antisera, if they recognize only antibodies capable of reacting against a limited range of related antigenic determinants are called anti-idiotypic antisera.

slg.

The Ig heavy chain which forms an integral part of the B cell membrane differs from that in secreted Ig by addition of a short stretch of hydrophobic amino acids at its carboxy-terminal end traversing the plasma membrane, and three terminal amino acids in the interior of the cell. Cell slg has been detected by i) anti-Ig mediated B cell cytotoxicity (1); ii) radioiodination and characterization of labelled surface proteins (31-33); and membrane binding of

labelled anti-Ig antibodies (34-36). The predominant sIg initially isolated from surface membranes of normal murine B cells was a 200,000 dalton mIgM. Subsequently, another surface Ig with a molecular weight of 170,000 daltons was identified as IgD (37, 38). Other isotypes are expressed on the surface of murine spleen cells, but the incidence of cells bearing these isotypes is low (39).

T cell recognition. It is generally accepted that T cells do not bear detectable endogenously synthesized immunoglobulin of any of the classes known to be expressed on B cells, but they do bear idiotypes similar to those expressed in clonally restricted antibody responses. Recent work with T cell clones (40) has led to the view that Vh regions form part of the T cell receptor for antigen. Thus T cells and B cells share a common cell surface receptor that recognizes antigen. However, as will be discussed later, T cells "see" antigen differently from B cells and in relation to their association with immune response gene products encoded for by non-Ig gene loci. Nevertheless, there are some reports that antigen specific molecules isolated from T cells are Ig-like: (i) Krawinkle et al have isolated idiotypic positive receptor material which does not bear Ia determinants capable of neutralizing haptenated bacteriophage (41). They claim that it is similar to IgG because of its relationship to an IgG allotype. However, the "heavy chain" class of this material is unique to T cells and its light chains are antigenically unique. ii) Marchalonis has pointed out that anti-u has been shown to inhibit antigen binding by T cells in many experimental systems but not all anti-u antisera are effective (42). He has isolated a μ -like chain

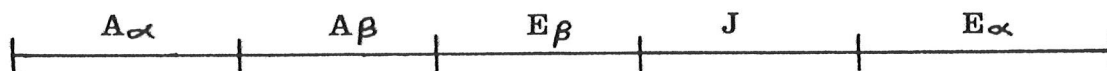
from Th cells with a molecular weight of 200,000 daltons. In general the low incidence of demonstrable Ig on T cell surfaces has been attributed to 1) inaccessibility, 2) a propensity for being shed rapidly as complexes of antigen and antibody and 3) the existence of an uncharacterized Ig isotype (IgT).

Ig components of murine humoral immunity.

There are 5 major isotypes in the mouse (43-45): IgA, IgE, IgD, IgM and IgG of which the latter can be further subdivided into IgG1, IgG2a, IgG2b and IgG3 (46). The serum concentration of each Ig class is dependent upon the age, strain and immunological experience of the mouse (43, 47-49). At the time of birth the bulk of the Ig is IgG1 of maternal origin and is acquired by placental transfer to the offspring, and through colostrum and milk. At the time of weaning (3 weeks post-partum) endogenous synthesis of Ig in the young mouse is evident with IgG1 predominating. Whereas normal, adult mice have high IgG1 and IgG2b levels (6.6 mg/ml and 1.2 mg/ml respectively in BALB/c mice), germfree mice have low levels of serum Ig of all classes and congenitally athymic BALB/c nu/nu mice show normal IgM levels (1.0 mg/ml in BALB/c) with decreased IgA, IgG1, IgG2a and IgG2b levels [14, 3, 27 and 14% of nu/+(normal phenotype) littermate control levels].

Antigenic stimulation elicits antibody in each of the Ig classes not necessarily in proportion to normal serum Ig levels. Many of the factors dictating the isotype of the antibody elicited include the concentration of antigen, presence or absence of adjuvant, intrinsic properties of the antigen and the involvement of T cells.

The ability to induce an immune response in the presence or absence of a thymus has established two major categories of antigens (50): 1) thymus independent⁴ (TI) antigens such as bacterial lipopolysaccharides (LPS), polyclonal activators, pneumococcal polysaccharides and their haptened derivatives, which in most instances fail to induce cell-mediated immunity (e.g., DTH). CBA/N mice possess a dominant X chromosome gene (Xid) which renders this strain and its F1 hybrids unresponsive to most soluble polysaccharide antigens and their haptened derivatives. The unresponsiveness of Xid mice to this subset of TI antigens has led to their designation as a unique group of antigens called Type-2 TI antigens (51); and, 2) thymus dependent (TD) antigens such as proteins, and histocompatibility antigens. In the mouse, immune responses to TD antigens are strictly controlled in an antigen-specific manner by a group of genes which map in the murine histocompatibility complex (MHC). The MHC controls a large number of immunological functions and is divided into 5 regions (K, I, S, D and L). The K, D and L regions code for membrane glycoproteins which are expressed on most cells of the body and appear to be the principal targets of cytotoxic T cells. The S region regulates the serum level of complement components. The I region which is divided into subregions (see below) contains immune response (I_r) genes.



⁴ There is a growing body of evidence (52,53) which has established that TI antigens require some form of T cell influence and so these antigens are now simply referred to as type-1 and type-2 antigens.

The humoral immune response typically elicited by TI antigens consists almost exclusively of IgM antibodies (54-57). The production of other Ig classes by TI antigens has been shown but not consistently so; i.e., Type III pneumococcal polysaccharide will elicit weak IgG and IgA antibody responses (58) which seem to arise in synchrony with the IgM response. Both dextran (59) and ficoll (60) are able to elicit IgG antibody responses to haptens covalently bound to them. LPS has been shown to contain a mitogenic moiety (61,62) which has a stimulatory effect on B cells and can act as an adjuvant but only with respect to TD antigens (63-65). Humoral immune responses elicited by TD antigens are primarily IgG (66). The influence of the T cell on Ig production appears to be variable within each class (67, 68). Torrigiani investigated the relative susceptibility of the IgG1, IgG2a, IgG2b, IgA and IgM murine immune responses to T cell depletion (68) and found that with the exception of IgM all of these isotypes demonstrated a dependence upon T cells (68). Later studies which included IgE found that of all the isotypes IgE antibody responses demonstrated the strictest requirement for T cell involvement (69-72).

Ontogeny of T Cells and B Cells

Haematopoietic origins of T Cells and B Cells.

It has been generally accepted since the pioneering work of Becker et al (73) and Wu et al (74) that a single haematopoietic stem cell, after extensive proliferation following transplanation into lethally irradiated syngeneic mice, can restore all of the haematopoietic cells

including polymorphonuclear (PMN) cells, mast cells, lymphocytes and $M\phi$ s of donor cell origin. This haematopoietic stem cell and the colony forming unit in the spleen (CFU-S) described by Becker et al are related, the major difference being that some of the progeny do not form distinct colonies in the recipient spleen (e.g., mast cells, $M\phi$ s and lymphocytes). Nevertheless, CFU-S can generate immunologically competent cells (75) and it is felt that CFU-S cells can generate a lymphoid stem cell which is the progenitor of both T cells and B cells distinct from the progenitor cells of the $M\phi$, PMN or erythrocyte lineage [reviewed in (76)]. By injecting low cell numbers (10^3 - 10^5) from various organs of adults and neonates into lethally irradiated hosts, Till and McCulloch found that the major organ generating CFU-S shifted from the liver to the bone marrow as the mouse matured. For example, 72% of all the CFU-S derived from neonatal tissue originated from the liver whereas less than 1% of the CFU-S generated using adult tissue originated from liver cells. In contrast 85% of the adult derived CFU-S originated in the bone marrow, while the neonatal bone marrow accounted for only 8% of the CFU-S from newborn tissues. For both neonates and adults the spleen accounted for 18% and 14%, respectively, of the remaining CFU-S.

T cell ontogeny. The earliest identifiable stem cell is a prothymocyte and is characterized by its propensity to home to the fetal thymus. This stem cell originates in the liver, the spleen or the bone marrow (depending upon the age of the mouse) and migrates to the thymus. While the primary function of the thymus is to produce thymocytes only about 1-5% leave the thymus, with the remainder being locally destroyed. The thymus is composed of lobules, each of which

can be divided into a cortical and medullary area; the former can be differentiated from the latter by the presence of closely packed lymphocytes and epithelial reticular cells (cortical epithelial cells). The cortex produces large numbers of thymocytes from immigrant bone marrow cells. Cells leave the thymus via draining veins and lymphatics to enter the peripheral lymphatics. Thymocytes differ from peripheral T cells in many respects, one of which is the density of a differentiation marker called theta (θ , Thy1 in the mouse) on the cell membrane. The density of θ decreases as the thymocyte matures to a T cell. Thy.1 is present at high concentration on cortical lymphocytes, at lower concentration on medullary lymphocytes and still lower concentrations at the time of export. Two populations of T cells can be characterized in terms of their migration patterns and longevity; (i) the long-lived recirculating T cell which enters the lymphatic circulation from the blood stream and goes back into the blood stream via the thoracic duct and, (ii) a short-lived T cell found predominately cycling through the spleen. Circulating T cells enter lymph nodes directly from the blood stream via specialized post-capillary, high walled endothelial venules to reside for a time in the paracortical zones. Afferent lymphatics draining through the cortex establish the flow of T cells into the medullary sinusoids and then into the efferent lymphatics. In the spleen T cells are located in an area around the central arterioles called the periarteriolar sheath in the white pulp. The spleen contains no lymphatic vessels (77) so T cells must enter by the blood system. Blood arterioles curve around the white pulp to create a marginal sinus or zone district from the red pulp. The white pulp is

positioned as a sleeve around the arterioles. T cells enter the white pulp by traversing the marginal sinus and can return to the blood stream by veins which drain the marginal sinus.

T cell differentiation continues in the peripheral lymphatics. Different subpopulations of T cells have been identified in the mouse by surface markers, particularly the Ly (Ly1, Ly2 and Ly3) and the Qa series of antigens (78-81). Since the Ly2 and Ly3 glycoproteins appear to be coexpressed and controlled by genes mapping closely together the notation 23(+) will replace 2(+)3(+). Cells expressing Ly1(+) 23(+) are first seen on cortisone sensitive cortical thymocytes but this differentiation marker disappears until it is reexpressed on peripheral T cells which appear to be the precursor cell for Ly1(-)23(+) cells (82,83): Ly1(+)23(+) cells are detectable in the spleens of mice within the first week of life in the absence of Ly1(+)23(-) and Ly1(-)23(+) cells. Ly1+23(-) cells are involved in inducing B cells to produce antibody (Th cells and amplifying the activity of Mφs in DTH. Ly1(+)23(-) T cells also induce Ly1+23(+) precursor cells to differentiate into Ly1(-)23(+) Ts cells (84). The ability of Ly1(-)23(+) cells to activate effector cells (B cells and Mφs) and induce the production of Ts cells has been associated with the presence or absence of the Qa1 antigen: only inducer cells co-express Qa1 and Ly1 [60% of the Ly1(+)23(-) cells]. Ly1(-)23(+) cells are involved in suppression of both cellular and humoral immunity (Ts cells) as well as mediation of cytotoxic activity (Tct cells), however only Ts cells express I-J region encoded determinants (see below) (85). The functional effects of these I-subregion genes

have been identified by using congenic and recombinant mouse strains in studies on the antibody response and T cell proliferation experiments:

I-region Facet of immune response controlled by this locus

$I-A_{\alpha}$ }
 $I-A_{\beta}$ } Immune responses to various TD antigens

$I-J$ Production of a suppressor factor for IgG responses

$I-E_{\alpha}$ }
 $I-E_{\beta}$ } Production of a suppressor factor for IgE responses

T cell Factors

The following properties are shared by antigen specific T cell factors, be they helper or suppressor factors (40):

- i) they are obtained only from antigen primed cells.
- ii) they can be selectively removed by antigen immunosorbents.
- iii) they bear I-region encoded determinants⁵ ; helper factors bear determinants encoded for by I-A and suppressor factors bear determinants encoded for by I-J.

⁵ The I-region has been shown to code for a number of serologically detectable cell surface antigens which are also present on T cell factors. These I-region associated (Ia) antigens are found on most B cells, some T cells and some MΦ's. Two distinct classes of Ia determinants exist (86), one class of determinants found associated with T cells are composed of carbohydrate and the other determinants, found on B cells and MΦ's, are protein in nature.

The most extensively studied antigen specific T cell factors are the suppressors factors (TsF). The technique of cell fusion to produce hybridoma derived antigen specific factors has been used to fuse activated T cells to an established T cell lymphoma line. The hybridoma cell obtained expresses the T cell function of the non-lymphoma partner (40) which includes the production of TsF. (87, 88). These factors consist of two polypeptide chains in non-covalent association: the large chain (85,000 daltons) possesses the antigen binding activity and the small chain (25,000 daltons) expresses the I-J region determinants. TsF from activated T cells resembles the hybridoma products by their simultaneous expression of idiotype, antigen binding capacity and I-J region determinants (89, 90).

B cell ontogeny.

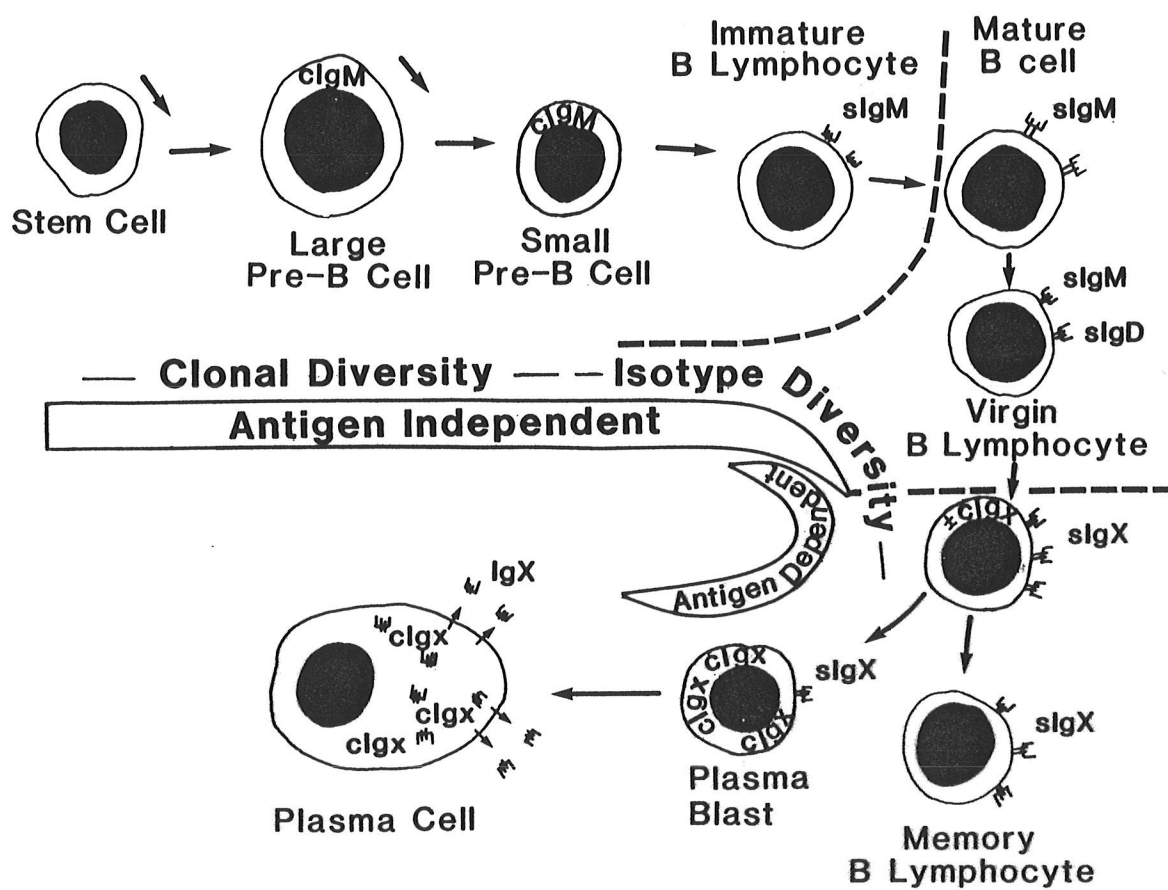
The development of B cells can be divided into two major sequences; i) antigen independent and ii) antigen dependent maturation. The former involves the differentiation steps from a haematopoietic stem cell to a virgin B cell, i.e., a cell with the potential to react to antigen but has not previously been exposed to antigen. The latter stage includes the antigen dependent production of memory cells and AFC. These stages are represented in Figure 2.

Antigen-independent maturation.

B cells are derived from pluripotent hematopoietic stem cells which also serve as precursors for the myeloid, megakaryocytic and erythroid cell line. The most primitive identifiable member of the B cell lineage is a large lymphoid cell containing intra-cytoplasmic μ -chains, which is the major B cell type in murine fetal liver 12 days

Figure 2.

An outline of the developmental sequence of murine B cells from pluripotent hemopoietic stem cell through to virgin B cell and then to antigen stimulated memory B cell and plasma cell. [Adapted from Lawton and Cooper (407).]



IgX = IgM, IgG, IgA or IgE

s = surface

c = cytoplasmic

after gestation (91). This cell type, called a pre-B cell does not produce light chains (92, 93) but does secrete μ chains. Pre-B cells, lacking surface μ chains (sIgM) produce smaller daughter cells which differentiate into immature B cells with the acquisition of sIgM within 36 hours of division (94). In adults pre-B cells are found exclusively in bone marrow. In the fetus pre-B cells can be found in liver, spleen and bone marrow, but as hematopoiesis shifts from liver to bone marrow the frequency of pre-B cells in the bone marrow increases. Studies in the rabbit (95) indicate that in contrast to mice their pre-B cells express light chains and that allelic exclusion has already begun; pre-B cells from heterozygous rabbits express either maternal or paternal allotypes.

The B cell which develops from the pre-B cell differs from a mature B cell in its sensitivity to surface interaction of its membrane IgM with crosslinking agents such as antigen or anti-IgM (96, 97). Modulation of sIgM from neonatal B cells, which are rich in immature B cells, occurs at a lower ligand concentration than for adult or mature B cells and, unlike in the mature B cell, is irreversible. It is thought that this process engenders in the neonate a higher susceptibility to inactivation of B cell clones, and accounts for the tolerance susceptibility of neonates (98). Up to this stage, B cells produce only IgM, but further developmental processes result in progeny committed to the synthesis of other isotypes (IgD, IgE, IgA and IgG) by a process referred to as isotype switching which will be described in more detail later on. Immature B cells are first seen in the developing fetus between days 16 and 17 following conception. The

progression from immature to mature B cell is associated with surface expression of IgD. Since neonatal mice acquire surface IgD and immunocompetence at about the same time, it is believed (99) that sIgD signifies a transition to a mature state wherein an encounter with antigen will lead to the development of this B cell into progeny which are either AFC or memory cells. Indeed sIgD(+) B cells appear to be less susceptible to tolerance induction by antigen (99). In the mouse the maturation from sIgM(+)/sIgD(-) B cells to mature sIgM(+)/sIgD(+) cells takes place in primary lymphoid follicles⁶ (100) in the peripheral lymphatics of both adults and neonates (101). The mature B cell with sIgM and sIgD expression represents the major peripheral B cell population.

Antigen-dependent maturation and class switching.

The results of several experiments support the concept that B cells consist of a large repertoire of cells, each with the potential to produce a unique antibody product identical to the sIg receptor in terms of its antigen binding specificity (103, 104). Clonal expansion of a B cell by antigen to AFC and memory cell stages has been shown to maintain the unique antigen binding specificity (105-107). Memory B cells differ from "virgin" IgM(+)/IgD(+) B Cells in several respects, such as surface charge (108), adherence to glass (108) and the degree

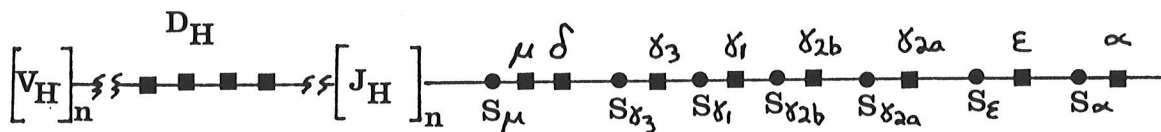
⁶ compact nodules of small lymphocytes within an interconnecting network of dendritic reticular cells which differ from the reticular cells associated with T-dependent zones by possessing Fc receptors (102). In lymph nodes, primary follicles are found only in the outer cortex; in the spleen they are found in the white pulp. B cells isolated from primary follicles home preferentially to follicles but enter the lymphoid tissue by the same vessels as T cells. This pattern is not evident in the adult until 2-3 days after birth and is independent of antigen stimulation.

of hapten substitution required for activation of these cells (106). With respect to the latter point, it was shown that memory B cells can be induced to proliferate at a lower epitope density which may indicate that the slg receptors are in closer proximity to each other than on virgin B cells. Virgin B cells also are unable to recirculate from the blood to the lymph as memory B cells do (100): thoracic duct cells have 3 to 5 times the memory cells activity of spleen cells, and bone marrow contains no memory cells.

With regard to the development of IgG AFC, Okudaira et al (109) demonstrated that immunized mice differed from unimmunized mice in the surface expression of IgG1, IgG2a and IgG2b. Prior to immunization, cell populations enriched for slgG1 or slgG2 expression by fluorescence activated cell sorting, were not able to produce an adoptive immune response, while cells from immunized mice were capable of antibody production. Moreover, only IgG2 antibody was obtained from IgG2 enriched cells and similarly the slgG1 bearing cells were found to generate only an IgG1 antibody response in an adoptive secondary immune response. It appears, therefore, that for IgG production the virgin B cell which is predominantly slgM(+)/slgD(+)/slgG(-) gives rise to a memory cell population which is slgM(-)/slgD(-)/slgG(+). Therefore, the major change in antigen induced maturation is the loss of slgM and slgD and the expression of slgG. Data obtained from radioactive suicide experiments (110) indicate that, for IgM production, two precursors are present: a virgin B cell expressing only slgM and another expressing both slgM and slgD. The former is postulated to be committed to IgM synthesis, while the latter can be recruited for expression of other isotypes.

The earliest explanation of the diversity in amino acid sequences of Ig molecules was that a gene existed for each antibody in the germline; i.e., thousands of heavy and light chain genes encoded with variable and constant regions adjacent to one another. The trouble with this model was that only the initial portion of each gene could vary while the terminal portion remained constant. In 1965 Dreyer and Bennett (111) proposed that V and C region genes were separately encoded and joined at some point in ontogeny. Their theory predicted that only a few Ch genes were present in the genome, and this was later confirmed (112). It was also shown that, as predicted, V and C region genes for a murine light chain were separated in germline DNA, but not in the genome of an immunoglobulin secreting tumor cell, (myeloma) (113-115). Combining the tools of recombinant DNA technology and sequencing studies it was found that the germline gene coded for fewer amino acids than were contained in the conventional V region and that the missing amino acid sequence contained a hypervariable region (116). This segment is now known as the J region, a specific site at which V region and C region genes join to form an active gene, viz. Ig gene expression involves translocation of V-region genes to C region genes to generate a transcription unit for the entire chain. Genes for κ , λ and H chains appear to follow this pattern (116). Examination of the DNA from a series of myelomas indicates that the formation of an active Ig gene involves rearrangement and deletion of germline DNA (117, 118). It was found that δ 2b plasmacytomas were lacking μ and δ 1 genes but not α genes, while δ 2a producing cells were lacking μ , δ 1, and δ 2b but not α . These results and others

(116, 119) have led to a gene map (see below) of the CH locus based on the premise that the organization of a myeloma cell genome is similar to that of normal antibody forming cells and is not a peculiarity of the malignant state.



This map reflects the DNA sequence believed to exist in the germline. During differentiation of B cells, Vh and Ch genes are brought together by at least two types of gene rearrangement: i) the first recombination takes place between a given Vh gene, a D segment (encoding for amino acids 98-105 of which only 4 sequences have been identified) and a Jh segment to complete the V-D-J sequence; ii) the second recombination involves the class switch from expression of IgM and IgD to expression of another isotype without alteration of the V-D-J sequence. The class switch starts with S_μ (S is functionally responsible for the recombination event) and involves its recombination with the S region of another Ch gene. Both types of recombination are accompanied by deletion of the intervening DNA sequence of the chromosome.

Ig diversity is currently believed to result from a combination of germline diversity, (provided by all possible combinations of V, D and J regions) and somatic mutation. The latter source of diversity has been suggested to fit the following findings: i) the germline Vk locus is comprised of only enough DNA to accommodate 90-320 genes; and ii)

Vh genes map in clusters comprising only 3 families to date (120). As outlined above, B cell differentiation progresses from a pre-B cell containing μ chains in the cytoplasm and no slg to an immature B cell bearing slgM and then to a virgin B cell with the addition of IgD on its surface⁷ (see Figure 2). Given that the signal for expression of isotypes other than IgM and IgD involves a recombination event, B cells may enter a stage of development in which they are not committed to any other isotype. At this stage, the possibility exists of suppressing the B cells ability to express any of the other isotypes. Further maturation, usually driven by exposure to antigen, results in a loss of slgD and exclusive production of either IgM, IgA, or IgG. It is generally accepted that a B cell is committed early in its development and, in the absence of antigen, to the expression of a given Vh-Vl receptor which is expressed in all of its progeny. This working hypothesis has received support from experiments in which identical specificity and idiotypy were discovered on the slgM and slgD of the same cell (38, 110, 122). Investigation of somatic DNA rearrangements which involve the fusion of V, D and J segments in each developing B cell is expected to shed light on allelic exclusion (the mechanism by which the cell is restricted to the production of only one allele) and class switching. Allelic exclusion is thought to be the by-product of errors in the rearrangement process. With respect to class switching, since S μ does not appear to have any special affinity for the other S loci there may be multiple S μ sites for specific switching to particular Ch genes (120) or certain V-D-J

⁷ the simultaneous production of μ and δ appears to be a special case of H-chain synthesis and does not involve deletion Ch DNA.

sequences select for specific Ch regions (119).

B cell activation.

The mechanism by which antigen stimulates sIgM(+)/sIgD(+) B cells to differentiate into AFC of a specific isotype or into memory cells committed to a certain isotype is not known. It is clear however that T cells are instrumental in the process. As shown by Gearhart et al using the splenic focus assay (123), that when T cell help is non-limiting IgM, IgG and IgA AFC can be generated from the same clone of antigen stimulated B cells. Under these conditions there were no temporal restrictions upon the expression of any isotype.

The role of sIg in the activation process is unknown. The simple act of binding antigen does not activate the cell, for unless certain other conditions are met the interaction will result in clonal deletion (124-127). Therefore antigen can stimulate only those B cells whose sIg receptors and state of development permit activation. Several models have been postulated to define the requirements for the generation of an activation signal with regard to the interaction of antigen with sIg: a) The two signal hypothesis. In 1970 Bretscher and Cohn (128) postulated that virgin B cells are activated by antigen only in the presence of a second signal. The interaction of an antigen with sIg changes the B cell in such a way that it must receive a second signal or it becomes unable to differentiate into an AFC or a memory cell. According to this model, all TI antigens supply the second signal themselves by virtue of the mitogenicity inherent to the antigen in question while TD antigens require the second signal to be generated by a Th cell. b) The one non-specific signal hypothesis.

Möller and Coutinho (129) postulated that slg merely concentrated antigen on the surface of the B cell and that the activation signal is provided by a mitogenic moiety on the antigen or by T cells. The basic difference between these two alternatives is that B cell tolerance cannot be induced by the latter mechanism. c) The matrix hypothesis. As originally presented by Feldmann et al (130), this model postulates that proliferation is induced by a critical cross-linking of surface Ig either by an antigen with repeating determinants or by an organized matrix formed by arrangement of antigen bound to T cell factors on the surface of an accessory cell. There is also some evidence that resting B cells may not require the same signals as virgin B cells and that the main function of slg is to bring the B cell to a point in its cell cycle that is most easily activated (128-131).

Influences of slg expression on B cell development..

The fate of a B cell may be dictated by the slg isotype expressed by it. Some examples of this form of selection are:

- i) IgE bearing B cells are preferentially influenced by a factor or factors which appear subsequent to infection of rats with the nematode *Nippostrongylus brasiliensis* (132). The IgE B cell generating factor isolated by Urban et al induces IgM bearing B cells to express surface IgE. This factor is produced preferentially by IgE, IgM, IgD triple bearing B cells.
- ii) IgA bearing B cells exhibit distinct migration patterns (133-137). There is evidence for the existence of a mucosal

immunological system interconnected by the migration of B_{α} blasts into intestinal, respiratory and genital tissues and, in the case of the female, mammary glands. After oral immunization IgA antibodies appear in bronchial and lacrimal secretions and IgA producing cells can be isolated from the mammary glands of pregnant and lactating mice (133, 134). B_{α} blasts are believed to migrate from Peyer's patches to mesenteric lymph nodes after exposure to antigen from the lumen of the gut. These blasts are precommitted to IgA production and, while resident in the mesenteric lymph nodes they do not bear sIgM. Mesenteric lymph nodes are also believed to serve an as yet unidentified special function in the life history of IgA and IgE B cell blasts after exposure to antigen (135). Experiments in the rat indicate that the precursors to IgE and IgA antibody forming cells may have similar migration patterns. The migration pattern after oral immunization is from the Peyer's patches^a to mesenteric lymph. Few sIg(+) cells are found in the Peyer's patches of conventionally reared rats, however, commitment to IgE and IgA production is felt to take place in the Peyer's patches (135). This stimulus may be a structural element of the

^a Peyer's patches have been thought to function as nonbursal lymphoid tissue which supports B cell differentiation from stem cells in the chicken, accounting for the fact that surgical removal of the avian bursa does not lead to an absolute deficiency in the capacity of the chicken to produce antibody. Other gut associated tissues appear to support B cell differentiation, such as cecal tonsils, and Peyers patches (136). The mammalian GALT may have been derived from these corresponding tissues in the chicken and became endowed with the ability to promote the antigen independent phase(s) of B cell development.

microenvironment of the Peyer's patch or it may be antigenic, mitogenic, enzymatic or hormonal. Peyer's patches of germ-free rats carry B cells which coexpress sIgA and sIgE but not sIgM (135), resembling a finding in the avian bursa of B cells coexpressing sIgY and sIgA.

In addition to gut associated lymphoid tissue (GALT), there is also a broncho-associated lymphoid tissue (BALT) present in chickens and mammals (138). Both BALT and GALT appear to be interrelated in terms of the ability of each to repopulate both with IgA containing cells (137). The evidence for the involvement of IgE in BALT is limited in the mouse, however, some indirect data from Gerbrandy and Bienenstock (139) in which mice immunized by the intratracheal or intraperitoneal route produce similar patterns of response in bronchial lymph nodes shows that murine IgE (IgEm) activity was highest in these nodes compared to spleen and peripheral lymph nodes.

- iii) IgE class specific TsF are capable of binding IgE and of exerting their effects directly on sIgE bearing B cells (140-143).

Effects of T Cells on B Cell Development

Introduction.

There is increasing evidence that the intensity and duration of an immune response is determined by a series of interactions among the

different T cell compartments: Th and Ts. Indeed antibody responses to complex TD antigens (e.g., SRBC, ovalbumin, keyhole limpet hemocyanin) irrespective of their being transient or long-lived contain both Th and Ts cells specific for the antigen (144-147). The role of antigen specific T cells and their factors in regulating immune responses has been studied most effectively by Sercarz et al (148-151): using inbred strains of mice and molecularly well defined low molecular weight TD-antigens such as hens egg white lysozyme (HEL).

HEL is a globular protein whose amino acid sequence and three dimensional structure is known (148). The N-terminal and C-terminal peptide are in close proximity (N-C peptide) and appear to present a conformational determinant recognized by Ts cells preferentially in non-responder C57Bl/10 mice and by Th cells in congenic B10.A responder mice. The rest of the molecule, the LII peptide (amino acids 13-105) induces Th cells in both strains (149). At the level of the B cell most of the secondary anti-HEL antibody responses are directed against the N-C peptide (150) with most of the antibodies bearing common idiotypic determinants. Three types of T cells are generated: i) a carrier specific Th cell induced by the LII peptide and the N-C peptide (152); ii) a carrier specific Ts cell specific to the N-C peptide (151); iii) an idiotypic specific Th cell induced by the activation of id(+)B cells (152). Induction of the anti-HEL antibody response is postulated to involve communication between B and T cells either by antigen bridges (153) by which carrier-specific Th cells and N-C specific id(+)B cells collaborate or by idotype:anti-

idiotype interactions (154) in which idiotype-specific Th cells and N-C specific B cells collaborate.

Suppression of both cellular and humoral immune responses can also involve idiotype anti-idiotype networks (155,156). In DTH (157) idiotype specific Ts cells (called Ts2 cells) which suppress the function of effector id(+)Tdth cells have been shown to be induced by id(+)Ts cells (Ts1) (157). Similar types of interactions between idiotype specific Ts cells and id(+)Th cells are believed to regulate the humoral immune responses to synthetic co-polymers of Glycine, Alanine and Tyrosine, and to azobenzene-arsonate (157).

Effect of Ts cells on B cell development and Ig synthesis.

Suppressor T cells share the following characteristics: i) they are radiosensitive, short-lived cells which are found in the spleen in high numbers; ii) they express Ly1(-)23(+) cell surface phenotype; iii) they are regulated by the I-J subregion of MHC and express on their surface I-J encoded determinants; and iv) the target cell (either T or B cell) must share the same histocompatibility haplotype at the I-J locus.

Allotype suppression.

In most strains of mice perinatal exposure to antibody against specific allotypic determinants expressed mainly on H-chains delays the production of the immunoglobulin class bearing the allotype. This suppression is totally dependent upon the relative concentrations of anti-allotype antibody, and the allotype bearing B cells. Continual replacement of suppressed cell types from the stem cell pool and gradual reduction of the levels of anti-allotype antibodies will lead to a breakdown of the suppression (158). Short term suppression is

the rule for all strains of mice, except those containing the SJL haplotype. In SJL x BALB/c hybrids, perinatal exposure to anti-(Ig-Ib) antibodies induces chronic suppression. This is the only haplotype-allotype combination for which chronic suppression can be induced; however, a less pronounced suppression does occur with respect to the Ig-Ia allotype in these hybrids. Moreover, anti-(Ig-Ib) antibody induced chronic suppression has been found to be mediated by an $Lyl(-)23(+)$ Ts cell (159). The presence of IgIb specific Ts cells in suppressed mice does not impair survival of IgIb B memory cells although it was found that the rapid transition from IgD(+) to IgD(-) memory cell in normal memory cell development and the accompanying avidity maturation of the memory cell pool is retarded in these mice and that the suppressed mice retained a high number of low affinity IgD(+) memory cells (160).

