THE UNIVERSITY OF MANITOBA

Investigation of the Existence of Anti-Idiotypic Antibodies in House Dust Mite Allergic Individuals

by

JIANGPING WU

A Thesis

submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

Department of Immunology

Winnipeg, Manitoba, Canada

May 1986

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TO MY COUNTRY, CHINA

TABLE OF CONTENTS

Page No.

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ABSTRACT

With an aim to investigate the existence of idiotype-anti-idiotype interactions in house dust mite allergic individuals, monoclonal antibodies (Mab) against antigen P1, the major allergen in house dust mite (HDM), were prepared. Crossinhibition between sera from HDM allergic individuals and Mabs suggested that a large portion of human anti-P1 IgE antibodies recognized the same antigenic moiety as that recognized by Mabs. The Mabs were employed to screen auto-anti-idiotypic antibodies (auto-anti-Id) against P1 specific antibody. Indeed, auto-anti-Id positive sera were detected and the majority of them were from HDM allergic individuals. An extensive characterization showed that the auto-anti-Id antibody was idiotope specific, and carried an internal image of a P1 epitope. Analysis of the affinity purified auto-anti-Id confirmed that it was an IgG. This study is the first step that might lead to further study of the regulatory effects of anti-Id in HDM allergy in human.

CHAPTER I

INTRODUCTION

Abbreviations used in this Chapter:

Ab	antibody
Ab1	idiotype antibody
Ab2	anti-idiotype antibody
ABA	azobenzen arsonate
A-CHO	group A streptococcus carbohydrates
Anti-A antibody	anti-hog gastric A substance antibody
Anti-AchR	anti-acetylcholine receptor antibody
Anti-Id	anti-idiotype, anti-idiotype antibody, anti-idiotope antibody
BPO	benzylpenicilloyl
CPE	cytopathic effect
CRI	crossreactive idiotope (idiotype)
DNA	deoxyribonucleic acid
DTH	delayed type hypersensitivity
ELISA	enzyme-linked immunosorbent assay
Formyl-Met-Leu-Phe	formyl methioyl-leucyl-phenylalanine
Gppp	guanosine triphosphate
HA	hemagglutinin
HBS Ag	hepatitis B surface antigen
HSV	herpes simplex virus
i	idiotope, idiotype
IFN A	leukocyte A interferon
IgE	immunoglobulin E
IgG	immunoglobulin G

Igh	immunoglobulin heavy chain
i.p.	intraperitoneally
KLH	keyhole limpet hemocynine
NP	4-hydoxy-3-nitrophenyl acetyl
NZB	New Zealand black (mouse)
OVA	ovalbumin
P	paratope, parotope
Pl	the major allergen in house dust mite (D. pteronyssinus)
PC	phosphoryl choline
PFC	plaque forming cell
PMN	polymorphonuclear (leukocyte)
RBP	retinol-binding protein
Rye I	rye grass pollen antigen I
SLE	systemic lupus erythematosus
T_H	T helper cell
TMA	trimethylammonium
TNP	trinitrophenyl
TR_β	ß chain of T cell antigen receptor
T_S	T suppressor cell
TsF	T suppressor cell factor
TSH	thyrotropin
TT	tetanus toxoid

In 1974, Dr. N.K. Jerne proposed a theory which depicted the immune system as a network of interacting idiotypes (Jerne 1974). He postulated that the immune system must play an eigen-behavior mainly resulting from paratope-idiotope interactions within the system, and this system achieves a dynamic steady state by the interactions of its elements. The invasion of a foreign antigen will disturb the equilibrium, and push the system to a new state of dynamic steady state along with the manifestations of the commonly observed immune response. This network theory has had a fundamental impact on the thinking of scientists interested in the nature and regulation of the immune response. Numerous findings on the response and regulation of the immune system have been interpreted with the network theory and many experiments have been specially designed to test this hypothesis. Among the early key supportive data are idiotype suppression (Cosenza et al 1972), neonatally induced idiotype suppression (Strayer et al 1975), the finding of auto-anti-idiotypic responses (Kluskens et al 1974) and the induction of mirror imaging antibodies (Sege et al 1978; Urbain 1977). The regulation of the IgE antibody response by idiotype-anti-idiotype network was reported by Blaser et al (1982a) in the phosphorylcholine (PC) and benzylpenicilloyl (BPO) systems. Most of these studies were carried out in animal models, and we are still far from being able to visualize the intact picture of the network system. The study in human model is in a preliminary stage due to various ethical reasons, and most of the investigations are still searching for the evidence that auto-anti-idiotype antibodies exist.

Such antibodies have been reported to occur during the immune response to tetanus toxoid (Geha 1982), DNA (Abdou et al 1981), acetylcholine receptor (Dwyer et al 1983), casein (Cunningham-Rundles et al 1982), and Rye I (Bose et al 1984). It is apparent that this kind of search is the first and essential step to establish the network theory in the human model.

IgE mediated allergy to house dust mite is a very common disease in many parts of the world (Morrison-Smith et al 1969), and a major allergen, named P1, has been identified and isolated (Chapman et al 1980) from house dust mite Dermatophagoides pteronyssinus, which is one of the dominant species in the human residences (to be reviewed later). The immune regulation of anti-P1 IgE response is poorly understood. The effects of desensitization treatment are controversial and no clearcut decrease of the production of specific IgE has been observed (Pauli et al 1984; Delaunois et al 1985). It will be interesting to know if the immune network operates in the human response to P1, how it regulates the IgE response to P1 and if it could be manipulated to eliminate the allergic status.

The purpose of the present study is to investigate the existence of human auto-anti-idiotype specific to anti-P1 antibodies. It might be the first step leading toward a better understanding of the mechanism of house dust mite allergy and toward a better way of treatment. As an introduction, a literature review of the immune network and of house dust mite allergy are presented.

I. The Immune Network

Immunology was once primarily a part of microbiology, devoted to the establishment of bacterial serotypes and vaccination. It was gradually realized that the immune repertoire is infinite and complex. The immune system is able to respond to an enormous array of unforeseen antigenic stimuli such as artificially synthesized haptens. Under the influence of the receptor theory proposed by Ehrlich et al in 1897 and the natural selection theory of antibody formation by Jerne in 1955, the Australian immunologist Burnet extended the Darwinism to the cellular level and proposed the clonal selection theory in 1958. The discovery of thymus-bone marrow cooperation (Claman et al 1966) and hapten-carrier cooperation (Mitchison et al 1971) led to the conclusion that there must be communication between lymphocytes in the process of the immune response and that such communication is regulatory. A fundamental and comprehensive theory became necessary to interpret, describe and predict the interactions between different components of the immune system. Therefore, postulated by Jerne (1974), the immune network theory, which shed a brilliant light onto the current generation of immunologists, emerged from the horizon with the discovery of idiotype by Oudin et al (1963), Kunkel et al (1963) and Kelus et al (1968) as the prelude.

Oudin et al (1963,1969) immunized allotype matched rabbits with different Salmonella antigens, and harvested the antigen specific antibodies which were used as antigens to immunize the second batch of

allotype matched rabbits. The anti-anti-sera thus prepared could only precipitate antibodies against one given Salmonella antigen but not antibodies against another noncrossreacting Salmonella antigen, nor antibodies from the nonimmunized rabbits. The second feature of anti-antibodies was that the anti-antibody prepared with antibodies of a given rabbit immunized with S. typhi would only react with the S. typhi specific antibodies of that individual rabbit, but not with anti-S. typhi antibodies prepared in other allotype matched rabbits. It was then concluded that the rabbit antibody to Salmonella antigen carried a kind of peculiar allotype in which each allotypic specificity would be restricted to a single clone of antibody or a single individual. Later, a new word "idiotype" was coined (Oudin et al 1969) to describe this kind of newly found allotype. The first part of the word "idio", which was from the Greek work "peculiar", was justified by the extreme peculiarity of the antigenic specificity present in the antibody in question, while the anti-anti-serum in this context was named anti-idiotype antibody (anti-Id). At the same period, Kunkel et al (1963) made similar observations by employing human antibodies. Human individuals were immunized with hog gastric A substance, levan or dextran. The specific antibodies were purified and used as antigens to immunize rabbits. The rabbit anti-anti-sera were extensively absorbed with normal human serum or normal human IgG and the absorbed preparations were used to react with different human antibodies. The individual antigenic specificity of human antibody was demonstrated for two anti-A antibodies, one anti-dextran antibody,

and one anti-levan antibody. These individual antigenic specificities of the antibody were idiotype in nature. From their close correlation with antibody specificity, one would expect that idiotypic determinants are localized on the variable portion of the immunoglobulin molecules and determined by the variable amino acid sequences. This was proved later (Somme et al 1982). Oudin and Kunkel's work realized the elusive dream of early immunologists who speculated that the unique configuration of the antigen-combining site determined not only its specificity for antigen binding but also its own antigenic specificity and that it would therefore be possible to raise antibodies directed against the antigen-combining site of an antibody molecule.

In order to describe his theory, Jerne (1974) proposed several terms. An epitope was a patch, i.e., the antigenic determinant, on an antigen molecule and a paratope was the antigen-combining site of an antibody molecule. Since a determinant of the variable region of an antibody molecule could also be antigenic, an epitope of this nature was denoted as idiotope, and an idiotype was a set of idiotopes displayed by a set of antibody molecules. Therefore, each Fab arm of an antibody molecule displayed one paratope and a small set of idiotopes which in Jerne's model was located away from the combining site.

There were three major postulates in Jerne's network theory (Fig.1, adopted from Jerne, 1974). First, since the repertoire of an

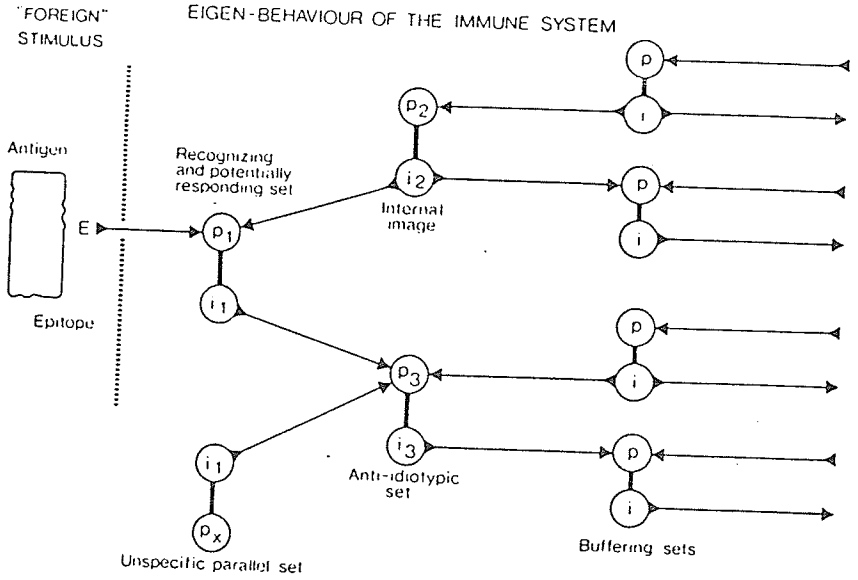


FIG. 1. — *Eigen-behaviour of the immune system.*

The immune system contains a set p_1 of combining sites (paratopes) on immunoglobulin molecules and on cell receptors which recognize a given epitope (E) of an antigen. This recognizing set includes the potentially responding lymphocytes. The molecules of set p_1 also display a set i_1 of idiotopes. Apart from recognizing the foreign epitope, the set p_1 likewise recognizes a set i_2 of idiotopes which thus constitutes, within the immune system, a kind of internal image of the foreign epitope. This set i_2 occurs in molecular association with a set p_2 of paratopes. Likewise, the set i_1 is recognized, within the immune system, by a set p_3 of paratopes which represent anti-idiotypic antibodies. Beside the recognizing set p_1i_1 there is a parallel set p_Xi_1 of immunoglobulins and cell receptors which display idiotopes of the set i_1 in molecular association with combining sites that do not fit the foreign epitope. As a first approximation, the arrows indicate a stimulatory effect when idiotopes are recognized by paratopes on cell receptors and a suppressive effect when paratopes recognize idiotopes on cell receptors. Successive groups of ever larger sets encompass the entire network of the immune system.

antibody was enormous and complete, it would be reasonable to assume that within the immune system of one given individual, any idiotope (i_1) on an antibody molecule (Ab1), which possesses its paratope (P_1), could be recognized by a set of paratopes (P_2) on another antibody (Ab2) which also expressed its own idiotypes (i_2). The second major postulate was that any paratope could recognize a set of idiotopes within the immune system of a given individual. In other words, in each individual there exists a set of antibodies carrying idiotypes which had a configuration similar to that of the determinants on antigens involved in natural or experimental immunization. Such idiotypes were designated as the internal images of the antigen. By the above postulates, it would be easy to view the immune system as a network in which idiotopes and paratopes recognize each other and form endless chains or nets, and this system would play an eigen-behavior mainly resulting from the interactions between idiotopes and paratopes even in the absence of foreign antigens. Since the immune system was observed to be in a stable status in the absence of foreign disturbances, there came the third postulate that the essence of the network was repression of its lymphocytes in order to achieve dynamic equilibrium. In such equilibrium, the elements (molecules and cells) of the internal image P_2i_2 were largely stimulatory towards the potentially responsive P_1i_1, whereas the elements of the parallel set P_3i_3 (Fig. 1) were largely inhibitory. These opposing forces resulted in a balanced suppression which must be overcome in order to obtain an immune response to a foreign antigen.

This network theory drew great attention from the immunologists and many experiments were designed to test the theory. As a logical process, the investigators started from testing the existence of anti-idiotype and proceeded to investigate the regulation of the network. The experiments were progressing from animal to human models. Currently, a large body of evidence supports the theory and naturally the theory has been constantly modified to cope with the new phenomena encountered during more than ten years exploration.

A key prediction of the network concept was that idiotypic determinants are autoimmunogens. This has been proven to be true by many investigators. The synthesis of anti-Id antibodies against self-idiotypes was first demonstratd by Rodkey (1974). Rabbits were immunized for a period of 189 days with the hapten trimethylammonium (TMA) and anti-TMA antiserum was collected. The rabbits were rested for 432 days, and the affinity purified anti-TMA Fab'_2 fragments were used to immunize the rabbit from which the antiserum was collected. The auto-anti-Id was detected by radioimmunoassay. This auto-anti-Id was specific for autologous antibodies, and no crossreactivity was detected for antibodies of the same specificity but from other littermates. Interestingly, the idiotype-auto-anti-Id interactions were hapten inhibitable. This indicated that the

idiotype was located in or close to the combining site of the antibody. This idiotype differed from the idiotype in the original sense of Jerne who hypothesized that idiotype and paratope are separately located. Thus, the theory had to undergo a modification. This kind of newly defined idiotype was named combining site idiotype and the corresponding anti-Id was designated as $Ab2_{\beta}$ (Kohler 1984). The location of such a combining site idiotype was not fully established. According to the known three dimensional structure of Fab fragments and to the calculation of the binding energy contributed by the combining site, Kabat (1984) suggested that the so called combining site idiotope is located on the rim of the cavity-type combining site of the antibody, and that it will undergo a conformational change when antigen binds to the combining site. However, I believe that this suggestion should not be overgeneralized, because this suggestion is only based on three experiments, and does not exclude the possibility that the combining site idiotope locates within the combining site.

The auto-anti-Id was subsequently found in many systems. Most of them were artificially induced by immunization with antigen or antibody, like in the initial experiments by Rodkey. Sakato et al (1975) immunized syngenic mice with myeloma protein, and anti-Id was later detected. Cosenza (1976) detected auto-anti-Id positive cells in phosphorylcholine immunized Balb/c mice. Schrater et al (1979) and Cowdery (1981) reported the presence of auto-anti-Id during the normal

immune response of mice to TNP-Ficoll or polyinosinic- polycytidylic acid. Rodkey et al (1983) demonstrated the occurrence of auto-anti-Id when rabbits were hyperimmunized with the gram positive bacterium micrococcus lysodeikticus. McKearn et al (1974) discovered auto-anti-Id in rats repeatedly immunized with alloantigens.

Recently, auto-anti-idiotype antibody has been found in several other animal models. Rabbits immunized with thyroid-stimulating hormone could produce auto-anti-Id antibodies (Beall et al 1985). Mice infected with Schistosoma mansoni generated splenic auto-anti-Id plaque-forming cells (Powell et al 1985).

The spontaneously occurring auto-anti-Id seemed more convincing in supporting the network theory since most of those artificially induced auto-anti-idiotype antibodies were generated under drastic immunization procedures which were not close to physiological conditions. In a few cases, a spontaneous auto-anti-Id response was also demonstrated during a natural immune response. Coomb's antibodies to erythrocytes produce severe hemolytic anemia in New Zealand Black (NZB) mice. The F1 hybrids of NZB and normal strains develop a milder form of disease. This correlates with the presence of auto-anti-idiotypic antibodies that specifically recognize the idiotype of the NZB Coombs' anti-erythrocyte antibody (Cohen et al 1982; Eisenberg et al 1985). Rodkey et al (1983) mated a heterozygous

female rabbit with a homozygous male rabbit. The offsprings were exposed via in utero transplacental and later by colostral Ig transfer to immunoglobulins of an allotype which was foreign to them, and developed anti-a1 antibodies. The purified anti-a1 Fab fragment was able to detect the auto-anti-Id antibodies in the offspring.

In the human system, only a few well documented examples have been described demonstrating auto-anti-idiotype antibodies. Cunningham-Rundles (1982) found that individuals with IgA deficiency would develop anti-casein antibodies and subsequently would develop anti-anti-casein antibodies. Geha (1982) described auto-anti-idiotypic antibodies after tetanus toxoid hyperimmunization. The anti-Id was highly specific, and no crossreactions between different individuals were observed. This anti-Id was detectable 10 days after booster immunization. Several studies (Abdou et al 1981; Zouali et al 1983) have shown the presence of anti-Id against anti-DNA antibody in systemic lupus erythematosus (SLE) patients and in some of normal individuals. Dwyer et al (1983) discovered naturally occurring anti-idiotypic antibodies against anti-acetylcholine receptor antibodies (anti-AChR) in myasthenia gravis patients. It is interesting to note that his finding was made incidentally, when he was trying to set up an enzyme-linked immunoabsorbant assay (ELISA) to measure the level of anti-AChR antibodies in the patients. Bose et al (1984) reported the presence of auto-anti-Id to anti-Rye I antibodies in rye grass pollen allergic individuals.

Most recently, Troisi et al (1985) detected an IgM auto-anti-idiotype antibody against anti-hepatitis B surface antigen (HBS Ag) antibody in hepatitis B patients. Naturally occurring serum anti-idiotypic antibody against anti-liver-specific membrane lipoprotein in patients with hepatitis was also demonstrated (Tsubouchi et al 1985).

The presence of auto-anti-idiotypic antibody is a strong evidence that idiotype determinants of immunoglobulin can function as auto-immunogens, and has fulfilled the prerequisite of the existence of the network. Since each Ab1 can elicit the production of Ab2, by inference, the idiotopes of Ab2 molecules should be able to cause the production of Ab3, which in turn will cause the production of Ab4 and so on. This has been shown to be true in some cases. Bona et al (1981) reported that syngeneic mice immunized with autologous levan-binding myeloma protein (Ab1) led to the appearance of auto-anti-anti-Id antibodies (Ab3), which could bind Ab2 but did not bind levan. These results seemed to support the classical view of open-ended network. Urbain et al (1977) and Wikler et al (1984) developed an immunization cascade in which a group of rabbits were immunized with Micrococcus luteus, and the purified antibody from the group against a carbohydrate isolated from M. luteus was used to immunize the second group of allotype matched rabbits to produce Ab2. Ab2 was used to produce Ab3 in the third group of rabbits, and Ab3 was used to produce Ab4 in the fourth group of rabbits. It was found that

Ab4 recognized Ab1, like Ab2, but only about 5% of the total amount of Ab3 made by individual rabbits bound antigen, as Ab1 did. Bona et al (1981) used another myeloma binding to fructosan to immunize syngenic mice in a protocol of cascade immunization similar to that of Urbain, and prepared Ab1,2,3 and Ab4. The results suggested that Ab4 resembled Ab2 because both bound to Ab1, while Ab3 did not bind to antigen as Ab1 did. This result was similar to what Wikler et al (1984) observed. These observations have been interpreted to indicate that the idiotype network system is not open-ended, and that there might be asymmetry in the system.

Although Jerne never excluded T cells in his network theory, his emphasis was on the B cells and their products. Many experiments later indicated that T cells and their products were also an important component of the network. A cascade of idiotype-anti-idiotype of T cells and their factors has been described in the antibody response to the azobenzen arsonate (ABA) epitope by Germain et al (1981) and in delayed type hyersensitivity (DTH) response to the hapten 4-hydroxy-3-nitrophenyl acetyl (NP) by Weinberger et al (1980). Intravenous injection of hapten derivatized syngeneic cell can induce T suppressor cells (T_{s1}) and its factor $T_{s1}F$. The T_{s1} and $T_{s1}F$ are antigen specific and share idiotypic determinants with antibodies. $T_{s1}F$ can induce T_{s2} suppressor T cells which secrete a T_{s2} factor ($T_{s2}F$). This factor does not bind to the antigen or anti-idiotype but can be removed by the idiotype, therefore the $T_{s2}F$ carries an anti-

idiotype. The $T_{s2}F$ with the help of antigen can activate T_{s3}, which is an idiotype positive and antigen binding effector cell. It is now known that T cell is utilizing a different set of genes for Ag receptors than does the B cell, therefore it is interesting that T cells or their factors bear crossreactive idiotypes with antibodies. Most likely, this crossreactivity between T and B idiotypes is due to the similarity in conformation but not the primary structure of the idiotype, and the basic reason for this conformational crossreactivity could be that T cells are positively selected by the idiotypes and internal images carried by B cells during the thymus education as hypothesized by McNamara et al (1984) and Sy et al (1984). There are certain similarities between the V gene structures of Igh and that of ß chain of T cell antigen receptors ($TR_ß$) (Siu et al 1984). They all have clusters of V, D and J exons; in both cases, these exons undergo rearrangement to form the final gene; in both cases, there are highly preserved hepta- and decanucleotides in the vicinity of V, D and J exons; and in both cases, the rearrangement of V, D and J follows a similar 11 + 23 rule. There are 7 high variable regions in the V gene of $TR_ß$ (Patten 1984) and 3 of them are located in the correspondent places of the classical immunoglobulin high variable region of κ chain. All these similarities seem to indicate that V gene of Ig and $TR_ß$ might be derived from a primordial gene, and this might be an additional factor contributing to the crossreactivity between idiotype antibodies and T cell receptors.

It is worthwhile to point out that the existence of auto-anti-idiotype antibodies or idiotype-anti-idiotype cascades have only been demonstrated in a limited number of antigen systems or animal models among a large number of antigens or animal models tested. This should not surprise us since we know how diverse the idiotypes or anti-Id is and subsequently how sparse a certain idiotype or anti-Id will be in the huge repertoire of idiotypes existing in a given animal or human. Probably the Id and anti-Id are present in many, if not all, antigen systems but the signals are so low that the methods currently employed are not sensitive enough to detect them. When the artificial immunization protocol, which is usually quite drastic, is applied, the concentration of certain idiotypes is considerably elevated and the subsequent components of the cascade is then induced to a detectable level.

One important concept in the network theory is that each paratope recognizes an idiotype. This requires that foreign antigenic determinants crossreact with some of the idiotypes and these crossreactive idiotopes will be the internal images of foreign epitopes. The first direct evidence suggesting the concept of internal image was provided by the studies of Sege et al (1978a) in the insulin system. Rabbits were immunized with rat anti-insulin antibodies, the anti-Id thus prepared could bind to rat-anti-insulin antibodies, and the binding could be totally inhibited by insulin.

Conversely, the anti-Id could cause almost complete inhibition of insulin binding to the insulin receptors on rat epididymal fat cells. The anti-Id could also increase α-aminoisobutyric acid uptake by young rat thymocytes as did insulin. Nepom et al (1982) studied internal image in the reovirus system. It was found that the immune response to the mammalian reovirus was specific to the hemagglutinin (HA) of the virus, and antibodies generated to HA were of limited heterogeneity. A monoclonal anti-Id antibody was produced against a monoclonal anti-HA antibody. It was found that the monoclonal anti-Id and the reovirus type 3 could both bind the same type of receptor on the target cells of the reovirus. Furthermore, it was found that the HA-specific cytotoxic T lymphocytes could lyse the hybridoma producing anti-Id antibody, and that the anti-Id Mab could trigger immune response to a certain extent against reovirus. It was suggested from all these results that anti-Id antibodies represented the internal image of virus HA. Schreiber et al (1980) raised anti-idiotypic antibodies against the Ig fraction of the rabbits immunized with alprenolol, which is a potent antagonist of the ß-adrenergic catecholamine hormones. The anti-Id could bind to the ß-adrenergic receptors and activate the adenylate cyclase of turkey erythrocyte. Sege et al (1978b) prepared antibody to retinol-binding protein (RBP) in rats, and anti-Id antibody to RBP specific antibody was raised in rabbits. The anti-Id could bind the receptor for RBP on the epithelial cell of intestine and impede RBP-mediated ^{3}H-retinol uptake of intestine epithelial cells. Farid et al (1982) studied

anti-Id in thyrotropin (TSH) system. Rabbits immunized with rat anti-human TSH antibodies produced immunoglobulin which did not bind the hormone but inhibited the binding of bovine TSH to the porcine thyroid receptor in a dose-dependent manner. In the presence of Gpp(NH)p, the anti-Id antibody increased the thyroid membrane adenylate cyclase activity 40% over the enzymic activity induced by nonspecific Ig. The rate of incorporation of ^{131}I-Na into cultured thyrocytes was also increased in a dose dependent manner by the anti-Id which further induced the organization of these cells into follicles after five to seven days culture. Thus, the anti-Id was mimicing TSH both structurally and functionally. In a study by Marasco et al (1982) anti-idiotypic antibodies were raised in mice, guinea pigs and goats against rabbit antibodies to the chemoattractant peptide formyl methioyl-leucyl-phenylalanine (formyl Met-Leu-Phe). The anti-Id could bind to rabbit polymorphonuclear (PMN) leukocytes which carried receptors for formyl Met-Leu-Phe. The $(Fab')_2$ of the anti-Id could partially inhibit the binding of the formyl Met-Leu-Phe peptide to the receptor on PMN. However, the authors could not demonstrate that the anti-Id mimiced the biologic activity of this peptide. Kennedy et al (1983a,b) have shown that injecting Balb/c mice with soluble anti-Id antibodies specific for an interspecies crossreactive idiotype associated with the combining site of antibodies to hepatitis B surface (HBS) antigen could elicit an IgM anti-HBS antigen antibody response without subsequent antigen

exposure, and administration of alum precipitated anti-Id enhanced IgG class idiotype production.

Recently, the role of internal image of anti-Id was demonstrated in some more instances. Thanavala et al (1985) produced monoclonal anti-Id against a monoclonal antibody (idiotype) to the determinants of HBS antigen. The two clones of the anti-Id mimiced HBS antigen in their ability to bind polyclonal anti-HBS antigen prepared in a variety of species. Glasel et al (1985) reported that rabbit anti-idiotypic antibodies raised against monoclonal anti-morphine IgG could block the binding of morphine or naloxon to rat brain opiate receptor. This suggested that the anti-Id carried the internal image of morphine. An anti-idiotypic hybridoma antibody directed against rabbit antibody to the recombinant human leukocyte A interferon (IFN-A) was prepared by Osheroff et al (1985). Interestingly, the anti-Id demonstrated interferon-like anti-viral activity as shown by a cytopathic effect (CPE) inhibition assay employing vesicular stomatitis virus and it targets bovine kidney (MDBK) cells. The monoclonal anti-Id was also shown to be able to bind interferon receptor bearing MDBC cells, and to compete with ^{125}I- IFN- A for binding to rabbit anti-interferon antibody. Therefore, this anti-Id seemed to be a functional internal image. The existence and function of internal image is not only related to humoral immune response, but is involved in cellular response as well. Ertl et al (1984a,b) used a monoclonal anti-idiotypic antibody directed against a Sendai

virus-specific mouse helper T cell clone, to induce an anti-viral immune response in vivo. Mouse splenocytes primed with the anti-idiotype antibody mediated an antigen-specific cytolytic response and preimmunization of mice with the anti-idiotypic antibody resulted in protection against a subsequent lethal infection with Sendai virus. In this study, anti-Id was against a T cell idiotype and was able to trigger cellular immune responses.

In the human system, most of the studies of internal image were focused on the similarity of determinants between anti-Id and epitope of antigen. Very little work of the function of the internal image was carried out in vivo, again due to ethical concerns. The auto-anti-Id described previously, i.e., in the casein system by Cunningham-Rundles (1982), in the tetanus toxoid system by Geha (1982), in the SLE system by Abdou et al (1981), in the myasthenia gravis system by Dwyer et al (1983), in the Rye I system by Bose et al (1984) and in the hepatitis B system by Troisi et al (1985) are all internal images of an antigen, at least conformationally, because their binding to the idiotype could be inhibited by epitopes of their respective antigens. Naturally, studies on biological function are necessary to finally prove that these anti-idiotypic antibodies behave as internal images.

It is noted that in Jerne's original network model, the internal image (i_2) is carried by a set of anti-Id, the paratope of which

(P_2) can be specific to some other unrelated epitope (Fig.1). In other words, Jerne suggested that the idiotopes are located away from the combining sites, i.e., the paratopes of the antibodies. This is not necessarily always the case. In most of the studies discussed above, the Ab2 carrying internal image reacts with Ab1 which is specific for the original antigen. This suggested that the paratope and idiotope, at least those in Ab2 (P_2i_2), are in the same location, and that the idiotope is a part of the paratope.

The biological function of the anti-Id carrying internal image of foreign antigen under the natural conditions needs to be further investigated. According to Jerne, the internal image has a stimulatory effect on Ab1 production. This effect could play a role in the priming, boosting or long term memory of the immune response. Besides this, internal image might be essential in the establishment of the T cell repertoire during early differentiation of immune competent cells in the thymus as hypothesized by McNamara et al (1984), and the transplacental transfer of maternal internal images might influence the fetal idiotype profile (Rothstein et al 1984).

Most auto-anti-idiotypes and internal images discussed previously only bind to the idiotypic antibodies with the same gross specificity to their respective antigens. However, Oudin et al (1971) showed that rabbit anti-Id antibodies for anti-chicken ovalbumin antibodies bind idiotopes of anti-turkey and anti-duck ovalbumin antibodies. This

indicates that shared idiotopes exist on antibodies of different specificities. In Jerne's model, these antibodies are named parallel sets (Fig.1). This phenomenon has been confirmed in many systems. Wisocki et al (1981) demonstrated that the sera of non-immune A/J mice contained low but detectable levels of immunoglobulins bearing the crossreactive idiotypes (CRI_A) that are normally the dominant crossreactive idiotypes in the anti-arsonate response of strain A mice. These immunoglobulins could not, however, be absorbed onto arsonate coupled to an affinity column and thus appeared not to be specific for arsonate. The level of CRI_A positive arsonate-non-binding Ig remained low even after hyperimmunization with arsonate conjugated proteins, whereas monoclonal anti-CRI_A antibodies could elicit high concentrations of both arsonate-binding and arsonate-non-binding Ig. The CRI positive arsonate-non-binding hybridoma antibodies have now been isolated. Based on these and other similar observations, Paul and Bona (1982) proposed the concept of regulatory idiotypes. The idiotypes shared by antibodies with different specificity are referred as regulatory idiotypes and at least some of the dominant crossreactive idiotopes are actually regulatory idiotopes. These idiotopes are more auto-immunogenic than the conventional idiotopes because they exist in higher concentrations, and therefore quite reasonably are able to boost the pre-existing regulatory cells which bear complementary paratopes. Hence, the activation of B cells (and, presumably T cells) bearing such regulatory idiotopes is likely to be regulated by the

pre-existing idiotope specific regulatory system. Moreover, this control is extended on B cells with different antigen specificity as long as they bear the same regulatory idiotope. This concept based on experimental data from several systems, is another modification of Jerne's original theory. Some subsequent studies have been carried out to examine the regulatory idiotype model, and will be reviewed in the following paragraph. Since the proposers of the concept themselves, did not expect that all the antibodies expressed regulatory idiotopes, especially in the instances where responses to complex proteins are involved (Paul et al 1982), it is not surprising that not all the results support the model.

We have reviewed studies about idiotype auto-anti-Id, internal images and crossreactive idiotypes. These are the essential constituents of the immune network. The next logical question to be asked is whether the immune network is functional? To be functional it means that the network is self regulatory and can maintain a dynamic equilibrium. Any foreign disturbance, e.g., disturbance by epitope, idiotope, or anti-idiotope, of the immune system should cause subsequent changes of different but interconnected components of the network, and a new equilibrium will be reached through activation or suppression among those components. The effect of epitope or idiotope on immune system has long been studied, however, the results can be interpreted by many other theories besides the network hypothesis. It seems better to dissect out the anti-idiotype from the entity of the

network, and to study its regulatory effect in order to elucidate to some extent the regulatory mechanism of the network including that of regulatory idiotype and to verify that the network is functional.

Numerous studies have been carried out to study the regulatory effect of anti-idiotype and most of them indicate that the anti-Id play a inhibitory role toward either the humoral or cellular arm of the immune response. The first study was by Cosenza et al (1972). More than 80% of the phosphorylcholine (PC)-specific antibodies produced by Balb/c mice express idiotypic determinants crossreacting with those found on the TEPC-15 group of PC-specific myeloma protein. These crossreactive idiotype are named T15. Adult Balb/c mice were injected with anti-T15 antiserum prepared in A/He mice, and challenged with a PC antigen (R36A) on the following day. The plaque forming cell (PFC) response was specifically inhibited after 4 days. It was then concluded that the anti-Id to T15 can suppress the formation of anti-PC humoral response. The mechanism of the anti-Id suppression in the PC system was studied later by Bottomly et al (1978). Adoptive secondary antibody response to PC could be generated by the transfer of keyhole limpet hemocynine (KLH) primed T cells, PC-bovine-IgG-primed B cells and PC-KLH together into irradiated syngeneic Balb/c mice. If the KLH-primed T cell donors were pretreated with anti-Id to T15 raised in rabbits, their T cells were unable to collaborate effectively with PC-primed B cells. Moreover, they could suppress the helper activity of T cells from KLH-primed normal mice. These results

suggested that anti-Id induced T_S cells which could inhibit T helper cells. However, in a study by Kim (1979) different results were obtained. Balb/c spleen cells were cultured in vitro in the presence of anti-Id to T15 prepared in A/He mice and a PC containing antigen R36A. The antibody response to PC was determined by PFC assay with R36A coupled sheep red blood cells. The presence of anti-Id antibody in the culture for at least 2 days was necessary to achieve full suppression of anti-PC production in the presence of R36A. The anti-PC response of a nylon wool-separated B cell population was also greatly suppressed (94%) after 2 days treatment with anti-Id in the presence of antigen. It was thus concluded that B cells could also be the direct target of anti-Id suppression. Nevertheless, in this study the purity of B cells prepared was questionable and the contamination by T cells could not be excluded.

Another early study about the regulatory effect of anti-Id was by Hart et al (1972) in the arsonate system. A significant percentage of the arsonate-specific antibodies produced by A/J mice express at least some members of a family of crossreacting idiotypes referred to as the CRI_A idiotype. Adult A/J mice were injected i.p. twice within 3 days with rabbit anti-Id against CRI_A and challenged after 2, 4, 6 and 10 weeks with arsonate conjugated keyhole limpet hemocyanine (Ar-KLH). A suppression of CRI positive anti-arsonate antibody was observed in all the bleedings at week 5, 7 and 11. When A/J mice were

injected twice i.p. with rabbit anti-Id against CRI_A two weeks after the first immunization with Ar-KLH (Pawlak et al 1976), the production of anti-arsonate CRI_A positive antibody upon challenging with Ar-KLH was suppressed and this suppression lasted up to 7 weeks after the administration of anti-Id. This suppression on the secondary antibody response indicated that the memory cells were inhibited. Owen et al (1978) tried to elucidate the mechanism of such anti-Id induced suppression. A/J mice were injected i.p. with rabbit anti-T15 anti-Id and then hyperimmunized with hemocyanine-arsonate. The purified T cell from these mice were transferred to irradiated naive mice. It was found that the CRI_A positive anti-arsonate antibody response of the recipient mice was inhibited. The T cells which formed rosettes with CRI_A coated mouse RBC were responsible for the suppression. Thus, the inhibition by anti-Id was via idiotypic-specific T_S cells. It was noted that anti-arsonate antibody response of the recipient primed with protein-Ar conjugate prior to the adoptive transfer of T cells were not suppressed. This was taken to suggest that the activated B cells were rather resistant to T_S cells. This explanation seemed to be contradictory to Pawlak's observation that the secondary antibody response to arsonate could be inhibited by anti-Id for more than one month. Maybe another unknown mechanism was involved.

An interesting study was carried out by Eichmann (1974) in another antigen system. All A/J mice immunized with Group A

streptococcus carbohydrates (A-CHO) responded by the production of an idiotype which is crossreactive with the monoclonal antibody A5A against A-CHO. This crossreactive idiotype is referred to as A5A. A/J mice were injected i.v. with guinea pig anti-idiotype antibody against A5A 10 days before receiving a series of A-CHO intravenous immunization. The titre of antibody carrying A5A idiotype was tested by radioimmunoassay. It was found that low dose (< 10 ng) and high dose (> 1 μg) of anti-Id achieved only a transient suppression of A5A positive antibody and that the suppression was dose- dependent. However, the intermediate dose of anti-Id could cause a long lasting suppression which was not dose-dependent. Surprisingly, the inhibitory effect resided exclusively in the IgG_2 subclass of anti-Id while the IgG_1 subclass had a slight enhancing effect on A5A positive antibody production.

Hence, crossreactive idiotypes exist in all the three antigen systems discussed above. Both enhancing and suppressive effect of anti-Id were observed in different instances, although more studies are needed to conclude that in these cases the regulation was exclusively mediated via those crossreactive idiotopes and that these idiotypes were indeed regulatory idiotopes, as hypothesized by Bona. Recently, the regulatory effect of anti-Id was studied in several other animal models, in some of which no crossreactive idiotype was demonstrated. Kennedy et al (1983a,1984a) reported that Balb/c mice receiving i.p. rabbit anti-Id before the injection of HBS Ag generated

an enhanced anti-HBS antigen antibody response which was monitored with radioimmunoassay. On the contrary, when rabbit anti-Id against monoclonal antibody to herpes simplex virus type 2 (HSV_2) was injected into Balb/c mice before challenge with a 50% lethal dose of HSV_2 there was a shorter survival time than that in the control animals treated with normal rabbit IgG (Kennedy et al 1984b). Hanh (1984) studied the effect of anti-Id on anti-DNA antibody production. NZB/NZW F1 mice spontaneously develop nephritis and anti-DNA antibodies carrying a crossreactive idiotope. When mice were treated with repeated injections of a monoclonal anti-Id that was widely crossreactive with NZB/NZW antibodies to DNA, the crossreactive idiotope positive anti-DNA IgG became undetectable in their serum, total antibody to DNA were reduced in quantity and the survival was prolonged. However, suppression of the total anti-DNA antibody was transient due to the emergence of large quantities of anti-DNA antibody that did not bear the crossreactive Id. Hence, other mechanisms may counteract the anti-Id regulation and it is necessary to take this into consideration in attempting to manipulate the immune response with anti-idiotypic antibodies.

Several studies have been carried out to study the anti-Id regulation in the human model. Geha (1984) raised rabbit anti-Id to tetanus toxoid (TT) specific human antibody. The preincubation of T cells from TT hyperimmunized individuals with anti-Id for 48 hr resulted in the generation of antigen-specific suppressor cells that

inhibited in vitro T cell proliferation in response to TT. These suppressor cells also inhibited the in vitro synthesis of IgG anti-TT in response to TT antigen stimulation. These suppressor cells are idiotype positive since adsorption of T cells over plates coated with rabbit anti-Id removed suppressive effects of the T cells. In a similar model, Saxon et al (1984) reported that in the TT hyperimmunized human individuals, the occurrence of auto-anti-Id in the serum was correlated with the development of a cutaneous anergy to TT and with a suppressed in vitro peripheral blood mononuclear cell blastogenic response to soluble TT. In a clinical study by Rankin et al (1985), mouse anti-Id was administered to 2 patients with advanced B cell lymphoma. A transient (several hrs) reduction of the number of circulatory Id positive lymphocytes was observed, however, the mechanism was not studied and the possibility of relocation of these malignant cells was not excluded. It was also understood that the idiotype bearing B cells were malignant and that the effect of anti-Id on these cells might be totally different from that on normal B or T cells. Another recent clinical study was carried out by Frommel (1985). He reported that a hemophilic patient developed auto-anti-idiotypic antibodies toward antibody against factor VIII C and this could account for the decrease of activity of autoantibody to factor VIII C. Like many clinical investigations, this study was difficult to control for various ethical reasons.

Since our major interest is the immune network in house dust mite allergy which is mediated by IgE, it is worthwhile to review the idiotype/anti-idiotype regulation of IgE response, which has been investigated so far, in animal models. In a study by Blaser et al (1983) Balb/c mice were first actively immunized with purified anti-benzylpenicilloyl (BPO) antibodies (Id) derived from syngeneic animals. These Id primed mice were then injected after several months with BPO_4-ovalbumin (OVA) in alum. The anti-BPO IgE antibody production was selectively suppressed for more than 20 weeks in such mice actively producing anti-Id. The production of IgE anti-carrier protein (OVA) in these mice was not impaired. The suppression of IgE anti-BPO could be transferred by whole spleen cells to irradiated mice. If the spleen cells were treated with monoclonal anti-Lyt2.2 antibody plus complement, the suppression could no longer be transferred. This suggested that anti-Id had induced T_S cells that were responsible for the suppression of the hapten specific IgE response. In similar experiments (Blaser et al 1981), mice were immunized repeatedly with anti-carrier (OVA) antibody, in order to produce anti-Id to anti-OVA antibody. These mice were then sensitized three months later with BPO_4-OVA or DNP_4-OVA. The production of IgE antibodies against both haptens was suppressed. If, however, they were sensitized with an antigen consisting of the same haptens, but bound to an unrelated carrier (such as ascaris protein extract, ASC), they showed normal levels of anti-hapten and anti-carrier IgE. This carrier specific suppression was not isotype specific, because IgG

antibodies were also suppressed. The exact mechanisms involved in the regulation by anti-carrier anti-Id were not fully understood. It is possible that the anti-Id had induced carrier-specific T_S cells and/or blocked carrier-specific T helper (T_H) cells. A similar regulation by anti-Id was also observed in the PC system (Blaser et al 1982). According to Blaser et al (1983), it seemed that IgE was more susceptible to anti-Id suppression than the other isotypes.

The theory of the immune network has now been consolidated after 12 years' extensive studies by many investigators. It is now understood that the immune response can be modulated through idiotype-anti-idiotype interactions, that anti-idiotype antibody can suppress or enhance the humoral or cellular response, and that T cells are, most likely, involved in such anti-Id-induced suppression. However, several questions are still open. Does the immune network exist universally, is it applicable to all antigens in the animal/human systems? To what extent does the network govern the immune system, part of it, or all of it? What is the mechanism of anti-Id regulation? Is there only one mechanism, or more? The immune system is so complicated that one can hardly believe that a single theory can describe all its facets, and that a single discipline can govern all its actions. Most likely, we will never get a perfect picture of the immune system just as we have not yet and will never get any perfect picture about anything in the universe. Nevertheless, by continuous exploration under the guidance of the existing theories, and by

constant modification of these theories, including network, based on the accumulated evidences, we might be able to approach the truth closer and closer. As one effort of exploration, the possibility for the existence of an immune network in house dust mite, allergic patients will be examined in the present study.

II. House Dust Allergy

Asthma, allergic rhinitis (hay fever), eczema and urticaria are clinical examples of one type of immediate hypersensitivity. These allergies are quite common, affecting about 10% of the population (Platts-Mills, 1982a). They are often referred to as atopic diseases and it is generally accepted that they are mediated by IgE antibodies. Serum IgE concentration is 3 to 7 logarithmic orders lower than that of IgG. The biological activity of IgE stems from its unique property to fix to tissue mast cells and basophils. Upon contact with specific allergens, chemical mediators, such as histamine are released from these cells, and induce clinical manifestations. Many materials of plant origin such as pollens, spores of mould, various kinds of woods, etc., and of animal origin such as some proteins of parasitic worms (Ascaris) or grasshoppers (Locusta), skin scales of various animals, etc., can induce IgE antibody synthesis in atopic patients (Spieksma 1969). House dust is one of the major allergens, and it was reported that in the United Kingdom, 5-10% of the population develop symptoms due to dust allergy (Morrison-Smith et al 1969). In addition to rhinitis, patients with house dust atopy most frequently suffer from

asthma (Spieksma 1967). In many parts of the world, up to 85% of patients with extrinsic asthma show positive skin tests to dust extracts (Kabasawa et al 1976; Biliotti et al 1972). House dust allergy might also play a role in atopic dermatitis (Mitchell 1982; Chapman et al 1983).

The existence of allergy to house dust was first suggested by Kern (1921) and subsequently by Cooke (1922) and many others, who showed the high frequency of positive skin tests to house dust extract in allergic patients. House dust is a mixture of many compounds, such as human or animal danders, moulds, bacteria, mites, rubbish and many inorganic materials. Voorhorst and his group (1962) searched for a major allergic factor in house dust, and various components of house dust were investigated. Human danders extract could cause high frequency of positive skin-reaction in dust allergic patients. Nevertheless, there was no quantitative or even qualitative correlation between the skin reactivity to dander and to house dust. Besides, the maximum dilution still giving positive reactions was about 0.01%, for both human dander and house dust, while a major allergen should be much more potent than house dust. Therefore, dander could not be considered as a major allergen in dust. Various ubiquitous moulds, such as Penicillium, Aspergillus, Alternaria and Streptomycetes, were also tested. Although some of them could induce skin-reaction in certain patients, they were less potent than house dust. Their seasonal variations were not in accordance with

that of house dust allergy, so the moulds were also ruled out. Voorhorst also examined several kinds of bacterial extracts, especially from aerobic spore-forming bacteria. Some of them could provoke positive skin-reactions, but these reactions could not be passively transferred by means of a Prausnitz-Kustner test, and were considered to have resulted from an irritative effect of the extract. Extracts prepared from flies, mosquitoes, moths, spiders and other arthropods would seldomly or never give positive skin-reactions. Therefore, all these components of dust were not the major allergen responsible for allergy to house dust. The extracts from three species of mites, i.e., Acarus siro, Tyrophagus putrescentiae and Glycyphagus domesticus, however, could give strong skin-reactions sometimes even at a concentration of 0.0001%. These mites were certainly producers of strong allergens. But their presence was not correlated to the skin reaction to house dust, so, the results of studies by Voorhorst did not really identify the major source of house dust allergen but did suggest, for the first time, that some species of mite might be responsible.

Many species of mite were found as inhabitants or invaders of the house. Oboussier (1939) reported 4 species and Solomon (1961) reported 12 species of mites in human inhabitated buildings. Oshima (1964) reported the finding of mites in the dust collected in Yokohama area, Japan. Of these mites, almost 90% appeared to belong to the genus Dermatophagoides. In an independent study by Voorhorst and

Spieksma (1964), it was also found that mites occurred regularly in the house dust and that the species of Dermatophagoides was much more represented than any of the other species found in house dust in Leiden, The Netherlands. In a large scale study carried out in 4 cities of Western Netherlands in September 1965 (Spieksma 1967), dust was collected from 150 houses, and the species of mites were examined. It was found that the mite Dermatophagoides was present in dust of all the 150 houses. It was the most abundant species and accounted for 69.9% of the total mites found in the dust samples. Among the Dermatophagoides counted, 87.6% were D. pteronyssinus, which was present in 100% of the houses, 11.2% was D. maynei, which was present in 53% of the houses, and 1.2% was D. farinae which was present in 2% of the houses. To find out whether there was a causal relation between the number of D. pteronyssinus and the allergen, 13 patients were tested with 4 house dust extracts containing different numbers of mite D. pteronyssinus. The results of the skin tests showed a good correlation between the number of mites and the strength of the extracts of the 4 dust samples. In order to establish absolutely the causal relationship between D. pteronyssinus and house dust allergy, the mites were inoculated into a dust sample which showed a low allergic activity, and incubated under a suitable condition. Three to four months later, the allergen activity of the dust increased 100 times along with a rising number of D. pteronyssinus, while the allergen activity of the same dust,

incubated under the same conditions but without inoculation of mites, remained the same. Therefore, after more than 40 years of research, it was established that at least in The Netherlands, the major allergen of house dust was D. pteronyssinus.

It was soon reported by many authors that D. pteronyssinus was the dominant mite species in house dust in many parts of the world, such as Cadiz, Spain (Calero et al 1971), Scotland (Sesay et al 1972) Strasbourg and Alsace-Vosges region, France (Araujo-Fontaine et al 1972,1973), Hawaii (Sharp et al 1970), South Africa (Ordman 1971), New Delhi, India (Lal et al 1973), Birmingham, England (Blythe et al 1974), North Zambia (Buchanan et al 1972), Switzerland (Mumcuoglu 1976), Uruguay (Schuhl 1977), Nigeria (Hunponu-Wusu et al 1978), U.S.S.R. (Dubinina et al 1978), Iran (Sepasgosarian et al 1979), Lisbon (Pinhao et al 1977), Havana, Cuba (Lopez et al 1981), Brunei, South East Asia (Woodcock et al 1982) and the West Coast of the United States (Bullock et al 1972). In some parts of the world, such as Japan, other species such as Dermatophagoides farinae is the most abundant species (Morita 1975).

The place of Dermaphagoides pteronyssinus in the animal kingdom is shown in Figure 2 (Spieksma 1969). The Acari, which is comprised of mite and ticks, forms an important division of the great Arthropodan class Arachnida. The Arachnida also include such

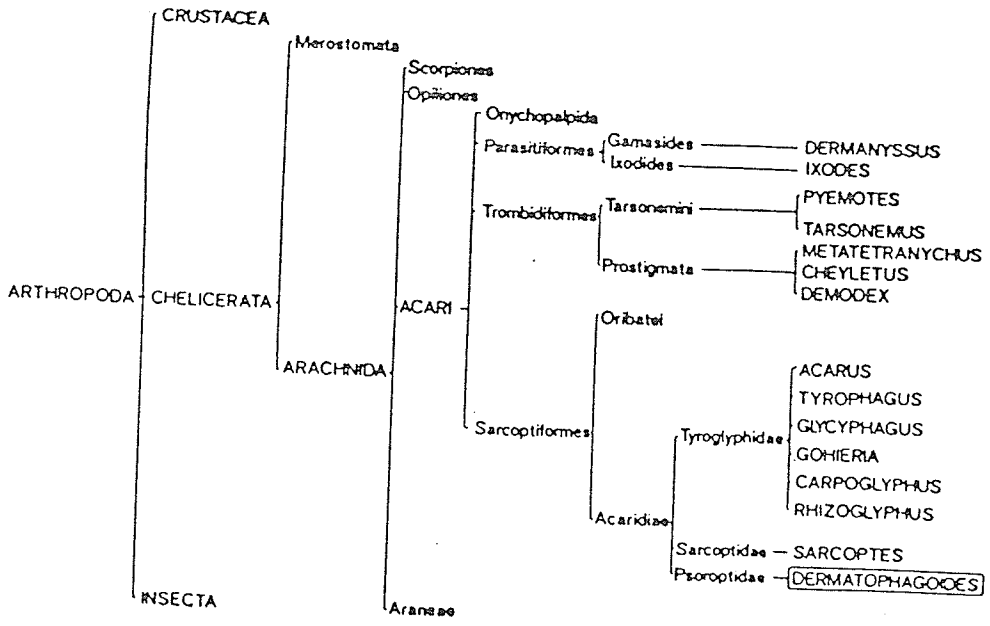


Fig. 2 Classification of some of the best known Acari (mites and ticks) in the phylum Arthropoda (jointed-legged animales)

animals as scorpions, spiders and harvestman. In 1897, Trouessart (in Berlese 1897) created the genus Mealia with two species: M. pteronyssina and M. longior. Baker and Wharton (1952) synonymized the genus Mealia with the genus Dermatophagoides. The genus Dermatophagoides had long been included in the family Epidermoptidae. Fain later transferred it to the Psoroptidae. D. pteronyssinus was redescribed by Fain (1966) from paratypes in the original preparations of Trouessart, the original types being lost.