A clue to the relationship between allotype suppression and isotype suppression and the sensitivity to suppression provided by the SJL haplotype was obtained by Black and Herzenberg (161), who documented the ability of anti- μ isotype suppression to induce Ts cells in SJL mice. This finding suggests that depleting Ig-Ib B cell precursors with anti- μ leads to a failure in Th induction which in SJL related mice establishes a long-lived Ts population committed to preventing the appearance of the missing Th population. Three important concepts are revealed in this experimental system: 1) B cell development regulates the levels of helper T cells responsive to allotypic determinants present on the B cell surface; 2) SJL-related strains spontaneously generate allotype (Ig-Ib and maybe also Ig-Ia) specific

Ts cells in the absence of allotype positive Th cells; and 3) the production of the Ig-1b allele and the Ig-1a allele are differentially responsive to these influences.

Idiotype Suppression.

Idiotypic determinants (located on the Fab portion of the antibody) can elicit specific anti-idiotypic antibodies in heterologous, isologous and syngeneic animals (162). In the autologous system idiotype presentation is probably regulated by means of anti-idiotypic antibodies (163), i.e., antigen administration stimulates some elements of a network, subsequent alterations to restore the perturbed steady state condition represent the cellular and molecular reactions that occur during the immune response. Suppressor T cells may be found in idiotypically suppressed mice (164), however non-T cell populations are also involved (164-166). Anti-idiotypic antibodies together with antigen [an essential requirement (162)] appear to induce two separate Ts cell populations which inhibit either B cells (165) or Th cells (167) depending upon the antigen providing the TI or TD stimulus. Idiotype specific Ts cells appear to directly recognize B cells bearing the corresponding idiotype (168). Idiotype positive Ts cells appear to regulate a population of antigen-specific idiotype specific Th cells, both of which may participate in an immunoregulatory circuit.

Isotype-specific Ts cells.

The only isotype for which isotype specific Ts cells have been clearly implicated in suppression is IgE (169-171): 1) The inability of SJL mice to form an IgE antibody response to most test antigens in

the face of an IgG antibody response has been related to an irradiation sensitive and cyclophosphamide sensitive antigen non-specific T cell. This population of cells can be expanded by treatment of mice with FCA and was shown to release a soluble IgE specific suppressor factor. The factor can be obtained from all strains of mice and is not strain specific in its action (172); 2) There are also a number of instances in which antigen specific Ts cells and their factors (40,89,173,174) are epsilon specific.

Effects of Th Cells on B Cell Development and Ig Synthesis

As outlined earlier there is a T cell requirement for the production of IgG, IgA and IgE antibody responses. Since collaboration between T and B cells is a prerequisite for the production of an antibody response to TD-antigens for these isotypes, one or both of these two cell types may control the Ig class contribution of the antibody response. The B cell may be differentially sensitive to T cell influences thereby influencing the differentiation pathway selected by the B cell, or the specificity may reside in the T cell whose influences are only directed at B cells which express the target isotype. Cudkowicz and Shearer evolved an elaborate system to investigate this facet of the immune response which involved transplanting limiting numbers of primed (175) or unprimed donor spleen cells (176) into irradiated syngeneic recipients. By statistical analysis and limiting dilution experiments they were able to arrive at these conclusions: 1) precursors of AFC are already differentiated

for IgM and IgG production, and 2) no shift occurs from IgM production to IgG production. They divided the responding units into T cells and B cells and separately limited one in unlimiting concentrations of the other prior to adoptive transfer (177-179). In this way, they showed that Th cells do not dictate the class of Ig synthesized and are capable of interacting with more than one B cell. Also, the AFC for IgM were derived from a precursor involved in one prior interaction with antigen with or without T cells (their study could not separate the two), while IgG AFC-precursors were derived from a more complex interaction.

B cells can be activated by antigen in the absence of T cells (180) and many mechanisms have been proposed; such as, a mandatory mitogenic stimulus; activated C3b; and/or the formation of an antigen-antibody complex of specific proportions on the surface of the B cell. Since the proliferative response can be elicited by many antigens in the absence of T help (112, 181) the activation mechanism involved may be similar for triggering proliferation and in some cases differentiation of B cells to Ig production. Separate signals have been postulated to cause proliferation and differentiation of B cells prior to Ig production. Dutton has shown that T cell help is required subsequent to proliferation or IgM and IgG production will not occur (182). Schimpl and Wecker postulated that three signals were involved in the differentiation of a virgin B cell to an AFC (183). Stage 1 involves the interaction of antigen with the resting B cell, via its Ig receptors, to render this cell temporarily receptive to other signals. Stage 2 is the proliferative signal which may come from a mitogenic

moiety in the antigen, or by factors produced by $M\phi$ s. This signal may also come from a matrix of antigen and antibody on the B cell surface. Stage 3 is antigen unspecific and involves active Ig synthesis. The signal for this step may be intrinsic to the molecule or may come from activated T cells. Williamson et al (184) have suggested that two signals act on the same precursor cell. One signal, signal D, causes immunoglobulin synthesis and is dependent upon the presence of signal P, a signal for proliferation. The interaction of signals P and D dictates Ig production and memory cell production. If signal P greatly exceeds signal D, the precursor cell becomes a memory cell, while a balance of the two signals leads to antibody secreting cells.

Since IgG forming cells can be elicited in the absence of helper T cells by TI-antigens, the presence of T cells may be more important for the proliferation of precursors of IgG production (185).

McMichael and Williamson (186) have analyzed a sustained anti-DNP clone (No. S13) and concluded that memory production occurs during the proliferative response. Memory, in this case, could be found either before, or concomitantly with, but not after the generation of AFC.

Janeway has studied the cellular interactions during the adoptive secondary anti-hapten antibody response to DNP on a heterologous carrier to define the role of Th cells. B cells [primed to DNP and one carrier] and T cells were titrated independently of one another and in the presence of non-limiting amounts of the other (187).

Janeway plotted the relationship between anti-DNP titer and the number of limiting cells involved. At all times, T cells were required for a response to take place but the relationship between the amount of

antibody produced divided by the number of cells transferred was dependent upon the cell type titrated. When B cells were limiting, the relationship between B and T cells was linear. When T cells were limiting, an increase in T cells increased the antibody response exponentially. Janeway interpreted his data to mean that carrier primed Th cells contain at least two populations which act synergistically. Since it proved impossible to differentiate between the cell populations by a variety of techniques (eg, X-irradiation, anti- θ , antibody cytotoxicity, anti-thymocyte antibody cytotoxicity and adult thymectomy) it was postulated that one cell population provided more than one signal to the B cell. One Th signal affected clonal activation and the other affected clonal expansion (the premium effect). Evidence for two distinct Th carrier primed cells was obtained using T cells from mice treated from birth with anti- μ ; only then did the data indicate the absence of synergism. Since Ts cells could not be detected it was postulated that the Th cell which was absent in the neonatally suppressed mice was responsive to Ig determinants as well as carrier determinants. The other Th cell was more akin to the classical Th cell by recognizing carrier determinants in association with I-region encoded determinants. Other instances of Ig determinant specific Th cells include: 1) The absence of an Ig1b specific Th-cell in chronically suppressed (SJL x BALB/c)F1 mice. 2) The existence of a Th cell (Ly1(+)23(-)) whose presence or absence was dependent upon the presence of an idio type bearing B cell (167,188,189). For example, the Th cell required for the production of antibodies to phosphorylcholine (PC), expressing the idio type

characteristics of the BALB/c myeloma protein TEPC-15, is absent in mice treated from birth with anti- μ . 3) Activated B cells trigger Lyl(+)23(-) T cells to induce Ts cells (190). The structure on the B cell blast responsible for activating these T cells was found to be either the slg or a structure associated with the slg.

In summary, it has been demonstrated in several systems that in the mouse there exists a sub-population of Th cells whose presence or absence is related to the level of Ig-bearing B cells, and in some instances the reduction in the level of this T cell is accompanied by the presence of a previously undetectable Ts cell which actively suppresses the production of the target Ig determinant.

Regulation of IgE Antibody Formation in the Mouse

It is generally agreed that in rodents the intensity of the IgE antibody response varies with i) the antigen, ii) the animal species, and iii) the adjuvant employed (191, 192). In the rat, immunization with a protein antigen (together with *Bordetella pertussis*) produces a transient IgE response and very brief if any, secondary IgE response even though a boosterable, prolonged IgG response can be generated. Infestation of immunized rats with the nematode *Nippostrongylus brasiliensis* elevates the IgE antibody titer. IgE levels persist at high values without a concomitant increase in IgG levels. Re-infestation induces a further elevation in IgE levels. Also in the rat, passively acquired IgG antibody can induce suppression of an ongoing IgE antibody response. In contrast to the rat

system, persistent high IgE antibody levels can be induced in various strains of mice (e.g., B6D2F1 mice) by repeated challenge with low doses of antigen in aluminum hydroxide gel. The adjuvant requirement of an IgE antibody response in the mouse is limited to the primary response (193); a booster injection of homologous antigen will induce a secondary IgE antibody response without adjuvant. In some cases, the level of aggregated material in the antigen preparation is high enough to induce a primary IgE antibody response without adjuvant (194).

In the mouse, as a rule, low antigen doses favor higher, more persistent IgE antibody responses. Early work in this area indicated that i) low (0.1 μ g) doses of protein antigen selected for strains of mice with the ability to produce an IgE antibody response, and, ii) responsiveness was related to the MHC haplotype. Higher doses of antigen, (100 μ g) induced transient IgE responses in all strains to all protein antigens tested. Moreover, the dose of antigen was found to be particularly important for induction of an anti-hapten IgE antibody response in mice responsive to the carrier (195) since too high a carrier dose would result in a transient anti-hapten response in the face of a persistent anti-carrier response. The reason for this is as yet unknown. In addition, it has been found that IgE antibody responses can be tolerized at a dose of antigen which does not affect the IgG antibody response (196,197). These observations have led to the suggestion that the precursors of IgE AFC (IgE B cells) may have a higher affinity for antigen than B cells of other Ig classes (191). To date there is no evidence to support this suggestion.

Using the hapten carrier system, it has been established that IgE synthesis requires collaboration between carrier primed T cells and hapten primed B cells (71, 195, 198, 199). The absolute requirement for T cells in IgE formation was shown by Michael and Bernstein (200) who reported that congenitally athymic (nu/nu) mice were unable to produce reaginic antibodies, and that a thymus graft or reconstitution by thymocytes initiated IgE antibody formation. TI antigens were unable to induce IgE in mice (191, 193, 196, 201). Katz has exploited the ability to convert low responder SJL mice, which produce poor IgE responses to most antigens (201), to high responders to study the parameters which influence IgE production (195). He and his collaborators have shown that SJL mice inoculated with Freund's complete adjuvant (FCA) possess in their serum 7 days later, a factor called suppressive factor of allergy (SFA) which exerts its effect in an antigen non-specific, IgE specific manner. Passive transfer of SFA across MHC will suppress IgE antibody responses. Another factor which has been characterized by Katz, is the enhancing factor of allergy (EFA), which can also be found in SJL mice inoculated with *Mycobacterium* in FCA. EFA appears earlier than SFA and its highest level is at 3-4 days post-inoculation. EFA and SFA are present in all mouse strains following FCA injections; their relative levels reflecting the phenotype of the mouse strain in which they are induced. High responders have a higher EFA to SFA ratio than low responders.

Idiotypic suppression has also been described for IgE antibody responses (202). BALB/c mice actively immunized with IgG anti-BPO or IgG anti-PC produced anti-idiotypic antibodies which suppressed the IgE anti-BPO or IgE anti-PC antibody responses in mice immunized with the

respective hapten (BPO or PC) coupled to ovalbumin (OA) as a carrier. On the other hand, anti-OA IgE antibody responses were not suppressed. However, BALB/c mice actively immunized against IgG anti-OA produced anti-idiotypic antibodies which suppressed both the anti-hapten and the anti-carrier IgE antibody responses. The IgG anti-OA responses were not as dramatically affected as the IgE antibody responses in all cases. It appears therefore that the repertoire of responsive IgE B cells is more clonally restricted than the IgG B cells.

B_ε cells appear to be more susceptible than B_γ cells to T cell influences, such as adult thymectomy, the presence of non-antigen specific Th cell factors and the allogeneic effect (203). The dissociation of IgG and IgE antibody responses has been examined by many groups and it has been suggested that either B_ε cells are more sensitive than B_γ cells to the regulatory influence of T cells or that different effector T cells exist for each isotype. Some of the differences seen between IgE and IgG antibody responses have been explained as being due to i) differences in the assay systems; IgG levels are usually compared on a linear scale while IgE levels are compared on logarithmic scale, and ii) differences in B cell numbers, such that the B_ε population may be of lower frequency than B_γ cells; when the B_ε population is expanded by adoptive transfer experiments the difference in apparent Th cell sensitivity decreases (201). Indeed, Hamaoka et al (203, 204) were unable to demonstrate the existence of distinct IgE or IgG specific Th cells. However others have established that IgE specific T cell influences do exist. The potentiation of IgE levels in mice by *Nippostrongylus brasiliensis*,

described above for low responders, has been attributed to stimulation of Th cells specifically aiding B_E cells to develop into IgE AFC (205). Kojima et al (205) have characterized a soluble substance released into tissue culture supernatants by spleen and lymph node cells from mice infected with *Nippostrongylus brasiliensis*. The factor is released in the presence of parasite antigen and selectively enhances IgE antibody production in vivo. In other studies Ts cell factors (TsF) specific for IgE antibody production have been isolated (206-210) from normal SJL spleen cells and mycobacterium inoculated mice. Both factors are antigen non-specific, but the latter is dependent upon antigen for its production. The TsF isolated from mycobacterium stimulated mice also bear I-region encoded determinants.

Effects of Anti-Ig Antisera on B Lymphocyte Ontogeny

One approach which has constituted a powerful tool for the investigation of H-chain isotype presentation on the surface of B cells has been the application of anti-heavy chain antibodies to inhibit or stimulate B cell function. The early in vitro experiments with anti-Ig antisera yielded variable results: some preparations induced proliferation of B cells; others had no effect. However, the majority of preparations induced suppression or inhibition of B cell activity. The ability of anti-Ig preparations to induce proliferation has been examined and it has been found that some of the activity was related to the Fc portion of the anti-Ig antisera (211). Under appropriate conditions, for example, covalent attachment of anti-Ig

antibodies to polyacrylamide beads (212), anti- μ , anti- δ and anti-k can activate adult B cells to proliferate.

Inhibition in vitro of immune responses.

The objective of most of these experiments has been to characterize the surface Ig on those B cells which are precursors of IgG and IgA secreting cells: the experimental approach has involved treatment of the cell with isotype specific antibody. Interaction of bivalent anti-Ig with B cell sIg induces: 1) capping of sIg, 2) cell motility, and 3) shedding and/or endocytosis of sIg (195) regardless of whether the effect is inhibitory or stimulatory for a given B cell activity. Factors which influence the ability of anti-Ig to modulate B cells include the concentration of anti-Ig, its specificity and the state of maturation of the B cell. For example, capping is more rapid on neonatal B cells and is induced with a lower concentration of anti- μ than is required for adult B cells (213). Also, B cells from neonates do not re-express sIg after a cycle of capping with anti-Ig antibodies, nor are they induced to proliferate (96,213,214). Fragment cultures of fetal liver in the presence of anti- μ were unable to generate immature sIgM(+) B cells, which normally develop at 16-17 days in the fetal liver (215), without blocking the expression of cytoplasmic μ -chains by pre-B cells (216).

In 1972, Pierce et al (217,218) carried out a systematic analysis of the effect of goat anti- μ , anti- δ 1, anti- δ 2 and anti- α on primary and secondary anti-SRBC responses in murine spleen cell cultures. For the primary immune response, the degree of suppression mediated by anti- μ was related to its concentration in the cultures. All classes

of antibody (IgM, IgG1, IgG2 and IgA) were suppressed and the suppression was complete in 48 hours, provided that IgM was not added to the cultures during this time. The capacity of anti- μ to suppress the secondary IgM and IgA immune responses was evident for up to 28 days after priming; however, the IgG1 response was resistant to the presence of anti- μ by day 10. Anti- γ 1 as well as anti- γ 2 each suppressed both the IgG1 and IgG2 primary immune responses as well as the early phase of the secondary IgG1 and IgG2 antibody responses. In the same experiment the IgM and IgA antibody responses were unaffected. Anti- α could inhibit only the IgA antibody response. These results were consistent with earlier observations (219, 220) and were confirmed later by Abney et al (221) and Yuan et al (222). Later studies with anti- δ (223-225) indicated that only anti- μ could block primary in vitro antibody responses to TI-antigens, while both anti- μ and anti- δ could block the immune response to TD-antigens. Also anti- δ is unable to block a secondary IgG response (221). The implications of these in vitro studies are that; a) both IgM and IgD are expressed on the surface of a B cell activated by a TD-antigen, b) IgD was neither essential nor expressed on the surface of B cells activated by a TI-antigen, (c) once a response has been induced the expression of slgs on the responding B cells changes, and the ability of anti- μ or anti- δ to prevent a primed B cell from responding to a TD-antigen is reduced, d) slgG and slgA appear on independent cell populations either in the presence or absence of slgM and slgD, and e) membrane expression of IgM and IgD is evident on precursor cells for IgG and IgA antibody responses.

Direct evidence that IgG or IgA secreting plasma cells are ultimately derived from precursors which express sIgM was obtained by i) examination of the ability of LPS to induce polyclonal proliferation of B cells, and ii) differentiation of these cells to mature plasma cells secreting IgM, IgG1, IgG2 and IgA in the presence of anti-Ig antibodies (226-231). It was found that anti- μ enhanced the proliferation of adult B cells stimulated by LPS and at the same time suppressed the appearance of plasma cells (226,227 and 231); neonatal cells did not demonstrate an enhanced proliferative response, even though the LPS induced development of plasma cells was inhibited. Less anti- μ was required to inhibit LPS-induced differentiation of neonatal B cells than was required for adult cells. In fact contrary to the requirement of adult cultures of continuous exposure to anti- μ for both enhanced proliferation and suppression of differentiation, neonatal cells could be suppressed with shorter exposures and a 300-fold lower concentration of anti- μ . The neonatal cells did not recover, i.e., continuous re-expression of sIgM does not occur on neonatal B cells and cross-linking of sIgM may completely inhibit further development of these cells. Using direct immunofluorescence to determine surface isotype and cytoplasmic Ig content of newborn and adult B cells before and after exposure to LPS (231) virgin IgG2 precursors were shown to contain IgM and IgG2. At a time when cytoplasmic IgG2 was accumulating, sIgM was decreasing until only sIgG2 could be detected. Both adult and neonatal B cell populations contained a small subset of sIgM(+)/sIgG2(+) cells, however only in the adult was it demonstrated that B cells expressed sIgG2 in the

absence of sIgM. These cells could not be induced to differentiate into plasma cells by LPS. It is evident, therefore, that activation of B cells by LPS requires sIgM and that IgG2 secreting plasma cells arise from either sIgM(+)/sIgG2(+) precursor B cells or sIgM(+)/sIgG(-) B cells precommitted to IgG2 synthesis. In any event anti- δ is not inhibitory until the cultures have begun to express sIgG (232). These experiments also indicate that i) for complete suppression of LPS-induced maturation by anti- μ , the sIgM which can be re-expressed must be modulated off the cell for more than one cell division, and ii) the earliest B cell, the immature B cell, is exquisitely sensitive to modulation of sIg by anti- μ . This is evident also for antigen-induced modulation (97, 233) in that much lower concentrations of antigen causes sIg to migrate to one pole of the cell leading to its removal by endocytosis. The cells are inactivated (234) and the effect is irreversible (96).

Inhibition in vivo of antibody responses.

Most in vivo investigations have involved administration of anti-Ig into young or neonatal mice prior to injection of antigen. The treatment regimens used involve antibodies directed against μ, δ, α (reviewed in 235) and δ (236) H-chain determinants. Mice treated from birth with anti- μ antibodies are unable to generate a primary IgM, IgG or IgA antibody response, demonstrate a complete loss of serum IgM and exhibit severe deficiencies in serum IgG1, IgG2 and IgA levels. The degree to which serum Ig levels are depressed and the duration of the suppression is dependent upon the total quantity of anti- μ antibody administered and the schedule of treatment (235,236). IgM serum

levels are the most easily and permanently suppressed, followed by IgG2a (237), with IgA and IgG1 tending to recover more rapidly. Inbred mice are more easily suppressed than outbred mice, and the recovery phase of the anti- μ mediated suppression is strain dependent (238). Administration of anti- μ causes severe depletion or elimination of IgM, IgG and IgA plasma cells (239,240), depletion of splenic lymphocytes bearing slg (241), diminished germinal center formation (240) and reduced spleen size (240, 241). In general, immunosuppression of a particular isotype by anti-Ig is more easily detected by monitoring the appearance of antibodies of the appropriate Ig class during a primary immune response than by measuring changes in the total serum levels of the isotype.

Mice treated from birth with antibodies directed against the other heavy chains do not exhibit as pronounced an immunosuppression as the anti- μ suppressed mice. Anti- δ antibodies suppress IgG immunity without affecting the IgM antibody responses (236) to both a TD and a TI-antigen (IgA was not examined). The appearance of slgD(+) cells was suppressed in spleen and lymph nodes. Anti- δ and anti- α antibodies suppress immune responses only in the Ig class to which they are directed (235). The level of suppression obtained with anti-Ig was directly related to the length of the treatment schedule and inversely related to both the age of the animal at the time of its administration and the time interval between the last injection of anti-Ig and the time of assessment of suppression. Mice exposed to anti-Ig prenatally by transplacental passage are more severely suppressed than mice exposed from birth (242). Treatment schedules

begun when the mice were 1 to 2 weeks old were less effective than treatment at birth (241,243).

The target cell of anti-heavy chain immunosuppression is not the $M\phi$ (238), the T cell (217,244-248), the pre-B cell (244,249) or the plasma cell (217), but is most probably the immature $slgM(+)$ B cell or the $slgM(+)/slgD(+)$ mature, virgin B cell. The target cell of anti- μ antibody mediated immunosuppression appears to be the virgin $slgM(+)/slgD(+)$ B cell. The target cells of anti- γ and anti- α antibody mediated immunosuppression have been shown to be mutually exclusive and since the existence of an $IgG(+)/IgA(+)$ B cell has not been recorded, the target cell must be more differentiated than the $slgM(+)/slgD(+)$ B cell. Taken together the expression of isotype diversity occurring within each clone of developing B cells subsequent to antigen stimulation can be pictorially represented as in Figure 3, which can serve to highlight the following summary of facts:

- i) $slgM(+)/slgD(+)$ cells have been shown to contain the progenitors of cells secreting IgM , IgG and IgA in both primary and secondary immune responses (250,251).
- ii) $slgD(+)$ and $slgG(+)$ B cells (IgA was not examined) with or without another surface isotype make only IgG , not IgM (101, 250, and 251).
- iii) slg isotype expression changes after mitogenic and antigenic stimulation such that cells expressing $slgG$ increase and $slgD(+)$ B cells decrease (171, 252-254). For example there are no detectable $slgG(+)$ TNP binding cells before immunization, compared to 45% of antigen binding cells after immunization (252).

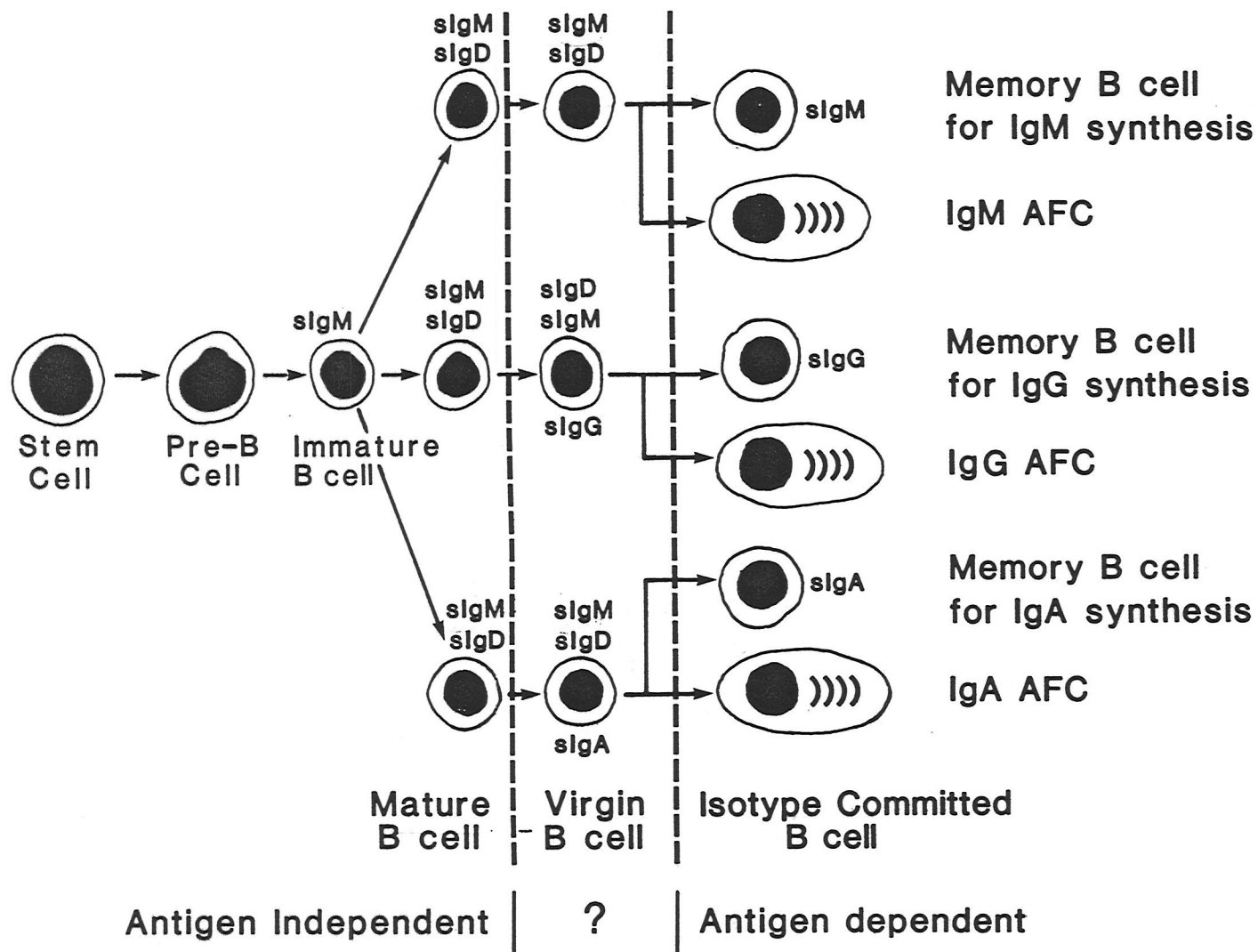
- iv) most sIgD(+) and sIgG(+) antigen binding cells (memory cells and virgin B cells) also express sIgM (252).

The development of the immature sIgM(+) B cell to a mature sIgM(+)/sIgD(+) virgin B cell has been shown to be easily blocked by cross-linking the sIgM with anti- μ . Also, mature sIgM(+)/sIgD(+) virgin B cells are capable of developing into cells with the potential to synthesize other Ig isotypes and their development cannot be so easily blocked.

Models of B Cell Differentiation Which Explain Isotype Switching

At the onset of this investigation in 1972, studies of the ontogeny of Ig producing cells were based on work in the chicken which showed a clearly identifiable focus of early B cell development --- the Bursa of Fabricius. It was found that the first population of B cells in the bursa synthesized only IgM and as this population expanded and the embryo matured IgG producing cells began to appear, some of which carried IgM on their surface. IgA bearing B cells were not detected until after hatching. Bursectomy at hatching did not affect IgM or IgY (IgG-like antibody) synthesis, but dramatically reduced IgA levels. Bursectomy performed early enough during fetal development rendered the chicken agammaglobulinemic, however, if the bursectomy was performed 2-3 days before hatching IgM levels were not affected but both IgG and IgA levels in the chicken were reduced. Development in the bursa appeared to follow the sequence: IgM $\longrightarrow$ IgY $\longrightarrow$ IgA. Birds given anti-u in ovo just before IgM synthesis was

Figure 3. Development of isotype diversity which occurs within each B cell clone before and after exposure to antigen [adapted from Lawton and Cooper (407)].



initiated in the bursa followed by bursectomy at hatching, had no detectable IgM, IgY or IgA. Chicks bursectomized at hatching and then treated with anti- μ showed pronounced reduction in IgM levels, no IgA but normal IgY levels. This was taken as further evidence for the following sequence of development of B cell clones: IgM $\longrightarrow$ IgY $\longrightarrow$ IgA.

In vivo studies in the mouse supported the concept that IgM bearing cells were progenitors for both IgG and IgA, however the switch from IgG to IgA was more difficult to show. Mice given anti- γ at birth were deficient only in IgG and mice treated with anti- α were deficient only in IgA. The mouse system differed from the chicken in that the sequence of isotype expression appeared to be: IgM $\longrightarrow$ IgG and IgM $\longrightarrow$ IgA.

Also, studies in the mouse were complicated by the lack of an identifiable bursa-like organ and a reduction in IgA levels in some cases after the neonatal administration of anti- γ . On the basis of the available data Cooper and Lawton (241) postulated that in both the chicken and the mouse the development of isotype diversity followed a precise order in which IgM bearing B cells produced progeny some of which expressed IgG. During this process of development the B cell would repress the $C\mu$ gene and switch to the expression of its $C\gamma$ gene. To account for the observed presence of IgM and IgG on the same cell surface (111) in the wake of a repressed $C\mu$ gene, they postulated that a long-lived mRNA maintained the expression of sIgM. They further postulated that IgA bearing cells developed from IgG bearing cells by switching from $C\gamma$ expression to $C\alpha$ expression. The

inability to demonstrate conclusively that in the mouse IgG precursors produced IgA bearing cells was attributed to the complete suppression of IgG levels by anti- χ treatments. IgD and IgE were not considered at the time since IgD had not yet been characterized and the existence of IgE in the mouse was just being established.

The inductive signal for isotype switching was believed by Lawton and Cooper (241) to be present in the microenvironment of the bursa (or bursa equivalent) and not to be antigen driven. Therefore, it was postulated, that IgM, IgG and IgA precursor B cells seeded the peripheral lymphoid tissues as they appeared in the bursa and interacted with antigen without further isotype switching. Antigen served only to induce proliferation of these B cells and their differentiation into plasma cells and/or into memory cells and played no apparent role in isotype switching. The findings by Herzenberg et al (30) which indicated that surface expression of IgG allotypes precedes exposure to antigen was taken as evidence for the universality of the model. However, in vitro experiments in mice indicated that i) class switching was induced by antigen, and ii) IgM appeared to be the exclusive sIg expressed on B cells in normal adult mice (128, 218). Lawton and Cooper explained this apparent contradiction to their theory by emphasizing the T cell requirement for IgG and IgA antibody synthesis. They postulated that the breakdown of lymph node and spleen physiology necessary for tissue culture would select against expression of any antibody forming precursor which required optimum T cell influence. Precursor cells co-expressing IgM would, therefore, be the only ones active in vitro and also sensitive to

anti- μ antibodies. To explain the lack of influence of anti- μ on antigen primed cells, Cooper and Lawton suggested that priming also increased the T cell effects and the affinity of the slgG and hence the efficiency of stimulation by antigen of slgM(-)slgG(+) as well as slgM(-)slgA(+) B cells would be enhanced.

While the studies in the chicken were most consistent with B cells expressing slg of each isotype in a systematic fashion prior to encountering antigen (see Figure 4a), there were those who believed that the requirements for B cell clonal ontogeny was quite different in mammals (185, 235) primarily as a result of the absence of a bursa-like organ. Indeed evidence that B cell development in the chicken did not apply in toto to the mammalian immune system and that each species had its unique requirements for isotype diversification came from in vitro studies by Kishimoto and Ishizaka (255) of rabbit secondary IgM, IgG and IgE responses. Their data suggested that B memory cells for IgG and IgM antibody formation both bore μ -chain determinants but that those for IgE formation did not.

On the basis of results obtained using mice, Manning postulated an alternate model of B cell clonal ontogeny (see Figure 4b). In his model IgM, IgG and IgA B cell lines develop from a common IgM bearing stem cell separately rather than sequentially. The precursors of IgA producing B cells were viewed as special cases in that some did not express surface IgM, since in some experiments neonatal administration of anti- μ had no effect on IgA production while anti- α was suppressive. It was felt that, in the mouse, (185,235) the development of the precursors to AFC of each isotype was determined instead by

stimulation by antigen and that the expression of each isotype had its own T cell requirement; however the precise role of each stimulus was unknown.

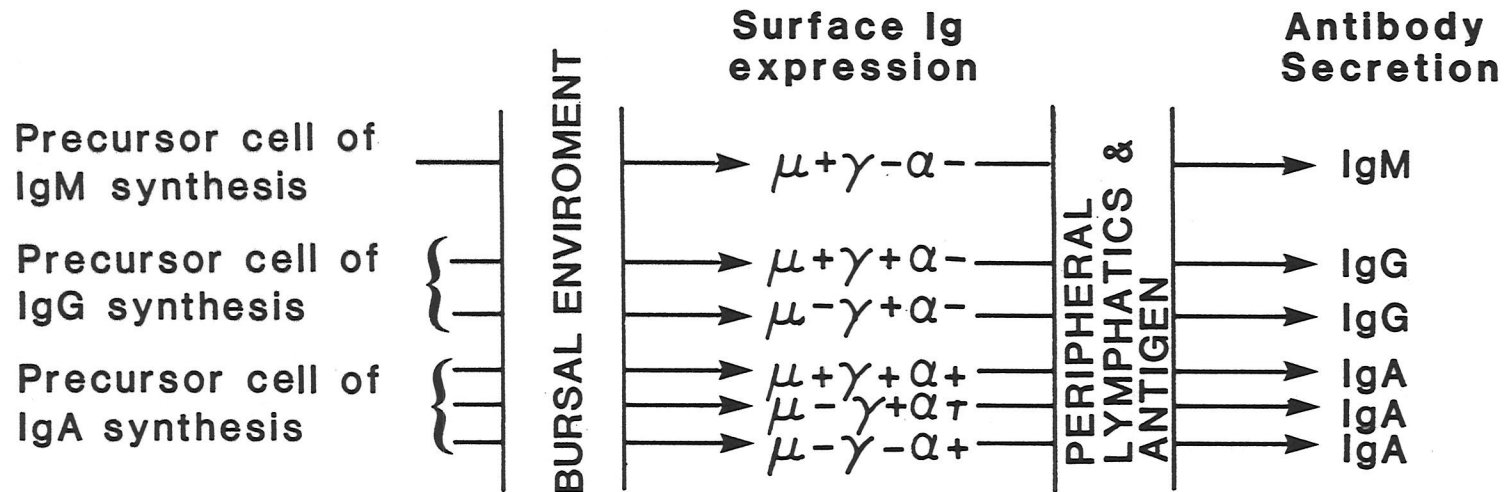
It can be seen that the proponents of each model of B cell differentiation relied heavily on the IgA data to support their hypothesis although the data with respect to IgA were in themselves inconclusive. While investigators were attempting to understand the murine IgA system, evidence was accumulating that the mouse like man possessed a fourth isotype: IgE.