The life cycle of D. pteronyssinus includes the egg stage which lasts 6 days, the larva stage which takes 5-6 days, the protonymphal stage which lasts 4-7 days, tritonymphal stage which is about 4-8 days, a resting form about 2 days and adult, male and female. The total immature life form from larva to adult lasts 14-20 days. Adult males live 60-80 days and females 100-150 days (Spieksma 1967). The illustrations of an adult male and female are given in Figure 3 and 4 (after Fain 1966). The idiosoma of the female is about 340 μm long and 230 μm wide. The male is smaller than the female, the length being about 280 μm and the width about 190 μm. Under laboratory culture conditions, 25°C and 80% humidity is optimal for the growth of D. pteronyssinus (Spieksma 1967).

The major allergen P1 of D. pteronyssinus was purified by Chapman et al (1980a) from culture mites by the combination of gel

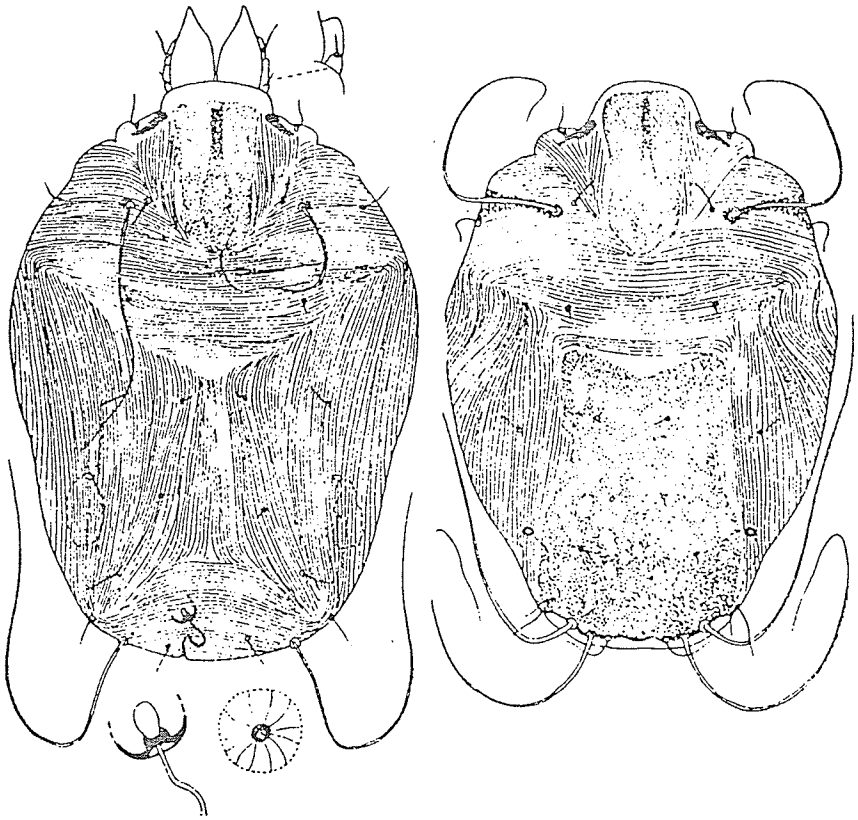


Fig. 3 *Dermatophagoides pteronyssinus* (Trouessart, 1897): dorsal view of female (left) and male (right) (after FAIN, 1966).

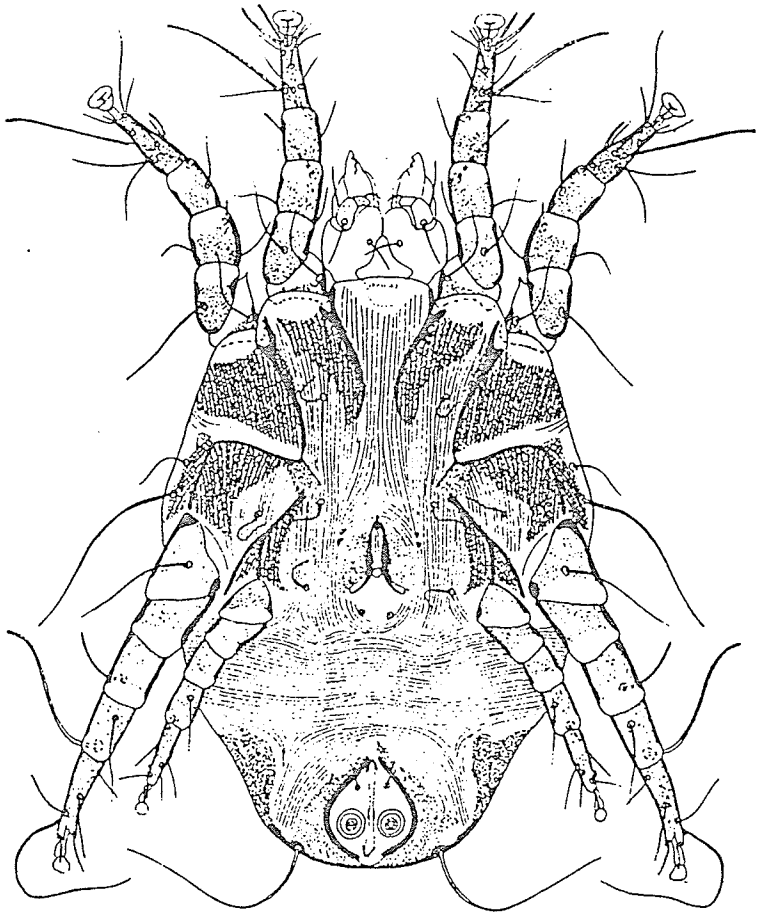


FIG. 4a *Dermatophagoides pteronyssinus* (Trouessart, 1897); ventral view of male (after FAIN, 1966).

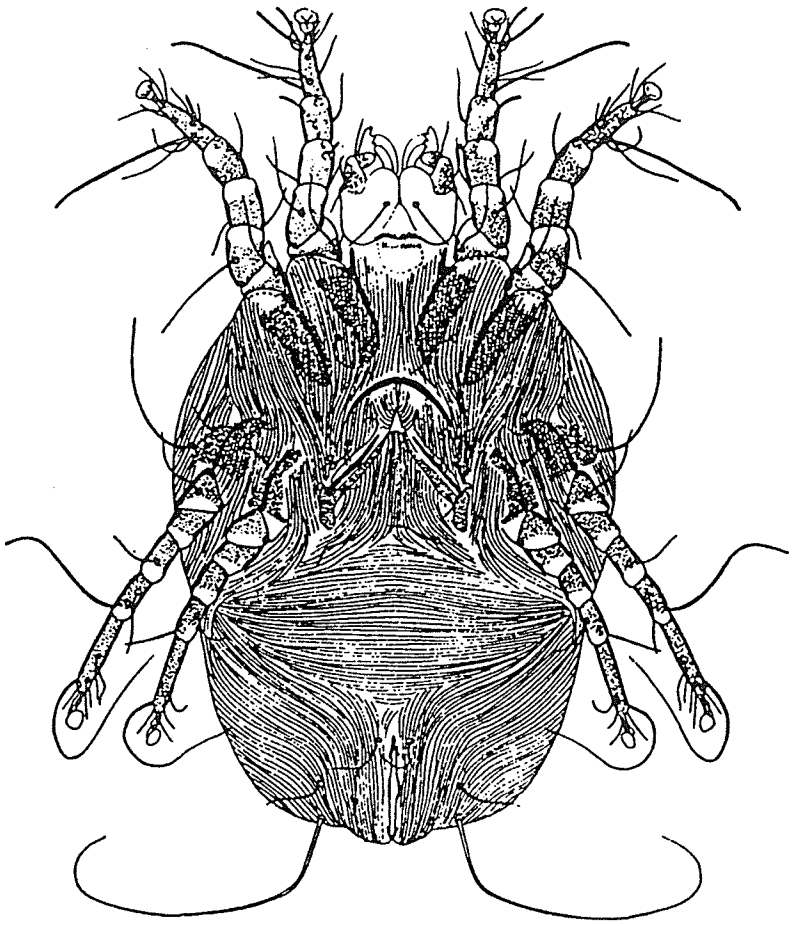


FIG. 4b *Dermatophagoides pteronyssinus* (Trouessart, 1897); ventral view of female (after FAIN, 1966).

filtration, electrophoresis and isoelectrofocusing. P1 is a glycoprotein with mol.wt. of 24,000 daltons. It is comprised of multiple isoallergens since its PI spreads from pH 4.7 to 7.1. Radioallergosorbent test showed that up to three-quarters of the IgE antibody to D. pteronyssinus from a group of house dust allergic patients was directed against P1, and that IgE antibody to P1 accounted for an average of 12% of the total serum IgE in a group of D. pteronyssinus allergic individuals. Different components of mite culture were studied for their P1 content by Tovey et al (1981). It was found that whole mites, cuticles and mite faeces contained antigen P1, but eggs contained very little. The concentration of P1 in faeces is as high as that of mite bodies, approximately 10 mg P1 per ml, and a mite can produce an average of 20 faecal particals per day, each of which is 10-40 μm in diameter. According to the calculations, after one month, faeces would account for more than 95% of the antigen P1 in cultures. Therefore, it was concluded that mite faeces are a major source of house dust allergens.

One of the proposed treatments for house dust allergy is dust mite avoidance, and the results are controversial. Platts-Mills et al (1982b,1985b) and Murray et al (1983) reported that if very strenuous efforts were made to clean the bedrooms, including removal of carpets, covering mattress and pillows with zippered vinyl bags and regular washing of bedding, the improvement of asthma symptoms and peak expiratory flow rate in asthmatic patients allergic to house dust mite

was observed. In other studies by Burr et al (1980) and Bowler (1985), the difference of improvement between dust avoided and control groups of asthmatic patients was not significant. This discrepancy is possibly due to insufficient cleaning measures in the latter two studies. Whatever the results are, the dust free treatment does not seem to be very practical considering the fact that mites are ubiquitous and a year round tremendous effort of cleaning is required. Besides, Korsgaard (1982) concluded that if the relative humidity in the house remains above 50%, which is the case in many parts of the world, it is not possible to reduce mite numbers sufficiently to help patients.

Another disputable therapy for house dust allergy is desensitization. In controlled trials, it has been demonstrated that injections of extract of house dust mite culture can modify the symptoms caused by natural exposure to house dust mite allergen (Davies et al 1983; D'Souza et al 1973; Gabriel et al 1977; Maunsell et al 1971; Smith 1971; Price et al 1984; Delaunois et al 1985). In most trials, the differences between the placebo-treated and the extract-treated groups were not very large, the therapy was not effective in all the patients. A prolonged treatment was needed, and there was only some relief of the symptom but no cure of the disease. There are as well many trials where no significant improvement by the desensitization therapy was observed (Gaddie et al 1976; Smith et al 1971, 1972; British Thoratic Association 1979). In the cases where

some beneficial effect of desensitization is observed, the mechanism is still not understood. Desensitization therapy was initially used to treat hay fever. At that time, Noon (1911) thought he was raising immunity against pollen toxin. This mechanism was of course ruled out when it was later known that atopy was caused by heat labile reagin (Spivake et al 1925). Subsequently, it was found that desensitizing injections would give rise to heat-stable antibodies which could "block" the skin reactivity of an allergen extract and which were distinct from reagins (Cooke 1935). Therefore, the generation of "blocking antibody" appeared a likely explanation for the effect of desensitization. A rough correlation between blocking antibody and symptomatic improvement has been found in some cases (Osler et al 1968), but generally the correlation was poor (Norman 1980) and most authors remain unconvinced that there is a direct relationship between serum blocking antibody and symptom improvement. The increase of the "blocking antibody" in the nasal secretion (IgG and IgA) after allergen injections is lower than that in the serum, where "blocking antibody" are mainly IgG (Platts-Mills et al 1976). Since only local IgA or IgG antibody in the secretions can compete to bind air-borne allergen in atopic asthma or rhinitis, it is not difficult to understand that high serum specific IgG titre might not correlate with improvement of symptoms. Moreover, IgE on the surface of mast cells or basophils has a great advantage to bind allergen because it is close to other IgE molecules on the cell surface and is effectively polyvalent, and the interaction of allergen with mast cells or

basophils is very rapid (i.e., less than one min) both in vitro and in the nose (Hastie 1971, Mygind 1979). By contrast, direct studies on the binding of allergens with excess antibodies from serum or nasal secretions have shown that with concentration of antigen below 1 ng/ml which is possibly the range for house dust allergen (Platts-Mills et al 1985), even 50% binding can take more than 30 min (Chapman et al 1980b). A similar conclusion has been reached using rabbit antibody to bind low concentrations of allergens in vitro (Schumacher et al 1979). Therefore, there are good reasons for doubting that any free antibody in the secretions of respiratory tract could prevent or "block" the allergen to reach the mast cells or basophils.

The effect of desensitization on IgE production is, if there is any, slow and modest. Initially, within the first two months, IgE antibody levels often rise and then progressively fall after 1 or 2 years of treatment (Lichtenstein et al 1973). However, Turner et al (1984) reported that the absence of any consistent pattern of change of P1 specific IgE antibody levels after one year desensitization with D. pteronyssinus antigen. In either case, the clinical improvement is not correlated to the change of serum IgE antibody level.

In several studies, a decrease in reactivity of basophils or mast cells has been observed during the desensitization. For example, 50% histamine release requires a higher concentration of antigen;

similarly the maximum percentage of histamine that can be released may fall and the percentage of basophils reacting to anti-IgE decreases (Sadan et al 1969; Kimura et al 1985). However, the relationship between clinical improvement and changes in sensitivity or reactivity of basophils and mast cells in most cases has been poor or even absent (Irons et al 1975; Norman 1980).

House dust mite allergy is an immunological disorder. As reviewed above, various treatments have been tried. Dust avoidance is not very practical. The results of desensitization are not conclusive and the mechanism remains obscure. Pharmacological treatment, which is out of the scope of the present study and is not reviewed, can relieve symptoms but only transiently, not to mention various side effects. Recently, many evidences have indicated that at least for some antigens, the immune response is subjected to idiotype-anti-idiotype regulation. It is therefore interesting to investigate the existence of idiotype-anti-idiotype interactions in house dust mite allergic individuals. This might possibly be the first step of a research program aiming to manipulate the network in order to downregulate the abnormal immune response to house dust allergen.

CHAPTER II

PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO THE MAJOR ALLERGEN OF HOUSE DUST MITE

Abbreviations used in this Chapter:

AchR	acetylcholine receptor
Auto-anti-Id	auto-anti-idiotype antibody
BSA	bovine serum albumin
Cpm	count per minute
CRI	crossreactive idiotope or idiotype
DNA	deoxyribonucleic acid
FCS	fetal calf serum
GAM	goat anti-mouse (Ig)
HAT	hypoxantin, aminopterin, thymidine
HDM	house dust mite
HPLC	high performance liquid chromatography
HT	hypoxantin, thymidine
IgE	immunoglobulin E
IgG	immunoglobulin G
i.p.	intraperitoneally
LPS	lipopolysaccharide
Mab	monoclonal antibody
MAH	mouse anti-human (Ig)
NMS	normal mouse serum
P1	the major allergen of house dust mite (D. pteronyssinus)

PBS	phosphate buffered saline
PC	L-α-phosphatidyl choline
PEG	polyethylene glycol
RAM	rabbit anti-mouse (Ig)
RIA	radioimmunoassay
Rpm	revolutions per minute
Rye I	rye grass pollen antigen I
SAS	saturated ammonium sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TT	tetanus toxoid

Abstract

Mab were raised against antigen P1, the major allergen of the house dust mite (Dermatophagoides pteronyssinus). The Mabs were P1 specific, and the majority of them were of the γ_1^{κ} isotype. Crossinhibition experiments, using Mab to inhibit the binding of radiolabeled P1 to Mab, suggested that 9 Mabs could be grouped into 4 families, with some degree of overlapping. It was postulated that each epitope was composed of a "public region" which was recognized by all the Mab families and of a "private region" which was only recognized by one family. Based on the concentrations of Mabs needed to give 50% inhibition in the crossinhibition assay, a linear and a two-dimensional topography of epitopes on P1 were proposed. Human sera from HDM allergic individuals could inhibit completely (100%) the binding of Mab to P1, and Mab could inhibit partially (with inhibitions ranging from 14-100%) the binding of serum IgE to P1. This suggested that a large portion of human IgE was against the same antigenic moiety as that recognized by Mabs.

Introduction

The house dust mite (HDM) Dermatophagoides pteronyssinus is an important cause of IgE-mediated asthma, rhinitis (Voorhorst 1967; Spieksma 1969), and possibly atopic dermatitis (Mitchell 1982; Chapman 1983) in many parts of the world. The allergen P1 derived from this species has been purified from extracts of mite culture, and the main source of antigen P1 in both mite culture or house dust is mite feces (Chapman et al 1980). P1 is a heat-labile glycoprotein with a molecular weight of 24,000 daltons and exhibiting a wide range of isoelectric points. Allergen P1 appears to comprise a substantial portion of the protein in crude mite extract (10 to 20%), and it was estimated in one study that up to three-quarters of the IgE antibodies to D. pteronyssinus from HDM allergic individuals were against P1 (Chapman et al 1980). Therefore, P1 is considered as the major allergen in house dust mite by most investigators. However, the determinants on P1 have not been fully characterized in terms of their immunogenicity and operational topography.

Natural exposure to HDM antigen leads to the production of specific IgG and IgE antibodies in individuals with allergic symptoms (Chapman et al 1978), and in infants at risk of allergy (Rowntree et al 1985). Moreover, the major HDM antigen P1 could stimulate the in vitro proliferation of T cells from HDM allergic patients (Rawle et al

1984). It will be interesting to know if idiotype-anti-idiotype interactions play a role in the immune response to HDM antigen. Such interactions have been proven to exist in many animals as well as human models, which has been reviewed extensively in Chapter I. Moreover, auto-anti-Id has been demonstrated in several human models indicating the existence of such interactions. For example, Abdou et al (1981) found auto-anti-idiotypes (auto-aId) against anti-DNA antibodies in the serum of patients with Systemic Lupus Erythematosus. Geha (1982) described auto-aId after tetanus toxoid immunization of normal individuals; Dwyer et al (1983) reported the existence in myasthenia gravis sera of auto-aId, that were detected by means of mouse monoclonal antibodies to the acetylcholine receptor. Bose et al (1984) demonstrated auto-aId in rye grass sensitive patients.

The landmark paper of Kohler and Milstein (1975), describing the production and characterization of the first somatic cell hybrids capable of indefinite production of antibody of predetermined specificity against a certain antigen, heralded a new era in biology. This technique has been applied to a wide variety of antigens and for a wide variety of purposes ever since. One of the important applications of monoclonal antibody (Mab) is to study the epitopes and their topography on antigen molecules since Mab is assumed monospecific and each clone only binds to one specific epitope. Monoclonal antibodies were prepared against human growth hormone (Ivanyi 1982), hemagglutinin of influenza virus (Gerhard et al 1981),

allergen of rye grass pollen (Smart et al 1983), Reovirus group 3 hemagglutinin (Spriggs et al 1983), and the antigenic structures of these molecules were defined by the Mab. In the last case, the topography of epitopes on group 3 hemagglutinin molecule was also described according to the results of crossinhibition between different clones of Mab. Monoclonal antibodies to P1 have been produced and their specificity was fully characterized by Chapman et al (1984). By means of their Mab, these authors were able to identify two distinct epitopes on P1, however, the topography study of the determinants was not carried out. Another possible application of Mab is to examine the immune network theory and to identify anti-idiotype antibody in human or animal. This approach will be feasible only if interstrain or interspecies crossreactive idiotypes (CRI) exist in the immune response to a certain antigen. Several studies showed that CRI between species do exist in certain cases. Karol et al (1977) reported that the anti-human sickle cell hemoglobulin antibody from a goat and from a sheep shared a CRI detected by a rabbit anti-idiotype antibody. CRI was also detected in murine and human anti-phosphoryl choline antibody (Riesen 1979; Bach et al 1981), rabbit and human anti-tetanus toxoid antibody (Hoffman et al 1985), murine and human anti-timothy allergen antibody (Malley et al 1983a), murine and human anti-DNA auto-antibody (Morgen et al 1985), and murine and human anti-acetylcholine receptor (AChR) antibody (Dwyer et al 1983). In the last case, Mab against AChR was successfully employed as a probe to demonstrate and even to isolate human auto-anti-idiotypic antibody against anti-AChR antibody.

In the present study, we have produced several monoclonal antibodies specific to P1 and reacting with a contiguous region on the P1 molecule. A linear map was constructed to depict the topography of this region on P1 according to the crossinhibition between monoclonal antibodies. Moreover, the Mabs prepared were found to react with epitopes that were also identified by IgE anti-P1 from several HDM allergic donors. These Mab will be used as probes to detect auto-anti-idiotypic antibody in human serum.

Materials and Methods

Radioimmunoassay for the detection of mouse anti-P1 antibodies (referred hereafter as RIA #1)

This assay was employed in monitoring mouse serum IgG response to P1 and in screening IgG antibody to P1 in culture supernatants of hybridoma. Since no mouse antibody to P1 was available at the time of optimizing assay conditions, this assay was set up according to a reference antibody and antigen system, i.e., monoclonal mouse-anti-human-IgE (MAH-IgE, clone #89, prepared in this laboratory) and human IgE (P.S.). One hundred µl per well of different dilutions of affinity-purified goat-anti-mouse IgG antibody (GAM-IgG; Calbiochem, La Jolla, CA) in sodium bicarbonate buffer (0.01M, pH 9.0) were used to coat flexible polyvinyl chloride microtitre plates (Dynatech, Alexandria, VA) by overnight incubation at room temperature

in a humid chamber. The solutions were then decanted and plates were blocked with 150 µl/well Hank's solution containing 10% fetal calf serum (FCS, Gibco, Burlington, ON) at room temperature for 2 hrs. Plates were washed 10 times with PBS with PHD cell harvester (Cambridge Technology Inc., Cambridge, MA) and air dried. Different amounts of monoclonal IgG MAH-IgE (clone #89) were diluted in normal mouse serum (NMS) or Hank's solution containing 15% FCS, and added to GAM-IgG coated plates at a volume of 100 µl/well. After overnight incubation at room temperature in a humid chamber, plates were washed 10 times with PBS, and 100 µl/well of radioiodinated human IgE (about 150,000 cpm) in assay buffer (PBS containing 0.5% Tween 20, 0.1% bovine serum albumin) was then added. Plates were again incubated in a humid chamber at room temperature overnight, and washed with PBS 10 times. Wells were cut individually and counted in a Beckman gamma 8000 γ-counter (Fullerton, CA).

After the conditions were optimized, this assay was used in monitoring the mouse antibody response after immunization with Pl, and in screening of hybridoma for anti-Pl antibody production. According to the results of the reference system, 20 µg/ml GAM-IgG was used to coat plates, and 150,000 cpm/100 µl radioiodinated Pl in assay buffer was employed instead of radioiodinated human IgE. Ten percent FCS was added to assay buffer which was used to dilute ^{125}I-Pl throughout assays except the first one, since it was found later that FCS in assay buffer could reduce background considerably in Pl and anti-Pl assay system.

Radioimmunoassay to demonstrate the specificity of anti-Pl Mab (referred hereafter as RIA #2)

It was a modification of RIA #1. The protocols and materials were basically the same as those in RIA #1, except for the last incubation. Briefly, plates were coated with GAM-IgG antibody and blocked with Hank's solution containing 10% FCS, and then washed with PBS. Test samples (Mabs) were added to wells (150 µl/well), and incubated overnight. The plates were washed again with PBS. For the final incubation, instead of using 100 µl of ^{125}I-Pl per well, 20 µl of different inhibitors in PBS and 80 µl of ^{125}I-Pl (about 150,000 cpm) in assay buffer containing 10% FCS were added to each well. After overnight incubation at room temperature, plates were washed again with PBS, and wells were cut and counted. Pl from Dr. T. Platts-Mills (University of Virginia, Charlottesville, VI), Rye I from Dr. D. Marsh (Johns Hopkins University, Baltimore, MD), Lipopolysaccharide (LPS) of E. coli from Fisher, L-α-phosphatidyl choline (PC) from Sigma, tetanus toxoid (TT) from Connaught Lab, and crude house dust mite D. pteronyssinus extract batch #1 from Beecham Pharmaceutical Co. and batch #3 from Dr. H. Lowenstein (University of Copenhagen, Denmark), were used as inhibitors. The background was mean cpm of 4 wells incubated with unrelated Mab (anti-Rye I #18), and the percentage inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{Mean cpm of wells with inhibitor - background}}{\text{Mean cpm of wells without inhibitor - background}} \times 100\%$$

Radioimmunoassay to determine the subclasses of anti-Pl Mab (referred hereafter as RIA #3)

All the steps and materials were basically the same as in RIA #1 except that different anti-mouse-IgG-subclass antibodies were used to coat plates. Briefly, goat-anti-mouse (GAM) γ1 chain or γ3 chain (Nordic Immunology, Tilburg, The Netherlands), goat-anti-mouse 2a chain or γ2b chain (Meloy, Springfield, VA), or rabbit-anti-mouse (RAM) κ chain or λ chain (Litton Bionetics Inc., Kensington, MD) were used at 1:100 dilution in 0.01M carbonate buffer, pH 9.0 to coat the plates. After overnight incubation, plates were blocked with Hank's solution containing 10% FCS for 2 hrs. Plates were washed and incubated with different monoclonal antibodies (0.5 µg/ml in PBS, 100 µl/well), which were purified by two precipitations with 50% saturated ammonium sulphate (SAS) followed by HPLC DEAE ion exchange chromatography. Plates were washed again, and ^{125}I-Pl in assay buffer containing 10% FCS were added to wells. After incubation, plates were washed, and wells counted in a gamma counter.

Radioimmunoassay to determine the epitope specificity (referred hereafter as RIA #4)

This assay was used in studying families of monoclonal antibodies to Pl and crossreactivity between Mab to Pl and human antibodies to Pl. It was also employed in testing the specificity of Mab. Monoclonal antibodies purified by HPLC DEAE ion exchange chromatography were used to coat polyvinyl chloride

microtitre plates (12.5 μg/ml in 0.01M carbonate buffer, pH 9.0; at a volume of 100 μl/well). After overnight incubation at room temperature, plates were blocked with Hank's solution containing 10% FCS for 2 hrs. Plates were then washed 10 times with PBS and air dried. Twenty μl of different inhibitor in PBS and 80 μl of ^{125}I-P1 (about 2 ng, in about 150,000 cpm) were added to each well. After another overnight incubation at room temperature in a humid chamber, plates were washed again with PBS 10 times, and wells were cut individually and counted in a gamma counter. The inhibitors employed in these assays were: (1) Different clones of Mab which were partially purified by two 50% SAS precipitations, (2) Lipopolysaccharide (LPS) from E. coli, (3) Rye I, (4) L-α-phosphatidyl choline, (5) crude house dust mite D. pteronyssinus extract batch #3, (6) human myeloma IgG partially purified by 50% SAS precipitation, and (7) pooled allergic human sera of seven patients who were allergic to house dust mite and were positive in RAST test to D. pteronyssinus antigen (obtained from Dr. R. Pauwels, Gent, Belgium). Normal serum pool from 10 nonatopic donors, who had no allergic history or symptoms and were negative in serum IgG antibody to P1, were employed as controls. The percentage inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{Mean cpm of wells with inhibitor - background}}{\text{Mean cpm of wells without inhibitor - background}} \times 100\%$$

Radioimmunoassay to determine the crossreactivity between Mab and human IgE anti-Pl antibodies (referred hereafter as RIA #5)

This assay was a modified RIA #2. Briefly, ascitic fluid of Mab clone #89 to human IgE was diluted 400 times in 0.01M carbonate buffer, pH 9.0 and was employed to coat microtitre plates at a volume of 100 µl/well. Plates were blocked with Hank's containing 10% FCS and washed. Human sera (from Dr. Pawels, Gent, Belgium) from patients with allergic symptoms to house dust mite and positive RAST test to D. pteronyssinus or pooled sera from 10 nonatopic donors, who had no allergic history or symptoms and were negative in serum anti-Pl IgG, were diluted 10 times in PBS and incubated in plates (100 µl/well) overnight at room temperature. Plates were then washed with PBS. Twenty µl of different Mab to Pl and 80 µl of ^{125}I-Pl (about 15,000 cpm) in assay buffer containing 10% FCS were added to wells and incubated overnight. Plates were washed with PBS again, cut, and finally counted. The percentage inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{Mean cpm of wells with inhibitor - background}}{\text{Mean cpm of wells without inhibitor - background}} \times 100\%$$

Radioiodination of Antigen or Antibody

A modified chloramine T method (Johnstone et al 1982) was employed to radioiodinate antigen or antibody. Ten µg - 100 µg antigen or antibody in 100 µl, 0.05M phosphate buffer (PB), pH 7.5, was mixed with 10 µl (1 mCi) of sodium iodine (Na ^{125}I, Amersham, U.K.). Twenty-five µl of chloramine T (Sigma, St. Louis, MO; 2.4

mg/ml in 0.05M, pH 7.5 PB) were added and mixed vigorously for 45 sec. The iodination was stopped by adding 100 µl of $Na_2S_2O_5$ (Sigma, St. Louis, MO; 4 mg/ml in 0.05M, pH 7.5 PB) and 200 µl of KI (Sigma, St. Louis, MO; 10 mg/ml in 0.05M, pH 7.5 PB). Labeled materials were diluted into 2 ml of assay buffer containing 10% bovine serum albumin (BSA, Sigma, St. Louis, MO) and dialyzed at 4°C against PBS in dialyzing tubing (Spectrum Medical Industries, Los Angeles, CA) with cut-off point of 10,000 daltons. After three changes of dialyzing buffer, the labeled antibody or antigen was stored at 4°C.

Immunization of Mice

Two female, 2-4 weeks old, Balb/c mice were immunized with P1. For immunization, each mouse received a subcutaneous injection of 5 µg P1 in 50 µl PBS emulsified with 50 µl complete Freund's adjuvant (Difco, Detroit, MI). There was a 4 week interval between the first immunization and the second, and a 2 week interval between the second and the third. Six weeks after the third immunization, a final boost of 10 µg P1 in 300 µl PBS without adjuvant was administered intraperitoneally, and the fusion was conducted 3 days after the boost. The mice were bled 10 days after the second and third immunization and at the day of sacrifice. The blood was collected into tubes containing 500 µl PBS and 1 drop of heparin (Gibco, Burlington, ON). After centrifugation, the supernatants were assayed for anti-P1-antibody with RIA #1.

Retrieving of NS-1 Cells from Liquid Nitrogen

Mouse myeloma cells NS-1, from Balb/c mice, were used as fusion partners. Vials containing NS-1 cells were taken out from a liquid nitrogen tank, and immediately submerged into 37°C water bath. After the cells were thawed, cell suspension was transferred to a 15 ml tube in sterile condition under a Laminar hood (Nuaire Inc., Plymouth, MA). Five ml complete medium, which was composed of 83% RMPI 1640 (Flow Laboratories, McLean, VA), 15% heat inactivated FCS (Flow Laboratories), 1% penicillin-streptomycin solution (penicillin - 10,000 U/ml, streptomycin - 10,000 mcg/ml; Gibco Laboratories, Chagrin Falls, OH), and 1% L-glutamine solution (29.2 mg/ml; Gibco Laboratories, Chagrin Falls, OH), were added to the tube and mixed. Cells were spun down by centrifugation at 1500 rpm for 5 min, supernatant was aspirated and cells were washed once more. Cell pellet was resuspended in 1 ml complete medium, and cell concentration and viability were examined by trypan blue exclusion (Mishell et al 1980). Briefly, 50 µl cell suspension was mixed with 450 µl of 0.01% trypan blue in PBS, and cells were counted in a hemocytometer. Viability was calculated as follows:

$$\text{Viability} = \frac{\text{Number of total cells - number of stained cells}}{\text{Number of total cells}} \times 100\%$$

Cell density was adjusted to 0.5 x 10^6 viable cells/ml, and the cells were cultured in 24 well flat bottom culture plate (Becton-Dickinson, Oxnard, CA) at a volume of 1 ml/well in an incubator (Forma Scientific, Marietta, OH) at 37°C in a humidified atmosphere containing 5% CO_2. The cell culture was split every 2-3 days depending on the rate of growth of cells, and was later transferred to 25 cm^2 and finally 75 cm^2 tissue culture flask (Becton-Dickinson, Oxnard, CA) which could hold 10 ml and 50 ml medium, respectively.

Preparation of Reagents for Fusion

(1) Littlefield's HAT medium (HAT, hypoxantin, aminopterin and thymidine; Littlefield 1964).

H, A and T were all purchased from Sigma (St. Louis, MO); 272.7 mg of hypoxantin and 76.5 mg of thymidine were dissolved in 200 ml double distilled water, pH was adjusted to 8.1-8.5 with NaOH; and 3.82 mg of aminopterin was dissolved in 200 ml double distilled water. These were 100 times concentrated HT and A stock solutions. HAT medium was prepared by adding 1/100 HT and 1/100 A stock solution to the complete medium, and HT medium was prepared by adding 1/100 HT stock solution to the complete medium.

(2) Polyethylene glycol solution (PEG, mol.wt. 1540, from Sigma, St. Louis, MO).

PEG was preweighted, then melted in the autoclave for 20 min, and dissolved in an appropriate amount of RMPI 1640 medium containing 15% DMSO (Fisher, NJ) to make a 50% solution (weight/volume).

Preparation of Feeder Cells for Fusion or Limiting Dilution

Feeder cells were prepared 1-3 days before the fusion or the limiting dilution. Normal Balb/c female mice were sacrificed by neck dislocation, and splenectomy was carried out. Spleens were put in a petri dish containing 10 ml complete medium and were teased with needles. Spleen cells were squeezed out of capsules and dispersed by repeated passing of the cell suspension through a gauge 22 needle. The cell suspension was then passed through a fine stainless steel mesh, and let to stand in a 15 ml sterile tissue culture tube for 10 min to sediment tissue clumps. The supernatant was transferred to another 15 ml tube, the viable white cells were counted by trypan blue exclusion, and cell density was adjusted to 1×10^6 viable cells/ml with HAT medium for fusion or HT medium for limiting dilution. The cell suspension was distributed to 24 well or 96 well Falcon flat bottom tissue culture plates (Becton-Dickinson, Oxnard, CA) at the volume of 1 ml/well or 0.1 ml/well, respectively. The former was used for fusion, while the latter for limiting dilution. Cells were then incubated in the incubator at 37°C in a humidified atmosphere containing 5% CO_2.

Fusion of NS-1 Cells with P1-Primed Spleen Cells

NS-1 cells were counted by trypan blue exclusion and spun down by centrifugation at 400g for 5 min. The supernatant was decanted, the pellet was resuspended in complete medium, and cell density was adjusted to 5 x 10^6 viable cells/ml; 25 x 10^6 cells were then aliquoted to each 15 ml tube, and tubes were gently rotated at room temperature until P1 primed spleen cells were ready for fusion.

P1 immunized mouse #2 was sacrificed and spleen cells were prepared basically by the same way as described for the preparation of feeder cells. After the cell suspension was sedimented for 10 min to remove clumps of tissue, the supernatant was centrifuged at 350g for 5 min to bring down large lymphocytes, and the supernatant was centrifuged again at 400g for 5 min to bring down small lymphocytes. Pellets from both centrifugations were mixed and resuspended in 5 ml of complete medium. Viable cells were counted by trypan blue exclusion. About 100 x 10^6 viable cells were mixed with 25 x 10^6 NS-1 cells. The mixture was centrifuged at 370g for 5 min and supernatant was decanted. Two ml of prewarmed (37°C) 50 % wt/vol PEG in complete medium containing 15% DMSO was added to the pellet, and was mixed gently with a pipette for 30 sec. The cell suspension was allowed to stand still for another 30 sec, and 5 ml of warm (37°C) complete medium was added dropwise over a period of 90 sec while the tube was flicked gently. Another 5 ml complete medium was poured into the tube, and the tube was allowed to stand for 150 sec. Cells were

spun down at 370g for 5 min. The supernatant was discarded and the pellet was gently resuspended in 5 ml of HAT medium. Fifty µl of cell suspension was added to each well of 24 well culture plates in which each well contained 1 x 10^6 feeder cells in 1 ml HAT medium. The cells were then cultured in the incubator at 37°C in a humidified atmosphere containing 5% CO_2. The day of fusion was referred to as day 0. Half ml of HAT medium was added to each well on day 5, and 0.5 ml of medium in each well was replaced by fresh HAT medium on day 8. On day 10, another 0.5 ml medium per well was replaced and the culture supernatants were saved for the primary screening assays. From day 10 on, cell cultures were fed or split according to growth of cells in each individual well. The second screening assay was carried out on day 14, and HT medium was used instead of HAT medium to feed the cells thereafter. Thirty-two primary cultures were chosen as candidates for limiting dilution according to the results from screening assays. They were directly used for limiting dilution, or frozen and preserved at -70°C.

Freezing and Conservation of Cells at -70°C in Liquid Nitrogen

Two to eight million cells were centifuged down in 15 ml conical centrifuge tube (Becton-Dickinson, Oxnard, CA) and the supernatants were discarded. The pellets were suspended in 1 ml of FCS containing 15% DMSO. The cell suspension was transferred to a Nunc freezing vial (Gibco, Burlington, ON) or a glass freezing vial (Wheaton Laboratories, Millville, NJ). Vials were sealed with caps for plastic

ones or by burner for glass ones. To store at -70°C, vials were placed into the freezer immediately, and to store in liquid nitrogen, vials were first placed in the gas phase of liquid nitrogen tank for 2 hrs and then submerged into the liquid phase.

Limiting Dilution

Viability of hybridoma cells directly from primary wells or from 2-3 day culture of cells retrieved from -70°C storage was determined by trypan blue exclusion. Cell density was adjusted to 10 viable cells/ml with HT medium. Fifty µl of cell suspension was dispensed into each well of 96 well flat bottom culture plates in which feeder cells were placed on the previous day. Thus, theoretically, each well would contain 0.5 hybridoma cell . For each primary culture, limiting dilution was carried out in 192 wells (2 plates). Cells were then cultured in the incubator at 37°C in a humidified atmosphere containing 5% CO_2. The day of limiting dilution was designated as day 0. Cultures were examined on day 7 under an inverted microscope, and wells containing one colony of cells per well were marked and fed with 50 µl HT medium per well, while wells having no colony were ignored thereafter; 120 µl medium per well was replaced by fresh HT medium on day 10, and the replaced supernatants were saved for the first screening assay. Cultures were fed or split depending on the growth of cells and complete medium was used instead of HT medium after day 10; 250 µl/clone of supernatants were taken from the cultures on day 13 for the secondary screening. Six positive clones

per primary culture were chosen according to screening assay and expanded into flasks, while other clones were discarded. A part of the cells of these selected clones were frozen and kept in liquid nitrogen when enough cells were obtained. Three out of these six clones per primary culture were further expanded and later injected into the peritoneal cavity of Balb/c mice.

Preparation of Ascitic Fluid Containing Monoclonal Antibodies

Two to four week old Balb/c female mice (University of Manitoba Animal Breeding Center) were injected i.p. with 0.5 ml pristane (2,6, 10, 14 tetramethylpentadecane, Sigma, St. Louis, MO) 3-5 weeks prior to the injection of hybridoma cell lines. Four million hybrid cells in 0.5 ml complete medium were injected i.p. into each mouse, and ascites were tapped twice with a 20 gauge needle between day 9 to day 13.

Purification of Monoclonal Antibodies from Ascitic Fluid

One volume of ascites was diluted with 1 volume of PBS and precipitated dropwise over a period of 4 hr with 2 volumes of saturated ammonium sulphate, the pH of which had been adjusted to 7.5 by ammonium hydroxide. The final mixture was centrifuged at 10,000 rpm for 20 min at room temperature in Sorvall centrifuge (Ivan Sorvall Inc., Norwalk, CO). The supernatants were discarded and the pellets were redissolved in 1 volume of PBS. The solution was precipitated once more in the same way. After centrifugation, the precipitates

were dissolved in about 10 ml PBS, and dialyzed in Spectrapor dialysis tubing (Spectrum Medical Industries Inc., Los Angeles, CA) against double distilled water. After 3 changes of water with interval of about 6-12 hrs each, the euglobulin which precipitated out during dialysis was removed by centrifugation at 900g for 10 min, and proteins in solution were freeze dried in Virtis Lyophilizer (Virtis Company, Gardiner, NY).

A portion of those freeze dried hybridoma proteins of each clone was further purified on DEAE ion exchange column (Ultrapac DEAE SK545, 7.5 x 150 mm, LKB, Bromma, Sweden) by HPLC apparatus. The regenerated column was equilibrated in 0.05M Tris-HCl, pH 8.0 designated as buffer A; 15-20 mg sample protein was dissolved in 0.2 ml buffer A and injected into the column at time 0. The gradient was expressed in percentage buffer B which was 1M NaCl in buffer A, and the turn points of the gradient were given in the following table.

Time(minute)	% B
0-7	0
22	50
24	100
27	100
32	0
40	0

The flow rate was 1 ml/min, chart speed 2mm/min, wavelength of spectraphotometer 280 nm, range of optical density monitored 0-2.0, and each fraction was 1 ml.

The activity of fractions were tested by RIA #1, and the purity was examined in 10% SDS-PAGE. The appropriate fractions were pooled and dialysed against double distilled water in Spectrapor cellulose dialysis tubing with molecular weight cut-off of 12,000-14,000 daltons. The protein content of dialysed solution was measured by the Lowry test (Lowry 1951). Antibodies thus purified were then freeze dried with Virtis Lyopholizer.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Ten percent SDS-PAGE was carried out to examine the relative purity of Mab fractions from HPLC ion exchange column. The detailed method is described in Chapter IV.

Results

Concentration of GAM-IgG for coating the solid-phase in RIA #1

The concentration of GAM-IgG for coating was decided in a reference system, i.e., human IgE and mouse monoclonal IgG against

human IgE (GAH-IgE). Four different dilutions of GAM-IgG were used to coat plates, while GAH-IgE ascites were at fixed concentration of 4 µl/ml in Hank's solution and ^{125}I-IgE was at fixed concentration of about 150,000 cpm/100 µl. The result is given in Figure 1. At 20 µg/ml of GAM-IgG, the ^{125}I-IgE bound was close to the plateau of the dilution curve in full logarithmic scale, and high signals were obtained. Therefore, this concentration of GAM-IgG was chosen for coating the wells in all the subsequent assays.

Sensitivity of RIA #1

The sensitivity of RIA #1 was tested with IgE and anti-IgE, and served as a rough estimation for that of the anti-Pl antibody assay. Different amounts of MAH-IgE were added to Hank's containing 15% FCS, or to normal mouse serum, while the concentration of GAM-IgG for coating was fixed at 20 µg/ml, and the concentration of ^{125}I-IgE was fixed at 150,000 cpm/100 µl. The results are given in Figure 2. In both Hank's containing 15% FCS, or in normal mouse serum, specific mouse antibody at the concentration of 78 ng/ml could be detected by RIA #1 in IgE and anti-IgE system. The background was 437 cpm which was determined by adding no MAH-IgE in Hank's.

Antibody Titre of Sera from Pl Immunized Mice

To minimize stress, mice were bled only after the 2nd and 3rd immunization, and on the day of sacrifice. Antibody titre to Pl was

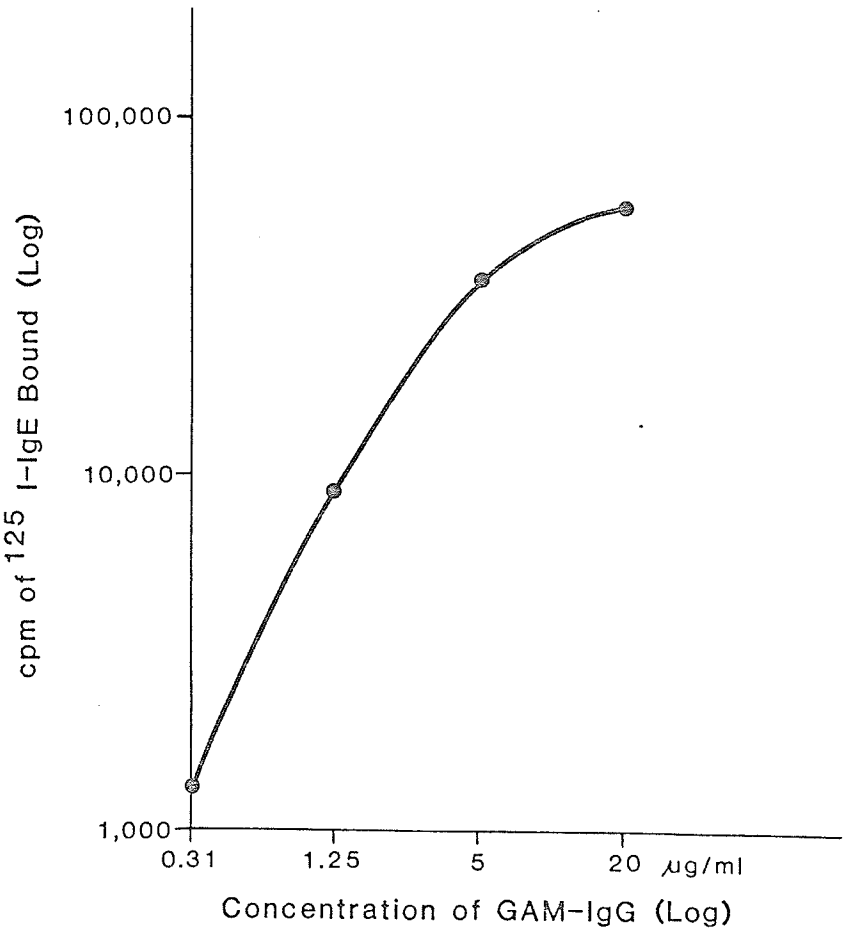


Figure 1: Determination of the optimal concentration of GAM IgG to be used for coating the wells in RIA #1.

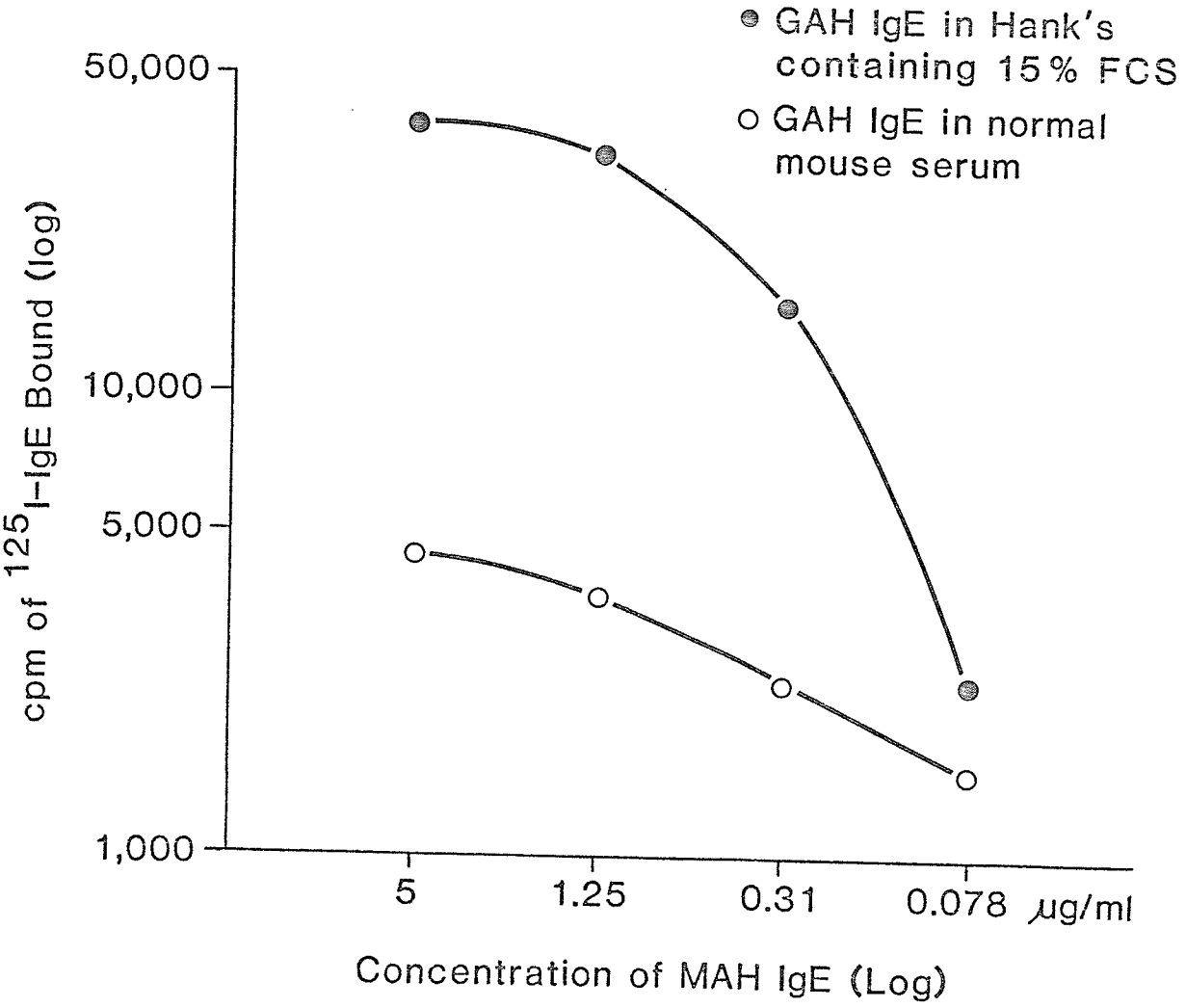


Figure 2: Standardization of RIA #1 by means of Mab anti-IgE and ^{125}I-IgE.

assayed by RIA #1. Samples were in duplicate. The background of the assay was determined by using serum from nonimmunized Balb/c mice. The assay was arbitrarily considered as positive if the cpm were twice the background. Both mouse #1 and #2 gave a positive response at titres of 1:10240 and 1:40960, respectively, after the second immunization. The serum titre from the bleeding after the third immunization and at the time of sacrifice was tested in the same assay as illustrated in Figure 3. Mouse #2 gave a better response, with a titer of 1:102,400 after the third immunization and 1:6400 on the day of sacrifice. The decline was expected due to the 6 week interval between the third immunization and the sacrifice. Spleen cells of mouse #2 were used for fusion.