Discovery of IgEm

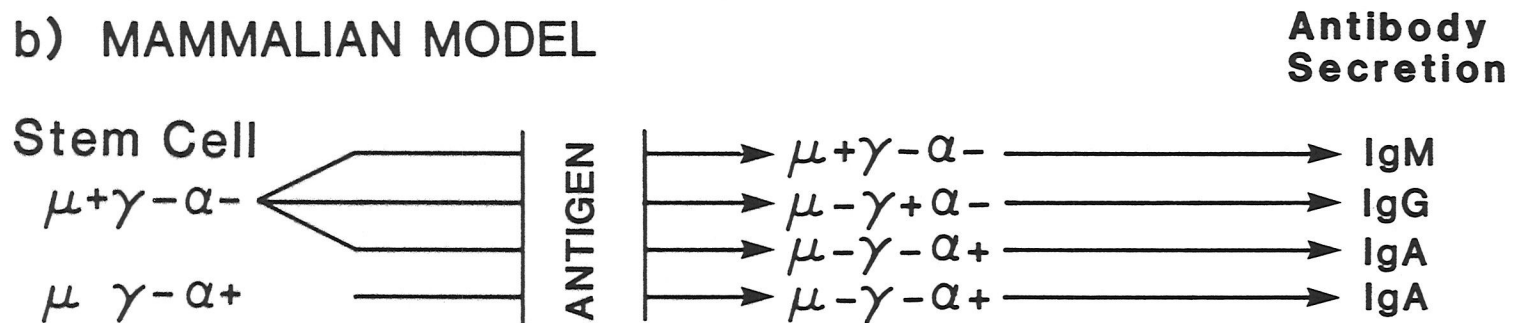
The IgG1 isotype was once thought to be the only murine Ig class able to sensitize murine tissues for anaphylaxis (256,257). However, Munoz was not able to correlate the hemagglutinating antibody titer and susceptibility of immunized mice to anaphylactic shock (258). He proposed that a new antibody isotype existed which was detectable only by its ability to mediate anaphylaxis. Mota, who had discovered in the rat a unique "mast cell sensitizing" antibody (259), the salient features of which were heat lability and a propensity to reside in the skin for long periods of time, reasoned that a similar antibody might be found in the mouse (260). Mota called this antibody "mouse early antibody" because of its tendency to appear before the other isotypes and then disappear from the circulation (260). Gradually the presence of two biologically distinct murine anaphylactogenic antibodies began to be accepted (261-263): 1) one of these gave maximal passive

Figure 4. The developmental requirements for isotype diversity in two widely studied classes of vertebrates: birds and mammals.

a) AVIAN MODEL



b) MAMMALIAN MODEL



cutaneous anaphylaxis (PCA) titers after a latent period of only 4 to 6 hours, was heat stable and correlated with the levels of IgG1 antibody; and 2) the other, resided in the skin for at least 72 hours, was heat labile and did not correlate with IgM, IgG1, IgA or IgG2 antibody levels. Murine IgG1 could be separated from this reagin-like⁹ antibody by rabbit anti- γ_1 (264,265), fixation of reagin to mouse peritoneal mast cells (266), DEAE-cellulose chromatography (207,210,212), gel filtration (268) and selective absorption of murine reagin after passage for 24 hours through a normal mouse (265). Furthermore only murine reagin was sensitive to reduction followed by alkylation (269).

In order to characterize the reaginic antibody further, its presence in various strains of mice in response to various antigen doses, adjuvant types and routes of immunization was studied. Prouvost-Danon and Binaghi found that antigen plus Al(OH)₃ induced reagin in all 6 strains of mice examined (270). However Revoltella and Ovary (271) found that only 3 out of the 6 strains (SWR/J, C57B1/6 and A/HeJ) responded to produce reaginic antibodies under the same conditions. Clausen et al (268) found that a saline extract of B. pertussis to be superior to Freund's incomplete adjuvant (or saline) for the induction of murine reagin. Further studies by Clausen et al (272) detected strain variation in the reagin response with C57B1/6 producing the highest reagin-mediated PCA titers. Levine et al (273-275) were the first to demonstrate the importance of low antigen dose for reagin production. Thus, these investigations established

⁹ The property of skin persistence provided the label of 'reagin' in the early literature (267).

that repeated immunizations with low doses of antigen induced persistent and boosterable reagin levels in certain mouse strains but not in others. In this way responder strains were distinguishable from non-responder strains for each antigen. The magnitude of the reagin responses induced by low doses of antigen was found to be related to the MHC haplotype, such that H-2k and H-2a mice mounted anti-BPO responses while H-2b, H-2d, H-2s and H-2q did not; in contrast H-2b, H-2q mice responded with an intense and persistent reagin response to OA. In addition all strains regardless of H-2 type were able to mount a secondary anti-OA reagin response

By the end of 1972 it had been recognized that several animal species produced reagin (262,276-279) and murine reagin was becoming accepted as the mouse equivalent of human IgE, even though Schwartz and Levine (263) could not demonstrate any antigenic or biologic crossreactivity between human IgE and murine reagin.

MATERIALS AND METHODS

General Procedures

Animals.

Adult 6 - 8 week old Swiss Webster (SW) mice were obtained from either North American Laboratory Supplies (NALS) Gunton, Manitoba or from Biobreeding, Ottawa, Ontario. Adult 6 - 8 week old CFW mice were purchased from Carworth Farms, Wilmington, Mass. Both strains of mice were random bred from closed colonies. Adult inbred [C57B1/6 x DBA/2]F1 hybrid (B6D2F1) mice were obtained from NALS; all other inbred strains of mice (BALB/c, AKR and A) were purchased as needed from Jackson Laboratories, Bar Harbor, ME.

250g Wistar/Lewis rats were purchased from Canadian Breeding Laboratories, Montreal, Quebec and 250g hooded (COBS Long-Evans) were obtained from either Canadian Breeding Labs or NALS. New Zealand white rabbits (2.2 kilos) were purchased from the University of Alberta, Canada, and outbred guinea pigs were purchased from High Oak Ranch, Quebec, Canada.

Bleeding procedures for mice, rats, guinea pigs and rabbits.

Mice. Two bleeding schemes were used:

- i) To obtain individual serum samples mice were lightly anesthetized and bled from the orbital sinus into heparinized

blood collecting capillary tubes. (Natelson, American Hospital Supply Corporation, Miami, Fl.). The tubes were centrifuged to separate white and red cells from the plasma and then broken at the cell-plasma interface. The section containing the cells was discarded and the plasma was collected in 100 μ l capillary pipettes. The exact volume of plasma was determined by measuring the length of the pipette filled with plasma and expressing this length as a fraction of the total length of the pipette and then multiplying this value by 100 μ l. The plasma was then diluted 10-fold with phosphate buffered saline (PBS), pH 7.2. Plasma collected in this way was always tested on the same day.

- ii) When sera were to be pooled, the animals were bled from the lateral tail vein. Mice were placed under a heat lamp to cause dilation of the tail veins; the subsequent cut by a scalpel blade allows for quick blood collection. Efforts were made to collect an equal number of drops from each mouse and the blood from each group was collected in the same tube. The blood was allowed to clot and then centrifuged and the resultant supernatant serum was removed by a pasteur pipette. Unless the serum was to be used directly, it was stored at -20°C .

Rats. Rats were anesthetized in a chamber saturated with ether vapour (Mallinckrodt, anesthesia grade, Paris, Kentucky) and then bled by orbital sinus puncture (280) through a shortened 100 μ l heparinized glass capillary tube. Usually 5 to 10 ml of blood were collected in this manner.

Guinea pigs. Cardiac puncture, as described in Methods in Immunology Vol. I (280) was routinely used on anesthetized animals.

Rabbits. Rabbits were routinely bled from the marginal ear vein into a 50 ml centrifuge tube (280).

In all cases the blood was allowed to clot for 1 hour at 37°C and then left at 4°C overnight. The next day the serum was separated from the clot, by centrifugation, and stored at -20°C until required.

Preparation of deaggregated protein solutions.

Material to be deaggregated was filtered through a 0.22 μ millipore filter and then ultracentrifuged at 105,000 x g for 2 hours at 4°C. The clear liquid situated between the lipoprotein pellicle and the sedimented aggregates was carefully pipetted from the centrifuge tube.

Adjuvants.

Both FCA and Freund's incomplete adjuvant (FIA) were obtained from Difco Labs, Detroit, Mich. and stored until needed at 4°C. The $Al(OH)_3$ gel was prepared as required.

PCA.

Introduction.

The term PCA was first coined by Ovary (281) to describe a cutaneous reaction involving increased vascular permeability which was mediated by combination of passively administered antibody with the corresponding antigen. The reaction site is visualized as a consequence of the extravasation of a dye such as Evans blue which is injected intravenously (i.v.) with the challenge antigen. The reaction, which occurs within minutes, is due to the release of

vasoactive substances as a consequence of the antigen-antibody interaction. The dye, bound to circulating albumin, leaks out of the vasculature into the surrounding skin tissue to produce a stained area of variable size which may be accurately determined by reflecting the skin and measuring the diameter of the reaction on the everted surface.

The main pharmacological mediators released during anaphylactic reactions include histamine, serotonin, heparin and the leukotrienes; the mediators however vary in importance from species to species. For example, histamine plays an important role in guinea pig PCA (282) but serotonin and histamine are implicated in rat PCA (283).

Not all classes of antibodies are capable of sensitizing the skin for PCA reactions but generally each species has one IgG subclass which can mediate PCA in the homologous species (i.e., the IgG1 isotype of the mouse) and a different IgG subclass which can mediate PCA in a heterologous species (282), (i.e., the IgG2a isotype of the mouse mediates PCA in guinea pig skin). The first definitive example of two different Ig classes capable of mediating homologous PCA was found in the mouse (44, 261, 263): the IgG1 subclass and reaginic antibodies. One important difference between the two is that the former appears to bind to peritoneal mast cells only after it has bound antigen (284) while the latter sensitizes mast cells in the absence of antigen (285). Consequently to distinguish IgE mediated PCA from IgG1 mediated PCA it is necessary to wait until all of the IgG1 antibody has diffused out of the skin site leaving behind the tenaciously bound IgE antibody (260). However, sera with very high

IgG1 antibody levels provide sufficient residual antibody 24 to 48 hours after the initial sensitization to participate also in the late PCA (286). To avoid the complication due to IgG1 in 24 to 48 hour PCA assays advantage may be taken of the observation that only mouse IgE and not IgG1 mediates PCA in rat skin (264).

Generally speaking, the PCA reaction involves antibody molecules which fix to some tissue constituent(s), with the subsequent release of vasoactive amines and other pharmacologic mediators when the antibodies are cross-linked by the specific antigen. The ability to induce an anti-(sheep red blood cell) PCA reaction with red cell stroma (287) has raised the possibility that circulating antigen first encounters antibody fixed to or adjacent to the internal side of the endothelial cells of the small veins and this contact induces local permeability providing antigen access to deeper tissues. This theory is supported by histologic examination (286) which demonstrated edema and swelling of endothelial cells. The most likely tissue source of mediators of anaphylaxis is the mast cell since antigen-antibody complexes which are capable of triggering the release of mediators from peritoneal mast cells are also able to trigger a PCA reaction (284, 285). However, since M ϕ 's have shown also to possess IgE receptors (288), it is still an open question whether or not other cells in the skin are sensitized and whether other mechanisms play a role in PCA. In this regard evidence provided by Goose and Blair (289) suggests that not all PCA reactions are associated with mast cell degranulation, and the release of histamine or serotonin.

By using rabbit IgG mediated guinea pig PCA, Ovary established a model system for the study of quantitative parameters of the IgG mediated PCA reaction (282). Thus, in general the minimum amount of a complex homologous antigen required to induce the maximal PCA titer attainable is directly related to the molecular weight of the antigen (282) and inversely related to both the amount (282) and the affinity of the antibody (290,291). According to the laws of mass action, the bimolecular reversible reaction between antibody fixed to receptors on the mast cell and antigen dictates that antibodies, which bind weakly to their respective antigens, will require higher concentrations of antigen than antibodies that bind antigen more avidly to provide the necessary degree of cross-linking required to trigger mediator release. In practice the recommended dose of antigen for optimum PCA titer is 10 to 100 times the amount necessary for titration of sera from hyperimmunized animals (282). While the proper dose must be determined empirically, for protein antigens this value is usually 100 to 1000 $\mu\text{g/ml}$. Guided by these principles Ovary concluded that IgG mediated PCA in mouse skin was maximum when the antigen challenge was performed 3 hours after sensitization (292).

Mota et al (264,265 and 293) and Schwartz and Levine (263) established that 2 to 4 hour PCA in mice measured only IgG1 antibody and 24 to 72 hour PCA measured only IgE antibody. Although it was postulated (294) that high IgE antibody levels should be detectable at 2 to 4 hours, IgE containing samples from which the IgG1 had been selectively removed by incubation with an [anti- δ 1]-immunosorbent did not give PCA reactions at 2 hours (294).

Methods. Solutions were diluted in PBS. In general, the dilutions were geometric progressions of $1/2$, $1/3$ or $1/4$. For PCA in mouse skin, 30 μ l of each dilution was injected i.d. The back of each mouse was shaved 24 hours prior to the injection (268); no more than 4 sites could be accommodated on each mouse. Each dilution was tested on at least 4 backs and never repeated on the same mouse unless specifically mentioned in the test. For PCA in rats, the back of the rat was shaved just prior to i.d. injection. Each rat could accommodate 24 to 36 spots; 50 μ l was injected at each site.

After a defined interval of time, the PCA reaction was stimulated by challenging the sensitized site i.v. with antigen. Evans blue dye (Matheson, Coleman and Bell, Norwood, OH10; color index 23860), at a concentration of 0.25% w/v, was included in the injection to facilitate measurement of the PCA reaction. To titrate antibody in the mouse the antigen was administered with dye in 0.25 ml into the lateral tail vein. In the rat PCA was induced by injection of antigen and dye in 1.00 ml into the jugular vein of anesthetized animals. The reaction was allowed to develop for 20 minutes, the animals were then killed by cervical dislocation and the diameter of the blue spot measured on the everted skin surface. In both the mouse and the rat the reciprocal of the maximum dilution of the serum resulting in a 5 mm spot was deemed to represent the end-point of the titration. When the reactions were above 5 mm the test was repeated with increasing dilutions until a reaction below 5 mm was recorded.

Under some circumstances it was convenient to express the titer in terms of PCA units per ml. One unit is defined as the minimum amount

of antibody in the skin site able to elicit a 5 mm spot, thus, the number of PCA units per ml is equal to the reciprocal of the highest dilution divided by the exact volume in μ ls of that dilution injected i.d. For example, when IgE levels are determined in rat skin using 50 μ l of each dilution, the titer is multiplied by 20 (a factor which represents the number of 50 μ l aliquots per ml). In this manner total PCA activity can be monitored during fractionation procedures to determine the extent of recovery.

PCA in the absence of IgG1. IgG1 was removed from murine reaginic serum (MRS) by precipitation with anti- γ 1 antibodies which were obtained from a rabbit which had been immunized with a purified IgG1 myeloma protein (MOPC-21A). The rabbit anti- γ 1 antibody preparation used was obtained by precipitation from rabbit antiserum using Na_2SO_4 according to the method of Keckwick (295) followed by absorption with solid phase k light chains. To ensure maximal precipitation of IgG1 from MRS using this rabbit antibody preparation it was necessary first to determine by quantitative precipitation the appropriate conditions. Thus, the rabbit anti- γ 1 IgG fraction was diluted by one-half and incubated for 2 hours at 37°C with varying dilutions of MRS containing both IgG1 and IgE. After a further incubation at 4°C overnight, the precipitates were removed by centrifugation and washed 3 times in PBS. Then a constant volume of 1N HCl was added to each tube to dissolve the precipitate and the O.D. at 280 m μ was established. Figure 5 depicts the precipitin curve obtained; the maximum precipitate occurred when the mouse IgG1 containing solution was diluted 1/16.

Physicochemical Properties of IgEm

Reduction followed by alkylation.

The serum sample to be reduced was dialysed against 0.1M 2-mercaptoethanol (2-ME) in PBS at room temperature for three hours with constant stirring. The samples were then dialysed overnight at 4°C against 0.02M iodoacetamide. Non-specific loss of activity during dialysis was assessed by dialysing a sample of the serum against PBS without 2-ME or iodoacetamide.

Heat inactivation.

Serum samples were heated at 56°C in a water bath either before or after dilution for various lengths of time and their capacity to evoke PCA was compared to that of unheated samples.

Fractionation.

A reagin-rich fraction of the serum was obtained by precipitation at 4°C with ammonium sulphate at half-saturation (50% SAS). The 50% SAS precipitate was dissolved in a volume of PBS corresponding to the volume of the original serum and was subjected either to gel filtration on Sephadex G-200 (50% SAS-G200) or to chromatography on DEAE cellulose prior to gel filtration (50% SAS-DEAE-G200). Chromatography on DEAE-cellulose involved initially dialysis of the reagin-rich fraction against 0.02M phosphate, pH 8.0, then the fraction was applied to a Whatman DE52 column (2.5 x 30 cm) equilibrated with 0.02M phosphate, pH 8.0. Fractions were eluted sequentially using 0.02, 0.05 and 0.1M phosphate, pH 8.0. Figure 6 depicts a typical elution pattern. Each fraction was concentrated to the original serum volume

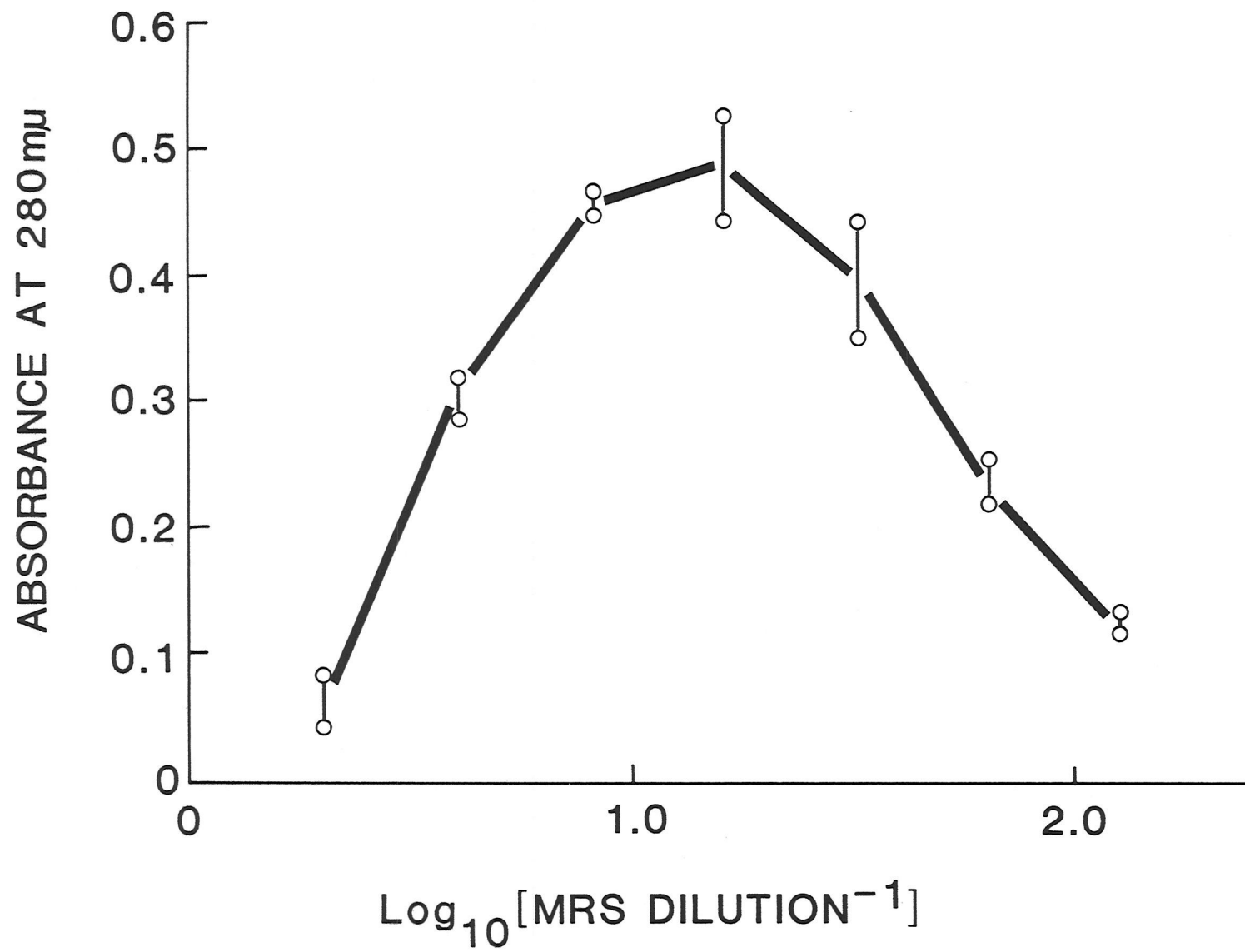
before testing. Four fractions were collected; the first two representing the bulk and trailing edge of the 0.02M fraction respectively. The first fraction was split up this way because Revoltella and Ovary detected IgE on the descending portion of the 0.02M fraction (271).

Production of High Titered IgEm Antibody Preparations

Preparation of the antigens. Three antigens were employed at the onset of this work; keyhole limpet hemocyanin (KLH, Calbiochem, A grade, California), OA (5X recrystallized, ICN Pharmaceuticals, Cleveland, Ohio), and adult *Ascaris suum* extract (ASC). All three antigens were maintained in a lyophilized state at 4°C until required, whereupon a stock solution of 2 mg per ml of PBS was prepared. Prior to immunization, the antigen was added to a solution of $\text{AlK}(\text{SO}_4)_2$ and then co-precipitated with $\text{Al}(\text{OH})_3$ at pH 7.2-7.4; this pH was reached by the dropwise addition of 1N NaOH (with constant stirring), using phenol red (3 drops) as a pH indicator. Optical density measurements of the supernatant taken at 280 m μ established that all of the antigen had been absorbed to the $\text{Al}(\text{OH})_3$ gel. For example, if a 10% $\text{AlK}(\text{SO}_4)_2$ solution is prepared and resuspended in 10 ml of PBS, it will yield 1 mg of $\text{Al}(\text{OH})_3$ per ml of suspension. The concentrations of $\text{Al}(\text{OH})_3$ and antigen varied throughout and are stated in the result section.

Immunization schedule. Mice received 0.5 ml of the $\text{Al}(\text{OH})_3$ gel containing antigen intraperitoneally (i.p.) by holding each mouse by the scruff of the neck and the base of the tail with ventral surface

Figure 5. Precipitin curve representing the precipitates obtained by mixing a given MRS at various dilutions with equal volumes of the rabbit anti- γ_1 antiserum. Each point represents one determination; two determinations were made at each dilution.



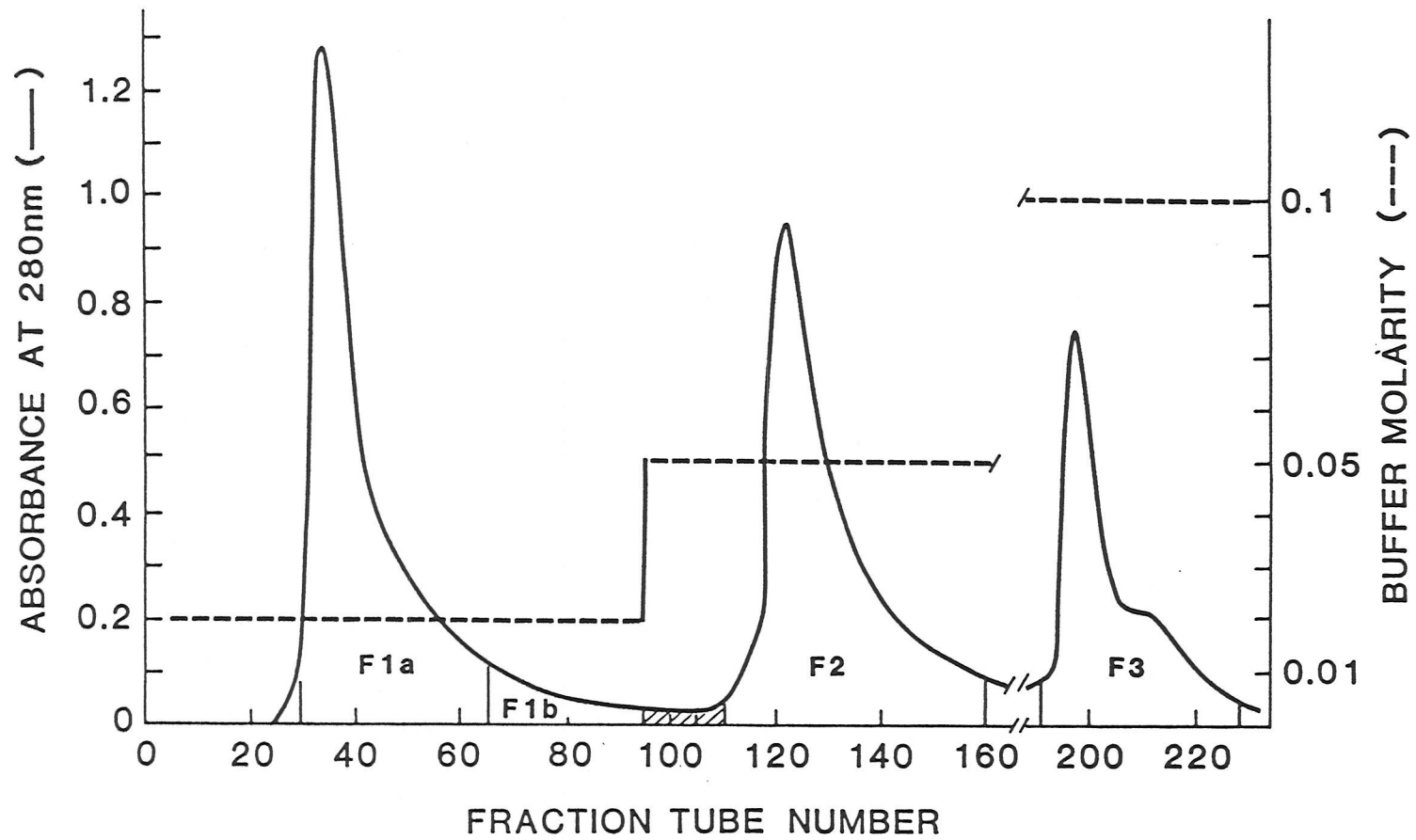
upward at an angle of about 45° . This has the effect of displacing the contents of the peritoneal cavity towards the diaphragm thus facilitating insertion of the needle at a shallow angle into the lower left quadrant without causing damage to any internal organs.

Mice were immunized by either a single injection or multiple monthly injections. In exploratory experiments it was found that injection of mice younger than 21 days old with 1 mg $\text{Al}(\text{OH})_3$ (the standard adult dose) resulted in high mortality. However, mortality was reduced considerably by decreasing the dose of $\text{Al}(\text{OH})_3$ to $60 \mu\text{g}$ of $\text{Al}(\text{OH})_3$ per gram of body weight.

Production of ascitic fluids.

The method used was a modification of the procedure recommended by Munoz (296). Mice already producing IgE were given two i.p. injections of 0.25 ml of FCA; the first, 7 days after the last injection of antigen in $\text{Al}(\text{OH})_3$ and the second 7 days later. Ascitic fluid usually began to accumulate within 14 days at which time the mice were given an additional injection of the antigen in $\text{Al}(\text{OH})_3$. Ascitic fluid was drained from the peritoneum 7 days later. The procedure involved injecting 5 ml of air into the peritoneum if it was not already greatly distended by fluid, and then using gentle suction to withdraw the fluid. If the peritoneum was already greatly distended, the fluid collection involved puncturing the abdominal wall with a 18 gauge needle and allowing the fluid to drip out. The volumes of ascitic fluid varied, ranging from less than 1 ml to 10 ml per mouse. The formation of clots in these fluids often occurred once or twice upon subsequent thawings. The fluid was separated by centrifugation and stored at -20°C until required.

Figure 6. Typical elution profile of murine Igs of MRS from a DEAE column using stepwise gradient elution; the molarities of the applied eluting buffers are indicated by the dotted line and the absorbance of the eluted material by the solid line. In this experiment 16 mls of an $(\text{NH}_4)_2\text{SO}_4$ precipitated fraction of an anti-OA antiserum were applied to the DEAE column. Four fractions were collected, Fla, Flb, F2 and F3; each was concentrated back to the applied sample volume and tested for IgG_1 and IgE antibody activity.



Spleen cell suspensions.

Spleens were removed and placed in ice-cold Hanks balanced salt solution (HBSS), and then disrupted with a loose-fitting teflon tissue homogenizer. The cell suspension was allowed to stand for 5 minutes for the sedimentation of the tissue debris, and the remaining cell suspension removed. An average of 2×10^8 cells were recovered from each spleen. Cells were counted with the aid of a Neubauer hemocytometer slide.

Adoptive transfer experiments.

Recipient mice were exposed to whole body γ -irradiation from a Cobalt-60 source (Eldorado Unit, Atomic Energy Canada) one day before transfer of syngeneic spleen cells. The dose of irradiation was varied for different groups of mice covering the range of 600R-1000R. In most cases, 5×10^7 cells were transferred by i.v. injection via the lateral tail vein. Antigen was then administered i.p. one day later.

Preparation of OA-coupled Sheep Red Blood Cells (OA-SRBC).

SRBC were purchased from NALS (Gunton, Manitoba) as sheep blood in Alsevers' solution (1:2, blood:Alsevers). The cells were washed three times in PBS and the supernatant removed by aspiration. To 10 ml of a 5 mg/ml solution of OA was added 0.4 ml of packed SRBC and 1 ml of a 2.5% glutaraldehyde solution in PBS. This mixture was stirred briefly at 10 minutes intervals for 1 hour at room temperature, and then washed 3 times in PBS containing 1% normal rabbit serum (PBS-NRS). Finally the OA-SRBC preparation was suspended in PBS-NRS to a concentration of 1% by volume.

Serum was 2-fold serially diluted in micro-hemagglutination trays, each well containing 50 μ l of PBS-NRS. To each well was added 25 μ l of a 1% OA-SRBC suspension. In each assay the quality of the conjugated SRBC was assessed by determining the hemagglutination (HA) titer of a standard anti-OA antiserum. In some tests, 0.1M 2-ME was incorporated into the dilution to inactivate IgE and IgM antibodies. Volume relationships were retained in wells without 2-ME by adding 25 μ l instead, of PBS-NRS.

Production of Antibodies Reactive to Murine Epsilon Chains (anti- ξ m)

Preparation of rat peritoneal mast cells (RPMC).

Rat mast cells were collected from rat peritoneal cavities by gentle massage in Bloom saline medium (297). For immunization purposes the cells were washed twice in Bloom saline by centrifugation and finally resuspended in 5 ml Bloom saline to which 1.5 ml of an IgE-rich solution was added. This suspension was incubated for 2 and 1/2 hours at 37°C, washed 5 times in Bloom saline and resuspended in a minimum volume of Bloom saline. Prior to use for immunization, RPMC were emulsified with an equal volume of FCA or FIA as required.

Immunization of rats, rabbits, guinea pigs and a goat with IgEm.

Rats. Three groups, of 4 to 6 rats each, received one of the following treatments one day prior to immunization with MRS: (i) 1 ml of deaggregated 50% SAS fraction of normal mouse serum (NMS), i.v., (ii) 1 ml of deaggregated NMS or (iii) no pretreatment. Each rat was immunized with 0.5 ml of MRS in 0.5 ml of FCA into its foot pads (0.4

ml per rear foot pad and 0.1 ml per front pad) on day 0 and 2 weeks later each rat received 4 equal i.d. injections of 0.1 ml of MRS in 0.1 ml of FCA and one month later 4 equal i.d. injections of 0.1 ml of MRS in 0.1 ml of FIA. This protocol represents an adaptation of that employed by Henney and Ishizaka (298) for the preparation of isotype specific antisera.

Goat. Two injections, each containing 10 mg of the reagin-rich fraction of the serum of B6D2F1 mice (50% SAS-G200 in 1.1 ml PBS emulsified with 1.6 ml FCA) were administered intramuscularly (i.m.) into a goat at two month intervals.

Rabbits. Each animal received at one day intervals three i.v. injections of 5 ml of deaggregated 50% SAS fraction of NMS prior to subcutaneous (s.c.) injection of 0.5 ml of MRS in 0.5 ml of FCA. The s.c. injections were repeated over 6 months at one month intervals.

Guinea Pigs. Injections consisted of 600 μ g of the reagin-rich fraction (50% SAS-DEAE-G200; isolated from the serum of SW mice in 0.5 ml PBS and 0.7 ml FCA were given i.m. into each guinea pig at two week intervals.

Immunosorbent purification of anti- ϵ antisera.

The animals, immunized as previously described, produced antibodies to different Ig isotypes in addition to IgE; removal of these contaminating antibodies was accomplished by treatment of each antiserum with immunosorbents prepared by coupling, to CNBr activated Sepharose-4B (299), the following proteins: (i) MOPC 21A, an IgG1 monoclonal protein; (ii) L4, an IgA monoclonal protein and (iii)

normal serum proteins (NSP). Both the MOPC 21A and L4 tumor cell lines were propagated in female BALB/c mice as an ascites and the corresponding myeloma proteins were isolated from the ascitic fluids according to the protocols established by Potter (300). For this purpose 178 ml of the MOPC 21A and 160 ml of L4 ascitic fluids were fractionated by salting out and subjected to dialysis against low ionic strength buffer. The L4 myeloma protein was further subjected to dialysis against deionized water. Immunosorbents prepared from each of these two myeloma proteins contained 10 mg of protein per ml of gel. NSP were isolated from NMS by salting out with 50% SAS. Immunosorbents prepared using NSP contained the protein equivalent of 30 ml of serum per 100 ml of gel. All anti- ϵ antisera were absorbed with the immunosorbents until the elimination of all antibodies to serum constituents other than IgEm, as judged by immunoelectrophoresis using NMS for the development of precipitin bands.