Screening of Primary Culture of Fusion

Spleen cells from mouse #2 were fused with NS-1 cells. This fusion was numbered as 237.32 according to the series record of this laboratory. The wells of primary cultures of the fusion were numbered as shown in the first column of Table 1. The alphabet indicated the position of the row of a 24 well plate; the arabic number was the number of the tissue culture plate, and the last digit was the number of the column of the plate. For example "A1.2" meant this well was on plate #1, row A, and column 2. The first screening was carried out on day 10 by RIA #1. Samples were tested in single due to limited

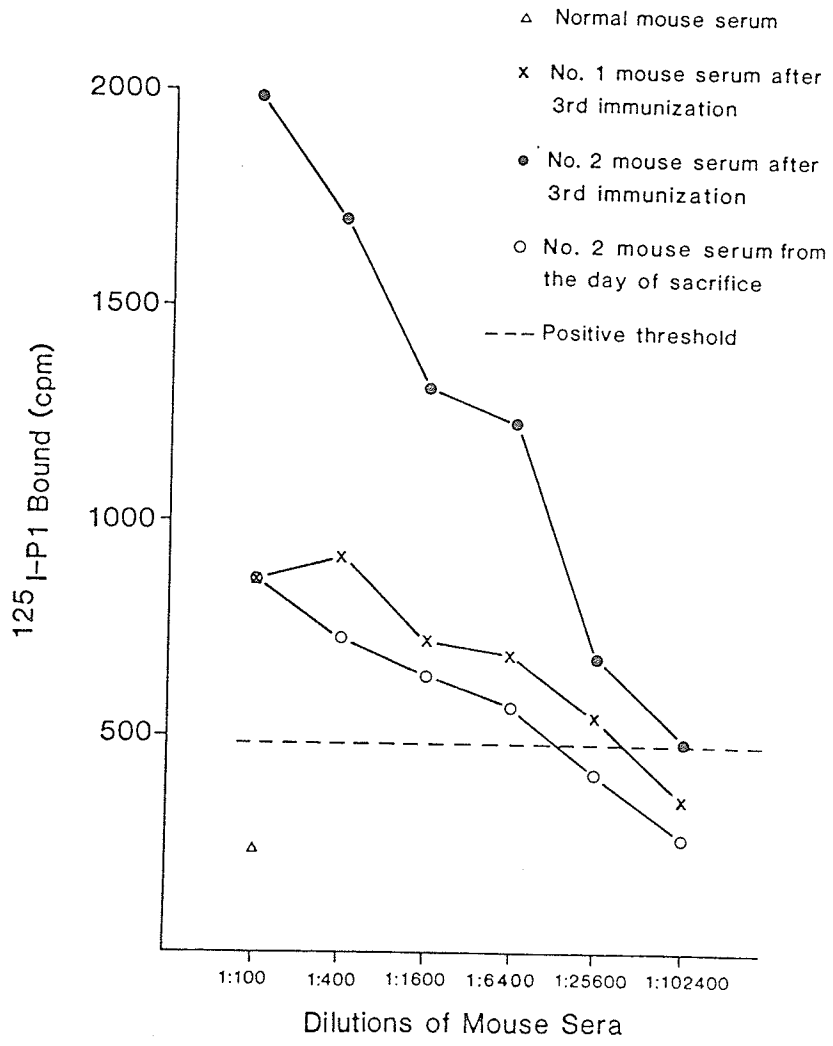


Figure 3: Titer of serum anti-Pl IgG in mice immunized with Pl.

Table 1. Screening Assay of Primary Hybridoma Cultures

Well of Primary Culture	^{125}I-Pl Bound (cpm)				
	First Screening at Day 10 by RIA #1	Second Screening at Day 16 by RIA #2		Third Screening on Day 14 Supernatants by RIA #2	
		No Inhibitor	Crude HDM Ag as an Inhibitor	No Inhibitor	Rye I as an Inhibitor
A1.1	1,204				
A1.2	9,610	1,592	898	ND	ND
A1.3	3,236				
A1.4	3,778				
A1.5	510				
A1.6	4,020				
B1.1	2,414				
B1.2	576				
B1.3	994				
B1.4	2,694				
B1.5	2,536				
B1.6	1,124				
C1.1	3,958				
C1.2	1,506				
C1.3	3,240				
C1.4	1,408				
C1.5	2,028				
C1.6	10,664				

Table 1 cont'd.....

D1.1	2,224				
D1.2	2,408				
D1.3	1,956				
D1.4	5,538				
D1.5	3,420				
D1.6	2,006				
A2.1	16,838	9,984	8,538	ND	ND
A2.2	4,098				
A2.3	14,470	11,360	6,672	11,680	13,268*
A2.4	8,880	5,318	3,682		
A2.5	3,416				
A2.6	1,462				
B2.1	28,236	20,090	16,384	21,863	22,713*
B2.2	20,759	18,096	13,856	20,362	18,000
B2.3	10,960	15,344	8,634	16,882	15,640
B2.4	4,208				
B2.5	4,166				
B2.6	6,036				
C2.1	4,058				
C2.2	4,400	9,176	4,388	8,482	7,332*
C2.3	5,084				
C2.4	10,954	7,332	3,454	8,974	9,310*
C2.5	4,102				
C2.6	1,514				
D2.1	5,496				
D2.2	3,188				
D2.3	2,584				
D2.4	1,716				
D2.5	1,716				
D2.6	3,848				

Table 1 cont'd.....

A3.1	4,954				
A3.2	2,956				
A3.3	7,332				
A3.4	8,856	2,802	2,128	ND	
A3.5	12,000	6,390	4,170	ND	
A3.6	5,288				
B3.1	14,944	12,112	8,834	14,446	17,778
B3.2	1,834				
B3.3	2,818				
B3.4	2,072				
B3.5	3,314	7,948	3,376	12,034	13,234*
B3.6	2,328				
C3.1	2,236				
C3.2	3,154				
C3.3	988				
C3.4	1,028				
C3.5	11,570	5,680	3,668	ND	ND
C3.6	11,470	7,942	3,082	8,810	10,200*
D3.1	11,626	5,694	4,342	ND	ND
D3.2	1,890				
D3.3	5,136				
D3.4	4,710				
D3.5	1,228				
D3.6	2,132				
A4.1	3,296				
A4.2	4,454				
A4.3	7,684	4,894	3,732	ND	ND
A4.4	7,226	5,958	3,328	4,460	5,158
A4.5	2,148				
A4.6	512				

Table 1 cont'd.....

B4.1	2,266				
B4.2	1,308				
B4.3	1,746				
B4.4	2,836				
B4.5	1,372				
B4.6	2,228				
C4.1	9,566	6,890	3,102	7,638	11,148*
C4.2	5,664	5,870	2,270	6,944	10,112*
C4.3	9,192	5,780	3,042	5,556	6,508*
C4.4	2,456				
C4.5	2,702				
C4.6	3,476				
D4.1	2,424				
D4.2	1,560				
C4.3	2,820				
C4.4	3,200				
C4.5	1,386				
C4.6	2,456				
Background (HAT Medium)	232	188		254	

Samples were assayed in a single determination.

Background was the mean cpm of 6 wells incubated with HAT medium.

Wells marked with "*" were chosen for limiting dilution.

ND - Not determined.

quantity of supernatants available. The background of the assay was determined by measuring the mean cpm of 6 wells incubated with HAT medium. The positivity limit was arbitrarily set at 462 cpm which was twice the background. All 96 primary wells were positive ranging from 512 cpm to 28,236 cpm. The wells giving less than 6000 cpm were all discarded except C2.2, B3.5 and C4.2. The 21 wells of culture thus selected were assayed a second time on day 14 by RIA #2 using 10 µg/ml crude HDM antigen batch #1 as an inhibitor. For the control, PBS was added in place of inhibitor. Binding of antibody to Pl from all 21 wells could be well inhibited by 200 ng of crude HDM antigen. Fifteen primary wells, which gave above 6000 cpm in the absence of inhibitors in the second assay, were chosen as candidates for limiting dilution, and were expanded and frozen to -70°C when enough cells were available. Twelve supernatants from day 14 of culture were further tested in a third assay to examine the crossreactivity of the hybrid antibody to Rye I antigen. RIA #2 was employed, and 10 µg/ml Rye I in PBS was used as an inhibitor. In all 12 supernatants the binding to ^{125}I-Pl could not be blocked by 200 ng of Rye I.

Limiting dilution was carried out on 9 primary cultures (A2.3, B2.1, C2.2, C2.4, B3.5, C3.6, C4.1, C4.2 and C4.3 = HY3) which are marked with "*" in Table I. Clones were selected according to their binding activity to labeled Pl and the inhibition by HDM antigen.

Results of Limiting Dilution

Limiting dilutions were carried out on 9 primary cultures (A2.3, B2.1, C2.2, C2.4, B3.5, C3.6, C4.1, C4.2 and HY3). For each primary culture, two 96 well plates were employed, and wells were numbered from 1-192. The final nomenclature of a clone was illustrated as follows:

C4.1/ 102

(1) (2)

(1) is the number of primary culture.

(2) is the clone number after limiting dilution.

Among 1728 wells of 18 plates, 404 wells had hybridoma cells growing, and of these 404 wells, 274 were clearly monoclonal as shown by examination under a microscope at day 7 after limiting dilution. Supernatants from those 274 clones were assayed for anti-P1 IgG antibody by RIA #1 at day 10. One hundred and thirteen clones were positive (Table 2). These clones were expanded, and their supernatants were assayed at day 13 by RIA #2 to examine the specificity of Mab and the crossreactivity with human antibody to P1. Twenty µl of crude HDM antigen, batch #1 (10 µg/ml) and 1:10 diluted human serum pool from 7 patients allergic to HDM antigen were employed as inhibitors. The inhibition ranged from 23-89% and 27-83%, respectively. All the assays were carried out with a single determination due to limited supply of supernatants. Twenty-seven clones (marked with "*" in Table 2) were chosen to be expanded and

Table 2. Screening Assays After Limiting Dilution

Clone		RIA #1 on Day 10 ^{125}I-Pl Bound (cpm)	RIA #2 on Day 13				
			No Inhibitor ^{125}I-Pl Bound (cpm)	HDM Ag as Inhibitor ^{125}I-Pl Bound (cpm)	Percent Inhibition	Allergic Serum as Inhibitor ^{125}I-Pl Bound (cpm)	Percent Inhibition
C4.1/	3	12,492	6,314	1,854	70	2,486	61
	5	13,310	6,272	1,704	73	3,140	50
	15	13,484	7,372	2,266	69	3,544	52
	24	12,956	6,214	2,090	66	3,376	46
	27	14,236	6,606	2,254	66	3,212	51
	35	14,662	6,694	2,324	65	3,114	53
	37	14,262	6,474	2,426	63	3,318	49
*#	40	15,068	6,962	2,614	62	3,242	53
	44	13,904	6,966	2,262	68	3,394	49
	58	11,934	6,734	1,964	71	3,204	52
	71	12,784	6,874	2,090	70	3,030	56
*	75	13,580	7,242	1,848	74	2,720	62
*	76	11,920	4,040	770	81	1,720	57
	77	12,994	6,474	1,830	72	2,880	55
	82	12,748	6,974	2,568	63	3,740	46
	86	11,598	6,688	2,252	66	3,398	49
	92	12,844	6,618	1,904	70	3,578	46
*#	96	14,136	7,190	2,110	71	3,752	48
	101	14,108	7,444	2,220	70	3,430	54
*#	102	17,488	10,852	6,646	39	5,994	45
	105	14,602	6,274	1,660	74	2,952	53
	108	13,844	6,190	1,782	71	2,498	60
	119	12,676	6,674	2,016	70	2,728	59
	123	11,180	5,468	1,528	72	2,408	56

Table 2 cont'd.....

*	125	12,558	6,664	2,042	69	3,348	50
*	137	12,504	6,288	2,070	67	3,554	46
	147	14,052	5,440	1,879	65	2,860	47
	157	13,022	6,364	2,288	64	3,486	45
	160	13,176	6,082	2,256	63	3,818	37
	165	13,234	6,538	2,410	63	3,720	43
	170	12,806	6,332	2,330	63	3,490	45
	171	13,778	6,490	2,492	62	3,590	45
	180	12,838	6,248	2,288	63	3,564	43
*A2.3/32		17,792	13,118	5,848	55	3,084	76
*	69	16,488	13,376	6,522	51	3,372	75
*	70	11,044	1,392	492	65	402	71
*#	79	16,780	13,428	7,782	42	4,072	70
	97	16,378	13,920	7,252	48	4,272	69
*	99	15,144	14,474	7,390	49	4,288	71
	177	16,022	12,168	5,494	55	3,620	70
	183	17,700	14,540	7,430	49	4,030	72
	184	15,830	14,888	7,626	49	3,430	77
*	188	15,724	13,794	4,888	65	2,758	80
*#	191	14,294	13,472	5,432	60	2,102	84
*#HY3/90		3,564	4,899	3,072	37	964	80
C3.6/	12	10,344	13,446	3,076	77	4,250	68
	14	1,088	2,030	474	77	554	73
*	17	9,786	13,646	2,812	80	4,412	68
	25	1,082	1,268	344	73	430	66
	28	10,394	12,138	2,500	80	3,902	68
	29	1,322	1,204	390	68	442	63
*	30	9,622	12,048	2,176	82	3,874	68
	31	1,098	1,454	462	68	598	59
	33	1,708	1,170	392	66	416	64
	34	2,666	1,098	456	58	440	60
	49	1,922	1,294	416	68	344	73

Table 2 cont'd.....

*	54	2,382	1,256	518	59	276	78
	55	1,972	1,838	1,248	32	372	80
*#	62	14,870	13,458	3,476	74	3,626	73
	66	2,402	1,696	484	71	550	68
	102	1,752	1,730	412	76	368	79
*#	104	14,762	13,498	2,626	81	4,082	70
	107	2,066	1,922	536	78	448	77
*	109	2,076	4,086	450	89	682	83
	110	2,252	1,222	516	58	414	66
	121	1,562	964	578	40	310	68
	122	1,790	1,196	752	37	402	66
	125	1,582	1,148	504	65	440	70
	146	1,922	1,654	484	71	450	73
*	151	2,286	1,582	340	79	436	72
	176	1,356	870	252	71	314	63
	188	1,670	1,212	342	72	308	75
	192	14,720	11,838	2,548	78	4,772	60
B2.1/	6	12,484					
	7	15,370					
	8	15,374					
	9	15,968					
	10	14,602					
	12	14,496					
	16	14,606					
	18	15,528					
	23	15,928					
	26	13,160					
	30	14,712					
	33	15,946					
*#	35	15,374	12,748	3,602	72	3,970	69
	47	13,902					
	49	15,390					
	51	14,534					
	55	15,830					
	62	14,404					

Table 2 cont'd.....

*	66	14,222	11,864	4,552	62	4,372	64
*#	81	11,454	12,390	1,668	87	3,660	70
	84	15,242					
	85	14,834					
	93	14,930					
*	97	14,172	12,022	4,544	62	4,260	65
	100	14,036					
	101	14,358					
	104	14,146					
	106	15,864					
	121	11,802					
*	125	8,574	8,982	1,508	83	2,608	71
	151	14,004					
	152	12,234					
	159	13,508					
	163	14,960					
	173	15,268					
	174	13,454					
	178	14,974					
	179	15,034					
	189	14,974					
*#	191	18,360	12,622	3,488	72	4,304	66
Background (HT Medium)		210	178	152		174	

Samples were tested by single determination due to the limited quantity of supernatant.
Background was the mean cpm of 6 wells incubated with HT medium.
Clones from C2.2, C2.4, B3.5 and C4.2 were all negative.
Clones from B2.1 were all strongly positive in RIA #1, only 6 clones were randomly selected to be tested in RIA #2.
Twenty µl of HDM (10 µg/ml) or 20 µl of 1:10 diluted human sera from allergic patients were used as inhibitors.
Clones marked with "*" were conserved in liquid nitrogen and clones marked with "#" were used to prepare ascitic fluid.

frozen in liquid nitrogen on the basis of the following criteria: (1) high binding to ^{125}I-P1, or (2) good inhibition by HDM antigen or allergic serum, and (3) good proliferation. Twelve (marked with "#") from these 27 clones were further expanded and later injected into the peritoneal cavity of female Balb/c mice to raise ascitic fluid.

Purification of Monoclonal Antibodies

Ascitic fluid was precipitated twice with 50% saturated ammonium sulphate, and then fractionated by HPLC ion exchange. The sample volume was 1 ml containing 20-40 mg of protein. The chromatography profiles of all clones were very similar, and that of clone C4.1/102 is illustrated in Figure 4. The anti-P1 activity of different fractions was tested with RIA #1, and was detected from fraction 17 to 40 (data not shown). The fractions were also analysed on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reduced conditions. The results obtained with clone C4.1/102 are shown in Figure 5. The protein bands started to appear from fraction 17, and the γ chain and L chain were mainly located from fraction 17-20. In these fractions the major bands were γ chain and L chain although some traces of impurity were also present. Since the monoclonal antibodies raised were of IgG class, the fractions 17 to 20 were pooled, and used for coating or radioiodination.

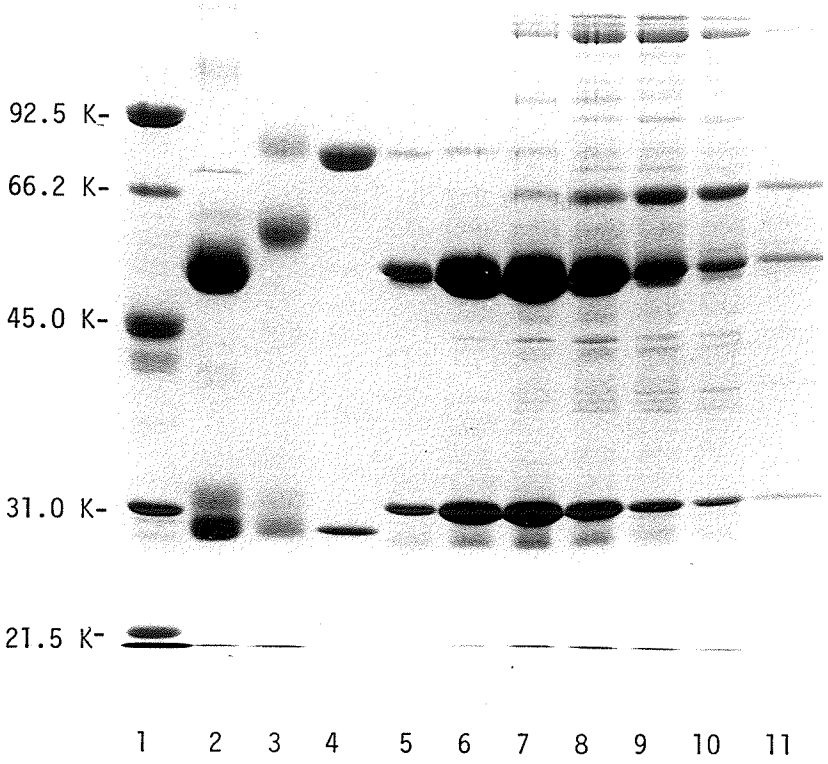


Figure 5: 10% SDS-PAGE analysis of ascitic fluid protein containing Mab C4.1/102 fractionated by ion exchange chromatography.
Lane 1 mol.wt. standard.
Lane 2 mouse IgG.
Lane 3 mouse IgA
Lane 4 mouse IgM
Lanes 5-11 fractions 17-23.

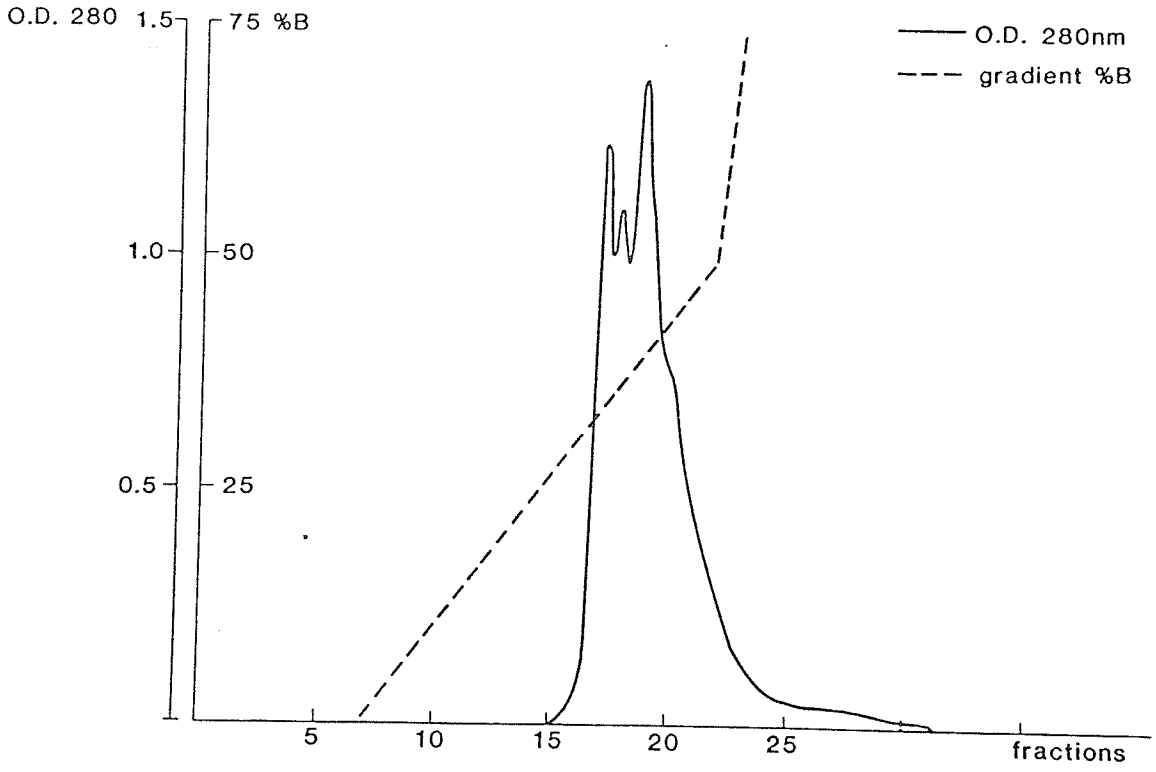


Figure 4: Ion exchange chromatography of ascitic protein containing Mab C4.1/102

Subclass of Monoclonal Antibodies

All monoclonal antibodies thus raised were IgG, since goat-anti-mouse-IgG antibodies were employed in the screening procedure. The subclass of each clone was determined by RIA #3. Goat or rabbit anti-mouse IgG subclass antibodies were used to coat plates, on which the HPLC purified Mab were then incubated, and ^{125}I-Pl were added for the last incubation. The results are given in Table 3. The background was calculated as the mean cpm of 4 wells incubated with normal Balb/c serum. Cpm double the background was arbitrarily set as the threshold of positivity. All the clones tested were $\gamma 1\kappa$ except B2.1/35 which was $\gamma 2b\kappa$. Since there was no positive control available for $\gamma 2a$, $\gamma 3$ and λ, 5 clones (C4.1/96, C4.1/102, C4.1/40, A2.3/79 and A2.3/191) were further tested in immunodiffusion where the same anti-subclass antibodies were used. The aim was to confirm the results from RIA #3, and to provide evidence that the anti-mouse sera employed were active. The results were in accordance with those from RIA #3 (Figure 6). All 5 clones tested formed precipitation bands with anti-$\gamma 1$ antibodies but not with other anti-IgG subclass antibodies. As positive controls, all anti-IgG subclass antibodies formed precipitation lines with normal mouse serum, indicating that the antisera were active. All five preparations of Mab were precipitated by both anti-λ and anti-κ antisera, suggesting that they were still contaminated with some antibodies from host Balb/c mice, and that both anti-light chain antisera were active.