In view of the inability to judge anti- ϵ antibody levels by classical means (no precipitating antigen-antibody complexes were detected on mixing, immunodiffusion or immunoelectrophoresis of the anti- ϵ with MRS), the presence of these antibodies was evaluated by their ability to neutralize IgEm mediated PCA.

Neutralization of PCA.

All anti- ϵ antibody preparations were evaluated with respect to their ability to neutralize the IgEm mediated PCA produced by a standard MRS of known PCA titer. To constant volumes of MRS were added different volumes of diluted or undiluted antiserum plus sufficient PBS to bring the reaction volume to 1 ml. The reaction

mixture was first incubated for 1 hour at 37°C and then overnight at 4°C. Any precipitate formed at this stage was removed by centrifugation and the supernatant was two-fold serially diluted with PBS. The control samples consisted of MRS mixed with appropriate volumes of normal serum of the species (NSS) used for production of the antiserum. Volumes of 50 µl of each mixture were injected i.d. into rats for the determination of PCA titer, as described earlier. To determine the degree of neutralization, the resulting PCA titer was expressed as a percent of the titer obtained in the controls in which NSS was substituted for the anti- ϵ preparation. The neutralization of the PCA was defined as a percent in the following way:

$$1 - \frac{\text{PCA titer after treatment with anti-}\epsilon}{\text{PCA titer after treatment with NSS}} \times 100\%$$

An estimate of the anti-IgE activity was ascertained by dividing the total number of PCA units present in the mixture by the minimum volume of undiluted anti- ϵ preparation (in µl) required to produce 100% neutralization.

This value was called the neutralization quotient (NQ).

$$\frac{\text{PCA units neutralized}}{\text{minimum amount of undiluted anti-}\epsilon \text{ (}\mu\text{l)}} = \text{NQ}$$

An NQ of 1000 indicates that 1 μ l of the anti- ϵ preparation neutralized completely 1000 PCA units (equivalent to 1 ml of MRS with a titer of 50).

Reagents required to examine antigenic crossreactivity between IgEm and rat IgE (IgEr).

Rat monoclonal IgE was isolated from the ascitic fluids of rats bearing either of the two IgE secreting immunocytomas, IR-162 or IR-331, and purified by Dr. Kelly, of this Department, as described by Conrad et al (301). The IgE isolated from IR-162 was used in most of the experiments unless otherwise stated and is referred to as IgEr. Normal rat immunoglobulins (NRtlg) were obtained from normal rat sera by ammonium sulfate precipitation at 50% saturation, fractionation on DEAE cellulose and gel filtration on Sephadex G-200. Normal rabbit immunoglobulins (NRblg) were obtained from normal rabbit serum by ammonium sulphate precipitation as described by Hebert et al (302) and either used as such or insolubilized by coupling to CNBr-activated Sepharose-4B (299).

Sheep antibodies to NRblg (SARb) obtained from Dr. Kelly were isolated by adsorption onto the NRblg-immunosorbent and then elution with glycine-HCl buffer (pH 2.8; 0.4M); the eluate was adjusted to pH 7.2 with 1N NaOH, dialysed against H₂O and then lyophilized. These antibodies were used either in the form of a solution or were insolubilized by coupling to CNBr-activated Sepharose-4B (299). Antisera against IgEr (anti- ϵ r), raised in rabbits, dogs, horses and sheep and rendered monospecific for ϵ r-chains were prepared (303) and provided by Dr. Kelly. Ig fractions were isolated from each monospecific anti- ϵ r antiserum by ammonium sulfate precipitation (302); the

immunoglobulin fraction of one rabbit antiserum referred to hereafter as Rb anti- ϵ r was used in most of the experiments in this section except where stated otherwise.

Inhibition of neutralization.

The ratio of MRS to the Rb anti- ϵ r was adjusted so as to result in 85% neutralization of IgEm after incubation for 1 hour at 37°C. Increasing amounts of IgEr were then added to this mixture of MRS and Rb anti- ϵ r and the final solutions were maintained first for 1 hour at 37°C and then overnight at 4°C. To remove Rb anti- ϵ antibodies and immune complexes, the appropriate amount of SARb-4B was added to the solutions and the suspensions were shaken for 1 hour at 37°C and then centrifuged. The residual IgEm in the supernatant was assayed for by heterologous PCA in rat skin.

Immunosuppression of IgE Antibody Responses

Anti-heavy chain antibodies.

Details of the techniques used for the production of antisera against murine and rat epsilon chains (ϵ_m and ϵ_r respectively) have been already described and published (303, 304). The Ig containing fractions from each anti- ϵ antiserum were prepared by precipitation with ammonium sulfate as described by Hebert (305). All Ig preparations were rendered aggregate free by centrifugation at $130,000 \times g$ for 20 hours and filtration through sterile millipore filters (0.22 μ); sera were stored in sterile vials at 4°C :

A rabbit anti- μ Ig preparation prepared by Dr. Rector, of this Department, against the IgM myeloma protein secreted by the MOPC-104E tumor cell line, was rendered aggregate-free as outlined for anti- ϵ preparations.

Igs were prepared from normal serum using similar methods and adjusted to the same protein concentration as the corresponding anti-heavy chain preparations.

Handling procedures for neonatal and young mice.

At the onset of these experiments pregnant female C57Bl/6 and SW mice, timed to be 16-18 days pregnant, were purchased from NALS (Gunton, Manitoba) but many of these mothers became cannibalistic within a week of delivering their litters. The frequency of cannibalism was decreased by allowing female C57Bl/6 and male DBA/2 mice, purchased from NALS, to mate in the Central Animal Facilities of the Faculty of Medicine (CAFFM). Also, to ensure a high rate of pregnan-

cies, female C57Bl/6 mice were exposed to DBA/2 males continually for 3 weeks in the same cage (copulation was interfered with by the presence of a metal screen). This exposure to the males has been reported to bring the female mice into synchronous oestrous cycles (personal communication, P. Boehm, Director of CAFFM). Males were then allowed to interact freely with females (at a ratio of 2 males per female) for 48 hours. Using this protocol 60% of the C57Bl/6 mice became pregnant and litters were delivered within one week of each other.

At the time of birth, littermates were kept in separate cages with their mother until they were weaned at four weeks, then males and females were identified and housed separately.

Each mouse in a given litter received the same treatment. Deaggregated heterologous Ig preparations and OA adsorbed to Al(OH)₃ gel were injected by routes which were dependent upon the age of the mouse. Within 24 hours of birth and up to 10 days of age, neonates received the injection via a 30 g needle passed through the muscles of the front limb and then into the peritoneal cavity. The extreme looseness and transparency of the skin facilitated this particular approach. Mice 11 to 18 days of age received the injection by inserting the needle first through the pectoralis muscles and then into the abdominal cavity. These techniques were introduced to prevent leaking of the injected material out of the peritoneal cavity; the above mentioned muscle groups serving as a seal, to compensate for immature development of the abdominal muscles which normally provides a natural seal once the needle had been removed. Mice older than 3

weeks of age received the injection i.p. directly. From 17 days of age up to 6 weeks of age 27 g needles were used. All neonatal and young mice up to 6 weeks of age were bled by orbital sinus puncture; mice older than 6 weeks were bled from the tail except when individual serum samples were required.

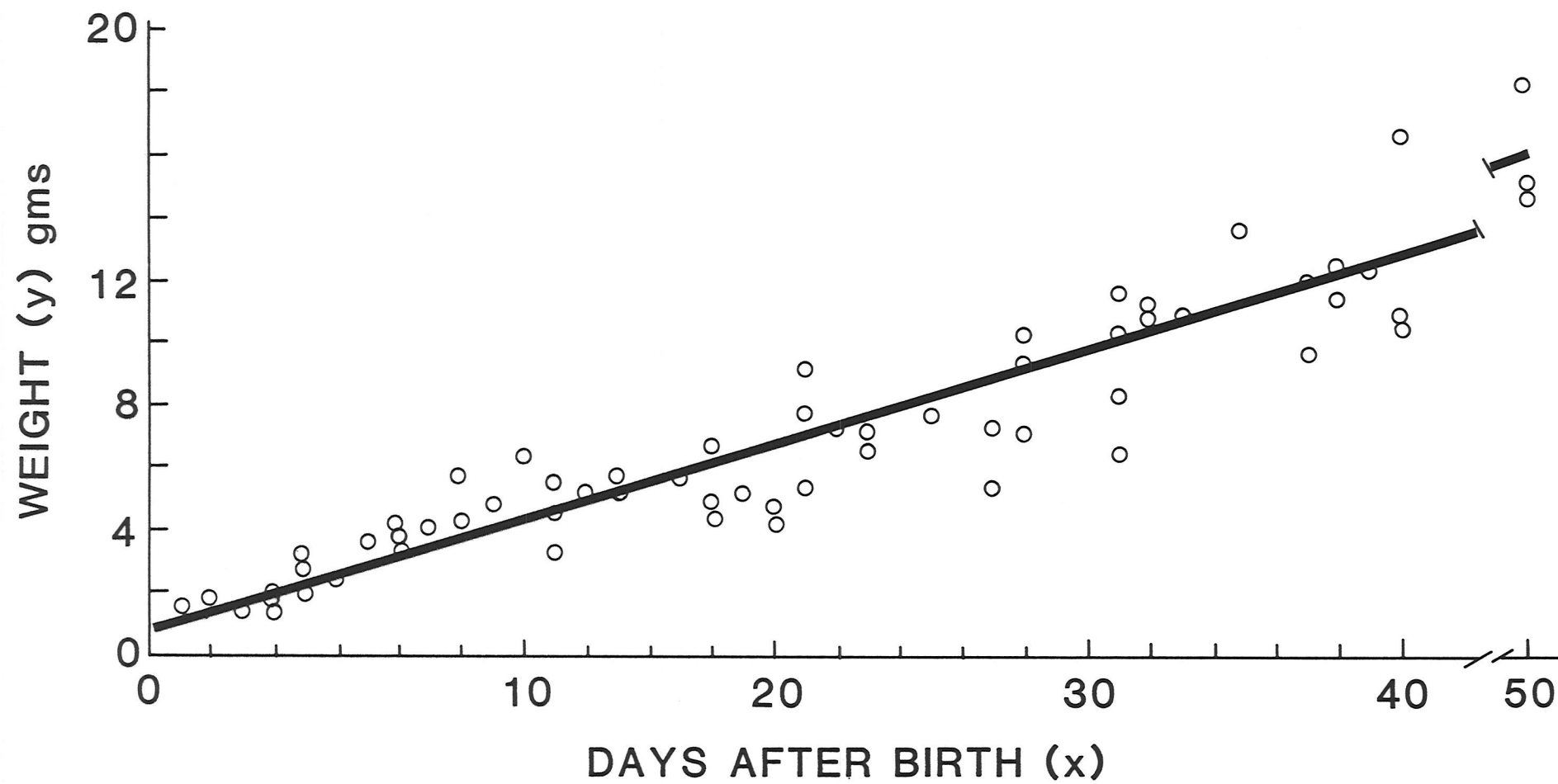
Calculation of the appropriate dose of $Al(OH)_3$ required for neonates.

It was observed that although an adult, 8 week old B6D2F1 mouse weighing approximately 18 gms can tolerate 1000 μg of $Al(OH)_3$ (60 μg of $Al(OH)_3$ per gram of body weight), this same amount caused distress and in many cases death among neonatal and young mice. In an effort to reduce littermate mortality, the feasibility of relating the dose of $Al(OH)_3$ to the average littermate weight was assessed. 82 litters of different ages were weighed and the mean littermate weight in gms (Y) was plotted against the age in days (X). An average of 6 mice were weighed per litter. A straight line was fitted to the data by using the method of least squares with the aid of the University of Manitoba computer department statistical package (Stat-pak). This statistical data was then subjected to linear regression analysis which generated the formula: $y=0.8+0.3x$. The correlation coefficient (r), a statistical measure of the confidence in the linearity of the points chosen, was 0.956; an r value close to 1.00 indicates that the individual points form a pattern which deviates little from the line drawn by the regression analysis. The data used for this calculation are graphically presented in Figure 7.

Using this formula, it was possible to calculate the weight in gms of any age B6D2F1 mouse. Based on the linearity of the growth curve

the younger mice were given this weight related dose. The use of doses of $\text{Al}(\text{OH})_3$ calculated using these formulae resulted in reduced mortality confirming the original assumption that $1000 \mu\text{g}$ of $\text{Al}(\text{OH})_3$ was too high for the young mice.

Figure 7. The weights of B6D2F1 littermates as a function of age. The mean littermate weight of each of 82 B6D2F1 litters ranging in age from 1 day to 56 days was determined and plotted against its age. The straight line fitted to this data by using the method of least squares and linear regression analysis is represented by the formula $y = .8 + 3x$.



RESULTS

Specific Aims

The main goal of this study was to determine the effects of administering anti- ξ antibodies to mice on the IgEm antibody response, and to compare the data with the results obtained using anti- μ and with published data on the effects of antibodies directed against heavy chains. It was thus hoped to unravel new information about the pattern of isotype expression on the B cell precursors of the IgE AFC. To accomplish this, the study was divided into the three phases as described below:

- i) The induction and collection of murine antisera containing high titres of IgE antibodies for use as a source of ξ -chains for the production of anti- ξ antibodies and for the establishment of immuno-assays for the detection and quantitation of IgEm.
- ii) The production of high-titered anti- ξ antibodies.
- iii) The administration of anti- ξ to neonatal and young mice and an evaluation of its effect(s) on the immune responsiveness of these animals.

Production of High-Titered IgEm Antisera

At the onset of this research program in 1972, it was generally accepted that the IgE levels in sensitized animals paralleled those of allergic patients (306), i.e., the total serum IgE levels were at least 1000 times lower than the IgG levels (being 1-10 $\mu\text{g/ml}$ in allergic adults). Consequently, it was envisaged that to produce IgEm in quantities sufficient for its isolation and for its use as an antigen, it would be necessary to sensitize large numbers of mice of a high responder strain. Moreover since all known immunization protocols for IgEm antibodies resulted in the formation of tissue sensitizing IgG1 antibodies, experiments were carried out to define the limits of the biological tests available for quantitation of these two distinct murine cytotoxic antibodies. In addition other procedures were explored in an attempt to induce specific IgEm antibody formation without a concomitant production of IgG1 antibodies in significant amounts.

Selection of an appropriate antigen.

Three antigens KLH, ASC and OA were chosen based on their proven ability to induce IgE [i.e., KLH had been shown to elicit IgE and IgG1 antibodies in SW mice (271,307), ASC had been shown to be a potent stimulator of IgE production in rats (308); and OA had been used by many investigators for the vigorous production of IgEm responses]. The SW mice were chosen because Dr. A. Prouvost-Danon had shown that this strain produced high IgE titers to many antigens (personal communication). For the earlier experiments each of these

three antigens was injected, at monthly intervals, into 3 groups of 300 SW mice each at doses of 0.1 μg of antigen in 500 μg of $\text{Al}(\text{OH})_3$ as recommended by Vaz and Levine (274), each group of 300 mice were further subdivided into 6 lots of 50 mice with the addition of 100 new mice per month. All mice in each group were bled seven days after each immunization, the sera were pooled and the IgE antibody levels determined. The serum pool from each group of 50 mice was tested separately unless otherwise stated; each serum pool was assayed in mice and rats for IgE mediated PCA. The results of this experiment are listed in Table I, from which it is evident that PCA titers obtained in rats were higher than titers obtained with mice. Moreover, from the data given in Table I it appears that OA stimulated the highest IgE antibody responses during the early bleedings but that after the fourth immunization all three antigens were equally effective in eliciting an IgE antibody response.

IgEm levels in ascitic fluids. Three groups of 50 mice each were immunized with KLH, OA and ASC respectively to test the feasibility of collecting murine reagin from ascitic fluid. The volume of fluid collected from the peritoneal cavity was routinely 4 to 8 times higher than the volume of serum collected by tail bleeding. Table II shows the results of this study, from which it can be seen that IgE antibody levels in peritoneal ascitic fluids were lower than the serum IgE levels, and that serum IgE antibody levels were lower in mice producing ascitic fluid. No appreciable increase in IgE was observed by the induction of ascites and therefore this technique was abandoned.

TABLE I

IgE PCA titers of MRS elicited against three distinct antigens*

Antigen 0.1 µg in 500 µg Al(OH) ₃	48 Hour Homologous PCA in CFW Mice			24 Hour Heterologous PCA in Rats		
	First Boost ^a	Second Boost ^a	Third Boost ^b	Third Boost ^c	Fourth Boost ^d	Fifth Boost ^b
Ascaris Suum Extract	10,2,24	35,12,12, 10,32	17	380,1400, 2560,224, 214	300 160	-- ---
Keyhole Limpet Hemocyanin	40,>30, >30,15	40,16,120, 64,15,20	59	320,320, 320,331, 214	490 335	-- ---
Ovalbumin	4,100, 90	30,100,50, 128,150, 100	91	334,400, 334,400. 364	368 400	338

* Sample bleedings taken seven days after each booster injection

^a Sera obtained from pools of 8 mice each^b Sera pooled from all mice in a group before testing^c Sera obtained from pools of 150 mice each

TABLE II

Comparison between the IgE_m content of
ascitic fluid and serum of immunized mice

Source of Fluid	Presence of ascites	IgE Mediated PCA Titers in Rats Against	
		ASC	OA
Serum	-	380,1400, 2560,224, 214	334,334, 400,364, 400,416, 640
Serum	+	112	80
Ascites	+	19	64,76

As stated earlier, the IgE levels in allergic humans and appropriately immunized animals (290, 291, 306, 309) are extremely low compared to the major serum isotypes (IgA, IgG and IgM); even IgE enriched fractions did not contain sufficient IgE to develop a visible precipitate on immunodiffusion in agar gels against anti- ϵ preparations. At the time of these studies, by contrast to the radioallergo-sorbent test used for the in vitro quantitation of human IgE, the only assay available to detect IgE antibody was PCA. Consequently, it was of paramount importance to define the limitations of the PCA assay and its specificity for IgEm.

Optimization of PCA.

While heterologous PCA in the rat as introduced by Mota and Wong (264) has been the standard assay for titration of levels of IgEm antibody the mechanism of IgE mediated PCA was assumed to be similar to that established by Ovary (282) and Levine (290, 291) for IgG mediated PCA. Consequently, doses of antigen which were found optimal for titration of IgG antibodies were used initially for IgE antibody titration.

Effect of antigen challenge dose. The criteria established by Ovary for IgG mediated PCA (reviewed in 282) indicate that the minimal amount of antigen required for maximal PCA titer is proportional to the molecular weight of the antigen. Since KLH is much larger than OA (at least 20 times larger; ref. 271) and the mean molecular weight of ASC was not known, it was necessary to ascertain whether or not the PCA titers displayed in Tables I and II reflected maximal values, especially since all PCA titrations had been performed thus far using

only one concentration of antigen regardless of the molecular weight of the antigen, i.e., 1 mg/ml [this concentration was originally selected because it was routinely employed by other investigators (270, 310, 311) for both IgE and IgG1 mediated PCA]. To study the effect of antigen dose at challenge on the resultant PCA titer, rats which had been sensitized with the appropriate MRS were challenged with OA, ASC or KLH at various doses. Thus, OA and ASC were examined at challenge doses of 0.01 mg/ml up to 10 mg/ml. However, since KLH would not completely dissolve at high concentration and since its extinction coefficient was not available at the time of this study, a highly concentrated "stock" solution was prepared by mixing 100 mg of KLH powder with 5 ml PBS (pH 7.2). Dilutions made from this "stock" were mixed with equal volumes of Evans blue dye prior to challenge.

The relationship between the PCA titer obtained and the challenge dose of antigen for each antigen is illustrated in Figure 8. It can be seen that with OA only some of the concentrations were high enough to give a zone of maximum PCA titer and that 1 mg/ml was in this region. With the other two antigens the PCA titer did not reach a well defined maximum, and both curves have a positive slope at the highest concentrations examined. When KLH was dissolved at pH >8.0, no insoluble component was detected but, as can be seen in Table III, the PCA titers elicited using this preparation of KLH were low or not present; this affect may be due to conformational changes at this pH resulting in masking or destruction of the antigenic determinants.

Optimum time between sensitization and antigen challenge. When Mota and Wong introduced the use of rat skin to titrate IgEm mediated

PCA (264), they reported that a 2 hour rat PCA gave a titer which was similar to 72 hour PCA in the mouse, and that the PCA titer in rat skin was similar at 2 hours and at 72 hours. However, Bach and Brashler (312) reported that at 72 hours the PCA titers in rat skin were twice as high as those determined in mouse skin. It is interesting to note that the results given in Table I indicate that the 24 hour PCA titer in rat skin was five times higher than the 48 hour PCA in mouse skin. To determine if the PCA titer in rat skin depended substantially on the time interval between sensitization and challenge this interval was varied. In Table III are given the PCA titers obtained at 2, 4 and 48 hours after sensitization using a challenge dose of 1 mg/ml of antigen. For all the sera tested, the PCA titers determined after 48 hours in rat skin were 2 to 4 times higher than at the two earlier times. Additional tests in which the PCA titers were determined after 4, 24 and 72 hours (data not presented) show no change in titer.

With respect to IgEm mediated PCA in rat skin there was an unexplained observation: a 48 hour sensitization period gave rise to PCA titers higher than at 2 hours and 4 hours (see Table III). This finding has also been seen by Ovary et al (313) in 2 out of 5 murine reagin containing antisera they examined. The most striking example of this phenomenon is illustrated by the IgE anti-KLH PCA titers in Table III (sera K243, K151 and K251) which, although routinely undetectable at 2 and 4 hours were detectable at 48 hours using KLH dissolved at pH 8.0.

Figure 8. Relationships between challenging dose of antigen and PCA titer. Various doses of KLH ($\square$ — $\square$), ASC (Δ — Δ) and OA ($\circ$ — $\circ$) were prepared for use in the titration of selected MRS raised against KLH, ASC and OA, respectively. Since the extinction coefficient of KLH was not known at the time of this experiment, the amount of KLH used, relative to a "stock" solution is indicated on the upper abscissa.

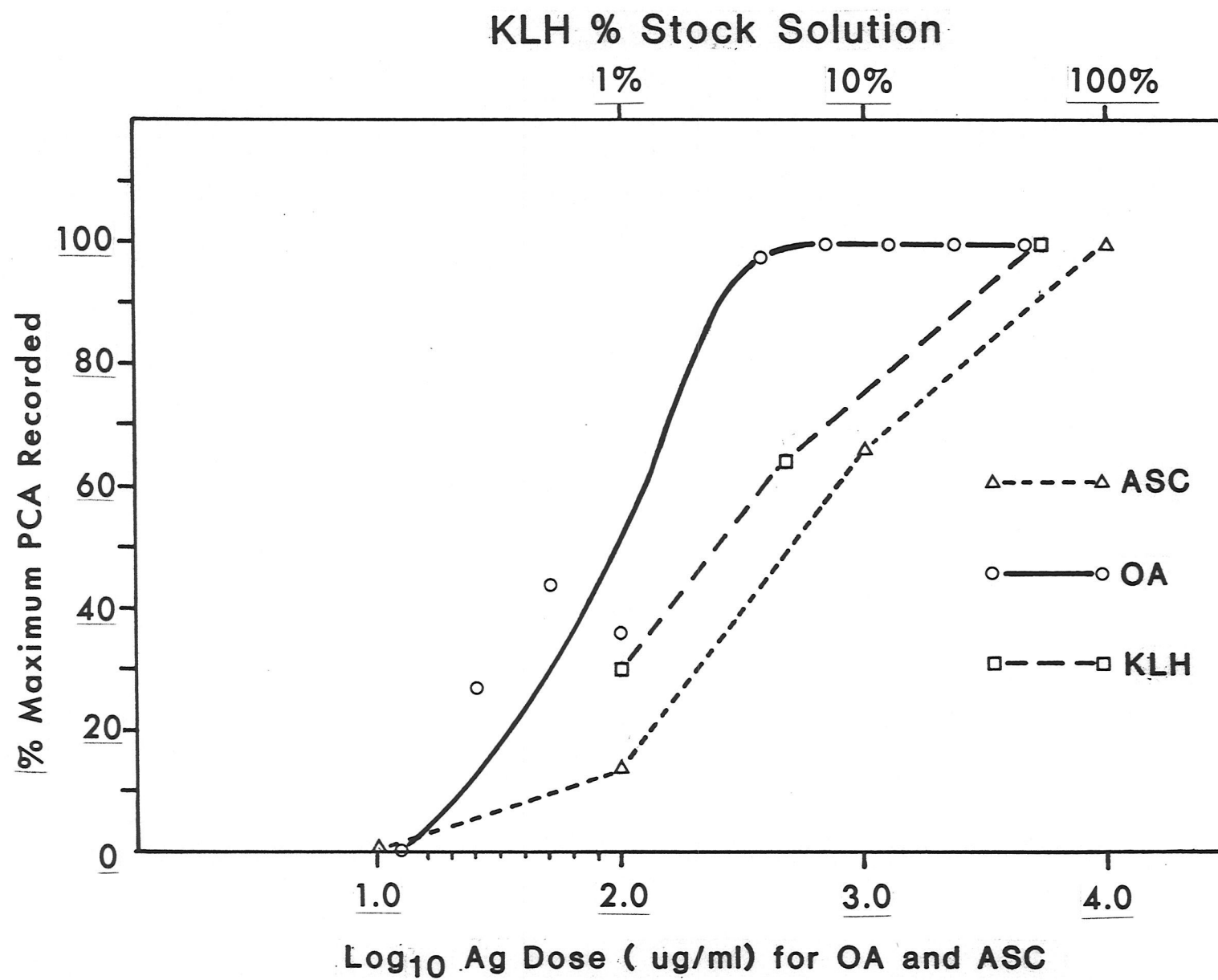


TABLE III

The Effect of Varying the Time Between Rat Skin Sensitization
and Antigen Challenge on IgE_m PCA Titers

MRS†	Challenge Antigen	Heterologous PCA Titer After		
		2 Hours	4 Hours	48 Hours
(Anti-ASC)-1	ASC	95	90	870
(Anti-ASC)-2	ASC	370	320	1220
(Anti-ASC)-3	ASC	100	160	950
(Anti-OA)-1	OA	420	370	1390
(Anti-OA)-2	OA	440	390	1260
(Anti-OA)-3	OA	350	340	795
(Anti-OA)-4	OA	430	375	1120
(Anti-KLH)-1	KLH	0	0	20††
(Anti-KLH)-2	KLH	0	0	40††
(Anti-KLH)-3	KLH	0	0	50††

†Bleedings collected from various groups of mice immunized with ascaris Suum antigen (ASC), ovalbumin (OA) or keyhole limpet hemocyanin (KLH).

††The KLH antigen was dissolved at pH 8.0 prior to i.v. challenge

Contribution of IgE and IgG1 to homologous and heterologous PCA.

Heat inactivation. Among the main characteristics of reagins one may cite their persistence in homologous skin, heat lability at 56°C and sensitivity to reduction (followed by iodoacetylation; 259, 260, 314). Several investigators (44,260,268,271,272,263) have demonstrated that the murine Ig isotype responsible for the 4 hour PCA in murine skin is not thermolabile, whereas the isotype responsible for the 24 to 72 hour PCA was [however, Clausen et al (268,272) found that the 72 hour PCA in mouse skin was protected if the serum was diluted in saline prior to heating]. In addition the murine Ig isotype responsible for the 24 to 72 hour PCA in mouse skin (268, 272) and PCA in rat skin (269) is sensitive to reduction with 2-ME and subsequent iodoacetylation, while the Ig isotype mediating the 4 hour PCA in mouse skin was not. Susceptibility of MRS to the effects of heat and reduction (followed by iodoacetylation) were examined to provide evidence that the 4 hour PCA in the mouse measured only murine IgG1 antibodies.

In an exploratory experiment, selected murine antisera were heated for 0.5 to 4 hours at 56°C at a 1/10 dilution prior to determination of their PCA titers in rats. All sera heated for 1.5 hours or longer lost their ability to mediate PCA in rats. Sera heated for 30 minutes lost on the average 80% of their activity and for 1 hour at 56°C lost 95% of their activity. In the subsequent experiment, an MRS was diluted 1/20 to 1/640 prior to heating for 4 hours at 56°C (Sample A) and compared to the same antiserum which was diluted 1/10, heated for 4 hours at 56°C and then diluted to 1/640 (Sample B). Both were compared to a serum which was thawed and diluted just before injection

into rat skin (sample C). The resultant PCA titers are given in Table IV. These results are similar to those of Clausen et al (268) in mouse skin in that diluting the antiserum prior to heating preserved the IgE mediated PCA activity. A similar test performed in mouse skin confirmed that the 48 hour PCA activity was destroyed at 56°C as can be seen in Table V. In this and other experiments the 48 hour PCA activity was destroyed after just 30 minutes at 56°C but the most surprising finding was that for the same time interval the 4 hour homologous PCA titer was also affected. It was also observed that while it took 3 hours to abolish completely the 4 hour homologous PCA titer, it was dramatically reduced after only 30 minutes.

<u>PCA TITER IN MOUSE SKIN</u>		
<u>TREATMENT</u>	<u>4 HOUR</u>	<u>48 HOUR</u>
30 min, 56°C	<10	<10
NONE	>80	54

In other experiments (not shown) it was found that the effect on the 4 hour homologous PCA was totally dependent upon the dilution of the serum at the time of heating. It was concluded that thermolability was not a reliable indicator of IgE activity in the 4 hour homologous PCA. As an alternate means for eliminating reagin, an examination was made of the effects of reduction (followed by iodoacetylation) on rat PCA mediated by murine reagins. Since it was found that only partial reduction in the IgE titer occurred, the analysis was not carried further.

The participation of IgG1 in PCA in mouse and rat skin. To determine the role of murine IgG1 antibodies in homologous and heterologous PCA, a sample of MRS was divided into two aliquots; one was treated with rabbit anti- γ 1 antiserum to remove IgG1 and the other was treated with an identical amount of normal rabbit serum. A comparison was then made of the ability of each aliquot to mediate PCA in rat and mouse skin at various times after sensitization. The results plotted in Figure 9a demonstrate that the PCA titers obtained in rat skin were unchanged by the removal of IgG1. However, as can be seen in Figure 9b there was a change in the 2 to 10 hour homologous PCA titers even though the anti- γ 1 did not remove all of the PCA activity occurring 4 to 6 hours after sensitization. In addition titrations performed at 48 hours (data not shown) showed that homologous PCA titers were similar for both aliquots. These results indicate that the effect was specific for the 2 to 10 hour homologous PCA.

In another series of experiments, an IgE-enriched fraction, isolated from MRS by ammonium sulfate precipitation was further fractionated by DEAE ion exchange chromatography. Four fractions were characteristically generated by the method employed. Since DEAE chromatography had been used by others (207,210,212,271) to separate IgG1 from IgE, the four fractions obtained were examined for PCA activity at 4 hours and at 72 hours after sensitization of mouse skin. Table VI illustrates the PCA titers of each fraction at 4 and 72 hours. From the data presented in this table it is evident that the 4 hour PCA activity of the first two fractions occurred in the absence

of the 72 hour PCA. These results add weight to the argument that 4 hour PCA in mouse skin is not mediated by IgE.

During these experiments considerable variation in the 2 to 4 hour PCA titers was observed among CFW mice (the only strain of mice used up to this point as recipients of MRS). In an attempt to obtain more consistent data BALB/cJ, A/J, AKR/J and SW strains of mice were tested for their usefulness for the titration of PCA activity using the same MRS. The AKR, BALB/c and A strains showed extreme sensitivity to OA which was manifested by spotty blueing in the skin surrounding the PCA reaction accompanied by an ill-defined PCA border and at times the whole back would become blue; hence these strains were not considered suitable for PCA tests. By contrast the SW mice provided consistent PCA reactions. However, occasionally, the skin of one or two of the SW mice was completely negative while the majority of mice injected with identical dilutions of MRS supported a PCA reaction and the data from these mice were not included in the determination of the average PCA diameter.

Variations inherent to the determination of PCA titers.

- i) To establish the variation inherent in the 4 hour homologous PCA in SW mice, 30 mice were injected i.d. with 2 PCA units (a 1/300 dilution of a serum with a titer of 600) in each of 4 sites. Table VII details the experiment and the results. As can be seen, there was little difference between the 4 sites and statistical analysis¹⁰ of this data confirmed this
-

¹⁰ To test if two random samples were representative of the same population, the ratio of their variances was calculated, which was achieved by placing the largest variance in the numerator and the

TABLE IV

Effect of Heating MRS on Heterologous PCA Titers

Sample [†]	PCA Titer ^{††}	Per cent Reduction
A	80	75%
B	<20	>94%
C	320	0%

[†]A = MRS Diluted from 1/20 to 1/640 prior to heating at 56°C for 4 hours.

B = MRS diluted 1/10, heated at 56°C for 4 hours then diluted from 1/20 to 1/640.

C = MRS diluted from 1/20 to 1/640 just before injection into rat skin.

^{††}All samples were examined at 1/20, 1/40, 1/80, 1/160, 1/320 and 1/640 dilutions.

TABLE V

Effect of Heating MRS on Homologous PCA Diameter

Heating time* (hours)	4 hour PCA** at 1/40 dil'n	48 hour PCA** at 1/40 dil'n
0	9 mm	12mm
0.5	7 mm	0
1	7 mm	0
1.5	6 mm	0
2	4 mm	0
2.5	3 mm	0
3	0 mm	0
3.5	0	0
4	0	0

*MRS was diluted 1/4 and then distributed into 9 tubes, each of which was heated for one of the indicated time periods.

**Ten mice were sensitized with the heated MRS after diluting tenfold after each time interval indicated. Five mice were challenged after 4 hours and 5 mice were challenged after 48 hours. Each diameter reported is the mean of 5 determinations.

TABLE VI

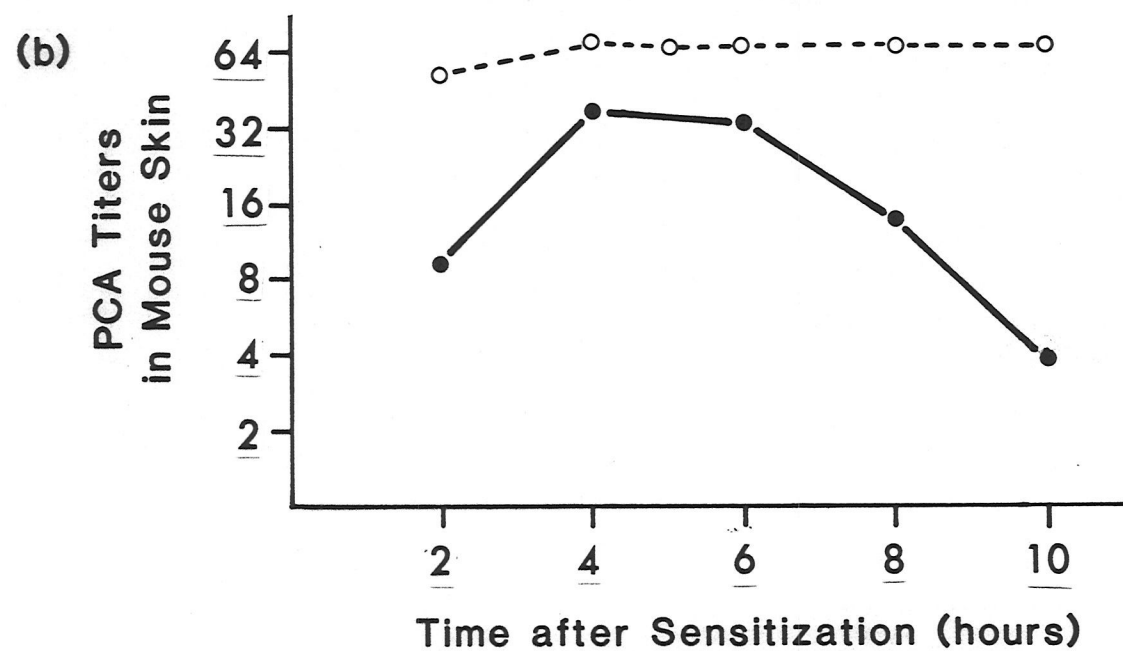
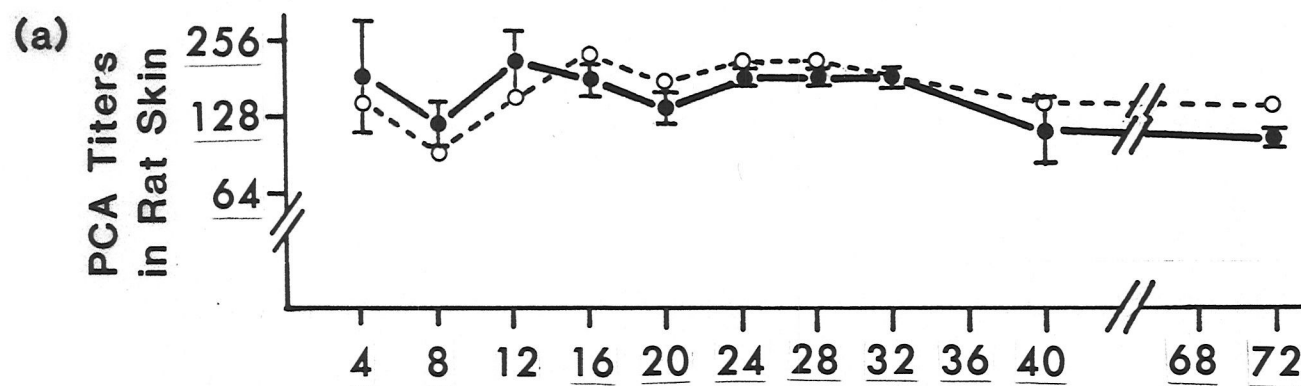
Homologous 4 Hour and 72 Hour PCA Titer of
Various DEAE Column Fractions

Column Fraction Examined †	PCA Activity††	
	4 Hour	72 Hour
F1a	>16	0
F1b	>16	0
F2	64	64
F3	9	<2

†Stepwise gradient elution by sequential application of 0.02M phosphate buffer, 0.05M phosphate buffer and 0.1M phosphate buffer generated three fractions; the material elution at 0.02M was split into two fractions.

††PCA titers in CFW mice of DEAE cellulose column fractions of Anti-OA MRS concentrated to the original volume.

Figure 9. Effect of removing IgG1 from an MRS on PCA titers determined in rat (a) and mouse (b) skin as a function of time: PCA titers of the MRS treated with rabbit anti- γ_1 antiserum (●—●); PCA titers of the MRS treated with an identical volume of normal rabbit serum (○----○).

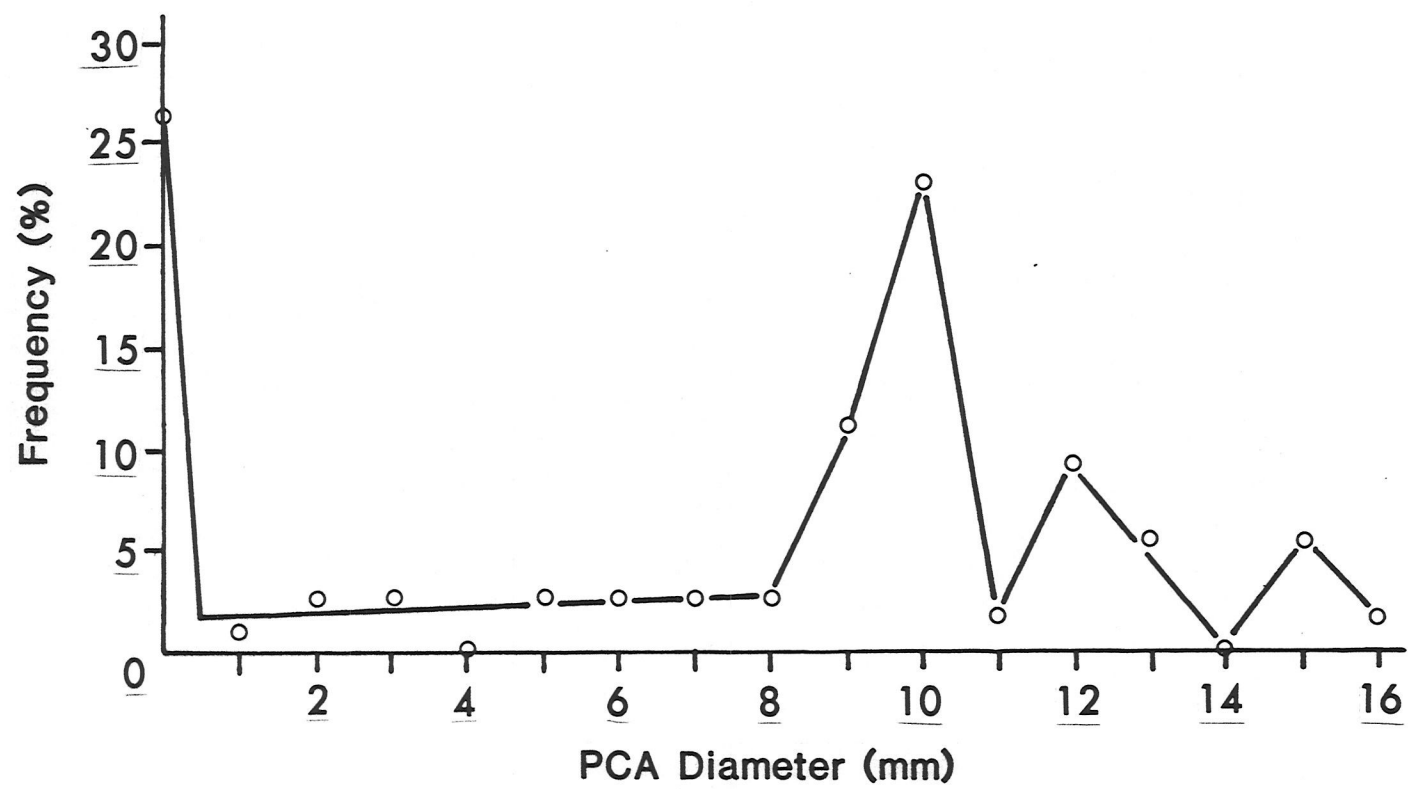


observation (an F statistic of 0.547 compared to a critical F statistic of 8.62). Considerable variation was seen between mice (an F statistic of 13.54 compared to a critical F statistic of 8.62). The mean PCA diameter was 7.4 ± 5 . The distribution of PCA diameters obtained has been plotted on a frequency graph (Figure 10). Of 120 observations 46% were between 8 and 13 mm in diameter, 23% of them were negative, and the other 31% were between 1.0 and 22 mm in diameter. Analysis of variance of the data ($p \leq 0.05$) indicated that the variation of the results obtained among different mice was extreme, while the difference among skin sites of the same mouse was negligible.

- ii) In view of the wide variation observed among mice, a similar experiment was conducted in rat skin in which the PCA titer of one murine reaginic serum was determined in the backs of 5 rats on 4 distinct regions of the same rat back, producing 20 separate titers. The diagram associated with Table VIII shows the location of the skin sites and the Table contains
-

smaller in the denominator. The ratio is called the variance ratio, or the F statistic after R.A. Fisher (who defined its distribution). F is calculated by the formula $F(Nn-1, Nd-1) = \frac{\text{large sample variance}}{\text{smaller sample variance}}$ where $Nn-1$ refers to the degrees of freedom of the numerator and $Nd-1$ refers to the degrees of freedom of the denominator. The experimentally derived F statistic may be compared to available Tables which give the expected critical values of F for 0.01; 0.01; 0.05; 0.1; and 0.2 percent probability. since the expected F distribution has a different shape for different numbers of degrees of freedom the tables are arranged such that at each percentage level a critical F statistic can be assigned for each $F(Nn-1, Nd-1)$. When the experimentally determined F statistic is higher than the reference F for a particular percentage value, the probability that the two sample are derived from the same population is less than the reference percentage value.

Figure 10. Frequency distribution of the PCA diameters obtained when a 1/300 dilution of MRS was injected into 4 distinct sites in each of 30 SW mice and then challenged 4 hours later to elicit PCA.



the PCA titers obtained at each of these sites (A→D). It can be seen that the mean titer was 295 ± 138 with titers ranging from 96 to 775; the analysis of variance data presented in Table IX is derived from these results. The critical F statistic (determined from the appropriate statistical table) was 5.91; any variance ratio above this denotes a significant departure from an expected biological variation of 5% (315). It can be seen that although there was a four-fold difference between rats, the variation could be explained in terms of acceptable biological variation. Consequently unless data are subjected to statistical analysis a difference of four-fold between two given groups will not be considered significant. On the graph in Figure 11 is represented the mean PCA diameter [± 1 standard deviation)] at each dilution. The mean PCA titer was obtained by drawing a straight line between the two PCA diameters on either side of the 5 mm cut-off. It can be seen that the largest variation in PCA diameters occurs at dilutions giving diameters close to 5 mm.

Summary. The results of these exploratory studies led to the following conclusions concerning the PCA assay system:

1. Of the three antigens chosen for evaluation---OA, KLH and ASC---only OA was able to induce optimal PCA reactions under the conditions tested. Furthermore, the lengthy purification process involved in preparing ASC and the solubility problems encountered with KLH led to the selection of OA for further investigations.

Figure 11. PCA titration curve of MRS in rat skin. The dotted line represents the 5 mm threshold diameter. Each point in this graph is the mean of 20 determinations $\pm$ one standard deviation. The PCA titer is interpolated by calculating the dilution indicated by the intersection of the titration curve and the threshold. In this instance it relates to a PCA titer of 280.

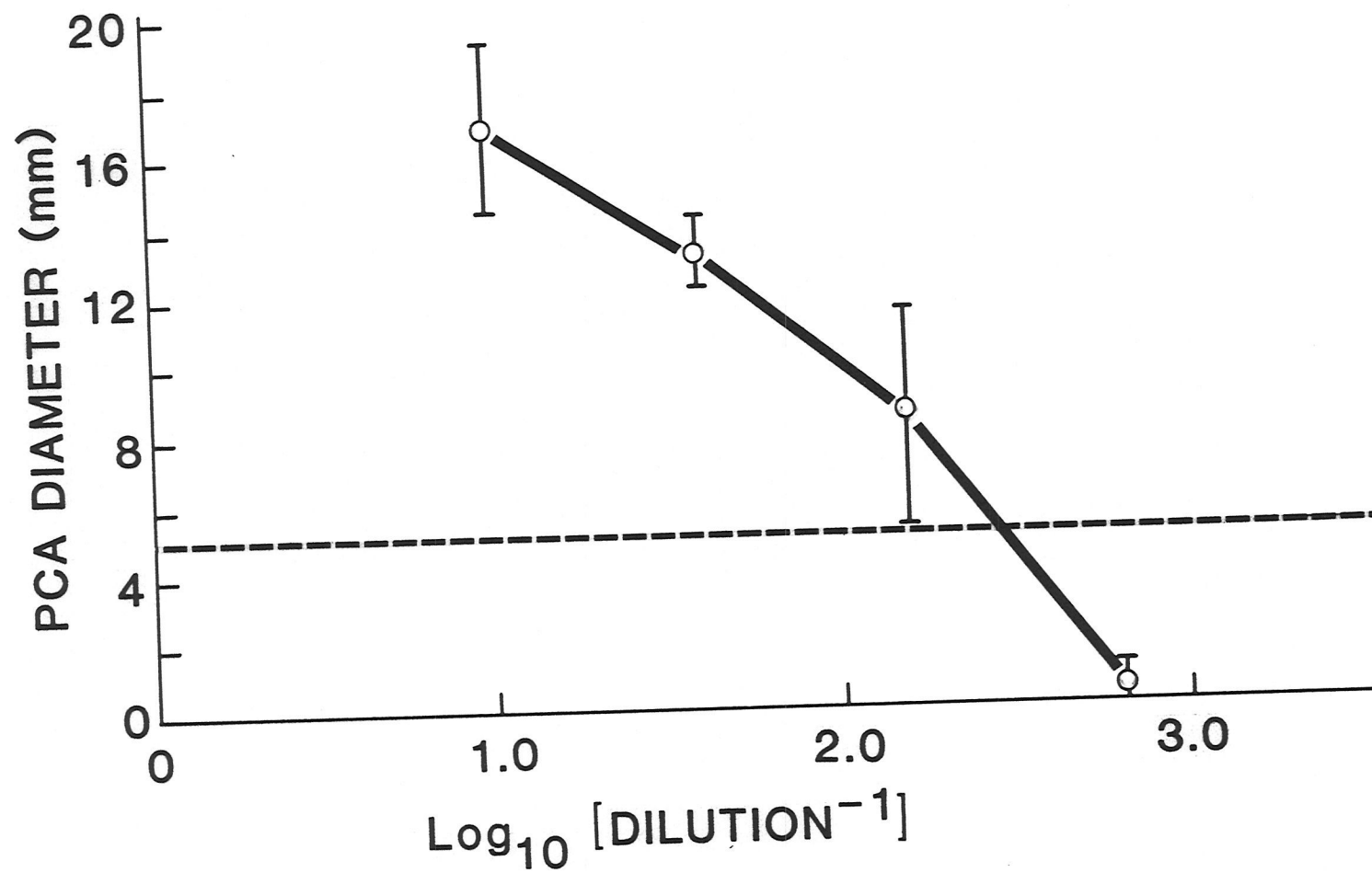


TABLE VII

Variation in Homologous 4 hour PCA diameters in SW miceDIAMETERS OF PCA REACTIONS IN INDIVIDUAL MICE ^(a)

Mouse No. Site(b)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26 to 30	AVERAGE
1	8	8	10	12	13	12	12	22	9	10	9	0	10	9	1	5	0	3	10	7	10	0	10	10	1	0	6.89
2	5	13	10	15	10	9	12	18	13	15	6	2	9	10	0	9	2	0	5	12	10	18	10	9	1	0	7.55
3	7	12	10	12	12	11	13	16	9	10	9	10	10	8	0	9	0	3	6	13	10	10	10	12	1	0	7.65
4	6	15	7	15	10	10	12	19	9	11	6	9	9	10	2	9	3	0	10	13	10	15	2	10	1	0	7.65
AVERAGE	6.5	13	10	13	11	10	12	19	10	12	8	5	10	9	1	8	1	2	8	1	10	10	8	10	1	0	

^aEach mouse was sensitized in 4 distinct sites with 2 PCA units

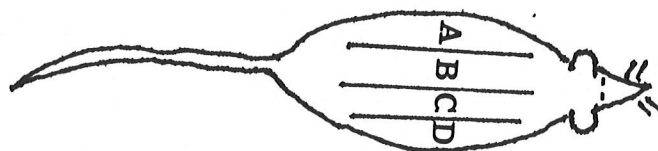
^bThe shaved back of each mouse was divided into quadrants:

Site 1 refers to the upper right quadrant and site 2, 3 and 4

refer to the lower right, upper left and lower left quadrants, respectively.

TABLE VIII

Variation Obtained in Heterologous PCA Titers Using
MRS in Rat Skin



Site	PCA Titer Per Rat					Site Average
	1	2	3	4	5	
A	288	775	228	96	335	277
B	312	312	335	120	305	276
C	312	305	312	200	335	292
D	251	320	312	100	346	265
Rat Average	291	428	297	129	330	

Range = 96 to 775; mean = 295 ± 138

TABLE IX

Analysis of Variance of Data Contained in Table VIII

Source	df	Sums of Squares	Mean Square	F	Critical F.95 (N,D)*
Column	4.0	186200	46550	3.49	5.91
Row	3.0	18095	6031	0.45	8.74
Error	12.0	159723	13310		
TOTAL	19.0	364020			

*When the experimentally derived value of F is lower than the value present in this column then the data fall within 95% confidence limits.

2. Of the mouse strains examined, the SW mice were found to provide the most suitable skin for homologous PCA.
3. The time interval between sensitization and challenge chosen for development of IgG1 mediated homologous PCA was 4 hours, since at this time maximum PCA titers were obtained with low levels of IgG1 and in the absence of IgE.
4. For titration of IgEm, heterologous PCA in rat skin was adopted over homologous PCA. The time interval between sensitization and challenge providing maximum PCA titer was found to be 48 hours.
5. Statistical analyses indicated that considerable variation was inherent in PCA determinations. Consequently it was decided that (i) the minimum number of replicates incorporated into each IgG1 mediated PCA assay be fixed at 5 mice and a minimum of 3 rats be used for determination of IgE mediated PCA. (ii) PCA titers determined at the same time on the same animals would be considered to be similar if they differed by no more than four-fold, unless statistical analysis indicated otherwise.
6. The reduction in 4 hour homologous PCA titers after removal of IgG1, the change seen in the profile of PCA titers obtained within the first 10 hours after sensitization with an IgG1-reduced sample and the ability to separate the molecules responsible for 72 hour homologous

PCA from those responsible for 4 hour homologous PCA on DEAE-cellulose all provide strong support for the conclusion that IgE does not participate in 4 hour homologous PCA.

Attempts to Increase Serum IgEm Levels Selectively

Effect of antigen dose. Experiments by Levine and Vaz (273,316) demonstrated different effects on the IgE and IgG1 antibody responses in mice depending upon the dose of the immunizing antigen. At 1 μ g of OA with 500 μ g Al(OH)₃ both IgE and IgG1 anti-OA antibodies were induced whereas 100 μ g of OA plus 500 μ g Al(OH)₃ elicited a strong IgG1 antibody response and a weak IgE antibody response (245). Lowering the antigen dose from 1.0 μ g to 0.1 μ g resulted in an increase in IgE titers and a decrease in IgG1 titers (213). Consequently experiments were designed to optimize the immunization schedule so as to elicit a strong IgE response in the presence of the lowest possible IgG1 response.

To this end five groups of SW mice were immunized with either 0.1 μ g of OA in 500 μ g Al(OH)₃ (Groups A and B) or 10 μ g of OA in 5 mg Al(OH)₃ (Groups C, D and E). Since monthly immunizations had been found to favour higher IgE production than weekly immunizations (317), each group was reimmunized 3 times at 4 week intervals. The mice were bled 7 days after each immunization. The dose of antigen and the amount of Al(OH)₃ used for each group are indicated in Figure 12, which also lists the resultant IgG1 and IgE titers from which it may be concluded that (i) there was no detectable IgG1 antibody response

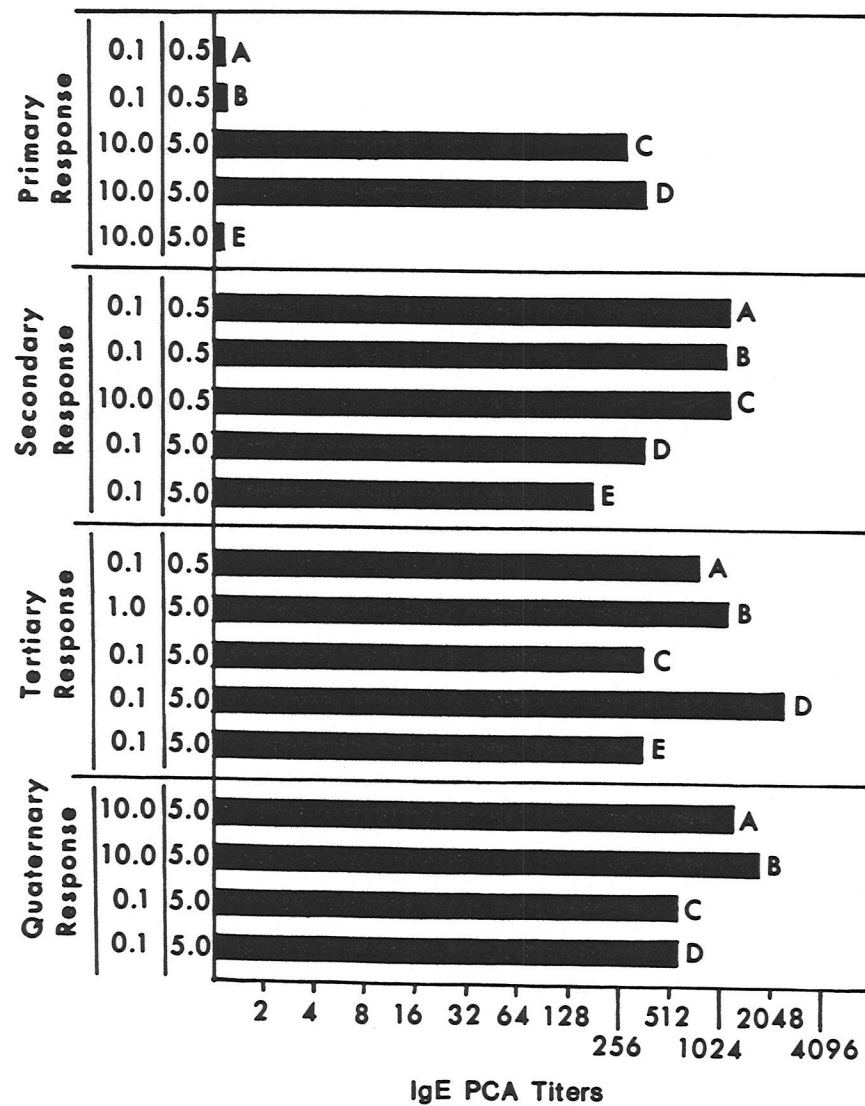
on day 7 after primary immunization irrespective of the dose of OA or Al(OH)₃ used but there was an IgE antibody response using 10 µg of OA and 5 mg Al(OH)₃ (ii) the antibody responses in the IgE and IgG1 classes appear to be differently affected by different doses of antigen. Hence it may be inferred that there exist unique requirements for optimal stimulation of an antibody response in each Ig class, and (iii) no significant increase in either IgE or IgG1 antibody levels were obtained with tertiary and quaternary immunizations.

Another experiment was performed in which three groups of 24 SW mice were immunized respectively, with 0.1, 1.0 or 10 µg of OA plus 5 mg Al(OH)₃. Twenty-eight days later each group was subdivided into three equal groups, one of which was reimmunized with 0.1µg of OA in 5 mg Al(OH)₃, another was reimmunized with 1.0µg of OA in 5 mg Al(OH)₃ and the remaining was reimmunized with 10µg of OA in 5 mg Al(OH)₃. The animals were bled at regular and frequent intervals after primary and secondary immunization for determination of their serum IgE, IgG1, IgG2a and HA anti-OA antibody levels. The data obtained can be summarized as follows:

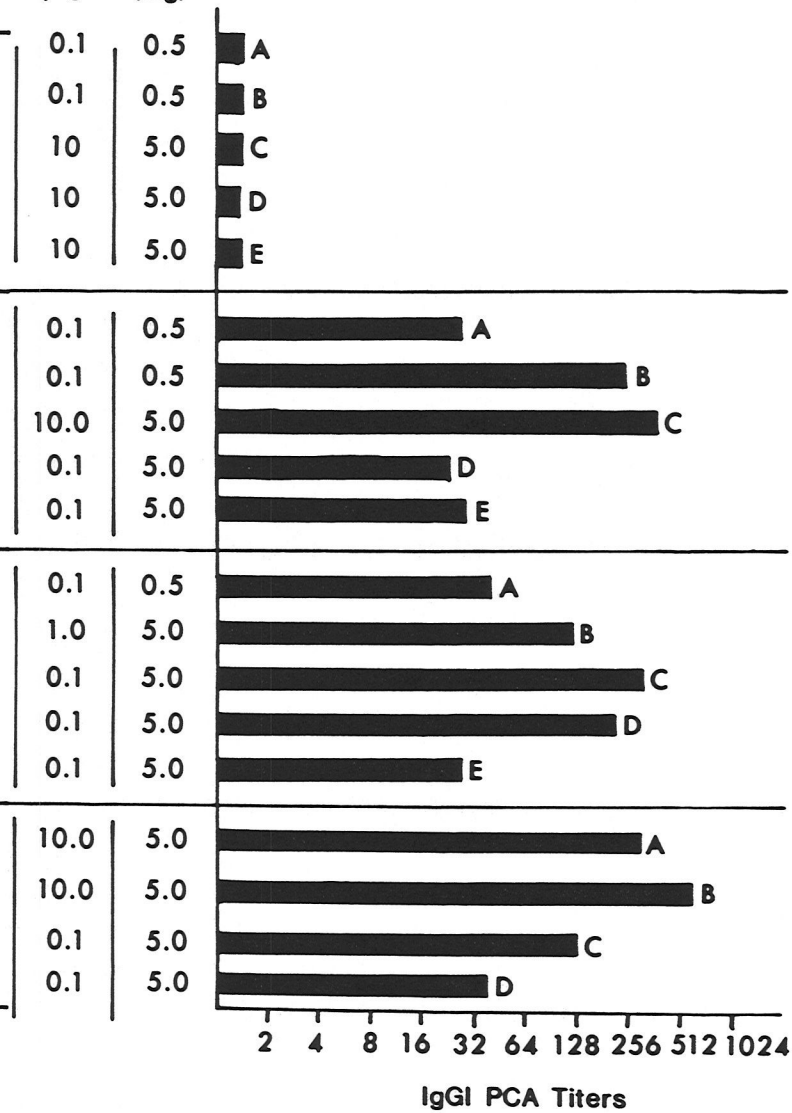
- i) The highest primary IgE antibody response was elicited by 10 µg of OA. The peak titer was 9 times higher than the peak titer obtained with 1 µg of OA and the peak appeared on day 8 which was two days earlier than the peak obtained with 1 µg of OA. This finding accounts for the presence of detectable IgE anti-OA titer on day 7 of the primary response elicited by 10 µg of OA (see Figure 12). The primary IgE response to

Figure 12. IgE (a) and IgG1(b) PCA titers seven days after the primary, secondary, tertiary and quaternary immunizations. Each re-immunization took place at 4 week intervals. The letter to the right of each bar corresponds to a group of 8 mice.

(a) OA Al(OH)₃
(μg) (mg)



(b) OA Al(OH)₃
(μg) (mg)



10 µg of OA dropped 16-fold after peaking and thereafter remained at a constant level until the mice were reimmunized. Primary IgE responses to 1.0 and 0.1 µg of OA had lower peak titers and smaller differences between the persistent titer and the peak titer. The secondary IgE response increased to a maximum earlier than the primary response and tended to remain at that level. All secondary IgE anti OA peak titers were higher than primary titers and similar to each other.

- ii) Both the primary and the secondary IgG1 antibody response were similar irrespective of the dose of antigen. The primary IgG1 antibody response was maximal by day 9 and remained at that level until the mice were reimmunized. The secondary IgG1 antibody response continued to increase with time over the 28 days examined.
- iii) There was no detectable IgG2a antibody response.
- iv) HA antibody titers were determined in the presence and absence of 2-ME, the 2-ME sensitive response was present only on days 7, 8 and 9 of the primary immune response; the 2-ME resistant response continued to rise until day 16 and thereafter remained constant.

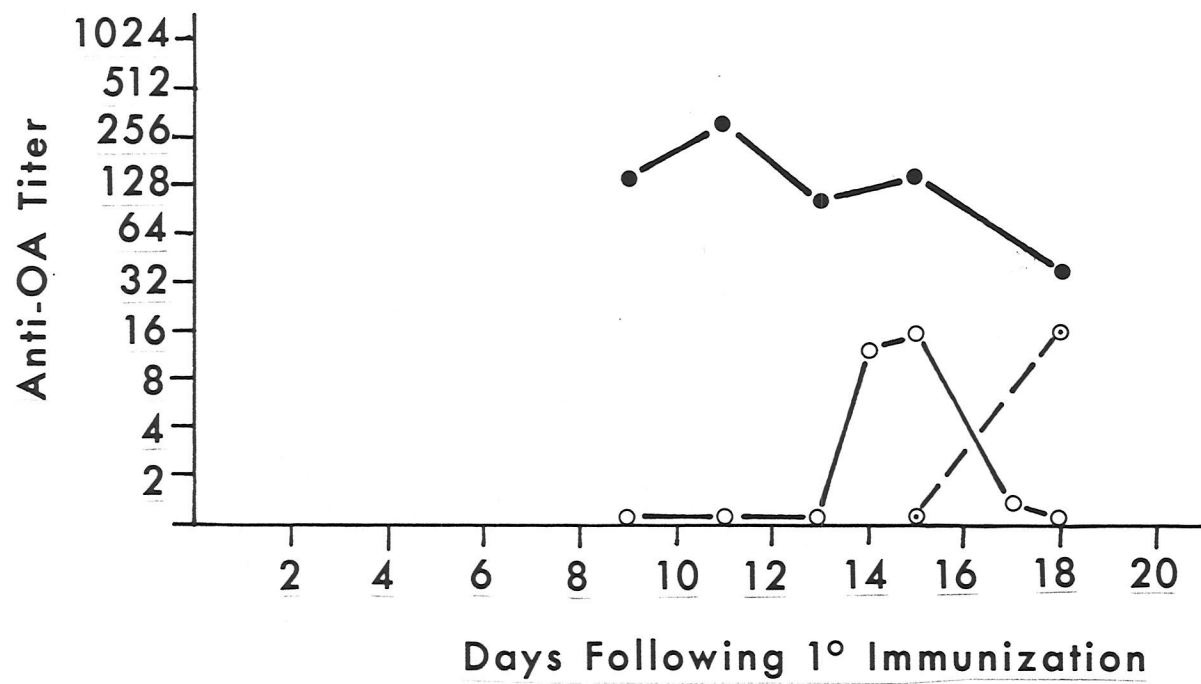
Adoptive transfer experiments. Hamaoka et al (318) had demonstrated that the magnitude of the IgE titers in irradiated recipients of primed cells after the cells had been challenged in the recipient were higher than those observed in secondary responses of intact animals. The possible usefulness of this system for the production of high IgE levels was therefore investigated. The first task was to establish

the dose of irradiation which was sufficient to abolish the recipients' ability to mount an immune response without transfer of cells. Five groups of SW mice were subjected to 500, 600, 700 and 800 rads of whole body γ -irradiation one day prior to receiving 10 μ g of OA in 5 mg of $\text{Al}(\text{OH})_3$. Mice were bled at intervals thereafter and their sera tested for IgE and IgG1 antibodies. The serum titers were compared to control groups which did not receive any irradiation. From the results shown in Figure 13 it may be concluded that exposure to 500 rads or 600 rads drastically reduced the IgE antibody response and that only a meager and short-lived response was seen on days 14 to 17 in mice exposed to 500 rads. The group exposed to 600 rads began to respond with an IgE response on day 18. No IgG1 response was detected in any of the irradiated mice. Hence a dose of 500 rads was chosen for further work because it would cause the least mortality.

The next experiment was designed to evaluate the adoptive secondary immune response as a source of high titered IgE. All recipient mice had been irradiated one day before the transfer of cells. Spleen cells obtained from SW mice immunized 47 days earlier against 10 μ g OA in 5 mg $\text{Al}(\text{OH})_3$ were washed twice in HBSS and injected i.v. into irradiated SW mice at a dose of 5×10^7 per recipient. At the time of cell transfer, the recipients were immunized i.p. with 10 μ g of OA in 5 mg $\text{Al}(\text{OH})_3$. Figure 14 depicts the results of this experiment. Although titers of both the IgE and IgG1 were not as high as had been anticipated these responses appeared to persist.

Summary. While several protocols were explored to produce high IgE antibody levels, the major source of IgE to this point was serum from

Figure 13. The effect of irradiation dose on the ability of SW mice to mount a primary IgE anti-OA antibody response to an i.p. injection of 10 μ g OA in 5 mg of $\text{Al}(\text{OH})_3$. The IgE responses of normal mice (●—●), mice subjected to 500 rads (○—○) and of mice subjected to 600 rads (⊗----⊗) are shown.



multiply-immunized SW mice. Table X lists the sera with highest IgE antibody levels, all of which have been raised against OA. They were kept frozen at -20°C until required for fractionation and isolation of IgE, and represented the 5 highest MRS pools available for fractionation. Many sera had been harvested in this study to provide IgE and IgG1 for standardization of assays, but all sera containing at least 5×10^5 PCA units were kept specifically for isolation of IgE. The following immunization protocols were employed:

- i) For routine primary anti-OA responses ... 1 μg OA in 1 mg $\text{Al}(\text{OH})_3$
- ii) For multiple immunizations ... 0.1 μg OA in 1 mg $\text{Al}(\text{OH})_3$

Except for the early stages of the primary immune response it was not possible to substantially raise IgE antibody levels without a concomitant increase in IgG1.