Table 3. Determine the Subclasses of Monoclonal Antibody (RIA #3)

	Coating Antibody						
	GAM_1	GAM_{2a}	GAM_{2b}	GAM_3	RAM	RAM	Subclass
Clone	^{125}I-Pl Bound (cpm)						
C4.1/96	1,982	Bkd	Bkd	Bkd	Bkd	3,806	$\gamma 1\kappa$
C4.1/102	3,260	Bkd	Bkd	Bkd	Bkd	6,625	$\gamma 1\kappa$
C4.1/40	1,911	Bkd	Bkd	Bkd	Bkd	4,254	$\gamma 1\kappa$
A2.3/79	3,322	Bkd	Bkd	Bkd	Bkd	7,072	$\gamma 1\kappa$
A2.3/191	2,926	Bkd	Bkd	Bkd	Bkd	6,567	$\gamma 1\kappa$
HY3/90	1,081	Bkd	Bkd	Bkd	Bkd	1,987	$\gamma 1\kappa$
C3.6/62	3,479	Bkd	Bkd	Bkd	Bkd	4,418	$\gamma 1\kappa$
C3.6/104	3,037	Bkd	Bkd	Bkd	Bkd	4,189	$\gamma 1\kappa$
B2.1/35	Bkd	Bkd	2,186	Bkd	Bkd	3,268	$\gamma 2b\kappa$
B2.1/81	860	Bkd	Bkd	Bkd	Bkd	1,645	$\gamma 1\kappa$
Background (Normal Mouse Sera)	285	462	326	147	155	559	

The background was the mean cpm of 4 wells coated with normal Balb/c serum.
Cpm doubling the background was set as positive threshold. Samples with cpm lower than threshold are marked as background (Bkd).
Samples were tested in duplicate.
GAM: goat-anti-mouse antibody RAM: rabbit-anti-mouse antibody

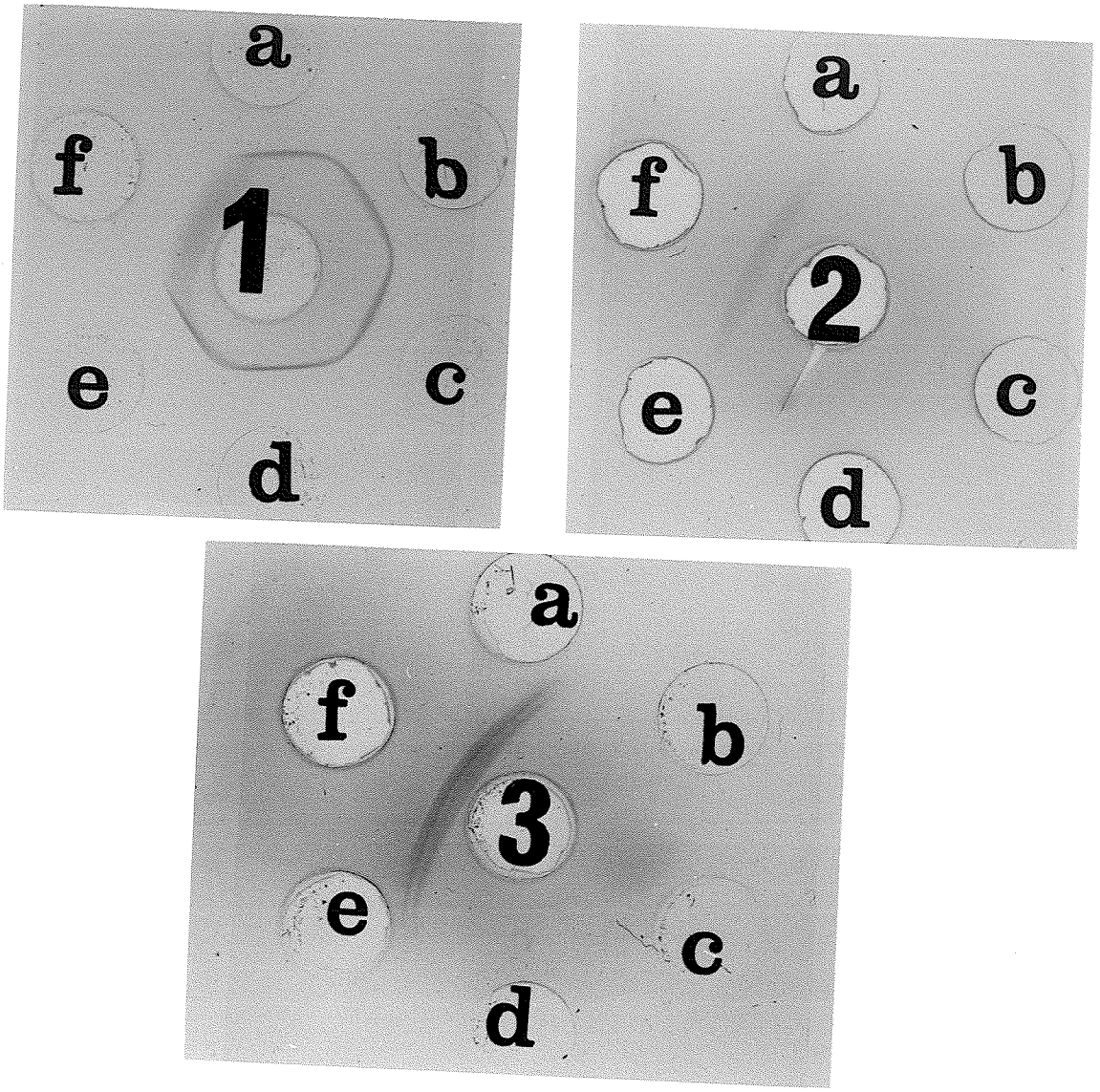


Figure 6: Ouchterlony assay for the isotype typing of Mab.

Well 1	goat-anti-mouse γ1
Well 2	goat-anti-mouse γ2a
Well 3	goat-anti-mouse γ2b
Well 4	goat-anti-mouse γ3
Well 5	rabbit-anti-mouse λ
Well 6	rabbit-anti-mouse κ
Well a	Mab C4.1/96
Well b	Mab C4.1/102
Well c	Mab C4.1/40
Well d	Mab A2.3/79
Well e	Mab A2.3/191
Well f	Normal Balb/c mouse serum

cont'd.....

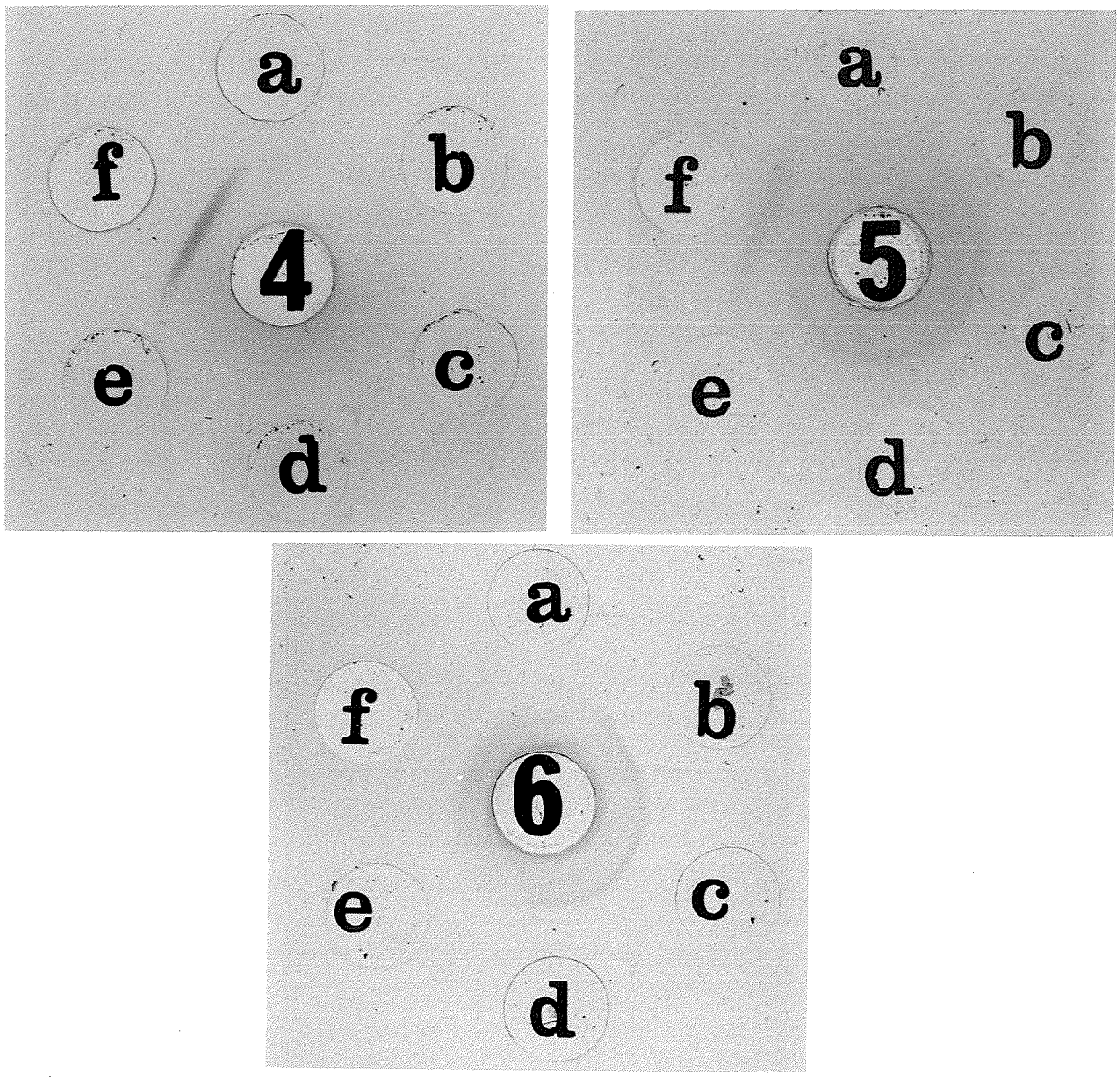


Figure 6: Ouchterlony assay for the isotype typing of Mab.

Well 1	goat-anti-mouse γ1
Well 2	goat-anti-mouse γ2a
Well 3	goat-anti-mouse γ2b
Well 4	goat-anti-mouse γ3
Well 5	rabbit-anti-mouse λ
Well 6	rabbit-anti-mouse μ
Well a	Mab C4.1/96
Well b	Mab C4.1/102
Well c	Mab C4.1/40
Well d	Mab A2.3/79
Well e	Mab A2.3/191
Well f	Normal Balb/c mouse serum

Optimization of the Total cpm of ^{125}I-Pl to be Added in RIA #2 and RIA #4

To ensure the sensitivity of RIA #2 and RIA #4, which were designed for inhibition assays, the amount of ^{125}I-Pl added should be such that the final ^{125}I-Pl bound in absence of inhibitor is below the plateau of the maximum binding. Different concentrations of ^{125}I-Pl were tested in both assay systems and results are shown in Tables 4 and 5; two representative sets of data from Table 4 and 5 are illustrated in Figure 7. In both assays and with all the clones investigated, when up to about 200,000 cpm of total ^{125}I-Pl was added, the bindings were still on the ascending parts of dilution curves. So, about 150,000 cpm per 80 µl of ^{125}I-Pl, were applied to each well throughout the RIA #2 and RIA #4 assays.

Specificity of Monoclonal Antibodies Against Pl

Different antigens were used to inhibit the binding of monoclonal antibodies to ^{125}I-Pl in RIA #2, where Mabs are bound to plates via GAM-IgG, and in RIA #4, where Mabs are directly bound to the plates. Five clones (C4.1/96, C4.1/102, C4.1/60, A2.3/79 and A2.3/191) were tested in RIA #2. Crude HDM antigen, Rye I, tetanus toxoid (TT), Lipopolysaccharide (LPS), L-α-phosphatidyl choline (PC) and Pl were used as inhibitors. The results are shown in Tables 6 and 7. At a final concentration of 0.2 µg/ml, crude HDM antigen caused 87-94% inhibition, while Rye I, LPS, or PC used at the same concentration, or tetanus toxid (1:1000 diluted), caused only 0-16% inhibition. The

Table 4. Titration of ^{125}I-Pl in RIA #2

Mab Incubated on GAM-IgG Coated Plates	Total ^{125}I-Pl Added (cpm)[a]			
	200,000	100,000	50,000	25,000
	^{125}I-Pl Bound (cpm)			
C4.1/96	10,081	4,895	2,495	1,427
C4.1/102	12,365	5,859	3,555	2,367
C4.1/40	9,378	5,428	3,368	1,497
A2.3/79	14,733	8,182	3,449	2,099
A2.3/191	15,838	6,395	3,081	2,330
Normal Mouse Serum	1,575	1,148	340	498

[a]Approximate values with ± 10% variation.

Background was the mean cpm of 4 wells incubated with normal Balb/c mouse serum.

Samples were tested in duplicate.

Table 5. Titration of ^{125}I-P1 in RIA #4

Clone coated on plates	Total ^{125}I-P1 Added (cpm)[a]				
	200,000	150,000	100,000	50,000	25,000
	^{125}I-P1 Bound				
C4.1/96	15,503	10,956	7,308	3,549	1,827
C4.1/102	15,114	13,294	7,696	3,714	1,978
C4.1/40	13,244	10,924	8,154	3,820	1,911
A2.3/79	10,885	9,112	6,702	4,294	1,526
A2.3/191	9,997	7,499	5,407	2,995	2,047
HY3/90	4,387	ND	2,974	1,664	960
C3.6/62	10,691	ND	5,428	2,997	1,462
C3.6/104	9,518	ND	4,010	1,839	886
B2.1/81	4,452	ND	2,429	1,476	1,111
Normal Mouse Serum	231	171	153	122	99

[a]Approximate values with ± 10% variation.
Background was the mean cpm of 4 wells coated with anti-Rye I Mab #8.
Samples were tested in duplicate.

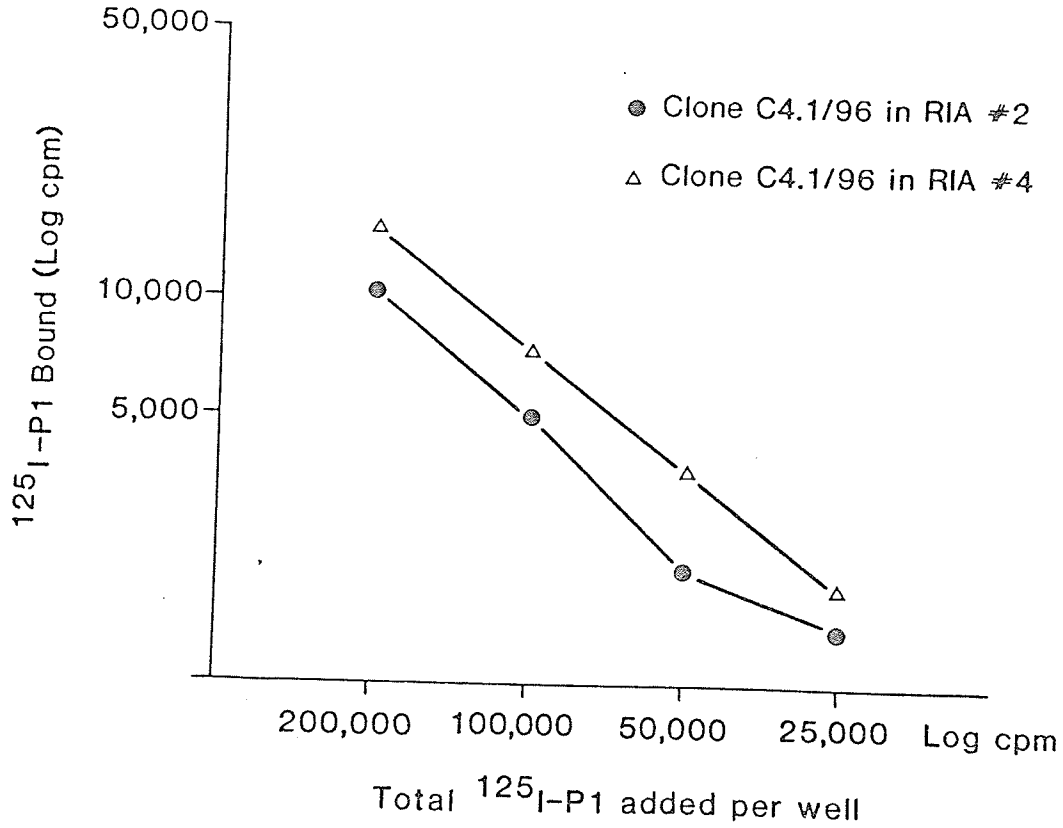


Figure 7: Titration of ^{125}I-P1 in RIA #2 and RIA #4.

Two representative curves based on data from Table 4 and Table 5 are shown in this graph.

Table 6. Inhibition of the Binding of Mab to ^{125}I-Pl by Different Antigens (RIA #2)

Mab	Percentage Inhibition by Different Antigens					Maximum Binding (cpm)	Background (cpm)
	Crude HDM Ag 0.2µg/ml[a]	Rye I 0.2µg/ml	Tetanus toxoid (1:1000)	LPS 0.2µg/ml	PC 0.2µg/ml		
C4.1/96	87	0	0	1	0	2,684	360
C4.1/102	88	16	0	9	0	6,026	
C4.1/40	92	5	3	12	3	4,426	
A2.3/79	90	4	0	14	0	7,457	
A2.3/191	94	5	10	15	0	6,580	

[a]Final concentrations of inhibitors.

Background was the mean cpm of 10 wells incubated with normal Balb/c mouse serum.

Samples were tested in duplicate.

Table 7. Inhibition of the Binding of Mab to ^{125}I-Pl by Pl (RIA #2)

Clone	Percentage Inhibition by Pl						Maximum Binding (cpm)	Background (cpm)
	200 ng/ml[a]	20 ng/ml	2 ng/ml	200 pg/ml	20 pg/ml	2 pg/ml		
C4.1/96	94	92	45	0	0	0	5,178	828
C4.1/102	98	91	34	0	0	0	10,125	
C4.1/40	98	90	42	6	10	6	6,736	
A2.3/79	94	79	0	11	0	0	5,427	
A2.3/191	96	93	54	13	11	1	5,713	

[a]Final concentrations. Background was the mean cpm of 10 wells incubated with normal Balb/c mouse serum.

Samples were tested in duplicate.

specificity was also demonstrated by the fact that cold P1, at final concentration of 20 ng/ml, gave 79-93% inhibition, indicating that Mab could bind natural determinants on P1 but not new determinants resulting from the radioiodination.

Nine clones including those 5 tested in RIA #2 were tested with RIA #4; 0.2 μg/ml crude HDM antigen could inhibit 78-100% binding of Mab to ^{125}I-P1, while TT at 1:1000 dilution, Rye I, LPS and PC up to 0.2 μg/ml could only give 0-21% inhibition (Table 8).

Epitope Specificity of Monoclonal Antibodies

Different Mabs in 6 dilution series (from 2 mg/ml to 2 x 10^{-5} mg/ml; final concentration) were used as inhibitors in RIA #4 to inhibit the binding of different Mab to ^{125}I-P1 in order to study the epitope specificity of each Mab. Ten Mabs were tested in two batches for the convenience of the experiments.

The batch I included C4.1/96, C4.1/102, C4.1/40, A2.3/79 and A2.3/191. Results of crossinhibition study are shown in Table 9, from which two representative sets of data are illustrated in Figure 8. C4.1/102 and A2.3/79 were employed to inhibit the binding of ^{125}I-P1 to 5 Mabs on the solid-phase. When inhibitors were in relatively low concentration (2 x 10^{-3} mg/ml), C4.1/102 could inhibit C4.1/79, C4.1/102 and C4.1/40 by 83-91%, but could only inhibit A2.3/79 and A2.3/191 by 19-25%. On the other hand, A2.3/79 could inhibit A2.3/79

Table 8. Inhibition of the Binding of Mab to ^{125}I-Pl by Different Antigens (RIA #4)

Mab	Percentage of Inhibition by Different Antigens							Maximum Binding (cpm)
	Crude HDM Ag 0.2µg/ml[a]	Rye I 0.2µg/ml	Tetanus toxoid 1:1000	LPS 200ng/ml	LPS 20ng/ml	PC 200ng/ml	PC 20ng/ml	
C4.1/96	84	1.2	0	0	ND	0	ND	6,886
C4.1/102	81	0	0	0	ND	0	ND	7,417
C4.1/40	87	4	0	7	ND	4	ND	7,129
A2.3/79	78	10	7	0	ND	4	ND	7,621
A2.3/191	79	21	13	10	ND	18	ND	7,913
HY3/90	100	0	16	8	0	2	15	1,332
C3.6/62	89	0	5	0	0	0	1	4,234
C3.6/104	99	0	0	1	0	0	1	5,131
B2.1/81	100	25	12	24	0	19	19	4,287

[a]Final concentrations.
Background (414 cpm) was the mean cpm of 18 wells coated with anti-Rye I Mab #8.
Samples were tested in duplicate.
ND - not determined.

Table 9. Crossinhibition among Five Anti-Pl Monoclonal Antibodies (RIA #4)

A.	% Inhibition by Mab C4.1/96 (mg/ml)					
Mab on solid phase	2	$2x10^{-1}$	$2x10^{-2}$	$2x10^{-3}$	$2x10^{-4}$	$2x10^{-5}$
C4.1/96	100	100	99	88	41	30
C4.1/102	100	99	97	85	25	16
C4.1/40	100	98	96	82	12	19
A2.3/79	97	68	67	18	0	10
A2.3/191	97	67	24	15	1	0

Table 9 cont'd.....

B.	% Inhibition by Mab C4.1/102 (mg/ml)					
Mab on solid phase	2	$2x10^{-1}$	$2x10^{-2}$	$2x10^{-3}$	$2x10^{-4}$	$2x10^{-5}$
C4.1/96	100	100	100	91	60	36
C4.1/102	100	100	98	87	44	10
C4.1/40	100	97	95	83	31	5
A2.3/79	46	33	21	19	0	11
A2.3/191	53	47	37	25	2	4

C.	% Inhibition by Mab C4.1/40 (mg/ml)					
Mab on solid phase	2	$2x10^{-1}$	$2x10^{-2}$	$2x10^{-3}$	$2x10^{-4}$	$2x10^{-5}$
C4.1/96	100	100	100	87	38	20
C4.1/102	100	100	98	84	36	15
C4.1/40	100	100	98	83	28	27
A2.3/79	100	98	89	39	8	17
A2.3/191	100	97	88	35	10	16

Table 9 cont'd.....

D.	% Inhibition by Mab A2.3/79 (mg/ml)					
Mab on solid phase	2	$2x10^{-1}$	$2x10^{-2}$	$2x10^{-3}$	$2x10^{-4}$	$2x10^{-5}$
C4.1/96	100	89	73	36	0	0
C4.1/102	99	86	70	35	0	8
C4.1/40	99	86	65	21	0	19
A2.3/79	100	100	98	90	38	5
A2.3/191	100	99	98	90	49	19

E.	% Inhibition by Mab A2.3/191 (mg/ml)					
Mab on solid phase	2	$2x10^{-1}$	$2x10^{-2}$	$2x10^{-3}$	$2x10^{-4}$	$2x10^{-5}$
C4.1/96	94	43	62	35	0	0
C4.1/102	85	58	62	29	0	8
C4.1/40	85	34	56	15	0	20
A2.3/79	100	98	97	92	35	15
A2.3/191	100	99	97	90	48	14

Table 9 cont'd.....

F.	Inhibition by Normal Human Serum Protein	Maximum Binding (cpm)
Mab on solid phase	2 mg/ml	
C4.1/96	11	9,977
C4.1/102	15	12,672
C4.1/40	3	7,554
A2.3/79	15	10,644
A2.3/191	26	10,370

Background (265 cpm) was the mean cpm of 4 wells coated with anti-Rye I Mab #8.

The maximum binding was determined by using PBS as an inhibitor.

The concentrations of inhibitors are the final concentrations.

This experiment was repeated with concordant results.

Samples were tested in duplicate.

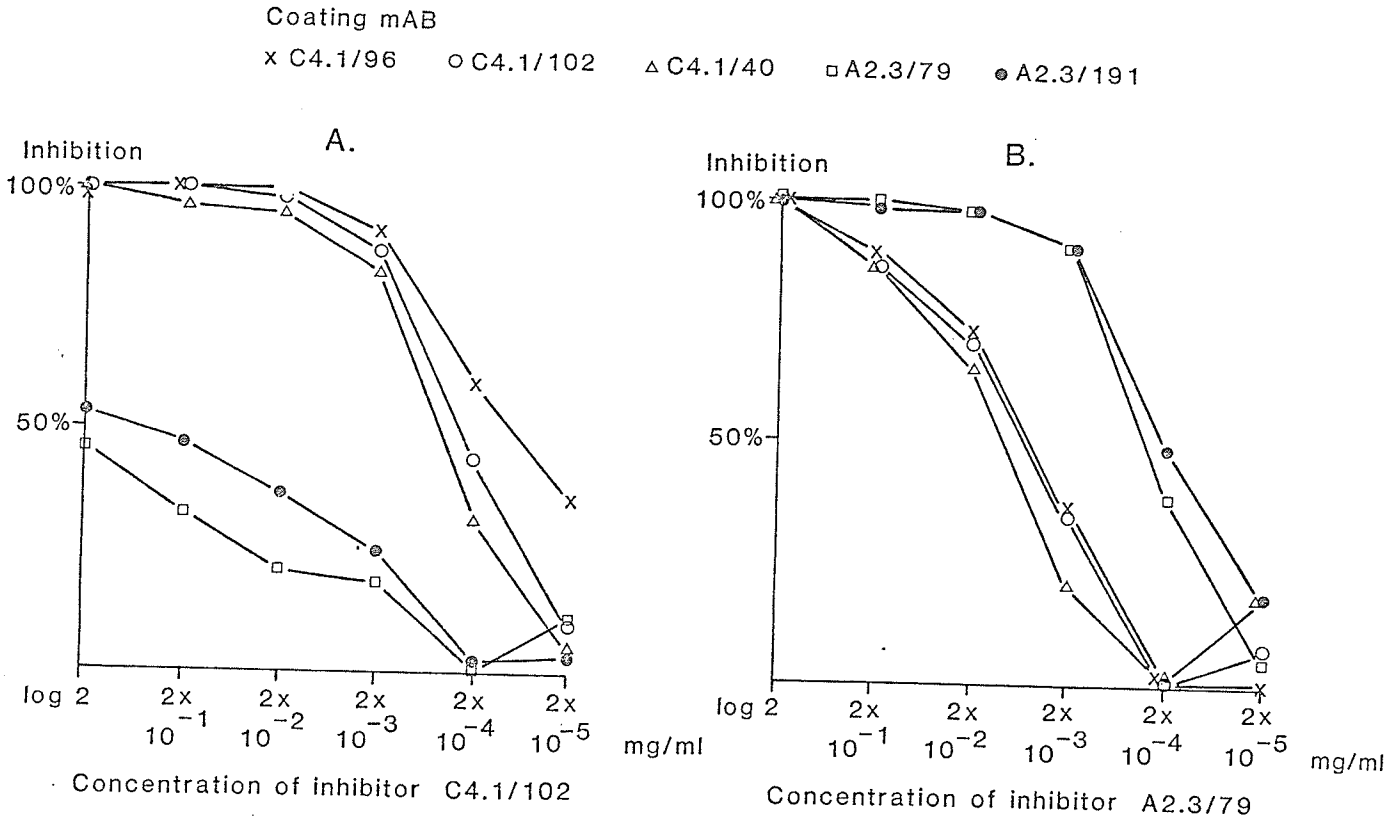


Figure 8: Crossinhibition among 5 Mab to Pl (Batch I) (RIA #4).

Panel A: Different dilutions of Mab C4.1/102 were used to inhibit binding of ^{125}I-Pl to 5 Mabs adsorbed on wells.

Panel B: Different dilutions of Mab A2.3/109 were used to inhibit binding of ^{125}I-Pl by 5 Mabs adsorbed on wells.

This graph is based on 2 sets of representative data from Table 9.

and A2.3/191 by 90%, but could only inhibit C4.1/79, C4.1/102 and C4.1/40 21-36%. Therefore, these 5 clones could rather clearly be grouped into 2 families: C4.1/96, C4.1/102 and C4.1/40 belonged to family I, and A2.3/79 and A2.3/191 belonged to family II. However, when the concentration of inhibitors increased, the difference of inhibitions caused by clones belonged to different families decreased. At 2 mg/ml, monoclonal antibodies from the other family could also cause 46-100% inhibition. This inhibition was not nonspecific, since normal human serum protein at the same concentration caused only 3-26% inhibition, which was considered as a nonspecific protein effect.

Batch II consisted of 4 clones of Mab (HY3/90, B2.1/81, C3.6/62 and C3.6/104). In one set of crossinhibition experiments with RIA #4 (Table 10 and Figure 9A), HY3/90 was used to inhibit the binding of ^{125}I-P1 by 4 different Mabs including HY3/90 on solid-phase. At concentration of 2×10^{-3} mg/ml, it could inhibit 88% and 92% for HY3/90 and B2.1/81, respectively, and 38% and 57% for C3.6/62 and C3.6/104, respectively. Thus, the former two were grouped into family III and the latter two into family IV. This way of grouping was verified to be appropriate by another set of crossinhibition study using also RIA #4, in which 5 different Mabs were used to inhibit the binding of ^{125}I-P1 by C3.6/62 on solid-phase (Table 11 and Figure 9b). At concentrations of 2×10^{-3} mg/ml, HY3/90 and B2.1/81, which belonged to a different family (family IV) than the coating Mab, gave 28% and 38% inhibition, respectively, while C3.6/62 and C3.6/104,

Table 10. Crossinhibition among Four Anti-P1 Monoclonal Antibodies (Batch II, RIA #4)

Mab on Solid-phase	% Inhibition by Mab HY3/90 (mg/ml)						Maximum Binding (cpm)
	1	2×10^{-1}	2×10^{-2}	2×10^{-3}	2×10^{-4}	2×10^{-5}	
HY3/90	100	100	100	88	65	25	1,332
B2.1/81	100	100	100	92	74	48	4,287
B3.6/62	100	92	70	38	8	0	4,234
B3.6/104	100	100	83	57	23	6	5,131

Background (480 cpm) was the mean cpm of 4 wells coated with anti-Rye I Mab #8.

The concentrations of inhibitors indicated in the table are the final concentrations. The background inhibition was determined by using 5 mg/ml of normal human serum protein; it ranged from 3 to 20%.

Samples were tested in duplicate.

Table 11. Crossinhibition among Seven Anti-P1 Monoclonal Antibodies (RIA #4)

Inhibitor	Concentration of inhibitors (mg/ml)	Mab on Solid Phase	
		C3.6/62	C3.6/104
		% Inhibition	
Mab HY3/90	1	100	100
	2×10^{-1}	92	100
	2×10^{-2}	70	83
	2×10^{-3}	38	57
	2×10^{-4}	8	23
	2×10^{-5}	0	6
Mab B2.1/81	1	100	100
	2×10^{-1}	84	93
	2×10^{-2}	53	71
	2×10^{-3}	28	46
	2×10^{-4}	5	37
	2×10^{-5}	6	30
Mab B2.1/35	1	93	100
	2×10^{-1}	74	83
	2×10^{-2}	39	64
	2×10^{-3}	17	42
	2×10^{-4}	6	36
	2×10^{-5}	1	30
Mab C3.6/62	1	100	100
	2×10^{-1}	100	100
	2×10^{-2}	100	100
	2×10^{-3}	97	100
	2×10^{-4}	84	96
	2×10^{-5}	64	60
Mab C3.6/104	1	100	100
	2×10^{-1}	100	100
	2×10^{-2}	100	100
	2×10^{-3}	95	100
	2×10^{-4}	83	92
	2×10^{-5}	60	75

Table 11 cont'd.....

Inhibitor	Concentration of inhibitors (mg/ml)	Mab on Solid Phase	
		C3.6/62	C3.6/104
		% Inhibition	
Mab C4.1/96	1	48	63
	2×10^{-1}	22	55
	2×10^{-2}	9	36
	2×10^{-3}	0	36
	2×10^{-4}	0	32
	2×10^{-5}	0	22
Mab A2.3/79	1	100	100
	2×10^{-1}	100	100
	2×10^{-2}	87	97
	2×10^{-3}	64	80
	2×10^{-4}	36	55
	2×10^{-5}	12	30
Human Serum Protein	1 mg/ml	3	10
Maximum Binding (cpm)		4,234	5,131

Background was 480 cpm, which were decided by mean cpm of 4 wells coated with anti-Rye I monoclonal antibody #8.

Cpm of 0% inhibition were decided by using PBS as an inhibitor.

Concentration of inhibitors indicated in the table were the final concentration in the well.

Samples were tested in duplicate.

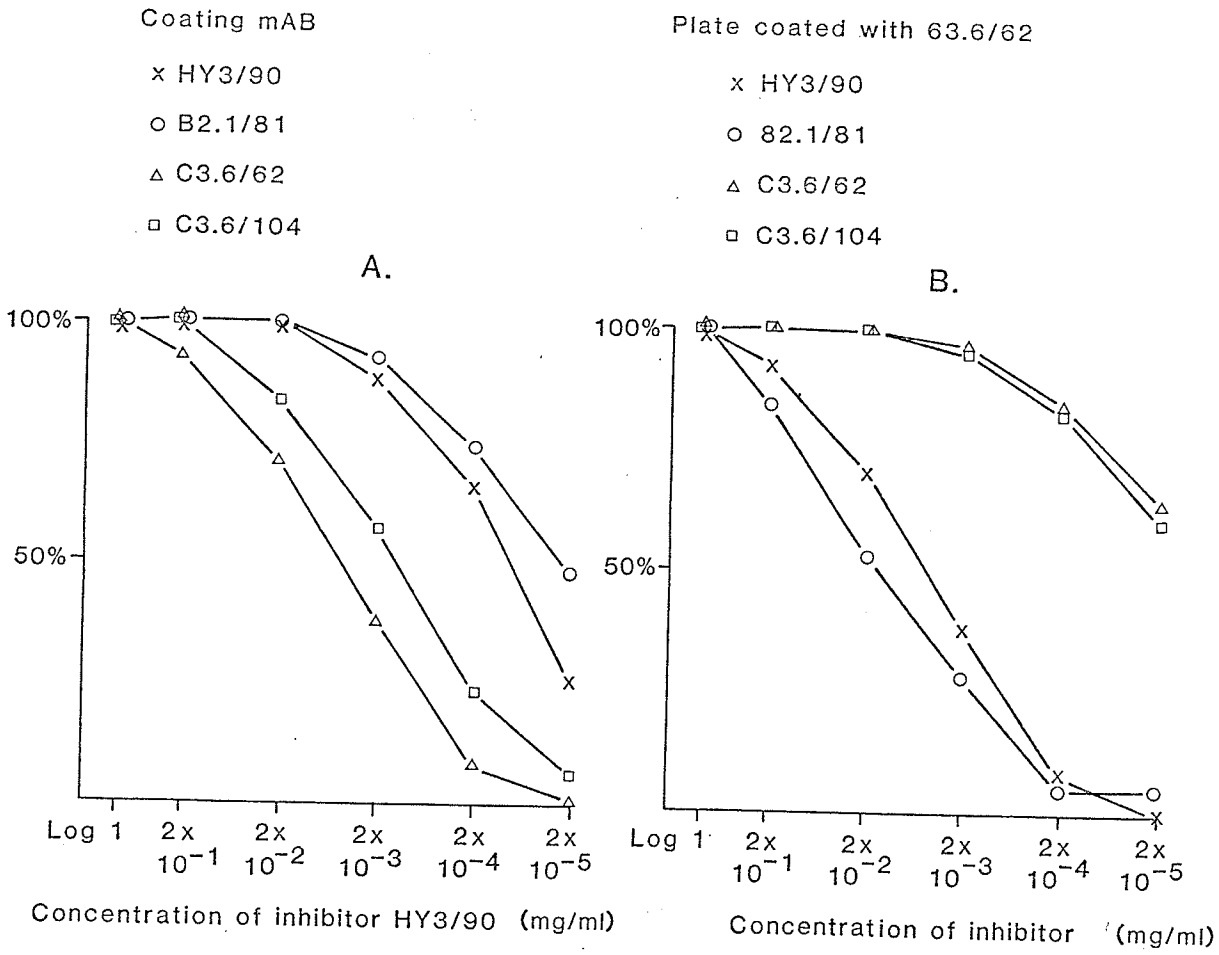


Figure 9: Crossinhibition among 4 Mab to P1 (Batch II) (RIA #4).

Panel A was graphed according to data from Table 10. Different dilutions of Mab HY3/90 were used to inhibit binding of ^{125}I-P1 to 4 different Mabs adsorbed on wells.

Panel B was graphed according to data from Table 11. Four different Mabs were used to inhibit the binding of ^{125}I-P1 to Mab C3.6/62 adsorbed wells.

This experiment was repeated with similar results.

which belonged to the same family (family IV) as the coating Mab C3.6/62, could give 95% and 97% inhibition, respectively. Similar to the results from batch I, when the concentration of inhibitors increased to 2 mg/ml, Mabs belonging to different families could crossinhibit up to 100% of each others binding to ^{125}I-Pl.

The relationship among 4 families from batch I and batch II was further examined. B3.6/62 and B3.6/104 were used to coat wells, and Mabs from 4 different families were used as inhibitors in RIA #4. Results are shown in Table 11. Concentrations of Mabs needed for 50% inhibition of the binding of ^{125}I-Pl to Mabs-coated well were calculated according to dilution curve based on data from Table 11, and are given in Table 12a and 12b. Two one-dimensional maps were drawn according to data from Table 12 (Figure 10). In the maps, the position of each clone was decided by the concentration needed for 50% inhibition of the binding of ^{125}I-Pl by Mabs 3.6/62 and C3.6/104. C4.1/96 was the representative Mab of family I and Mab A2.3/79 the representative of family II.

It was hypothesized that if the value of the concentrations on the axis of the maps was ignored and if only the distance between clones was considered, the latter could approximately reflect the relationships between the clones. The greater the distance between two clones on the map, the weaker should be their relationship, and the lowest should be their crossinhibition and vise versa.

Table 12.

A. Concentration[a] of Mabs for 50% Inhibition of the Binding of ^{125}I-Pl to C3.6/62

	Monoclonal Anti-Pl Antibodies						
	C3.6/62	C3.6/104	A2.3/79	HY3/90	B2.1/81	B2.1/35	C4.1/96
Concentration (log mg/ml) for 50% inhibition	-4.5	-4.4	-2.5	-1.6	-1.1	-0.7	0.7

B. Concentration of Mabs for 50% Inhibition of the Binding of ^{125}I-Pl to C3.6/104

	Monoclonal Anti-Pl Antibodies						
	C3.6/104	C3.6/62	A2.3/79	HY3/90	B2.1/81	B2.1/35	C4.1/96
Concentration (log mg/ml) for 50% inhibition	-4.7	-4.1	-3.2	-2.1	-1.8	-1.3	-0.2

[a]Refers to the concentration of the Mab employed for the assay.

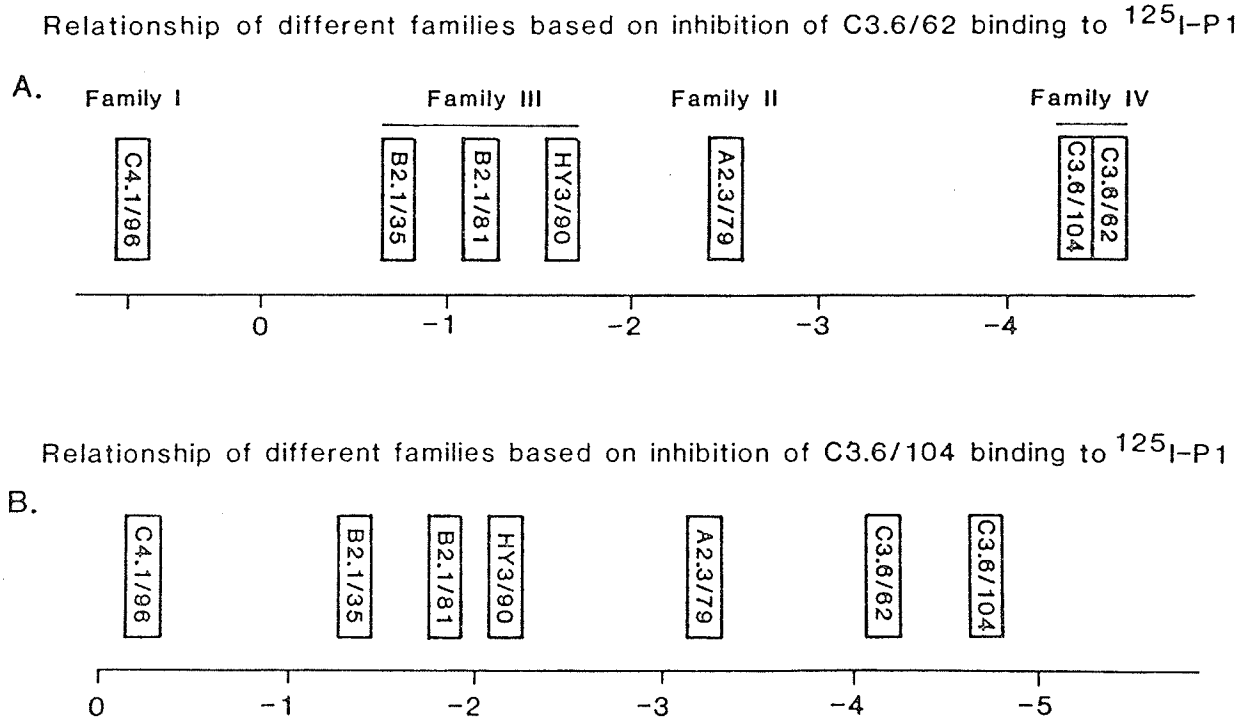


Figure 10: One-dimensional map depicting the relationship among families and clones of anti-P1 Mabs (based on data of Table 12).

The results in Figure 10a and 10b are concordant in supporting the hypothesis. If one made a prediction based on Figure 10a, and chose C3.6/104 as a reference clone, i.e., using different clones including C3.6/104 to inhibit binding of ^{125}I-P1 to C3.6/104, the order of strength for inhibition should be decided by the distance from each clone to C3.6/104, therefore, the order should be C3.6/104, C3.6/62, A2.3/79, HY3/90, B2.1/81, B2.1/35 and C4.1/96. This was proven to be the case in Figure 10b. If one started from Figure 9b, and chose C3.6/62 as the reference clone, the order should be C3.6/62, C3.6/104, A2.3/79, HY3/90, B2.1/81, B2.1/35 and C4.1/96, and this was again verified to be correct as shown in Figure 10a. Another two sets of experiments were carried out to further examine the validity of the maps and of the hypothesis.

In the first set, Mab HY3/90, B2.1/81, C3.6/62 and C3.6/104 were used to coat wells, and C4.1/96 from family I was employed as inhibitor in RIA #4 (Table 13). The strength of C4.1/96 in causing 50% inhibition on different clones should be in the order of B2.1/81, HY3/90, C3.6/104 and 3.6/62, from strongest to weakest according to map A in Figure 10; or in the order of B2.1/81, HY3/90, C3.6/62 and C3.6/104 from strongest to weakest according to map B in Figure 10. The results are given in Table 13 and Figure 11a. The order of concentration of C3.6/96 to give 50% inhibition on different clones was exactly that predicted by map A in Figure 10.

Table 13. Crossinhibition among Six Anti-Pl Monoclonal Antibodies (RIA #4)

A.	% Inhibition by Mab C4.1/96 (mg/ml)					
Mab on solid phase	5	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}
HY3/90	100	60	53	52	45	23
B2.1/81	100	100	100	100	93	93
C3.6/62	48	22	9	0	0	0
C3.6/104	63	55	36	36	32	22

B.	% Inhibition by Mab A2.3/79 (mg/ml)						Maximum Binding
Mab on solid phase	5	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	(cpm)
HY3/90	100	100	99	100	83	0	1,332
B2.1/81	100	100	84	84	78	63	4,287
C3.6/62	100	100	87	64	36	12	4,236
C3.6/104	100	100	97	80	55	30	5,131

Background was the mean cpm of 4 wells coated with anti-Rye I Mab #8.
Concentration of inhibitors indicated in the table were the concentrations of Mab employed.
Samples were tested in duplicate.

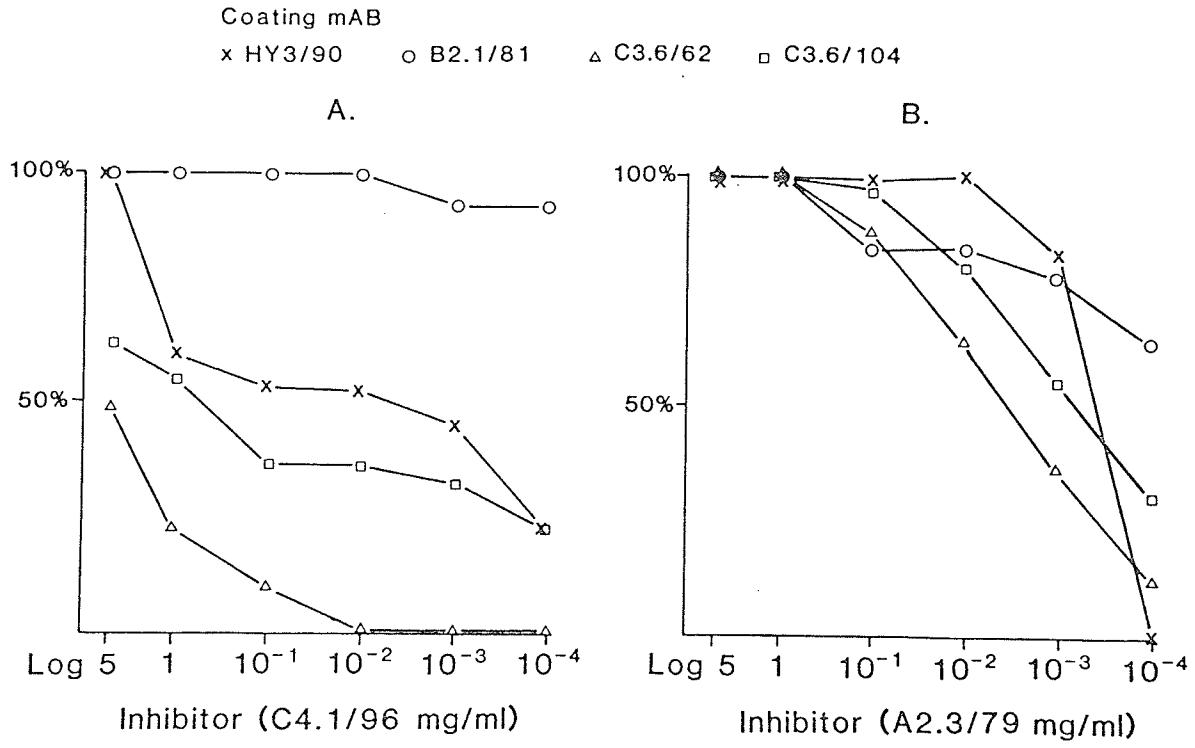


Figure 11: Crossinhibition among 6 clones from 4 families.
This graph was based on data in Table 13 (RIA #4).

Panel A: Mab C4.1/96 from family I was used to inhibit the binding of ^{125}I-P1 to 4 clones adsorbed on wells.

Panel B: Mab A2.3/79 from family II was used to inhibit the binding of ^{125}I-P1 to 4 clones adsorbed on wells.

In the second set of experiments, A2.3/79 from family I was used as an inhibitor to inhibit the same 4 Mab linked to the wells as in the first set of experiments (Table 13). The order of distance from A2.3/79 to other clones was HY3/90, B21/81, C3.6/104 and C3.6/62 from the nearest to the furthest according to map A in Figure 10. According to the results, the order of concentration of A2.3/79 to give 50% inhibition on those 4 clones was B2.1/81, HY3/90, C3.6/104 and C3.6/62, from the strongest to the weakest. This order of strength was basically the same as the order of distance except that HY3/90 and B2.1/81 switched positions with one another. Since HY3/90 and B2.1/81 belonged to the same family, they were very close in position in map A (Figure 10b), and this switch over was therefore considered as an experimental error. Although map A and map B were similar, map A could depict relationships of clones better according to the 2 experiments mentioned above. Another finding according to map A, as well as all the crossinhibition assays carried out, was that the 10 Mabs could only be grouped into several families on the basis of quantitative difference of their capacity in giving crossinhibition to other clones. When two groups of clones were about 3 units or more apart in the map, such as family I vs family II, and family II vs family IV, they could be rather clearly divided into different families in crossinhibition assays (Figure 8 and Figure 9); whereas clones separated apart less than about 3 units in the map could hardly be differentiated into different families. For example, when A2.3/79,

which belonged to family II, was used as a reference clone, since the difference of distance from family III to family II and that from family IV to family II was less than 1 unit in map A (Figure 10a), no clearcut families could be differentiated between family III and family IV in Figure 11b.

Crossreactivity of Mouse and Human Anti-Pl Antibodies

The crossreactivity was first examined by using sera from HDM allergic patients to inhibit binding of ^{125}I-Pl to mouse Mab in RIA #4. Nine different Mab were used to coat wells, and different dilutions of pooled serum from allergic or nonallergic individuals were employed as inhibitors. Results are shown in Table 14. Undiluted allergic sera gave 70-100% inhibition, while control nonallergic sera caused 13-50% inhibition. The difference is statistically significant ($P < 0.01$; by variance analysis). These results suggested that the epitopes recognized by mouse Mab were also recognized by human anti-Pl antibodies.

The crossreactivity was also tested by employing anti-Pl monoclonal antibodies as inhibitors of the binding of human Pl-specific IgE to ^{125}I-Pl (in RIA #5). In order to ensure the sensitivity of the assay, different cpm of total ^{125}I-Pl per well were pretested. According to results shown in Table 15, when 250,000 cpm were added to each well, the final ^{125}I-Pl bound in all 8 samples, which were to be employed later in the assays, were still in

Table 14. Crossreactivity of Mouse Monoclonal Antibodies and Human Antibodies Against P1

Mab on Solid-phase	Sera used as inhibitor	Serum Dilution[a] 1:1	1:10	1:100	1:1000	Maximum Binding (cpm)	Back-ground (cpm)
C4.1/96	Anti-P1 (+)[b]	94[c]	36	3	5	1,944	361
	(-)[d]	35	11	ND[e]	ND		
C4.1/102	Anti-P1 (+)	88	26	0	0	2,531	357
	(-)	50	15	ND	ND		
C4.1/40	Anti-P1 (+)	91	26	0	0	2,478	369
	(-)	32	3	ND	ND		
A2.3/79	Anti-P1 (+)	91	46	11	5	4,140	337
	(-)	31	15	ND	ND		
A2.3/191	Anti-P1 (+)	88	37	13	15	2,973	310
	(-)	30	26	ND	ND		
HY3/90	Anti-P1 (+)	92	58	20	17	1,842	356
	(-)	38	25	ND	ND		

Table 14 cont'd...

Mab on Solid-phase	Sera used as inhibitor	Serum Dilution[a] 1:1	1:10	1:100	1:1000	Maximum Binding (cpm)	Back-ground (cpm)
C3.6/62	Anti-Pl (+)	70	18	0	0	3,738	327
	(-)	13	3	ND	ND		
C3.6/104	Anti-Pl (+)	77	32	0	0	5,348	365
	(-)	17	10	ND	ND		
B2.1/81	Anti-Pl (+)	100	65	29	30	1,515	344
	(-)	30	9	ND	ND		

[a]Refers to serum dilution used to prepare the assay.

[b]Anti-Pl (+): serum pool from sera containing anti-Pl antibody.

[c]Percentage inhibition.

[d]Anti-Pl (-): serum pool from sera containing no anti-Pl antibody.

[e]ND - not determined.

Background (346 cpm) was the mean cpm of 18 wells coated with anti-Rye I Mab #18. This experiment was repeated with similar results.

Samples were tested in duplicate.

Table 15. Titration of ^{125}I-Pl in RIA #5

Serum sample[b]	Total ^{125}I-Pl Added (cpm)[a]			
	250,000	125,000	62,500	31,000
	^{125}I-Pl Bound (cpm)			
W77	2,812	2,027	1,611	953
W78	1,594	1,150	801	503
W122	10,743	5,673	3,219	1,744
W124	13,343	6,965	3,725	1,826
W125	10,389	5,328	3,309	1,783
W126	4,884	2,949	1,944	1,094
W127	4,011	2,489	1,948	1,078
Pool	5,933	3,578	2,238	1,235
Nonatopic serum	209	154	100	77

[a]Approximate values with about ± 10% variation.