Production of High Titered Anti-epsilon Antisera

Introduction

Early efforts to study the structure of reagins were hampered by the fact that only extremely small amounts of material were available. Depletion of IgA, IgG, IgM and IgD from reaginic antisera by solid phase antibodies directed against μ , κ , α and δ H-chains failed to remove reaginic activity (267,319-321). The exact immunoglobulin class to which reagins belonged was not definitively established until Ishizaka et al (17) succeeded in preparing a rabbit antiserum against a reagin-rich chromatographic fraction of human reaginic antisera from

TABLE X

Levels of Anti-OA IgE in Various Pools of MRS

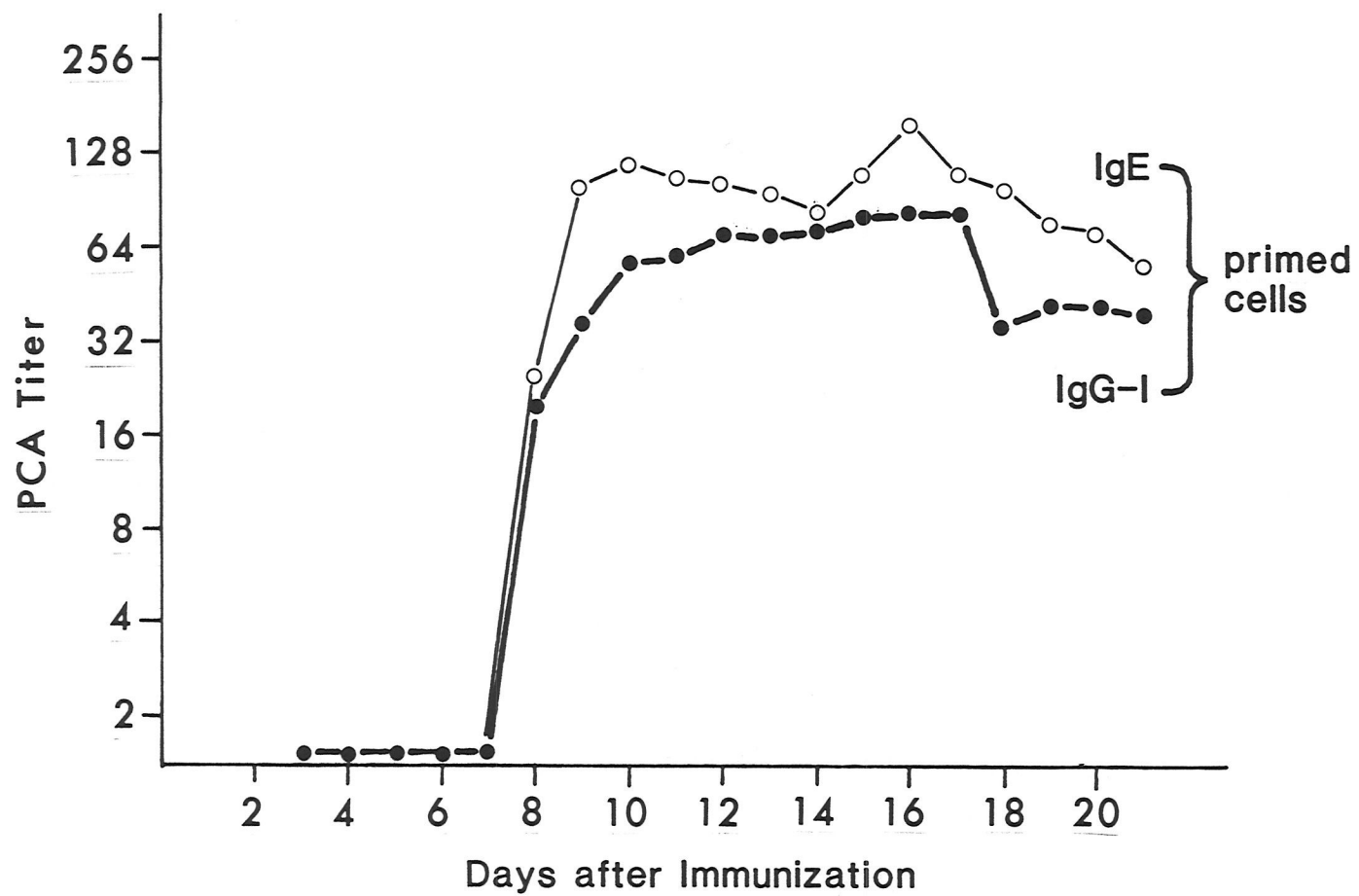
PCA Titer†	Vol	PCA Units
2900	12 ml	7×10^5
2200	90 ml	4×10^6
2600	45 ml	2×10^6
640	45 ml	5×10^5
360	75 ml	5×10^6

†All determinations were done in the same group of rats.

highly ragweed sensitive patients. The rabbit antiserum did not form a precipitate with normal human serum, but was capable of completely removing the reaginic activity from allergic sera as well as to produce a weak precipitate on radioimmunodiffusion against human reaginic serum. The precipitate, by virtue of its ability to bind radioactively labelled allergen, was shown to contain antibody which Ishizaka et al designated as IgE. The IgE content of human serum was found to represent only 0.05% of the total Ig. Shortly after the discovery of IgE it was demonstrated that a myeloma protein which had been characterized by Bennich and Johansson (279) possessed the same heavy chain determinants as human reagin, the availability of this myeloma protein (and a few additional IgE myelomas discovered since) facilitated greatly the production of anti-epsilon antibodies (322) and the emergence of the field of allergy from a medical practice of doubtful efficacy to a respected branch of bio-medical science.

Early studies in the rat by Mota demonstrated that severe, often fatal, anaphylactic shock could not be passively transferred by serum with the same effectiveness as in the donor (323-325). Only by altering the route and schedule of immunization were investigators successful in eliciting anaphylactic antibodies in sufficiently high levels in the donor to demonstrate their presence by serum transfer in the recipient (259,326). In addition, it was discovered (327,328) that infection of most species with the nematode *Nippostrongylus brasiliensis* led to high titers of anaphylactic antibodies directed against *Nippostrongylus brasiliensis* antigens (Nb). Rabbits immunized against complexes consisting of rat anti-Nb antibodies and Nb produced

Figure 14. Adoptive IgE and IgG1 antibody titers in recipients exposed to 500 rads one day prior to cell transfer. All recipients were immunized one day after cell transfer and bled on the days indicated. IgE titers were determined in rats (○—○) and IgG1 titers were determined in mice (●—●).



antisera which after absorption against rat IgG1 failed to form a precipitate against serum fractions high in reaginic activity, but were capable of reducing dramatically their reaginic activity (329). Bloch and Wilson interpreted these results to indicate that either the concentration of reagin in the complex was too low to stimulate a strong antibody response to its H-chain or that the concentration of reagin in serum from infected rats was too low to form a visible precipitate. Stechschulte et al (330) using radioimmuno-electrophoresis showed that an antiserum which they had produced against rat reagin not only removed the PCA activity from rat reaginic antisera (RRS) but also gave a precipitate against an IgE-rich fraction of RRS. Production of this antiserum required fractionation by salt precipitation, ion exchange chromatography and gel filtration of 240 ml RRS having an average PCA titer of greater than 50; the presence of the reagin in any of the fractions was demonstrated by PCA. Since the fractions containing reagin were heavily contaminated with IgG, Stechschulte et al utilized a procedure described by Henney and Ishizaka (298) for production of antibodies to human epsilon chains in guinea pigs. Briefly, guinea pigs were injected with IgG i.v. and were simultaneously immunized with reagin-rich fraction in FCA. This method tolerized the guinea pigs to the determinants of IgG and produced a strong antibody response to the H-chain of the reagin.

As with human and rat reagins, all of the physicochemical procedures used to fractionate MRS, such as ion exchange chromatography (269,271,272,309), gel filtration (272,309) zone electrophoresis (272,309) and density gradient separation (271) yielded reagin-rich

fractions contaminated with IgG1. Therefore Prouvost-Danon et al (309) exploited the strong affinity of IgEm to rat mast cells for the production of an antiserum to m, viz, RPMC were sensitized with MRS and then used to immunize rats. The presence of anti- ϵ m antibodies in the rat antisera was demonstrated after adequate absorption by the ability of the serum to neutralize IgE but not IgG1 mediated PCA, and its ability to degranulate mouse and RPMC sensitized with MRS. Utilizing these antibodies, these authors revealed by Ouchterlony analysis a precipitin band only with reaginic sera and reagin-rich fractions isolated from these sera. However, in the absence of an appropriate IgE standard (i.e., a IgEm myeloma for identification), it was not possible to state definitively that this precipitin band contained IgE, although its antibody activity was confirmed by its ability to bind radioactively labelled antigen.

As established earlier in Table VI, the chromatographic fraction eluted with 0.05M Phosphate buffer from a DEAE-52 column contained the bulk of the reaginic activity. Hence, the corresponding fraction isolated from 100ml of anti-OA MRS was used as a source of IgE for immunization of rats; the PCA activity of this fraction was 6000 units per ml.

Attempts to produce anti- ϵ m antibodies by a combination of the techniques employed by Prouvost-Danon et al (309) and Henney and Ishizaka (298)

The feasibility of combining a technique originally developed for guinea pigs (298) with the protocol described by Prouvost-Danon et al (309) for production of high-titered anti- ϵ antisera in rats was

investigated. In an exploratory experiment two groups of Lewis rats were immunized with RPMC (harvested from 3 Lewis rats) pretreated with the IgEm-rich 0.05M DEAE fraction; one of these groups received deaggregated NMS prior to immunization. The sera obtained from these two groups was examined for antibody activity by immunoelectrophoresis (IEP): NMS was separated electrophoretically in an agarose gel and the serum to be examined were placed in a trough cut into the gel in such a way that the rat serum could diffuse along the full area of fractionation. The IEP patterns obtained were compared to patterns obtained using a rabbit antiserum to NMS. It was found that the sera from rats receiving deaggregated NMS prior to immunization showed no detectable antibody activity, while the antisera produced in rats not given deaggregated NMS showed antibody activity some of which was directed against murine IgG (as judged by the position and the shape of the precipitin bands). Although these IEP data confirm the usefulness of the technique described by Henney and Ishizaka for induction of tolerance in rats to NMS constituents, none of the antisera were capable of neutralizing IgEm mediated PCA. This finding may have been due to an insufficient quantity of IgE to stimulate the production of anti- ξ antibodies.

In an effort to increase the concentration of reagin used as an immunogen, rats were immunized with high-titered MRS: prior to immunization one group of rats (Group I) received a deaggregated 50% SAS fraction of NMS; another group (Group II) received deaggregated unfractionated NMS and a third group (Group III) received saline. All groups received thereafter at least three monthly injections of MRS in

the foot pads; the first two emulsified in FCA and the latter injections in FIA. As was demonstrated previously with IEP, pretreatment of rats with deaggregated NMS resulted in suppression of their responsiveness to NMS, however in this experiment, some of the rats mounted a response to IgA presumably because of the less than tolerogenic levels of IgA in the deaggregated serum sample. Removal of anti-NMS activity was achieved by passage of these antisera through an immunosorbent composed of NMS to which an IgA myeloma protein (L4) had been added. Contrary to the previous experiment, all three groups of rats produced antisera capable of neutralizing IgEm mediated PCA. As can be seen below, rats injected with a 50% SAS fraction of NMS (Group I) made no detectable anti-IgG1 antibodies and produced the most specific antiserum to IgEm.

NEUTRALIZATION QUOTIENT

<u>Group</u>	<u>Before</u> <u>Absorption</u>	<u>After</u> <u>Absorption</u>
I	5	5
II	20	3
III	5	1.25

The reduction in anti-IgEm activity found after absorption in Group II and Group III antisera was presumably due to the removal of anti-Fab and anti-light chain activity. All three groups of rats produced anti-IgE activity by the 2nd injection with MRS. In addition

to these experiments in rats several guinea pigs and one goat were immunized with reagin-rich fractions of MRS; both species produced anti-IgEm by the 6th injection. The antiserum from each species was absorbed by passage through the NMS-IgA-immunosorbent until immunoprecipitin bands against NMS were removed. Each antiserum able to neutralize completely IgE mediated PCA after absorption was converted into a IgG - rich fraction by ammonium sulfate precipitation.

The exquisite specificity of the absorbed anti-IgEm sera for murine reagin, when compared to antisera against other Igs, is demonstrated by the results listed in Table XI. The IgEm-neutralizing capability of the three anti-IgEm sera listed in this Table is typical of all the sera raised against IgEm. Thus each anti-IgEm serum was capable of completely neutralizing MRS activity, even though mixing of these absorbed antisera with MRS did not result in any detectable precipitation. By contrast, the antisera produced against the other murine Igs caused heavy precipitates when mixed with MRS, but had only marginal reagin-neutralizing capacity, which was attributed to the non-specific trapping of some reaginic antibodies in these precipitates or to incomplete removal of anti-light chain antibodies.

In an attempt to isolate IgEm in a pure state from MRS, goat anti-IgEm was used to prepare a "reverse" immunosorbent by coupling its 50% SAS fraction to Sepharose-4B. A MRS with a PCA titer of 23,300 was applied to this anti-IgEm immunosorbent and, after washing the column with PBS, the specifically adsorbed material were eluted with 3 M KI, pH 7.2. The experimental procedure was repeated with a control immunosorbent prepared with a rabbit antiserum to NMS. As is

evident from Table XII, passage of MRS through a column of the anti-IgEm immunosorbent resulted in significant depletion of IgEm however most of the adsorbed reaginic antibodies lost their ability to mediate PCA after the elution step. This procedure, was therefore not pursued further. It was found that there was PCA activity in the effluent, probably due to the low capacity of the anti-IgEm immunosorbent used.

Crossreactivity between IgE and IgEr

The ability of IgEm to bind to RPMC and rat skin mast cells (264,309) was considered to be due to structural similarity between IgEm and IgEr. Indeed premixing of MRS with purified IgEr at the proper ratio before the injection of the MRS into rat skin resulted in the complete inhibition of IgEm mediated PCA. As shown in Table XIII the inhibition was dependent upon the amount of IgEr present: i.e., there was a dramatic drop in the PCA titer of IgEm in the presence of 51 μ g of IgEr; at slightly lower concentrations (e.g. 34 μ g) there was a reduction only in the intensity of the colour of the PCA reactions without any apparent change in the diameter of the reaction. Also as demonstrated in the lower section of this table, IgEr inhibits the homologous 48 hour PCA in the skin of CFW mice; however the effectiveness of inhibition appeared to be lower in mouse skin. This difference may be due to subtle differences in structural and biochemical properties of the tissue mast cells or to the differences in density of these cells in the skin of these two species. Both of these types of differences may be related to the consistent finding that the PCA titer of a given MRS is higher when determined in rat skin than in mouse skin.

TABLE XI

Effect on PCA Titers of Mixing 100 μ l MRS with
Antisera Against Murine Ig Determinants

Antisera to Murine Igs	Volume of Antisera	% Neutralization of PCA*
Guinea pig anti- ϵ	200 μ l	100
Goat anti- ϵ	200 μ l	100
Rat anti- ϵ	200 μ l	100
Rabbit anti- κ	600 μ l	21
Rabbit anti- μ	400 μ l	15
Rabbit anti- α	400 μ l	6
Rabbit anti- γ_1	400 μ l	16
Rabbit anti-Ig	600 μ l	23

*All mixtures were adjusted to 1 ml with buffered saline prior to in vitro mixing and centrifuged prior to injection into rat skin.

TABLE XII

Adsorption of IgE_m to an Anti-ε_m Immunosorbent

Immunosorbent	PCA Titer*	
	Effluent	Eluate
Seph-4B-anti-IgE _m	7,100	11,600
Seph-4B-anti-NMS	20,800	<10

*The effluents and eluates were concentrated to the original volume (2 ml) of the reaginic serum; the PCA titer of this serum was 23,300. For elution, a solution of 3M KI in PBS buffer (pH 7.2) was used.

It is also interesting to note in this Table that NRtIg did show a slight effect on the PCA titers at the highest concentrations tested. In view of the fact that NRtIg was not deaggregated the observed inhibition of PCA could have been due to one or all of the following: 1) desensitization of the site by aggregated IgG (331,332); 2) blocking of IgE binding by IgG2a¹¹ aggregates (333); and 3) inhibition by traces of IgEr present in NRtIg (334).

The above in vivo findings were extended in vitro (303) by examining the interactions between IgEm and IgEr and the epsilon receptor sites (FcER) on rat basophilic leukemia (RBL) cells and by comparing them to the interaction between IgEr (IR-162) and RBL cell FcER. It was thus demonstrated that IgEr and IgEm were similar with respect to their ability to bind to RBL cells. Studies were initiated to examine the crossreactivity between antisera produced against monoclonal rat IgE (anti- ϵ r) (335) and IgEm with a view to determining the feasibility of using anti- ϵ r antibodies as a reagent for immunosuppression experiments. The assumption was made that the degree of crossreactivity of a given anti- ϵ r antibody preparation could be determined by dividing its neutralizing capacity (expressed as an NQ value) for MRS by its NQ value defined for RRS¹². NQ values were determined for horse anti- ϵ r, dog anti- ϵ r and sheep anti- ϵ r for both MRS and RRS, these values and the degree of crossreactivity derived from these values are listed in Table XIV. It can be concluded from

¹¹ IgG2a is a subclass of rat IgG implicated in the homologous rat 4 hour PCA.

¹² RRS was obtained from Wistar Firth rats which had been immunized against Kentucky blue grass (336).

this data that the antisera were highly active against RRS compared to their activity against MRS; i.e., their crossreactivity with respect to MRS was in the range of only 0.3 to 3.5%.

To further demonstrate that anti- ϵ r antibodies combined with determinants common to rat and mouse epsilon chains, IgEr was tested for its ability to block the neutralization of IgEm by Rb anti- ϵ r antibodies. Thus, as shown by the data listed in Table XV, addition of increasing amounts of IgEr to a solution of Rb anti- ϵ r and IgEm (at a ratio calculated to yield 100% neutralization in the absence of inhibitor) resulted in increasing recovery of IgEm activity. Thus, addition of 20 μ g of IgEr to the test mixture resulted in almost total recovery of IgEm mediated PCA activity (in the order of 98%).

The antigenic crossreactivity between IgEm and IgEr was investigated in terms of the ability of the Rb anti- ϵ r antibodies to neutralize the PCA activity of MRS. In one series of experiments, Rb anti- ϵ r was mixed with MRS and the mixture was assayed for residual PCA activity. In the other experiments, any soluble complexes of Rb anti- ϵ and IgEm were removed from the mixture before testing for PCA activity, either by precipitation with SARb or by reaction with the immunosorbent SARb-4B. It must be pointed out that although the initial reaction between MRS and Rb anti- ϵ r was the same in these two experiments, different results were obtained in the two systems. From the results plotted in Fig. 15 it is evident that in either experimental system the degree of neutralization of IgEm mediated PCA depended directly on the amount of Rb anti- ϵ r added and that complete neutralization was achieved in both systems. However, at first sight, it would appear

that the Rb anti- ϵ r preparation was more effective in neutralizing IgEm in the absence, rather than in the presence, of SARb or SARb-4B. The basis for this apparent difference in the capacity of Rb anti- ϵ r to neutralize IgEm, depending on whether or not Rb anti- ϵ r was removed after complexing to IgEm, will be discussed later.

Summary

- i) Several anti- ϵ antisera were prepared which were capable of neutralizing IgEm mediated PCA. Each of these antisera was appropriately absorbed to render it ϵ -specific and none formed a detectable precipitate when mixed with NMS.
- ii) Antigenic similarity was demonstrated between murine and rat epsilon chains. However, the degree of crossreactivity determined by cross-neutralization experiments appeared to be less than 4% and the affinity for IgEm of antisera raised against IgEr was found to be low.
- iii) Although the specific activity for MRS of the anti- ϵ r antibody preparations, as indicated by NQ, was low when compared to RRS, they nevertheless were the most active antibody preparations available. For example an NQ > 20 was never seen with the anti- ϵ m antibody preparations examined, while the HARE antibody preparation gave an NQ value of 175 (see Table XIV).

TABLE XIII

Inhibition of Heterologous and Homologous IgE_m Mediated PCA
by IgE_r

Expt. No.	Inhibitor	Amounts of IgE Added (μg)	Heterologous PCA Titer	% Inhibition ^a of PCA
1	NIL	NIL	2150 ^b	0
1	IgE _r	8	2150	0
1	IgE _r	17	2150 ^c	0
1	IgE _r	34	2150 ^c	0
1	IgE _r	51	<10	100
1	IgE _r	68	<10	100
2	NIL	NIL	5800 ^b	0
2	NRtIg	50	5800	0
2	NRtIg	100	5800	0
2	NRtIg	200	4000	31
			Homologous PCA Titer	% Inhibition ^a of PCA
3	NIL	NIL	142	0
3	NRtIg	250	133	6
3	NRtIg	1000	122	15
3	IgE _r	8	115	19
3	IgE _r	25	32	76
3	IgE _r	50	<10	100

^a% Inhibition = $\left[1 - \frac{\text{PCA titer in the presence of inhibitor}}{\text{PCA titer in the absence of inhibitor}}\right] \times 100\%$

^bThe variation of PCA titers of the controls of expt. 1 and 2 is due to the fact that different groups of animals were used in these two experiments.

^cThe intensity of the blue color of these PCA reactions was much less than that of the controls, but the diameter of the reaction was equal to that of the control.

TABLE XIV

Comparison of the Capacity of Anti- ϵ_r Antibodies
to Neutralize IgE_m and IgE_r Mediated PCA

Anti- ϵ_r Ig ^a Preparations	NQ Values for		
	MRS	RRS ^b	% Crossreaction ^c
H A R E	60	2000	3.3
D A R E	43	13833	0.3
S H A R E	175	13833	1.26

^aHorse Anti- ϵ_r (HARE); dog anti- ϵ_r (DARE); and sheep anti- ϵ_r (SHARE)

^bPCA titers in rats obtained by homologous PCA in Long Evans rats as described by Ekramoddoullah et al (336).

^cCalculated by dividing the NQ for MRS by the NQ for RRS, i.e.,

$$\frac{\text{NQ against MRS}}{\text{NQ against RRS}} \times 100\%$$

TABLE XV

The Ability of IgE_r^a to Block the Capacity of Anti- ϵ_r to
Neutralize IgE_m Mediated PCA

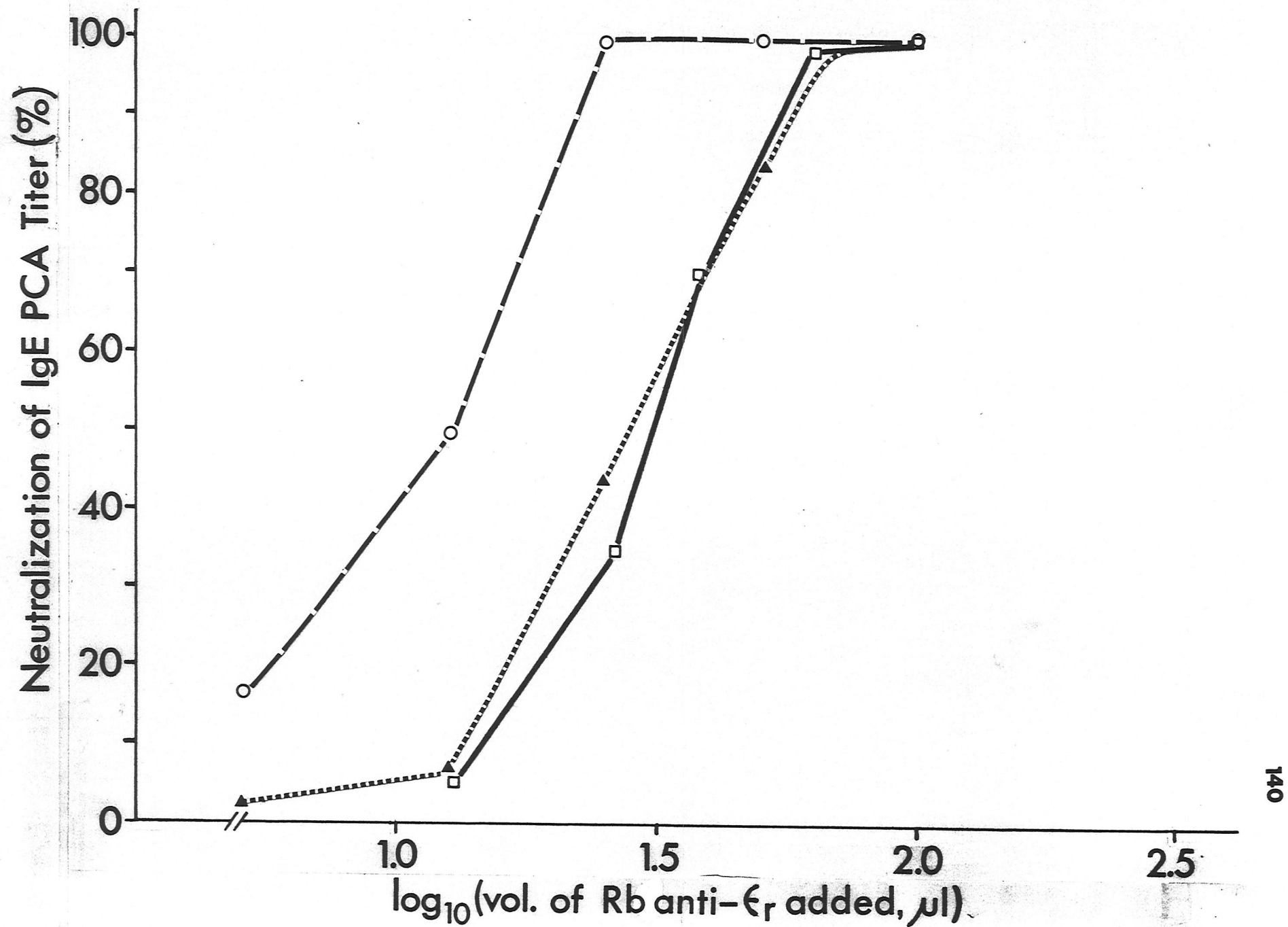
Inhibitor	Amount of Inhibitor Added (g)	PCA Titer ^b	% Recovery of PCA Activity ^c
NIL	NIL	415	0
NR Ig	40	650	7
NR Ig	400	510	3
IgE_r	0.4	560	6
IgE_r	4.0	1600	49
IgE_r	9.0	2600	89
IgE_r	20.0	2800	98

^aIsolated from ascitic fluids from rats bearing immunocytoma IR-331.

^bThe maximum heterologous PCA titer of the MRS standard was 2850 and represents 100% PCA activity.

$$\% \text{ recovery of PCA} = \left[\begin{array}{l} (\% \text{ neutralization of } \text{IgE}_m \text{ in the} \\ \text{absence of inhibitors}) - (\% \text{ neutralization} \\ \text{of } \text{IgE}_m \text{ in the presence of inhibitors}). \end{array} \right]$$

Figure 15. Neutralization of IgE_m by a Rb anti- ϵ_r Ig preparation in the absence and in the presence of SARb or SARb-4B. Increasing volumes of Rb anti- ϵ_r were mixed with 25 μl volumes of MRS and the final volume was made up to 1.0 ml with PBS. To these mixtures was added (a) an amount of SARb necessary to precipitate all of the rabbit immunoglobulin ($\square$ — $\square$) or (b) an amount of SARb-4B sufficient to remove all the rabbit immunoglobulins from the solution ($\blacktriangle$ ---- $\blacktriangle$), or (c) an amount of unconjugated Sepharose-4B equivalent to SARb-4B ($\bigcirc$ ---- $\bigcirc$). The precipitate or solid phase adsorbent was removed from each mixture by centrifugation and the supernatant was assayed for IgE_m by PCA in rat skin.



Effect of Administration of Anti- ϵ Antibodies to Neonatal Mice on the
Production of Anti-OA Antibodies

IgE responses as a function of age in SW and B6D2F1 mice

The IgE antibody response is known to be suppressible by the administration of unrelated antigens (337). Hence, to minimize the non-specific inhibition of IgE production in neonates by the injection of heterologous proteins, a search was made for a mouse strain which gave the highest IgE response, in anticipation that the relative effects of specific to non-specific inhibition would be still discernible in this type of strain. The non-specific inhibitory effect produced by the administration of foreign serum protein on IgE antibody synthesis in low responder strains was expected to mask completely any specific downregulation of the response. The two strains of mice chosen for these studies were the SW and B6D2F1; the former strain was selected because it had been used in the earlier experiments in this investigation and the latter because Vaz et al (338) had reported (at the time of initiation of these experiments) that B6D2F1 mice were among the highest responders of the various strains examined by them. These two strains were compared with respect to their ability to mount the highest IgE anti-OA responses and the earliest age at which each strain produced an IgE antibody response. In exploratory experiments, the littermates of each strain at different ages, ranging from 4 to 56 days after birth, were immunized with 1 μ g OA and Al(OH)₃. All the mice in each litter were treated in the same manner, and were bled 14 and 21 days after immunization. The blood collected at these times was pooled and the

levels of circulating IgE and IgG1 anti-OA antibodies were determined by PCA. In Table XVI are listed the maximum IgE and IgG1 anti-OA antibody titers obtained as a function of age when immunized. As can be seen, the PCA titers tended to increase with age and the IgE PCA titers were generally higher in B6D2F1 mice. Even the youngest mice tested were able to generate an IgE antibody response but not consistently. For further experiments B6D2F1 mice were selected because their responses were higher than that of SW mice.

A more detailed analysis of the primary anti-OA IgE and IgG1 antibody responses at selected ages was carried out by immunizing and bleeding larger numbers of litters with 1 μ g of OA in Al(OH)₃. Beginning with 8 day old B6D2F1 littermates, mice of different ages were immunized with 1 μ g OA and the appropriate dose of Al(OH)₃. Following immunization each mouse was bled at regular intervals by orbital sinus puncture and the bleedings of littermates were pooled for titration of both IgE and IgG1 anti-OA antibodies. The data plotted in Figures 16 and 17 demonstrate that B6D2F1 mice had the capacity to produce both an IgE and IgG1 anti-OA response even after one immunizing injection at all ages examined. It ought to be pointed out that mice immunized 4-7 days after birth showed considerable variation in their responses (IgE titers: < 10-250; IgG1; < 10-140) and that the IgE titers of older mice showed less variation and, as is evident from Figure 16, were higher and peaked earlier after immunization than those of young mice. Moreover, it appears that whereas the IgE anti-OA antibody response of adult mice declined after reaching peak levels, the IgE response in the younger animals reached gradually

TABLE XVI
IgE and IgG1 Anti-OA PCA Titers of SW
and B6D2F1 Mice at Various ages

Age When Immunized ^a (days)	Anti-OA PCA Titers			
	IgE		IgG1	
	SW	B6D2F1	SW	B6D2F1
4	70	40; 250; 0 ^b	0	10;140;0
5	0	0	0	N.D. ^c
6	N.D.	130	N.D.	20
7	50	N.D.	40	N.D.
8	190	2020	20	340
15	320	1000	200	150
22	250	200	200	100
42	1350	4000	400	310
Adult	N.D.	40000	N.D.	600

^aEach mouse in a litter received 1 μ g of OA in Al(OH)₃ on the same day.

^bLitters were bled on 14 and 21 days after immunization, the serum was pooled and then titrated. The titers listed represent the highest titer obtained on the two days examined for each litter.

^cN.D. = not determined.

a plateau level, which was maintained for a period of at least 10 days. Furthermore, although the IgG1 titers at a given time were significantly lower than the IgE titers, the kinetic features of the IgG1 response were similar to those of the IgE response. In further experiments designed to establish the specificity of the administration of anti- ϵ preparations, mice not less than 16 days old were chosen for immunization, unless otherwise stated.

Non-specific suppression of the IgE antibody response

Exploratory experiments in adult B6D2F1 mice suggested that the anti-OA IgE response could be suppressed by three injections of the guinea pig anti- ϵ m given on three consecutive days (data not shown). Therefore, littermates 8 to 13 days old were injected i.p. on three consecutive days with 200 μ l of saline, 200 μ l containing 1.4 mg of guinea pig anti- ϵ m Ig preparation, normal guinea pig Ig, or normal rabbit Ig. Coincident with the second injection all mice were immunized with 1 μ g OA in $Al(OH)_3$. Twelve days later blood samples were obtained from each mouse and the serum tested for the presence of IgE anti-OA antibody. From the results listed in Table XVII it is evident that while 35 out of 44 mice (80%), which had received saline alone, were able to mount an IgE response, not one of the mice receiving Ig responded. These results were similar to the observations of Hargis and Malkiel (337) in that the IgE responses were readily dampened by the presence of normal goat Igs. To establish some of the characteristics of the non-specific depression of the anti-OA IgE response, the experiment was repeated using various concentrations of deaggregated NRbIg from 200 μ g to 1650 μ g per mouse

on each of the three consecutive treatment days; in addition, each litter serum pool was assayed for the presence of IgE antibodies against both OA and NRS. The results of this experiment are plotted in Figure 18, from which it is obvious that administration of NRbIg over the range examined led to a marked depression of the anti-OA IgE response and resulted in the induction of an IgE anti-NRbIg response in all animals.

Since the depressive effect of NRS on the IgE anti-OA response was not affected significantly by reducing the quantity of protein injected, experiments were performed to establish if this effect could be avoided by introducing a longer time interval between injection of the Ig and immunization with 1 μ g OA in Al(OH)₃. To this end, groups of littermates were injected with either saline, a rabbit anti- μ Ig preparation, normal guinea pig Ig (Ngplg), or a guinea pig anti- ξ m Ig preparation for 3 consecutive days, and four days later animals were injected with 1 μ g of OA in Al(OH)₃. All mice were bled 12 and 23 days after immunization the sera of littermates were pooled and assayed for IgE antibodies. In Figure 19 are plotted the anti-OA IgE PCA titers, from which it can be seen that the insertion of a time interval between the injection of the Ig preparation and OA reduced the non-specific suppressive effect of normal Ig (compare the PCA titers of the saline group with the Ngplg group) such that by day 23 there was little difference between the two. It can be seen also that there was an indication that this preparation of guinea pig anti- ξ m induced a more pronounced suppression of the IgE response than did Ngplg. Thus, the administration of anti- ξ m antibodies reduced the day

TABLE XVII

The Effect of Heterologous Igs on the
Anti-OA IgE antibody Response

Treatment ^a	Total No. of Mice	No. of Mice ^b Producing Anti-OA IgE Antibody
Saline	44	35
Normal Guinea pig Ig	13	0
Normal rabbit Ig	9	0
Guinea pig anti- ϵ_m	16	0

^aAll mice in one litter received the same treatment. Mice (8-13 days old) received i.p. on three consecutive days 200 μ l of saline or 200 μ l containing 1.4 mg of a guinea pig anti- ϵ_m Ig preparation, normal guinea pig Ig or normal rabbit Ig. On the second treatment day each mouse received also 1 μ g OA in Al(OH)₃ i.p.

^bAnti-OA IgE positive sera 12 days after immunization.

Figure 16. Age response curves depicting the primary B6D2F1 IgE anti-OA antibody responses at various times after birth. Each age group was immunized with 1 μ g of OA in Al(OH)₃. Each point represents a pool of serum from 5 mice.

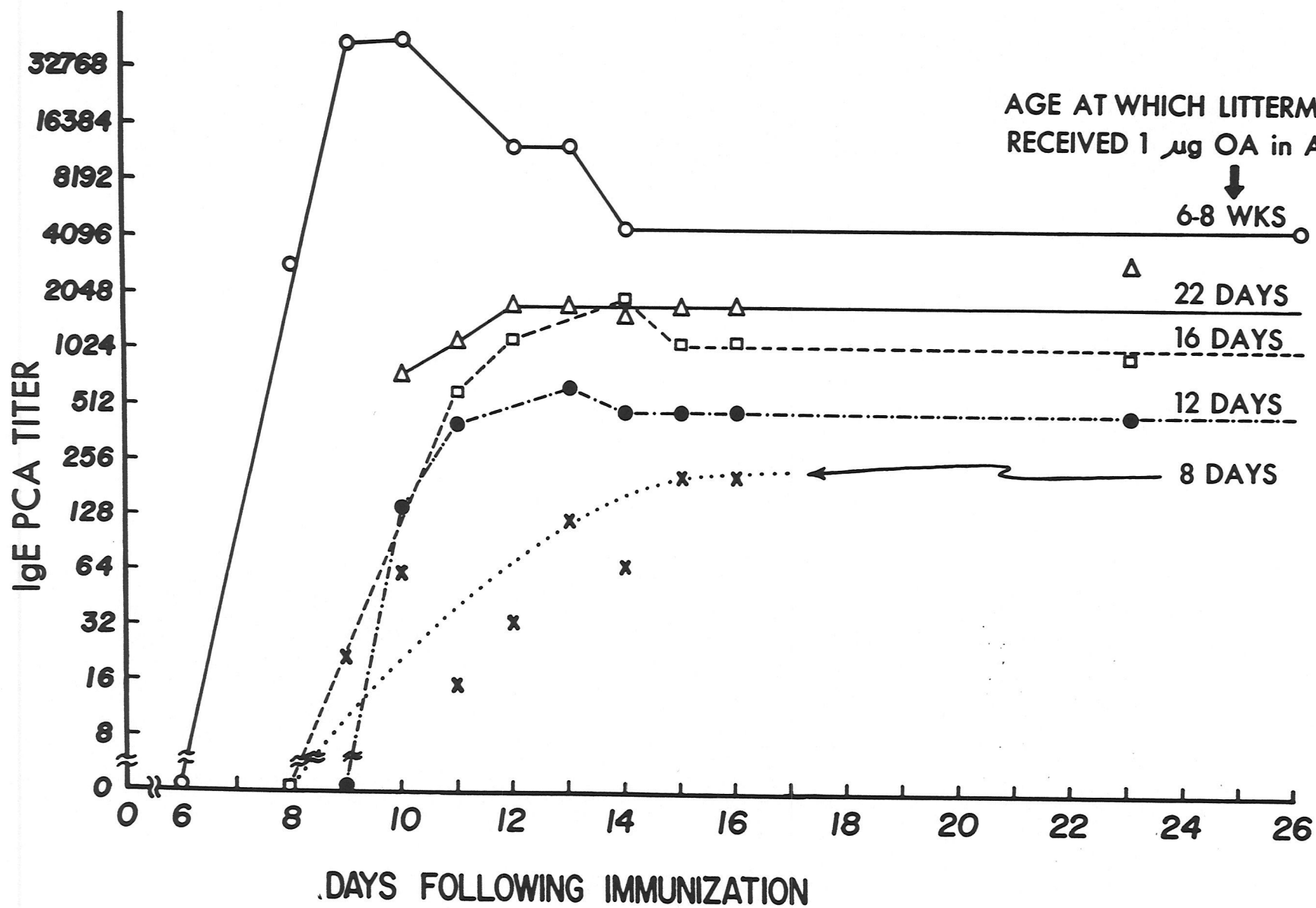
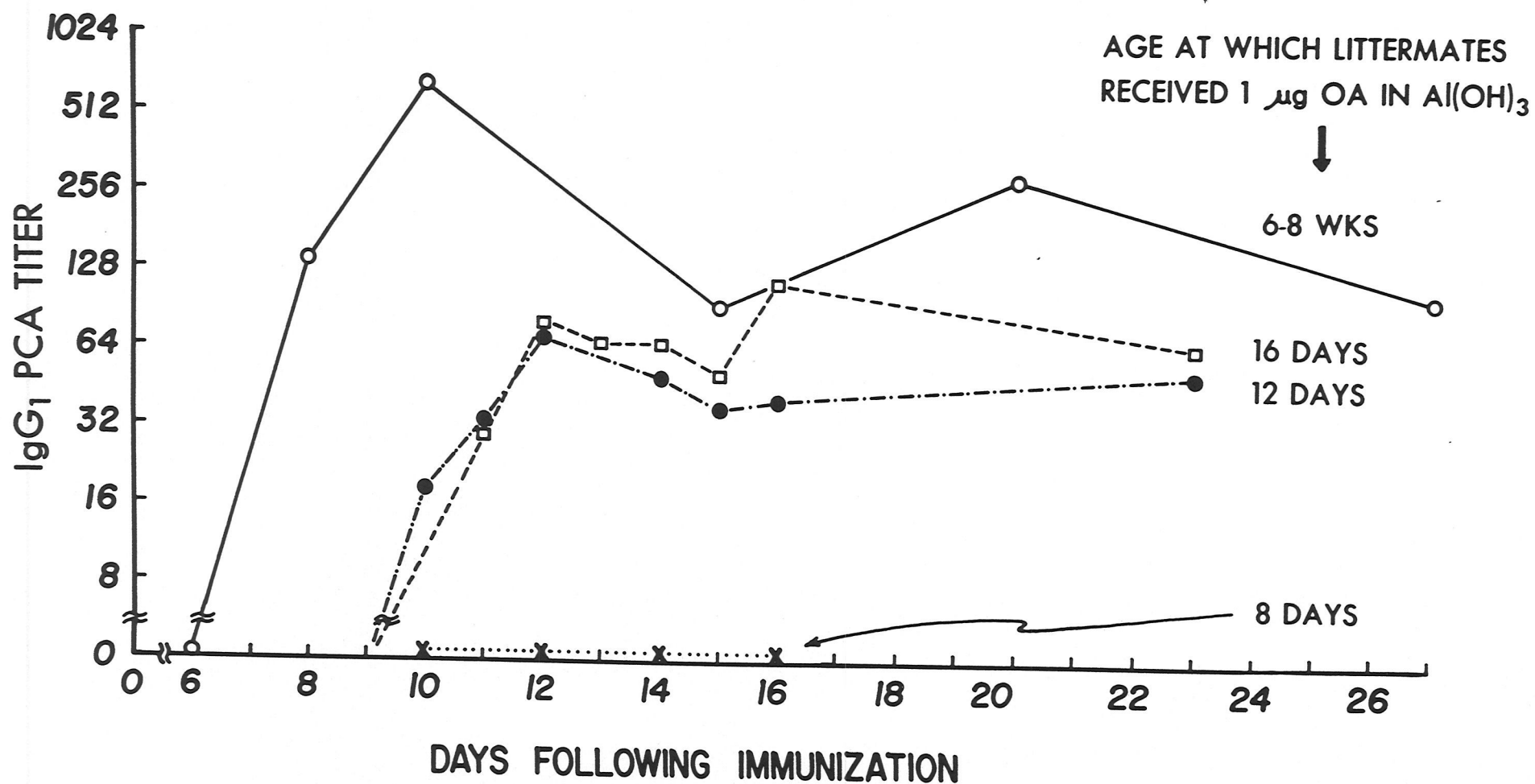


Figure 17. Age response curves depicting the primary B6D2F1 IgG1 anti-OA antibody response at various times after birth. Each group was immunized with 1 μ g of OA in $\text{Al}(\text{OH})_3$. Each point represents a pool of serum from 5 mice.



12 IgE anti-OA response to 20% of the response of mice treated with Ngplg. More to the point, examination of individual serum samples showed that this 20% figure was contributed by only 1 of the 13 mice treated with anti- ϵ m antibodies for it was the only mouse with a demonstrable anti-OA IgE titer compared to 13 out of 15 mice which had received Ngplg. Moreover, as is evident from the right-hand diagram in Figure 19 the depressive effect of the anti- ϵ m antibodies was transient and decreased with time, such that the IgE anti-OA responses were higher on day 23 than on day 12.

In contrast to the anti- ϵ treated group, the IgE response in the anti- μ treated mice was severely suppressed over the 23 day period examined. In Figure 20 are plotted the day 23 IgG1 anti-OA antibody titers from this same experiment from which it can be seen that only in anti- μ treated mice was the IgG1 antibody response suppressed below the level encountered in the Ngplg and anti- ϵ m treated mice. In conclusion: (i) brief exposure to anti- μ suppressed the primary IgE and IgG1 antibody responses for at least 23 days and (ii) only the IgE anti-OA response was inhibited by anti- ϵ m.

Specific suppression of the IgE response

In an effort to prolong the suppressive effect of the anti- ϵ m preparations, litters were injected within 36 hours of birth and then every 3 days until age 11 days with the most potent anti- ϵ m available (rat anti- ϵ m); control groups were injected with NRtlg. The volume of heterologous Ig was increased by 20 μ l at each injection from an initial 50 μ l dose. All mice were immunized with 1 μ g of OA in Al(OH)₃ at age 16 days and bled 12 and 23 days post-immunization. In Figure

Figure 18. Non-specific suppression of the primary IgE anti-OA antibody response by normal rabbit immunoglobulin (NRbIg) administered i.p. on three consecutive days. The figures on the abscissa indicate the amount of NRbIg injected on each of the three consecutive days. All littermates received the same dose of NRbIg and all mice were immunized with 1 μ g OA in Al(OH)₃ on the second of the three treatment days. Mice were bled on day 12 of the anti-OA immune response, sera from littermates were pooled and assayed for PCA activity against OA and NRb serum proteins.

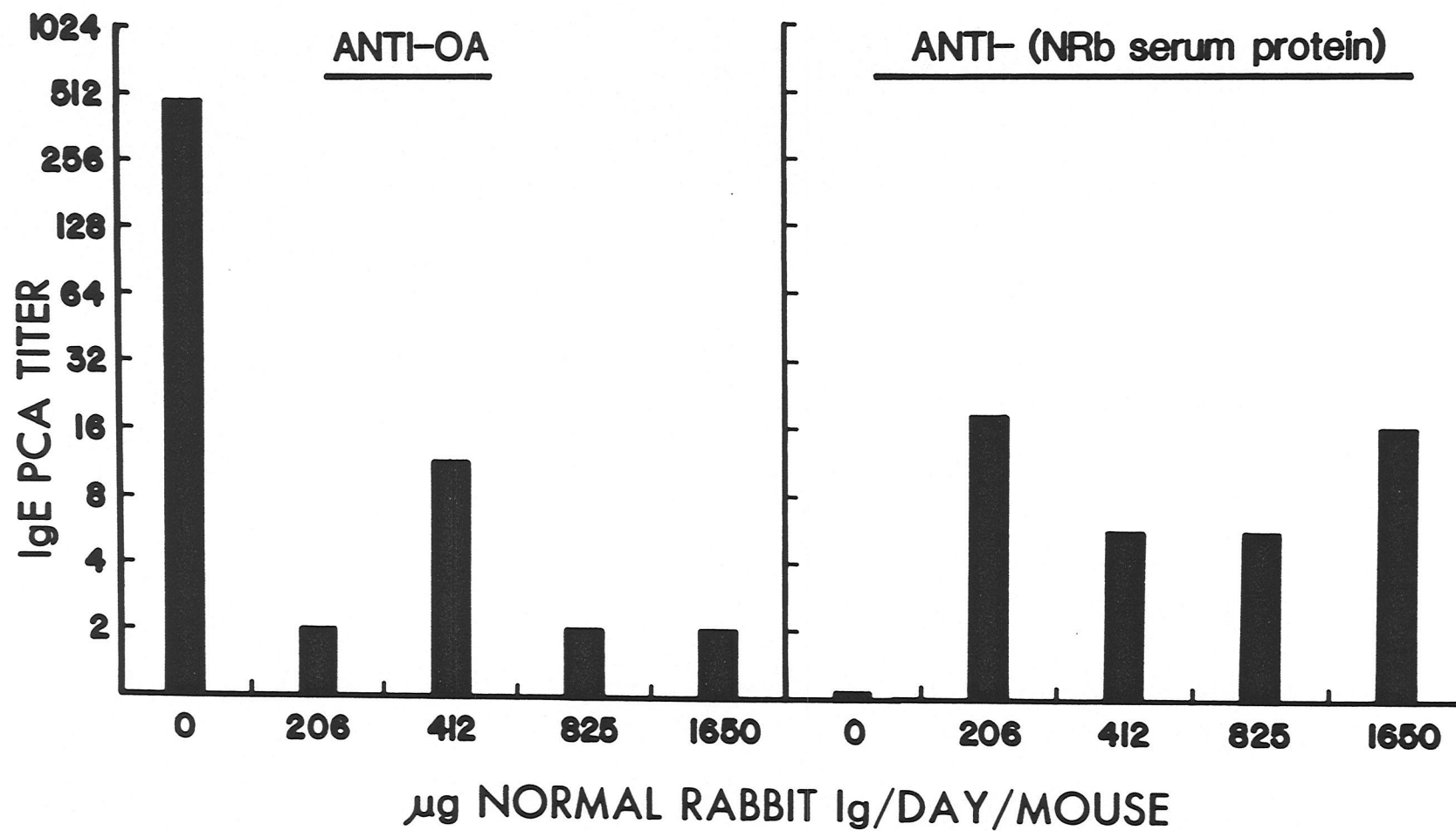


Figure 19. IgE anti-OA PCA titers of mice injected with saline, normal guinea pig Ig (NgpIg), rabbit anti- μ Ig or guinea pig anti- ϵ_m antibody prior to receiving 1 μ g OA in $\text{Al}(\text{OH})_3$. PCA titers were determined on serum pools from littermates.

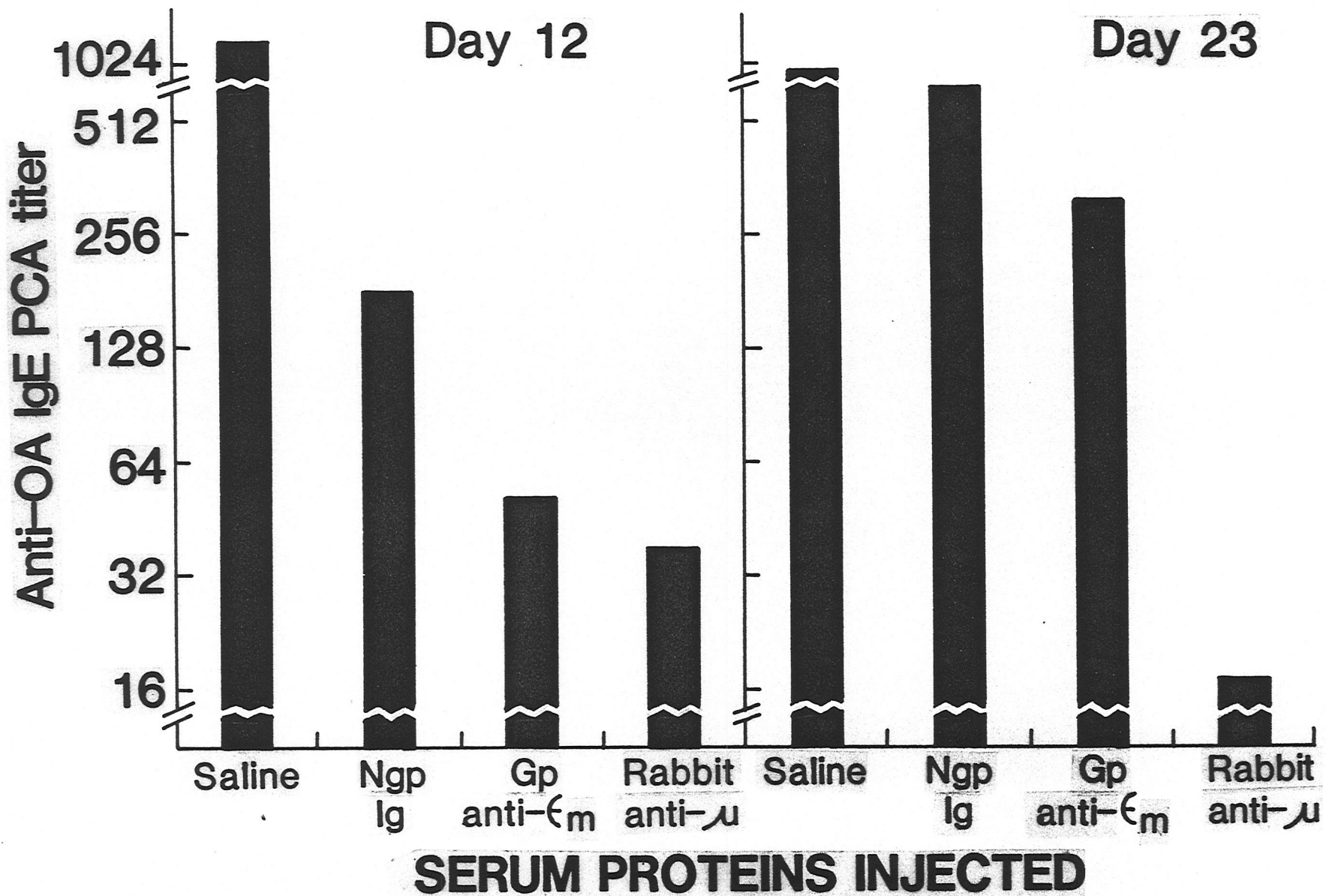
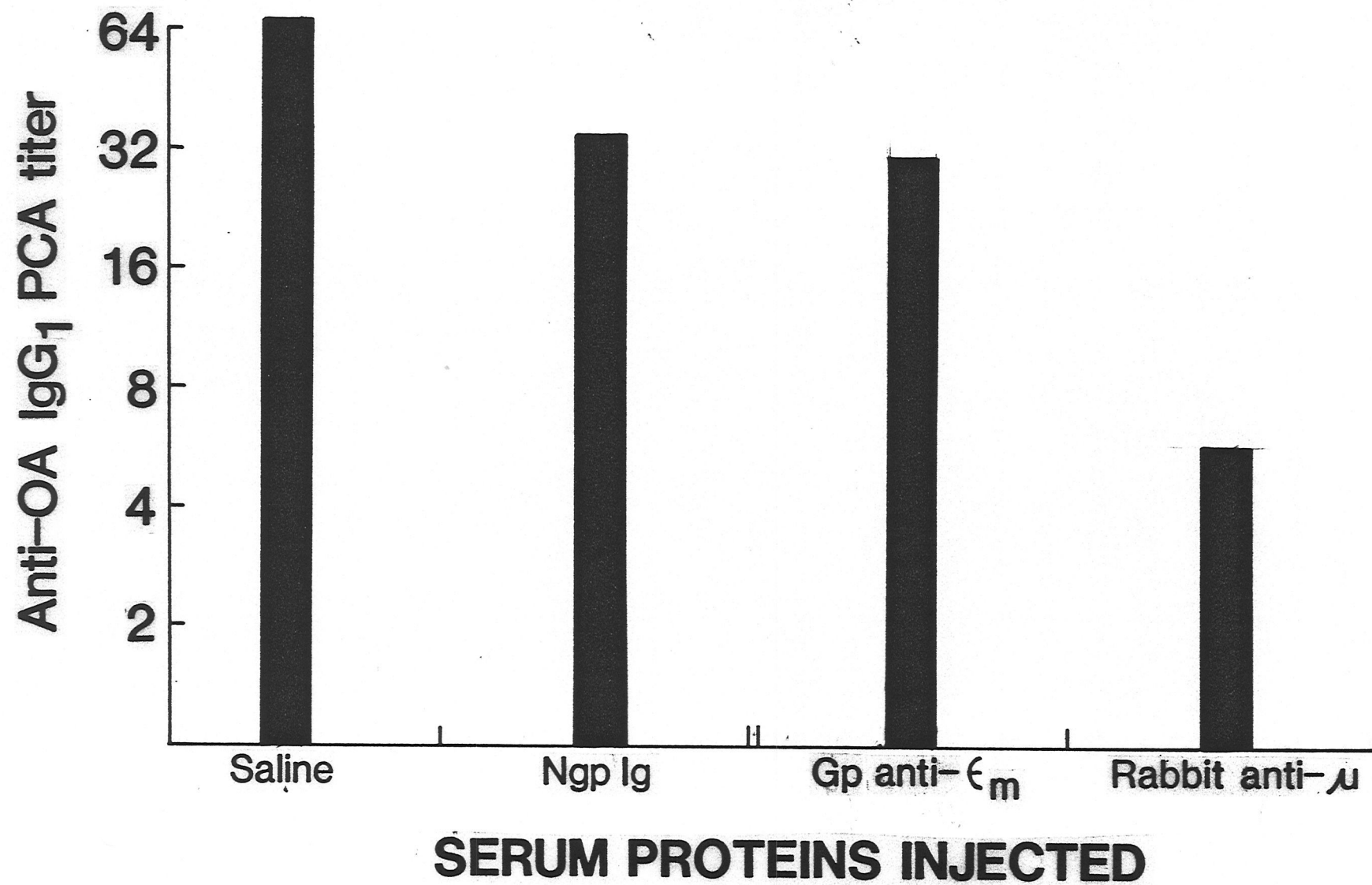


Figure 19. IgE anti-OA PCA titers of mice injected with saline, normal guinea pig Ig (NgpIg), rabbit anti- μ Ig or guinea pig anti- ϵ_m antibody prior to receiving 1 μ g OA in $\text{Al}(\text{OH})_3$. PCA titers were determined on serum pools from littermates.



21 are depicted the IgE and IgG1 anti-OA antibody titers. It is evident that the IgE response was markedly suppressed for the 23 days examined. Also suppressed in mice receiving anti- ξ_m (data not shown) were the IgE antibody responses to NRtlg compared to an anti-NRtlg response in mice receiving NRtlg. Interestingly, the IgG1 anti-OA response was transiently suppressed in this experiment, a phenomenon which may be attributable to trace amounts of anti- μ . The demonstration of antigenic similarities between IgEm and IgEr (303) prompted an investigation of the suppressive abilities of anti- ξ_r .

Suppression of Ig synthesis by anti-(heavy chain) antisera is critically dependent upon the amount of antibody administered (235). In published studies involving immunosuppression by anti- μ , anti- δ or anti- α antibodies, the concentration of specific antibody was determined by quantitative immunoassay. However, in our studies, measurement of anti- ξ activity depended entirely upon the capacity of these antibodies to interfere with the biologic expression of IgEm, e.g., neutralization of IgEm mediated PCA (303,304). A comparative titration (Fig. 22) of the three anti- ξ Ig preparations selected for suppression of the IgEm anti-OA response (dog anti- ξ_r , rat anti- ξ_m and sheep anti- ξ_r) revealed that less dog anti- ξ_r was required to neutralize 40 μ l of MRS than either rat anti- ξ_m or sheep anti- ξ_r , respectively. Littermates were injected 7 days after birth and then every two days until they were 17 days old with either anti- ξ_r , anti- ξ_m or normal Ig. The volume of Ig injected was increased on a daily basis by 20 μ l from an initial 100 μ l. All mice were immunized with 1 μ g OA in Al(OH)₃ 4 days later and bled 12 and 21 days post-immuniza-

Figure 21. Suppresssion of the primary B6D2F1 IgE anti-OA antibody response by rat anti- ϵ_m antibodies. Mice received either normal rat Ig or rat anti- ϵ_m every three days from birth until day 11 and then received 1 μ g of OA 6 days later. Each bleeding represents a serum pool from littermates. Both IgE () and IgG1 () anti-OA PCA titers were determined on each serum pool.

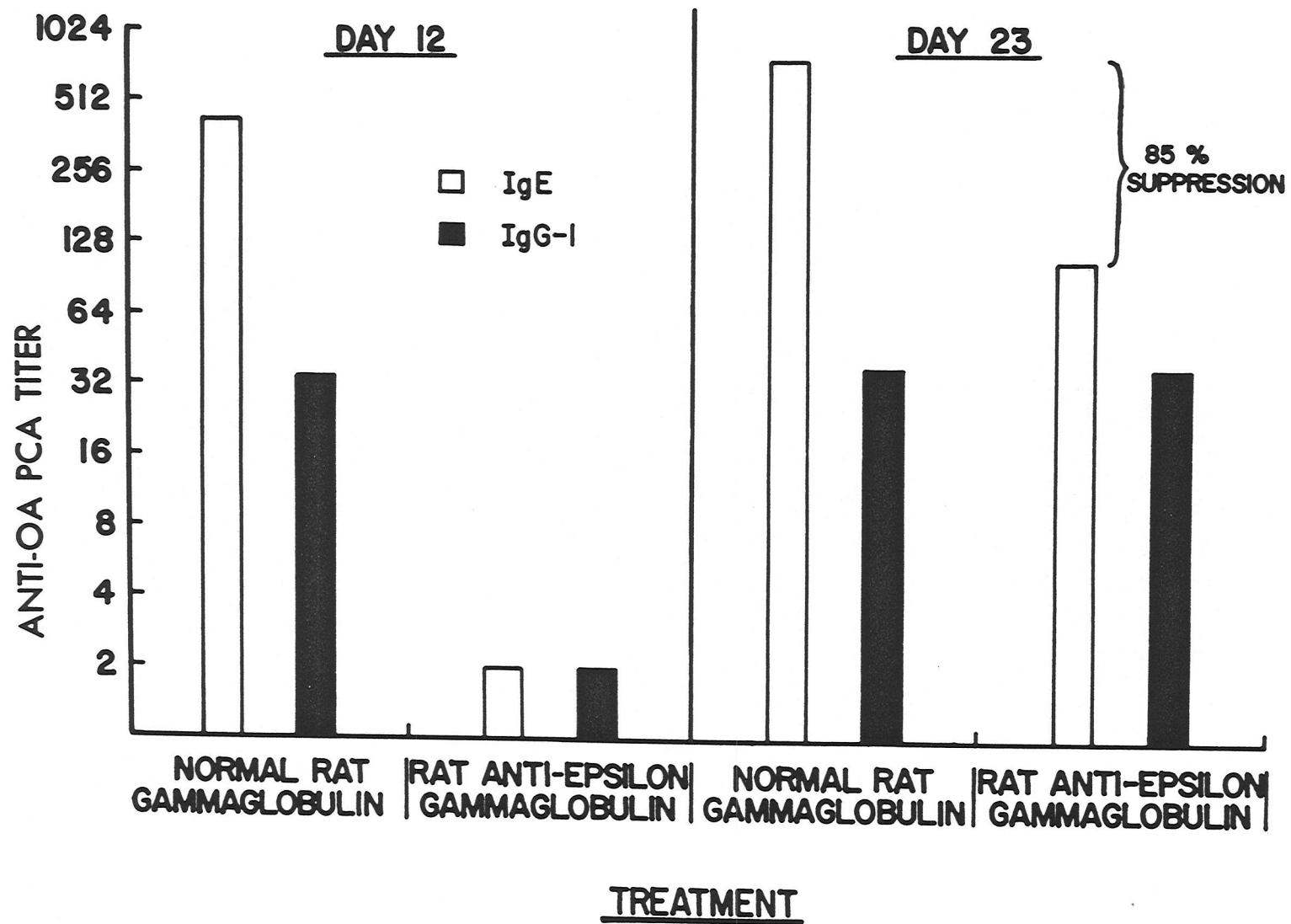
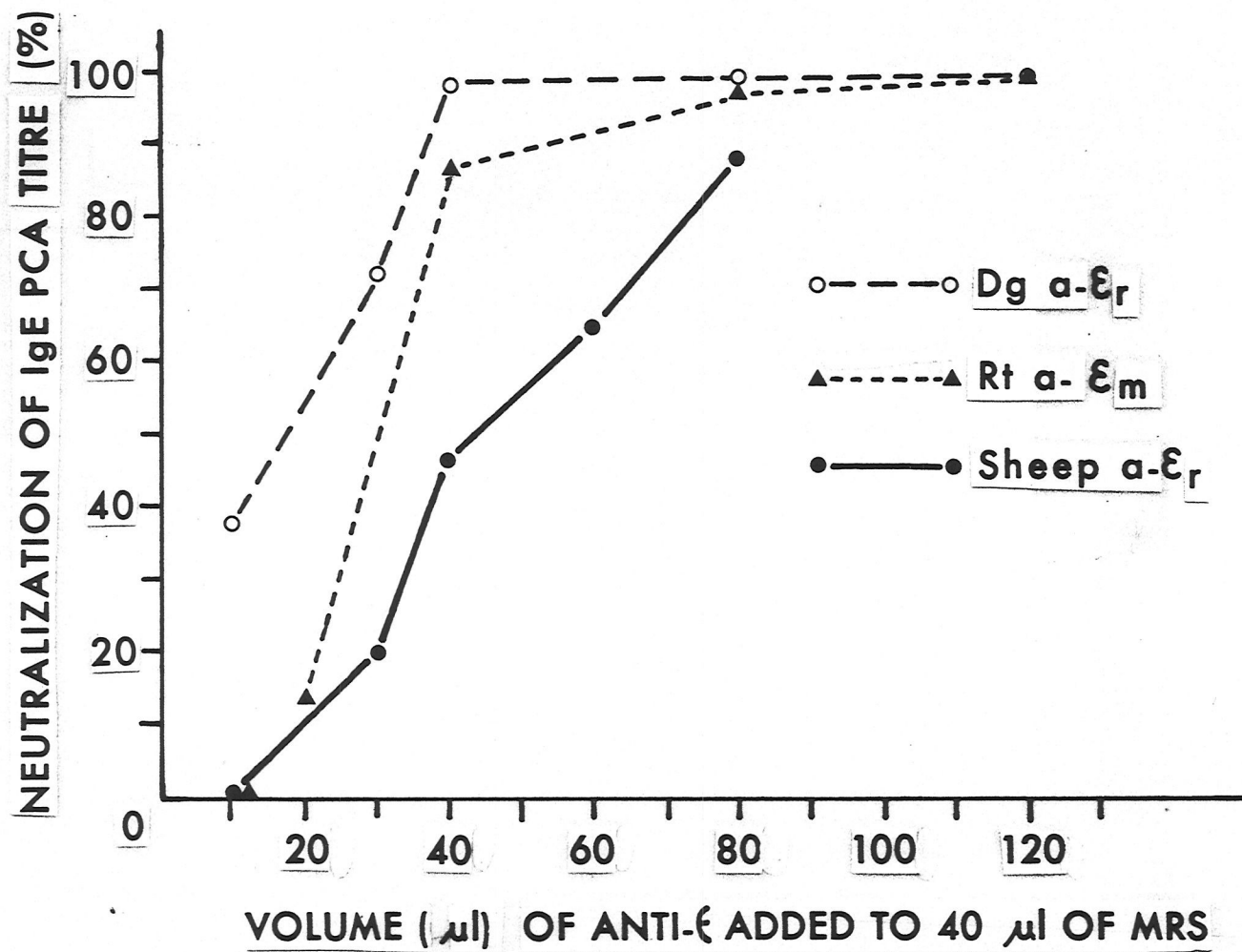


Figure 22. Titration curves of three anti- ϵ antisera used in suppression experiments. Various volumes of either dog anti- ϵ_r (O----O), sheep anti- ϵ_r (●——●) or rat anti- ϵ_m (▲-----▲) were incubated with 40 μ l of a standard mouse IgE anti-OA for 1 hour at 37°C and then overnight at 4°C. The % neutralization was calculated by relating the residual anti-OA IgE PCA titer to that of the standard according to the following formula:

$$\left[1 - \frac{\text{PCA titer in the presence of anti-}\epsilon}{\text{PCA titer in the absence of anti-}\epsilon} \right] \times 100\%$$



tion. In Table XVIII are listed the IgE and IgG1 anti-OA PCA titers from these mice. These results demonstrate that dog anti- ξ r antibody treatment completely suppressed mouse IgE expression for the 21 days examined and was more effective than either sheep anti- ξ r or rat anti- ξ_M , i.e., none of the antisera suppressed the IgG1 antibody response. The effect mediated by the sheep anti- ξ r was not striking and is reflected in the lower neutralization capacity of this antiserum (Fig. 22). Evidently the capacities of the anti- ξ antisera to neutralize in vitro correspond to their capacity to suppress IgE anti-OA production in vivo. It is clear that under appropriate conditions complete expression of murine IgE production can be achieved.

Summary

- a) It was possible to suppress IgE antibody production with anti- ξ antibody. The suppression was isotype specific and appeared to be directly related to the amount of anti- ξ administered and was more effective when given perinatally.
- b) Compared to other Ig isotypes, the IgE antibody response is easily suppressed by non-specific mechanisms. To reduce the magnitude of this non-specific suppression a period of several days was allowed to elapse after the last anti- ξ injection. Despite this requirement, which would tend to reduce the effects of the anti- ξ antibodies, one of the anti- ξ preparations, nevertheless, caused 100% suppression of the IgE response.

TABLE XVIII

Effect of Various Anti- ϵ Preparations on the
IgE and IgG1 anti-OA Responses of Young B6D2F1

Experiment No.	Treatment ^a	Anti-OA PCA Titers			
		Day 12		Day 21	
		IgE	IgG1	IgE	IgG1
1	Saline	2080	90	2300	640
2	Saline	600	90	2560	100
3	NShIg	1200	70	600	120
4	NDgIg	3000	100	1350	400
5	NDgIg	600	100	1390	600
6	SH anti- ϵ_r	600	70	340	120
7	Sh anti- ϵ_r	430	25	N.D.	N.D.
8	Dg anti- ϵ_r	<10	40	<10	90
9	Rt anti- ϵ_r	230	40	N.D. ^b	190

^aLittermates were injected 7 days after birth and every 2 days until they were 17 days of age with one of the following solutions: Saline, normal sheep immunoglobulin (NShIg), normal dog Ig (NDgIg), Sheep anti-rat IgE (Sh anti- ϵ_r), dog anti-rat IgE (Dg anti- ϵ_r) or rat anti-mouse IgE (Rt anti- ϵ_m). All mice were immunized against 1 μ g OA in Al(OH)₃ four days later.

^bN.D. = not determined.

DISCUSSION

The Manifestation of IgG1 and IgE in PCA Assays

An important observation concerning the roles of IgG1 and IgEm in PCA reactions arose from an experiment in which MRS was treated with an anti- γ 1 antiserum to remove the bulk of the IgG1 activity. Untreated serum was found to exhibit homologous PCA activity continuously from 4 hours to 72 hours with no clear cut distinction between IgG1 and IgE mediated PCA. The treated MRS, on the other hand, demonstrated two distinct optima: one with a maximum at between 4 to 6 hours associated with IgG1 activity and the other with a maximum occurring between 24 and 48 hours. The unexpected finding was that the IgEm mediated PCA did not manifest itself before 10 to 16 hours. The inability of IgEm to mediate PCA in homologous skin until 16 hours after sensitization contrasts with its ability to render heterologous rat skin triggerable for almost maximal PCA titers after only 2 hours. Two mechanisms may be postulated to account for the latent period in homologous IgEm mediated PCA.

1. IgG1 and IgG2 might be retained in the skin site after passive transfer by virtue of weak interactions with Fc ϵ -r receptors in receptors in the host's skin. If this were the case then binding of IgEm to adjacent Fc ϵ -receptors on murine skin mast cells would be interfered with by the relatively high local

IgG1 and IgG2 levels present at the injection site. The ability of injected IgG to inhibit IgEm mediated PCA would be expected to decline with time as a result of diffusion of the IgG from the injection site and the rearrangement of the retained IgEm by virtue of its higher affinity for the Fc ϵ -receptor. In the light of recent data obtained with monoclonal IgG1 and monoclonal IgEm, it appears that Fc ϵ -receptors for IgG1 and IgEm are functionally independent (340, 341) on mouse mast cells, and therefore IgEm mediated homologous PCA may not be competitively inhibited by IgG1. This data however does not rule out the possibility that weak interactions between IgG1 and the Fc ϵ -receptor can be augmented in the skin site within the first 10 hours when local IgG1 concentrations are far higher than IgE such that IgG1 can effectively compete.

2. Alternatively it may be visualized that antibody of the non-IgE isotypes, especially IgG which is present in excess in relation to IgEm, could inhibit the triggering of cell-bound IgE by sequestering the antigen and thus interfering with the establishment of the appropriate IgEm:antigen matrix on the mast cell necessary to unleash the mediators of anaphylaxis. This interpretation would also account for the observation by Mota et al (265) that very early antisera containing primarily IgEm gave homologous PCA after a 2 hour sensitization period, which was not affected by pretreatment of this antiserum with anti- δ 1 antibody, and for the observation by Hutchcroft et al (342) that 2 of 8 sera from allergic humans, whose IgG antibody

was low, were able to induce anaphylactic reactions in monkey skin after a 2 hour sensitization period. Hutchcroft et al succeeded also in sensitizing monkey skin with human allergic serum after 2 hours by removing the IgG with an anti-IgG immunosorbent. It can also be postulated that for IgG to inhibit the expression of IgEm for 10 hours or more that IgG is retained in the skin by binding to tissue Fc-receptors on mast cells (and other cells). Moreover, since IgE expresses itself later, i.e., after the diffusion of IgG out of the site it may be inferred that the affinity of Fc α -receptors for IgG is much lower than the affinity of Fc ϵ -receptors for IgEm. On the basis of these considerations I postulate that IgEm antibody free of IgG antibody should give a PCA reaction in mouse skin after a 2 to 4 hour sensitization period; indeed it was recently observed by Böttcher and Albring (340) that a 2 hour homologous IgEm mediated PCA reaction was obtained using purified hybridoma IgE.

In the process of characterizing the antibody molecules involved in the 4 to 6 hour homologous and 24 hours heterologous PCA reactions it was found, contrary to expectation, that the former was sensitive to heating at 56°C (Table V). Heat lability has been reported frequently as a unique property of IgE antibodies and as such was originally used as the principal criterion for distinguishing IgE from other classes of anaphylactic antibodies. However, heat stable forms or subclasses of IgE have been reported in the rabbit (343,344)

and the guinea pig (345). Indeed, new classes of Ig which mediate PCA (346,347) have been proposed on the strength of heat lability. While heat-induced structural changes have been detected in the Fc portion of human IgE (348,349), Clausen et al (268,272), Okudaira et al (350), Prouvost-Danon et al (351,352) and this author (Table IV) have found that dilution of IgEm in PBS prior to heating appeared to reduce the effect. Hence, it is evident that, with respect to IgE, isotype identification cannot be established solely on the basis of the heat lability of a particular biological property, such as fixation to the Fc ϵ -receptor.

From the present study emerge several basic data concerning the PCA reaction in mice and rats using MRS:

1. The minimal amount of OA required to trigger maximal IgE mediated PCA titer in rat skin was ascertained (Figure 4) and found to be of the same order of magnitude as that determined by Ovary (282) for IgG mediated PCA in mouse skin.
2. SW mice were found to be the best recipients for both IgG1 and IgE mediated homologous PCA, compared to BALB/cJ, A/J, AKR/J and CFW strains. In a larger study conducted by de Souza et al (353), in which 13 strains of mice were examined, including A/J and AKR/J, SW mice were also found to be the strain of choice for IgG1 mediated PCA, but not for IgEm mediated PCA, but not for IgEm mediated PCA. Interestingly, these authors found that mouse strains selected for their ability to host the highest IgG1 PCA titers did not necessarily host the highest IgE PCA

titers. For instance, the lowest IgG1 mediated PCA titers and the highest IgE mediated PCA titers were found using AKR/J mice as recipients.

3. PCA reactions in SW mice (Table VII) were more variable than PCA reactions in Long-Evans rats (Table VIII), the former having a standard deviation of 71% of the mean, while the latter only 46% of the mean.
4. The location of the injection site was not found to be a significant factor in PCA titration, which is in agreement with the observation of Okudaira et al (350).

In summary, it was concluded that the data obtained with the 4 hour PCA in mice and 24 hour PCA in rats provided reliable titration of IgG1 and IgE antibodies, respectively.

In terms of the sensitivity of the IgG1 and IgE PCA assays, it should be noted that at the time when this investigation was initiated, the levels of IgE antibody required to give a 5 mm PCA reaction were unknown. Recent work with IgE from hybridoma cell lines has established that the limit of detection by PCA was 100-300 pg (354,355), while others (356) have suggested that as little as 10 pg of IgE can elicit a 5 mm PCA reaction. Ovary has determined that the minimum amount of hybridoma IgG1 required to elicit a homologous 5 mm PCA reaction was 50 to 75 times higher than that for IgE (355). However, using IgG1 antibody from MRS Ovary determined in an earlier study that the minimum amount of IgG1 required to trigger a PCA reaction was 4 ng (357). The reason for this apparent discrepancy may be related to the average affinity of the IgG1 antibody molecules for the corresponding antigen.

Development of the Capacity to Produce IgE Antibodies at Different Ages After Birth

There is a paucity of published data concerning the ability of neonatal mice to generate an IgE response. In the earliest report in 1970 (337), Hargis and Malkiel described the use of tissue sensitizing antibody. They did not identify the class of antibody involved; nevertheless, tests using horse serum as antigen and *Bordetella pertussis* as adjuvant, led them to conclude that acute anaphylactic sensitivity occurred only in adults. In the same year Prouvost-Danon and Binaghi (270), using relatively high doses of hen's egg albumin (250 $\mu\text{g}/\text{mouse}$) in $\text{Al}(\text{OH})_3$ for immunization of a variant of SW mice called Copacabana, at various ages, demonstrated that immunization of 9 day old mice induced IgE and IgG1 antibodies and that the earliest serum antibody of both isotypes was detected at 17 days of age. On the other hand, Imada et al (359) using two-week old C57B1/6 mice were able to detect only IgE antibodies in the absence of IgG or IgM in response to 1 μg of OA in $\text{Al}(\text{OH})_3$ and were thus led to conclude that the capacity to produce IgE antibodies appeared earlier in ontogeny than IgG or IgM. Ngan and Kind (360) showed that immunization of 2 day old mice with OA in Concanavalin A and an alum-pertussis mixture induce anti-OA IgE antibodies detectable at 14 days of age. The results of the present study indicated that injection of 4 day old B6D2F1 mice with 1 μg OA in $\text{Al}(\text{OH})_3$ led to a substantial IgE antibody response which was detectable 12 days later. Although $\text{Al}(\text{OH})_3$ is a potent adjuvant for IgE production, antibody was not detected in the serum of mice before 14 days of age. Hence, it must be concluded that

in the initial two week post-natal period mice lack a system which is crucial to the development of IgE antibody production. Contrary to the findings of Imada et al (359), in the present study it was observed that there was no age at which an IgE response could be elicited at which an IgG1 antibody response could not also be elicited; i.e., developmental processes limiting the expression of antibody were common to both of these isotypes. A systematic appraisal of the possible contributing factors to the delayed onset of responsiveness of IgM and IgG antibody to SRBC in neonatal C57B1/6 mice led Carter and Rector (361) to conclude that one of the causative agents is a relatively immature antigen processing system.

The detection of IgE antibodies in mice of all ages studied preceded the detection of IgG1 antibodies by a few days. This may reflect differences in the sensitivity of the PCA assay which, as discussed above, is an unresolved issue. Alternatively, it may reflect differences in the effectiveness of stimulation of B_E and B₁ cells, i.e. B_E cells may proliferate and differentiate earlier than B₁ cells even though both types of B cells may coexist. Hamaoka et al (362) reported that, whereas B₁ memory cells develop independently of B₂ memory cells, the B₁ cell line was expanded earlier than the B₂ cell line. The reasons for this preferential stimulation are unclear but appear to be related to the T cell dependence of the response. In both of these instances the earliest isotype to be stimulated was the isotype found to have a greater degree of T cell dependence.

Production of High Titered IgEm Antisera

Several techniques, such as induction of ascitic fluid and experimentation with various antigen doses, adjuvant levels and multiple injections, were examined to establish a method for inducing the highest IgEm antibody levels. The IgEm antibody level in immunized mice appeared to decrease after the induction, by FCA injection, of ascitic fluid (Table II). Even though the presence of ascitic fluid greatly increased the volume recovered from an immunized mouse, the yield of IgE was offset by the reduced IgE antibody levels in the fluid probably as a result of the antagonistic effect of FCA: Katz et al (192) demonstrated that FCA induced the production of SFA (see introduction pages 45 to 49) which specifically suppressed IgEm production in all of the mouse strains examined. Ascitic fluid had been examined as a source for IgE by other investigators (271,272,363) and in all cases it has been found in levels lower than in the serum. Lehrer et al (363) compared the total IgE antibody content of ascites fluid to that of serum by normalizing each of the two sources to PCA units, and concluded that with some strains of mice IgE levels in serum and ascites were comparable.

In conclusion the highest IgEm antibody levels were obtained by repeated immunizations with 1.0 μ g OA in 5 mg Al(OH)₃ and in later experiments the aluminum hydroxide level was reduced to 1 mg without detectable loss in adjuvanticity. Serum was used as the sole source of IgEm.

Production of High Titered Anti- ϵ m Antisera

A significant problem intrinsic to this study was the isolation of purified antibodies to IgEm in quantities adequate for experimentation on the immunosuppressive effects of anti- ϵ m antibodies on the production of IgEm. The availability of IgE myelomas has facilitated the production of antibodies to human (322) and rat (335) epsilon chains; however as yet no corresponding IgEm secreting myeloma has been discovered. Nevertheless, it ought to be noted that, some six years since this thesis study was completed, several IgEm-secreting monoclonal antibodies have been produced from appropriate hybridomas created in this and other laboratories (354,364-369). However, the only source of IgEm throughout this study was the serum of immunized mice. By using a combination of ammonium sulfate precipitation, gel filtration and ion exchange chromatography IgEm-enriched fractions were isolated for immunization. Two techniques were successfully utilized (304) to produce anti- ϵ m antibodies in rats, guinea pigs and a goat: one approach investigated in rats involved tolerization of the rats with soluble deaggregated NMS prior to immunization against IgE enriched fractions; the other approach used in guinea pigs and a goat was to simply inject the IgE-enriched fraction without pretreatment with soluble deaggregated NMS. At about the same time, two other methods were published: one by Prouvost-Danon et al (370) and the other by Lehrer (363,371). The former group immunized rats, guinea pigs and rabbits with IgE:anti-IgE precipitin bands which were isolated from agar gels. It is interesting to note that the original

rat anti-IgEm antibodies were produced by immunizing with IgEm bound to rat mast cells (309). Lehrer et al (363) acknowledged the difficulties involved in isolating IgEm from MRS and chose to fractionate large pools of IgEm bearing ascitic fluids by ammonium sulfate precipitation, gel filtration and ion exchange chromatography. In this way Lehrer was able to produce an epsilon specific antiserum in rabbits. One year later, two other groups (372,373) reported the production of anti- ξ m antibodies: (i) Malley et al (372) incubated rabbit anti-NMS with MRS, centrifuged out the resultant precipitates and injected i.v. the IgEm-rich supernatant into rabbits; and (ii) Ito et al (373,374) infected BALB/c mice with *Nippostrongylus brasiliensis* and then immunized with both DNP-OA and Nb. DNP specific IgEm were isolated from the serum pool by ammonium sulfate precipitation, DEAE cellulose column chromatography and gel filtration. Guinea pigs were immunized s.c. with the IgEm containing fraction in FCA. Regardless of the immunization scheme used, all investigators found that their untreated antisera reacted against mouse proteins other than IgEm and, therefore, required absorption against NMS. Malley et al included one other absorption step which involved insolubilized mouse Fab, while Lehrer used three solid-phase immunosorbents, which contained NMS, Fab and mouse Ig. The major difference between this thesis work and the above mentioned results are the claims made by the others that their antisera produced visible precipitin bands against the IgEm contained within the IgE-enriched material. However, without the availability of an IgEm standard whose level of purity had been quantitatively established, it is not possible to state definitively that the

precipitin bands observed were indeed due to IgEm:anti-IgEm complexes. Lehrer did, however, ascertain that one of the constituents of the precipitate crossreacted with murine Fab (363). The positive indications that the Fab absorbed antisera contained anti- ϵ m antibodies were their ability (a) to induce degranulation of IgEm sensitized peritoneal mast cells as outlined by Prouvost-Danon et al (309) and (b) to neutralize IgEm mediated PCA.

As outlined earlier, Prouvost-Danon et al (309) succeeded in producing anti- ϵ m antibodies utilizing RPMC as a carrier of IgEm. This technique involved the selective removal of IgEm from the IgEm-enriched fractions by RPMC and then immunization of rats with the sensitized RPMC. However, remarkably, only one rat (rat A286) of the 4 immunized by Prouvost-Danon et al was able to mount an anti- ϵ m response. It is therefore not surprising that in this thesis study none of the rats immunized with sensitized RPMC responded with detectable anti- ϵ m antibodies.

In general it can be said that while it is possible to elicit an anti- ϵ m response by immunizing with cell bound IgEm, the most successful techniques have involved immunization with IgE-rich fractions.

Neutralization of PCA has been used by all investigators as a means to detect the presence of anti- ϵ m antibodies. These sera must be devoid of anti-light chain or anti-Fab activity for the assay to be meaningful, since as demonstrated in Table XI both anti-k and anti-lg antibodies inhibit PCA. In addition, the data presented in Fig. 15 indicate that the presence of anti- ϵ influenced the assay. It was found that less anti- ϵ was required to neutralize PCA if the anti- ϵ

was not removed. There are two possibilities which account for this observation:

1. Since the administration of anti- ϵ r antibodies into the skin of normal rats has been shown to induce reverse PCA (335), which is in accord with the presence of IgEr on the surface of mast cells of normal rats (375,376), the unbound anti- ϵ in the mixture may have induced mediator release from the IgE bearing mast cells present in the site, leading to a refractory state (377). The normal process of sensitization by IgEm would thus have been disturbed in this site, resulting in a smaller or undetectable PCA reaction which would lead to the paradoxical conclusion that, in the absence of SARb or SARb-4B, a smaller amount of Rb anti- ϵ antibody appeared to be sufficient for neutralization of IgEm mediated PCA.
2. The presence of soluble antigen-antibody complexes may have been an additional factor complicating the interpretation of these experiments. Thus, as is known (378), PCA-like reactions can be elicited by complexes of antigen and antibody, particularly in antigen excess. More importantly, aggregates of IgE have been implicated in the activation of complement through the alternate pathway (27). Hence, soluble immune aggregates containing IgEm may have partially desensitized the skin sites used for testing the presence of residual IgEm and may have thus interfered with the subsequent PCA reaction. This alternative mechanism may have, therefore, contributed to the apparently lower concentration of Rb anti- ϵ r antibodies required to neutralize IgEm in absence of SARb.

Consequently, for an unambiguous evaluation of the strength of the anti- ϵ antibody preparations, it is essential to use the double antibody procedure described in this study.

As described previously, several investigators claimed that their anti- ϵ m antisera had the capacity to form visible precipitates in agar gels against IgE containing samples. The reliability of these observations is dependent upon the level of IgEm actually present in NMS and MRS. Prouvost-Danon et al (370) calculated that the IgEm levels in 2 month old normal Swiss mice ranged from 500 to 2000 ng per ml, and that MRS contained 5000 to 50,000 ng per ml. It may be noted that serum IgE levels in normal rats fall also within the range of 500 to 2000 ng per ml (379,380). The assay described by Prouvost-Danon et al was based on the ability of known amounts of IgEr to inhibit the in vitro sensitization of mouse peritoneal mast cells by murine anti-OA IgE antibodies. The fact that inhibition plots in which various concentrations of IgEr were used as a standard for IgEm gave parallel linear curves when compared to solutions containing IgEm was used by Prouvost-Danon et al as an argument for the direct conversion of the levels of inhibition effected by IgEm to absolute IgEm levels. This conclusion is, however, considered to be erroneous since without an IgEm standard with which to assess the performance of IgEm at known concentrations, as discussed by Midgley et al (381), the finding of parallelism does not prove identity of equilibrium constants. Therefore, only if the lines are superimposable by virtue of a knowledge of the exact quantity of IgEm can this test yield unequivocal results.

Lehrer has estimated that the concentration of IgEm in NMS is at least 1000 ng/ml (382) based on his assay which indicated that 987 μ g of IgEm could be isolated from 855 ml of NMS, and in a recent article by Lehrer (387) in which he described an enzyme-linked immunoadsorbent assay for total IgEm levels, NMS contained 12-18 μ g of IgEm per ml. However, other investigators using radioimmunoassays (383,384) have found much lower IgEm levels. Indeed a competitive inhibition assay which developed out of this thesis study (385) established that NMS contained less than 5 ng of IgEm per ml. More recently, an RIA for total IgEm levels has substantiated this lower IgE level (386). The assays mentioned above for quantitation of IgEm levels are deficient in that they (i) lack an IgEm standard and (ii) utilize antisera from which anti-contaminant activity may not have been completely adsorbed. Consequently, until assays exist which employ specifically relevant anti- ϵ r antibodies which had been isolated on IgEm-immunosorbents, high values for IgE are subject to the criticism that the sample being assayed contains, in addition to IgE, other molecules to which the anti- ϵ m preparation is reactive.

It was found that an antigenic similarity existed, albeit a weak one, between IgEr and IgEm. These experimental findings were published (303) and confirmed by Prouvost-Danon et al (370) and Lehrer (371). Murine and rat IgE must therefore share common structural features which are responsible, on the one hand, for the biological properties of these Igs manifested in terms of their binding to RBL cells and mast cells in both species and, on the other, for their antigenic properties reflected in their competitive binding to anti- ϵ antibod-

ies. However, it is not possible to state at this time if identical or different regions of the two ϵ -chains are responsible for these distinct properties.

Suppression of IgEm Responses by Anti- ϵ Antisera

The suppression by antibodies to a given Ig H-chain of the corresponding antibody response is critically dependent upon the amount of antibody administered (235). In previous studies in which anti- δ , anti- μ or anti- α antisera were used, the specific antibody content was conveniently determined by radioimmunoassay or by radialimmunodiffusion. Regrettably, at the time when the experiments described herein were performed there was no known IgE myeloma. Hence the anti- ϵ antisera prepared for this study could be assayed only by their ability to neutralize IgEm mediated PCA. As a consequence of this limitation, it is conceivable that the levels of anti- ϵ antibodies injected into neonatal animals were quite different from the doses of anti- μ , anti- δ or anti- α used by other investigators. Nevertheless, specific suppression of the primary IgE anti-OA antibody response was obtained. Moreover, the amount and duration of this suppression was dependent upon the amount of anti- ϵ antibodies administered and the age of the animal at the initiation of the treatment regimen, i.e., parameters also affecting the suppression mediated by anti- μ , anti- δ and anti- α antibodies (240,243,388). The IgEm response was shown to be sensitive not only to anti- μ and anti- ϵ antibodies but also to the presence of heterologous proteins. Hargis

and Malkiel (337) were the first to report the ability of soluble heterologous proteins to suppress the production of acute anaphylactic sensitivity in mice by reducing the production of IgE in the treated mice. Manning et al (235,389) also describe a non-specific inhibition which interfered with their ability to study the specific effects of neonatal administration of anti- μ antibodies on the production of IgEm antibodies. Both of these groups found that normal serum was as effective as the specific antisera in suppressing the production of IgEm antibodies to protein antigens. The possibility that the non-specific suppression observed in this thesis study was the result of antigenic competition is based on the generally accepted finding (390) that antigenic competition will likely occur when the first antigen (i) is a TD antigen (ii) is injected at the same site 1 to 7 days before the second antigen, and (iii) is injected at a dose which is high compared to the second antigen. Several mechanisms for antigenic competition have been proposed, some of which include competition for essential nutrients, interference of the T and B cell interaction by non-specific inflammatory cell infiltration of the responding lymphatic organ, and also competition of helper factor for binding sites on M ϕ s.

In this thesis study, IgE and IgG1 antibodies to heterologous normal serum constituents were routinely demonstrated in the sera of mice receiving multiple injections of heterologous sera (see Fig. 18; data for IgG1 not shown). Lawton et al (239) also reported that mice receiving normal heterologous Ig from birth produced antibodies to the Ig. As a consequence of these observations, it was predicted that by

discontinuing the administration of heterologous serum and allowing some time to elapse before the injection of OA [in $Al(OH)_3$], the level of heterologous serum constituents actively engaged in producing the competitive effects would be reduced to a level which would accommodate an anti-OA antibody response. Indeed, under these conditions it was possible to distinguish specific suppression from other effects.

Administration of anti- ϵ antibodies by repeated i.v. injection was found to suppress the IgEm antibody response specifically. It could be argued that since the only assay available to measure the presence of IgEm antibody was PCA and since anti- ϵ antibodies interfere with the ability of IgE to mediate PCA, that the observed low IgE antibody titers in the mice treated with anti- ϵ antibodies was a consequence of the neutralization of circulating IgEm by the administered anti- ϵ antibodies. However, two independent observations argue against peripheral neutralization.

1. Treatment of mice with heterologous proteins was shown to elicit an IgG1 antibody response. This antibody class and others would be expected to bind to the anti- ϵ antibodies and remove them from circulation.
2. The total amount of dog anti- ϵ Ig preparation (selected here for an example) administered prior to immunization was 1.09 ml, which is equivalent to about twice the total blood volume of a 3-week old mouse. The data contained in Figure 22 demonstrate that to neutralize completely 40 μ l of MRS, 160 μ l of this antibody preparation was required. Clearly the amount of anti- ϵ administered was not sufficient to provide complete removal of

circulating and cell bound IgE even if the assumption is made that all of the anti- ϵ injected is still present 27 days after the last treatment (day 23 of the response).

On the basis of the above considerations, it was concluded that the development of IgE antibody production was specifically suppressed. Since the IgG1 antibody response was not similarly suppressed, the activation of sIgE bearing virgin B cells must be independent of the proliferation and differentiation of sIgG1 bearing virgin B cells. It was also shown that both of these B cells express IgM on their surface since 3 consecutive injections of anti- μ given 4 days before immunization (Figures 20 and 21) inhibited both IgE and IgG1 antibody responses to OA in the adult mouse. Taken together these data indicate that these B cells express IgM on their surface in addition to IgE and IgG1, respectively.

Ontogenesis of Isotype Diversity

Antigen-independent phase.

There is general agreement that a common sIgM bearing and anti- μ sensitive B cell exists in all animal species examined. This cell is thought to give rise to separate lines of B cells each committed to the synthesis of one Ig isotype. More controversial is the origin of the signal responsible for the switch from IgM to IgG, IgA or IgE synthesis. The first animal model used successfully to study isotype diversity was the chicken, largely because the two central tissues in which lymphopoiesis occurs (the thymus and the bursa) exist as

discrete and surgically accessible organs. It has been concluded from experiments in which bursectomy was performed at various times before and after hatching that, in the absence of antigen, there is an ordered sequence in the appearance of each isotype and that this sequence is directed by the bursa; i.e., IgM, then IgY (IgG) and then IgA. Isotype commitment is established in the bursa and then cells migrate to the peripheral lymphoid organs (e.g., the spleen) where in the presence of antigen, they are activated to develop into AFC. Evidence that in the chicken peripheral virgin B cells can express only one isotype is provided by experiments (391) in which bursectomy at hatching, followed by administration of anti-u and anti- α resulted in ablation of the animals ability to produce Igs other than IgY. It is evident from this kind of experiment that peripheral IgY B cells (i) do not express IgM on their surface, (ii) are committed to IgY synthesis, (iii) are no longer dependent upon the presence of a bursa, and (iv) do not give rise to IgA B cells.

Ontogenesis of isotype diversity in the mammal has been extensively explored in the rat as a result of the discovery of several Ig-secreting rat tumor cell lines. By contrast to the chicken, it has been found in the rat that at birth all B cells appear to express sIgM. Neonatal administration of anti- μ antibodies suppressed sIgM, sIgD, sIgA, sIgG (all 4 subclasses) and sIgE expression (392). Ishizaka et al (393) were unable to detect sIgE bearing cells which did not also express sIgM. In addition, based on the observed suppression of IgE antibody formation in rats receiving anti- δ from birth (392), it has been postulated that the precursor of an IgE AFC expresses also sIgD.

Taken together, these data indicate that, prior to IgE synthesis, the IgE B cell expresses both IgM and IgD on its surface. Antigen independent ontogenetic development of rodent B cells may parallel the avian system in possessing a temporal restriction on the expression of isotype. The findings by Ishizaka et al (393) that rat IgE-bearing cells are present within 24 hours after birth and precede IgG2a-bearing B cells by 3 days provides evidence for the ordered expression of different Ig isotypes on mammalian B cells.

Thus far in rats it appears that from the data available (i) expression of sIgG, sIgA or sIgE does not occur in the murine neonate without coexpression of sIgM and probably also sIgD, i.e., a B cell committed to IgEm synthesis would express sIgM and sIgD but not sIgG or sIgA, and (ii) precursors of IgG AFC would express sIgM, sIgD and sIgG but not sIgE or sIgA at the time of commitment to IgG synthesis. Indeed, the results of my thesis indicate that (i) B6D2F1 mice treated from birth with anti- ϵ antibodies are unable to mount a primary anti-OA IgE antibody response but were able to mount a primary anti-OA IgG1 antibody response, and (ii) B6D2F1 mice treated with anti- μ were suppressed with respect to their ability to produce both IgG1 and IgE anti-OA antibodies. The former finding is in agreement with data obtained for the rat (394) in which neonatal rats were treated with anti- ϵ r antibodies and for the mouse (395) in which neonatal mice were treated with anti- ϵ m antibodies; both studies were published after the program for this thesis had been completed and both relied on the use of myeloma or hybridoma derived IgE for the production and isolation of anti- ϵ antibodies. In agreement with the data obtained in this

study using anti- μ antibodies neonatal administration of anti- μ antibodies eliminated -- with one exception (396) -- the primary IgE response in both rats and mice (389,392 and 397). Manning et al (389) found that BALB/c mice treated with anti- μ antibodies were unable to mount an IgM, IgG and IgE antibody response to Nb, and that more anti- μ antibodies were required to suppress the IgE response than the IgG response. However, Dwyer et al (396) were unable to suppress the IgE anti-OA response of [C57Bl/10 X DBA/1]F₁ mice even when suppression of IgG antibody was successful. These seemingly contradictory data may be due to the choice of antigen. It has been shown that memory B cells are not sensitive to anti- μ suppression (129) and, therefore, it may be suggested that, if the mice in Dwyer et al's experiments had been exposed to a crossreacting antigen prior to treatment or during the treatment schedule, they would be less affected by anti- μ antibodies. Alternately, Dwyer et al may have failed to suppress IgE responses because their anti- μ preparation was produced in goats. Sera produced in goats have been shown to be inferior to those produced in rabbits (413) when used as murine isotype suppressive agents.

Antigen-dependent generation of B cell clonal diversity.

It is generally accepted that virgin B cells, in rats and mice, are unable to develop into AFC if they are exposed in vivo to anti- μ antibodies from birth. On the other hand, if treatment is delayed until after exposure to antigen, the susceptibility to anti- μ antibodies is confined to IgM antibody production. The ability of anti- χ , anti- α , and anti- ϵ to suppress specifically the primary

antibody response of the IgG, IgA and IgE class, respectively, strongly suggests, that in the mouse the commitment to isotype is established prior to exposure to antigen. This interpretation is strongly supported by the work of Shigemoto et al (398) in which depletion of μ -bearing or PC reactive cells completely abolished the induction of the anti-PC IgE response in CBA/N male mice; parenthetically it should be noted that these mice are unable to produce an anti-PC antibody response of other isotypes (399). The anti-PC IgE response was shown to be derived from a PC specific μ -bearing B cell, which had passed through an IgM, IgD and IgE triple bearing stage. It can be seen also from this work that commitment of B cells to IgE production probably occurs prior to the expression of IgE on the surface of the cell.

Mechanism(s) of Suppression Induced by Anti- ϵ Antibody

As discussed in the Introduction, administration of anti- μ antibodies has been shown to inhibit B cell development in neonatal mice and so prevent antibody responses in all classes. On the other hand anti- γ and anti- α antibodies induced class specific immunosuppression in both neonatal and adult animals which persisted only as long as the antibodies were present. While there is little information available concerning the mechanism by which anti- γ or anti- α antibodies induce suppression, there have been several reports published dealing with the mechanism of anti- μ antibody induced suppression. Anti- μ antibodies appear to induce in neonatal mice an

irreversible inhibitory effect on immature B cells which may be due to their inactivation or elimination (400). On the other hand, mature B cells are rendered unresponsive only as long as the anti- μ antibody is present. Friend et al found that slg density is reduced on the remaining B cells following administration of anti- μ antibodies in neonates (401). This finding indicates that (i) although slg is modulated off the cell the modulation is incomplete, and/or (ii) a subpopulation of B cells escapes suppression as a result of having insufficient slg expressed by virtue of their position in the cell cycle or their stage of development. Friend et al found also that ϕ positive cells were reduced in anti- μ immunosuppressed mice (401). In this context, Bottomly et al (188,189) demonstrated that with respect to antibody responses there existed a Th cell specific to Ig determinants whose presence or absence was dependent upon the presence or absence of the corresponding Ig determinant. Moreover, Black and Herzenberg (161) showed that mice chronically suppressed by treatment with anti-Ig1b antibodies were deficient in the Ig1b allotype-specific Th cell. Recent evidence in myeloma bearing mice (402-404) indicates that there exist separate T_γ , T_μ , T_ξ and T_α cell populations and that at least one them, T_α , is an IgA specific suppressor cell (402). It is, therefore, possible that treatment with anti- ξ antibody reduces not only the level of B_ξ cells but also modulates the levels of ξ -specific Th and Ts cells. Parenthetically it is to be noted that there is no published evidence to date for the existence of the ξ -specific Th cell.

With respect to the mechanism of anti- ϵ induced suppression, Nelson and Manning (405) demonstrated that in order for anti- μ antibodies to be suppressive it was essential they contained an intact Fc region; (Fab')₂ fragments of the goat anti- μ antibodies were without effect. From these results it has been inferred that two sites of attachment are involved for immunosuppression to be induced: one binding site involves the Fab portion of the anti- μ antibody as it attaches to the sIgM, and the other binding site involves the Fc portion of the anti- μ antibody which interacts with Fc receptors. The Fc-receptor may reside on the corresponding B cell, in which case crosslinking of the sIg may establish a critical matrix which inactivates the B cell. Similar mechanisms have been postulated for the induction of central B cell tolerance by polymeric antigens (408) by antigens in the presence of low levels of antibodies (409) as well as for immunosuppression mediated by tolerogenic compounds containing homologous IgG (410). On the other hand the B cell may be eliminated by antibody dependent cytotoxicity reaction (ADCC) (411).

No evidence was obtained in the present study to decide unequivocally on a particular mechanism. In principle it should be possible to study anti- ϵ binding to B cells and determine the numbers of anti-antibody molecules bound and the consequent effects on sIgE expression. However, the expected low frequency of sIgE(+) cells in normal mice would make such a study difficult but not impossible with the current availability of a FACS technique for cell sorting (412). Since isotype specific Ts cell-mediated suppression is usually found when the respective isotype is increased (402) and not when it is

decreased, it is unlikely that the observed suppression mechanism involves Ts cells. Plausible mechanisms underlying the immunosuppression induced by anti- ϵ antibody may be explained in terms of:

1. The formation of a rigid lattice on the surface of B_{ϵ} cells resulting from the simultaneous interactions of the antibodies with sIgE and with Fc-receptors on the corresponding B_{ϵ} cells results in deletion of this B cell type. With respect to deletion by anti-heavy chain antibody, it has been found in vitro (406) that while Lyb5(+) B cells proliferate in response to anti-u and anti- δ , most if not all Lyb5(-) B cells in the G₀ phase are stimulated to enter G₁ but not to progress to S. It is possible that if the virgin B_{ϵ} cells are Lyb5(-), they may be similarly induced to enter G₁ without further progression to the S phase of their cell cycle i.e. the normal cell cycle was blocked.
2. Elimination of the B_{ϵ} cell by ADCC. In this reaction the target B cell would be killed by an effector cell whose killing function is activated by binding of its Fc-receptor to the Fc portion of the anti- ϵ antibody.
3. The B_{ϵ} cell is not deleted but is inhibited from reacting with antigen by either blockade of its sIgE or modulation of the sIgE from its surface.

Since the immunosuppression induced by anti- ϵ antibodies in this study was found to be relatively short-lived it is obvious that the mechanism(s) underlying this suppression persisted for only as long as the anti- ϵ antibodies were present at some critical concentration.

Moreover the fact that the suppression was short-lived may be interpreted to indicate that the critical concentration of anti- ξ antibodies at the time of its administration was close to the minimum and that consequently the inactivation of the B_{ξ} cells was not irreversible, or that the deleted B_{ξ} cells were rapidly replaced from the bone marrow's pre-B cell population.

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