[b]All sera were diluted 1:10.

Samples were tested in duplicate.

the ascending part of dilution curves. Therefore, about 200,000 cpm of ^{125}I-Pl were added to each well. Eight serum samples composed of 7 sera from HDM allergic individuals and one serum pool from another 10 HDM allergic individuals were diluted 1:10 in PBS, and incubated in mouse-anti-human-IgE coated wells. Five anti-Pl Mab from 4 different families were used as inhibitors. The inhibition (Table 16) was less effective when compared with the results in Table 9, where mouse Mabs were used to inhibit mouse Mabs. At the level of 200 µg/ml of inhibitor, the mean inhibition ± 1SD by Mab of human IgE binding activity was 55.4 ± 21.3%, while that to autologous and heterologous mouse monoclonal was 94.1 ± 14.1% (Table 9). This suggested anti-Pl IgE in human sera recognized more than one epitope on Pl, compared to Mab. In Table 16, 24 out of 40 different combinations demonstrated more than 50% inhibition at the level of 200 µg/ml of inhibitors. This suggested the presence of a major antigenic region of Pl which was recognized by all mouse Mab families and by human IgE antibodies. The data of Table 16 were evaluated by two-way variance analysis. Comparing the inhibitory effect of different clones, the only significant difference was between HY3/90 and the other clones. Since HY3/90 and B2.1/81 were of the same family, the weak inhibition by HY3/90 could only be explained by its relatively weak affinity, as suggested by its low binding activity to ^{125}I-Pl (Table 13). It was noticed that 8 different human sera were inhibited by Mab to a different extent. The difference of inhibition between any of 2 human sera was compared statistically with variance analysis. There were

Table 16. Crossreactivity Between Mouse Mab and Human IgE Anti-Pl Antibodies

Serum on solid-phase	Inhibitor								
	Mab C4.1/96 (µg/ml)			Mab A2.3/79 (µg/ml)			Mab HY3/90 (µg/ml)		
	200	2	0.02	200	2	0.02	200	2	0.02
	% Inhibition								
W77	69	48	22	76	51	10	60	32	26
W78	90	36	0	83	57	0	69	32	21
W122	62	15	0	61	50	15	29	10	9
W124	42	7	0	26	13	0	14	11	0
W125	58	4	0	34	10	0	11	0	0
W126	51	2	0	31	15	0	42	9	0
W127	74	9	0	67	38	0	40	0	0
Serum pool of allergic donors	72	24	15	67	33	0	42	9	17

Table 16 cont'd.....

Serum on solid-phase	Inhibitor: Mab C3.6/104 (µg/ml) 200	2	0.02	Mab A2.3/79 (µg/ml) 200	2	0.02	Human Myeloma IgG (µg/ml) 200	20	2	0.2	0.02	Maximum Binding (cpm)
	% Inhibition											
W77	84	54	6	69	45	10	30	18	10	20	26	2,162
W78	100	61	0	91	62	0	3	3	0	10	0	1,298
W122	67	59	36	43	37	26	3	7	2	0	0	8,145
W124	33	23	15	33	24	15	0	0	0	5	0	8,504
W125	48	30	0	37	19	0	0	0	0	0	0	3.989
W126	46	21	0	41	41	0	0	0	0	0	4	2,597
W127	76	33	0	52	24	0	0	0	0	0	0	1,992
Serum pool of allergic donors	69	51	20	60	43	17	15	6	0	0	15	3,721

Serum samples were diluted 1:10 in final concentrations.
Background (486 cpm) was the mean cpm of 16 wells coated with nonatopic human serum pool diluted 1:10. This experiment was repeated with similar results.
Samples were tested in duplicate.

total (8-1) x (8-1) = 49 different combinations of pairs. About half (21/69) of the pairs differed significantly ($P<0.05$) between the 2 members of a pair in the extent of being inhibited by Mab (Table 17). Among all the sera, W77 was inhibited most strongly by Mabs, and W124 the least.

Comparison of the Inhibitory Effect of Individual Mab and Pooled Mab on the Binding of P1 to Human IgE Antibodies

Three monoclonal anti-P1 antibodies from 3 different families were employed as inhibitors, either individually or as a pool to inhibit the binding of P1 to human IgE antibodies. Three sera from HDM allergic donors were tested. The results (Table 18) were processed with Student-Newman-Keuls variance analysis, in order to compare the difference between inhibitory effect of single Mab to that of the pool. It was found that C4.1/102 was significantly stronger than B2.1/104 ($P < 0.05$), and pool Mab was significantly stronger than B2.1/104, while the difference between pooled Mab, C4.1/102 and A2.3/79 was not significant. It is therefore concluded that the pooled Mab did not induce a stronger inhibition than the individual Mab. That the pooled Mab gave stronger inhibition than B2.1/104 could be explained by the fact that pooled Mab contained C4.1/102 which was a significantly stronger inhibitor than B2.1/104.

Table 17. Comparison of the Differences of Inhibition of Individual Sera by Several Mab to P1

Serum	W124	W125	W126	W122	W127	Pool	W77	W78
W78	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05	
W77	p<0.05	p<0.05	p<0.05	p<0.05				
Pool	p<0.05	p<0.05	p<0.05					
W127	p<0.05	p<0.05	p<0.05					
W122	p<0.05	p<0.05	p<0.05					
W126	p<0.05							
W125								
W124								

This table shows the Student-Newman-Keuls analysis of the data in Table 16.

The difference between any of the two sera was compared and indicated as p<0.05 if the difference was statistically significant.

Table 18. Comparison of the Inhibitory Effect of Individual and Pooled Mab

Serum of HDM Allergic Donor on Solid-phase	MAB C4.1/102 (µg/ml)[a] 200	20	2	.2	Mab A2.3/79 (µg/ml) 200	20	2	.2
Pool	37[b]	27	7	0	44	21	11	9
W122	24	12	17	0	58	51	44	33
W126	39	29	21	4	38	30	24	3
W124	21	0	0	0	20	7	0	0

Serum of HDM Allergic Donor on Solid-phase	Mab B2.1/104 (µg/ml) 200	20	2	.2	Pool of Mabs[c] (µg/ml) 200	20	2	.2	Maximum Binding (Normal human IgG 10 mg/ml as an inhibitor)
Pool	23	27	0	0	48	38	26	5	9,690
W122	34	32	23	2	47	39	32	18	27,237
W126	20	15	18	0	52	40	31	0	9,853
W124	14	13	0	0	34	27	3	0	20,406

[a] Final concentration.
[b] Percentage inhibition.
[c] Pooled Mab was composed of C4.1/102, A2.3/79 and C3.6/104 in 1:1:1 ratio.
Background (337 cpm) was the mean cpm of 8 wells coated with nonatopic serum pool diluted
Samples were in duplicate.

Discussion

The purpose of this study was to produce monoclonal antibodies specific to P1 (the major allergen of the house dust mite, Dermatophagoides pteronyssinus), and to select those Mab that are reacting with epitope(s) which are identical or close to those identified by antibodies from HDM allergic patients. The Mab were also used in crossinhibition studies to analyse the topography of P1 antigenic determinants.

An essential prerequirement for the production of monoclonal antibodies is to establish a valid and efficient assay to monitor the antibody response of immunized mice and to screen the culture supernatants of hybridoma cell lines. Since the assay should be set up prior to the immunization of mice and since no P1-specific mouse Ig was available at the beginning of this study, a radioimmunoassay (RIA #1) was developed and validated by employing mouse IgG monoclonal antibodies to human IgE. RIA #1 allowed the detection of less than 0.1 µg/ml of mouse IgG anti-IgE in a culture medium comparable to that employed to grow hybridoma cell lines (Figure 2). This sensitivity was satisfactory since the Ig concentration in the culture supernatant of hybridoma was reported (Zola et al 1982) to be in the

range of 1 to 10 µg/ml. Since in the sera of immunized mice, the P1-specific IgG was expected to be only a small fraction of total IgG, enough binding capacity of goat-anti- mouse-IgG coated wells were needed to generate a reasonably high signal. To ensure that RIA #1 could be applied for the monitoring of mouse serum antibody response, we mixed a small amount of Mab anti-IgE with normal mouse serum to mimic the immunized mouse serum. The results (Figure 2) showed, that in this reference system, less than 0.1 µg/ml specific IgG Mab could be detected in mouse serum.

Different immunization schedules were used with comparable success by different investigators (Zola et al 1982; Spriggs et al 1983; Chapman et al 1984). We therefore employed an empirical schedule which was used in this laboratory for the production of several kinds of Mab. Balb/c mice were chosen since the fusion myeloma partner NS-1 was of Balb/c origin; hence, the resulting hybridomas will be easily grown in the peritoneal cavity of histocompatible Balb/c mice in order to obtain large quantities of ascitic fluid. The two mice immunized in the present study mounted a strong antibody response with serum IgG anti-P1 reaching a titer of 1:102,400. This response seems to be comparable or even better than that reported by Chapman et al (1984), who obtained a titer of 1:12,800, although it must be noted that these authors employed a different assay to measure IgG anti-P1.

After fusion, cells were cultured in aliquots of about 1×10^6 cells/well in a 96 well tissue culture plate. After 6 days, at least one colony of growing hybrid cells was found in each well. This fusion ratio is comparable to that (1-5 among 10^6 spleen cells) reported by Yelton et al (1978); the fusion was therefore considered successful.

Screening assays were carried out twice on day 10 and day 13. Due to limited amount of culture supernatants, each sample was tested singly in each assay. However, the previous (or subsequent) assay served as a duplicate and most of the results of the 2 assays were concordant. All the 96 primary cultures produced anti-Pl IgG antibody. This was not unusual, since each well contained several clones of hybrids and the reported frequency of hybrids producing the desired specific antibodies ranged from 11% to 57% (Yelton et al 1978). The binding of hybridoma antibodies to Pl could be inhibited by crude HDM antigen but not by an unrelated antigen like Rye I, the major allergen of rye grass pollen. These inhibition assays were essential because the labeled Pl used in the screening assays could express new antigenic determinants secondary to conformational changes due to radioiodination. Hence, the inhibition assay could distinguish the antibodies against natural determinants from those against modified determinants on Pl. By adding 200 ng of crude HDM antigen,

all the test samples were inhibitable and the inhibition was about 30-60% (Table 1). However, the same amount of crude antigen could give more than 80% inhibition in subsequent assays (Table 6 and 7). The relative low inhibition in this assay was, most likely, due to the long period (more than one month) of storage of the crude antigen in solution. It was a frequent observation by the author that the antigenicity of crude HDM solution would decrease during the storage even at low temperature. Ackland et al (1984) reported similar findings. He documented that esterase, lipase and alkaline phosphatase were present in whole mite extract. These enzymes could cause degradation of the antigen, and were probably responsible for the loss of antigenicity of crude HDM antigen during the storage. The primary cultures were selected for limiting dilution based on the high binding of activity of their culture supernatant to ^{125}I-Pl or to the strong inhibition by HDM antigen. Low binding to Pl could be due either to low affinity or to low concentration of the antibodies. In either case, the Pl-specific hybridoma antibody could still possibly share crossreactive idiotype with human anti-Pl antibodies. Therefore, some of these wells were also kept for future limiting dilution.

Nine primary cultures containing anti-Pl antibody inhibitable by crude HDM extract were cloned by limiting dilution. An average of 0.5 cell was dispensed into each well of 96 well tissue culture plates. According to Poisson distribution formula (Mishell et al 1980): Ln S

= m (where S is the theoretical fraction of negative cultures, and m is the mean number of cells applied per well), 30.7% of the wells should contain a growing clone. This value was quite close to that observed in this study, i.e., 23.4% (404 out of 1728 wells). One culture was assumed to be monoclonal when only one colony of cells could be identified in the corresponding well after examination under the microscrope 7 days after the limiting dilution. This assumption was valid in most of the cases unless two or more cells were stuck together during limiting dilution.

In 4 out of 9 primary cultures, the monoclonal hybridomas generated in limiting dilution did not secrete IgG anti-Pl antibody. There are several possible explanations: (1) the specific antibody producing cell was overgrown by other unrelated hybrids, (2) as pointed out by Yelton et al (1978), in some cases the hybrids making the largest amount of antibody may die after a number of generations in culture due to their terminal differentiation. On the other extreme, monoclonal hybrids derived from 2 other primary cultures (C4.1 and B2.1) were all secreting anti-Pl antibody. This was probably due to the fact that there was only one hybrid clone, which was an anti-Pl producing hybrid, in the primary culture. Alternatively, anti-Pl antibody producing clone(s) in the primary culture overgrew all other hybrids in the same well before the limiting dilution.

In order to select clones which were crossreactive with human anti-Pl antibody, pooled sera from HDM allergic patients were used as inhibitors in the screening assays. Two µl of allergic sera gave 27-83% inhibition of the binding of Mab to ^{125}I-Pl (Table 2). Normal human serum was not included for the control in the screening assays due to the limited quantity of supernatants. However, in subsequent similar assays (Table 14), 2 µl of normal human serum gave very little inhibition (3-14%). Therefore, it could be concluded that the screening procedure was efficient and that allergic serum specifically inhibited the binding of Mab to Pl. Several possibilities could account for this inhibition. The human antibody might bind to the same epitope of Pl as that recognized by the Mab and cause inhibition by true competition. Alternatively, human antibodies might bind to an area close to the epitope recognized by Mab and cause inhibition by steric hindrance. It is also possible that human antibodies bind to some totally different determinant and cause inhibition by allosteric effect as reported by Ivanyi et al (1980). There was no way to differentiate these three possibilities by means of the radioimmunoassays employed in this study. Therefore, it was logical at this stage to select the clones which were well inhibited by crude HDM antigen and human anti-Pl antibody, in a hope that at least some of the Mab were recognizing the same epitope as human anti-Pl antibody did. Indeed, the final goal of this part of the study was to obtain Mabs that share idiotype(s) with the human anti-Pl antibodies. It was assumed that the probability of mouse Mab to share

a crossreactive idiotype with human antibody should be much higher if the mouse and human antibodies were specific to the same epitope.

During the expansion of the selected monoclonal hybrids, 3 clones stopped producing anti-Pl antibody. This might be due to chromosome loss, or segregation of the genes for the antibody heavy and/or light chains as suggested by Gerhard et al (1976); alternatively, these hybrids might not have been monoclonal, and the anti-Pl antibody producing cells could have been overgrown by unrelated clones.

The subclass of 10 monoclonal antibodies was first tested by RIA #3 and clearcut results were obtained (Table 3). The data also support the view that these hybrids were monoclonal. However, there were no positive controls available for γ2a, γ3 and λ subclasses. An immunodiffusion assay was carried out to complement the results of the RIA by testing the activity of all the antisera employed in RIA. The results of this assay (Figure 4) were in accordance with those of the RIA. The five Mab clones identified as γ1κ by RIA, formed precipitation bands with anti-γ1 and anti-κ antisera, but not with anti-γ2a, anti-γ2b or anti-γ3 antisera, while as a control, anti-γ2a, anti-γ2b, anti-γ3 and anti-λ antisera could form bands against normal mouse serum. This indicated that the antisera were indeed active. The five Mab were also precipitated by antisera to λ chain. This was not too surprising, because (1) the monoclonal antibodies were isolated from ascitic fluid, in which unrelated Ig from the host mouse

could be present; (2) Mab were purified by ion exchange chromatography, a procedure that could not differentiate the Pl-specific IgG from the unrelated IgG; and moreover (3) some Ig of the other classes were copurified with the IgG anti-Pl, as shown by the SDS-PAGE analysis (Figure 4). Nine out of 10 Mab were of the $\gamma 1\kappa$ subclass, and one was of $\gamma 2b\kappa$. It was not unusual that all the Mab had κ light chains, since in mouse normal serum Ig, the ratio of κ to λ is 95:5 (Turner et al 1977). However, although IgG2a is the dominant IgG subclass in mouse, in this study as well as in that of Chapman et al (1984), the major IgG subclass of anti-Pl Mab was of IgG1. This could be due to the nature of Pl or to the immunization schedule; the exact reason is not known.

The specificity of Mab was tested in 2 assay systems, i.e., RIA #2 where Mab was bound to the solid-phase via goat-anti-mouse IgG, and RIA #4 where Mab were allowed to coat directly the solid-phase. The results were concordant and the binding of ^{125}I-Pl to monoclonal antibodies could be specifically inhibited by unlabeled crude HDM antigen or Pl, but not by unrelated antigen. Another investigator tried to prepare anti-Pl Mab, but later he found the Mabs were actually binding to the determinant of phosphatidyl choline (Dr. F. Alkan, personal communications). The explanation was that PC is ubiquitous, and is frequently present in Pl preparations. Therefore, PC was included as an inhibitor; no inhibition was found by adding 200 ng of PC per well, whereas at the same concentration crude HDM induced 81-100% inhibition.

The epitope specificity of the Mab was studied by cross-inhibition assays. Nine clones were divided into 2 batches according to the primary culture from which they were derived. Crossinhibition was first conducted within the batch. In the first batch 1, C4.1/96, C4.1/102 and C4.1/40 were from the same primary culture, and A2.3/79 and A2.3/191 were both from another primary culture. It was apparent from Figure 8a that C4.1/96, C4.1/40 and the autologous antibody C4.1/102 inhibited strongly and to a similar extent the binding of C4.1/102 to ^{125}I-P1, while the other two clones A2.3/79 and A2.3/191 were weak inhibitors. The difference of inhibition by these two groups of Mabs was statistically significant by variance analysis ($P<0.05$), and it was not due to the difference of affinity or difference of concentration of active antibody in the preparations. Indeed, when A2.3/79 was used to coat to the solid-phase (Figure 8b), A2.3/191 and the autologous A2.3/79 (both of which were weak inhibitors in the previous experiment in Figure 8a), became strong inhibitors. Thus, these two groups of Mabs were considered as members of two distinct families. C4.1/96, C4.1/102 and C4.1/40 belonged to family I, and A2.3/79 and A2.3/191 belonged to family II. By similar analysis, the second batch of Mab, which consisted of 4 clones, was shown to comprise another two families. Family III consisted of HY3/90 and B2.1/81, and family IV consisted of C3.6/62 and C3.6/104. As shown in Figure 9a, members of family III were strong inhibitors to autologous member (like HY3/90), but weak inhibitors for the member of family IV (like C3.6/62 (Figure 9b)). Conversely, members of family

IV inhibited less the member of family III HY3/90 than their autologous member C3.6/62 did. This again indicated that the difference of inhibition was not due to the relative difference of affinity. It was noticed that all the members of a given family were actually derived from the same primary culture in all cases, except family III, and that the inhibition curves (Figure 8 and 9) of members of the same family were very close and similar. This might suggest that all the clones of a family could be the progenies of one hybrid cell and they were recognizing the same epitope on the P1 molecule. Chapman et al (1984) produced anti-P1 Mab and conducted similar crossinhibition assays. The highest concentration of inhibitor employed in their studies was 50 µl of 1:100 diluted ascites which contained approximately 5 µg of Mab (based on the assumption that ascitic fluid contained 10 mg/ml of IgG). With this amount of inhibitor, it was found that the binding of antigen to some Mab could be inhibited completely (> 95%) by one group of Mab, but only partially inhibited (15-52%) by another group of Mab. They concluded that partial inhibition was nonspecific and that the two groups of Mab were directed against two distinct epitopes on the P1 molecule. It would be interesting to know the inhibition pattern when higher concentrations of Mab were employed. In our study, when 0.2 µg of Mabs was used as inhibitors, the results were comparable to that of Chapman's, i.e., one family would inhibit more than 80% while the other family only inhibit less than 55% in both batches (Figure 8 and 9). However, when the concentration of inhibitor Mab was further

increased to 20 μg/well, all the clones could give complete inhibition (> 85%) of the binding of Pl by Mab from either the same or a different family. We therefore consider that it is not appropriate to interpret the inhibition by some Mab as "true" while that by others as nonspecific.

Due to the fact that present anti-Pl monoclonal antibodies could all inhibit, albeit with different extents, the binding of Pl to a given clone of Mab, it was reasonable to assume that: (1) on the Pl molecule, there is a contiguous structural moiety which comprises several epitopes reacting with Mab, (2) there is a "public region" where these epitopes overlapped, and (3) each epitope was comprised of a public region plus a private region which only belong to a certain epitope. A tentative topographical model is illustrated in Figure 12 to depict this moiety on Pl. If we assume that the circles in the Figure were the center of different epitopes, and each epitope covers an area with 5 cm radius in the Figure, all the epitopes will overlap with one another. Among the area belonging to a certain epitope, the overlapped part is the "public region" and the unoverlapped part the "private region". The degree of overlapping between two epitopes will account for the degree of crossreactivity. This hypothesis could quantitatively describe the crossreactivity, which in many cases was not a yes or no event, but showed a gradual difference. Similar observations were made by others. For example, Ivanyi et al (1982) analysed the epitopes of human growth hormone with Mab. Some of the

Mabs were partially crossreactive with other Mabs as shown by crossinhibition assays. A similar phenomenon was also reported by Spriggs et al (1983) when the epitopes of Reovirus group 3 hemagglutinin was studied with Mabs.

According to this hypothesis, it was more reasonable to classify the Mabs into different families according to the pattern and degree of crossinhibition by a reference clone, instead of arbitrarily setting up a cut-off point to define specific or nonspecific inhibition and classify Mabs into families.

As mentioned earlier in our study, 9 clones were divided into 2 batches, and crossinhibition was first carried out within each batch. Family I and II were identified from batch 1, and family III and IV from batch 2. To further study the relationship among these 4 families, a crossinhibition assay was conducted among the members of the 4 families. The results were quite difficult to interpret at first glance (Figure 10), and it was more difficult to divide these Mabs into different families when A2.3/79 was used as a reference clone (Figure 9b). In this case, the differences of the inhibition by different clones were not statistically significant as assessed by Student-Newman-Keuls variance analysis ($P > 0.05$). However, when an innovative map (Figure 9) was constructed according to the concentration of different Mabs leading to 50% inhibition of the reference clone C3.6/62 or C3.6/104, the relationship between

different families became understandable. Different Mabs were lined up along a one dimensional map. The distance between each other reflected the relationship of the corresponding epitopes recognized by the Mab, and also reflected the degree of crossreactivity between different clones or families. In other words, the relative strength of inhibition of each clone against the reference clone was a function of the distance between them on the map. It was also found that 3 units or more on the map were the necessary distance for two clones to be clearly grouped into two distinct families. This suggested that the epitopes recognized by these Mabs were scattered in a contiguous region with quantitative difference of overlapping between the epitopes.

The validity of the map was tested with at least 4 clones (C4.1/96, A2.3/79, C3.6/104 and C3.6/62) as reference clones in cross-inhibition assays. When our clones were compared with the two families of anti-P1 Mab prepared by Chapman, it was found (personal communication) that our B2.3/35, HY3/90, A2.3/79, C3.6/62 and C3.6/104 were crossreactive with their 5H8C12 family (Chapman et al 1984), while our C4.1/96 did not crossreact with any of their two families. It was quite conceivable that the 5H8C12 family was located at the vicinity of the A2.3/79 family in our map (Figure 10). At this location, B2.1/35, HY3/90, C3.6/104 and C3.6/62 were all in the distance of less than 3 units, and, therefore, were crossreactive to a large extent with 5H8C12. This might be the reason why Chapman

considered that these clones do belong to their 5H8C12 family. On the contrary, C4.1/96 was far away at the left extreme of the map, and was thus considered by Chapman as binding to another "distinct" epitope. If we remember the fact that this author only graded the crossreactivity on the yes or no basis, instead of different degrees of crossinhibition, this could serve as another evidence to support the validity of the map.

It was found that not all the clones were equally good to be used as a reference to construct a map. For example, if A2.3/79 was used as a reference as shown in Figure 10b, i.e., different clones including A2.3/79 itself were used to inhibit the binding to P1 by A2.3/79, A2.3/79 would be located at the extreme left of the map since it was autologous to the reference clone and would give stronger crossinhibition; since the distances from family III and family IV to A2.3/79 were similar according to the map in Figure 10, although they were located at different sides of A2.3/79, they would give similar extent of inhibition to the reference clone A2.3/79 in the crossinhibition assay, and this is reflected in Figure 11b. Therefore, if we construct a map using A2.3/79 as a reference clone, the family III would superimpose on the position of family IV, and the map would fail to reflect the relationship between different families. This phenomenon could easily be explained by the fact that the map was only a one-dimensional projection of the complicated two or three-dimensional topography of P1 epitopes onto a line. The

relationship of the epitopes could only be reflected adequately if a suitable angle was chosen for the projection. Let us assume that the epitopes were located as illustrated in Figure 12, and parallel light beams were used to project the epitopes onto a linear map. When a reference clone is selected, this clone should be positioned at the left extreme on the linear map, because it is the autologous Mab and should give the strongest inhibition, therefore, the angle of the projection is approximately determined. In this case, if C3.6/62 was used as a reference, line AB would be the map, and if A2.3/79 was used as the reference, line CD would be the map. Apparently, AB would provide better reflection of the topography of the epitopes. Conceivably, if several linear maps were made using different Mabs as reference clones, and were orientated into suitable positions, a two-dimensional or even three-dimensional map of epitope topography might be constructed. It is worthwhile to point out that in the model shown in Figure 12, the circle might only represent the center of an epitope, and the corresponding antibody could cover a certain area in the vicinity either by true binding or by steric hindrance. These areas belonging to each epitope could overlap and, therefore, crossreactivity between clones recognizing different epitopes may occur.

The topographic map of epitopes thus constructed, either linear or two-dimensional, is an operational model, since it was prepared according to crossinhibition assays which could not distinguish the

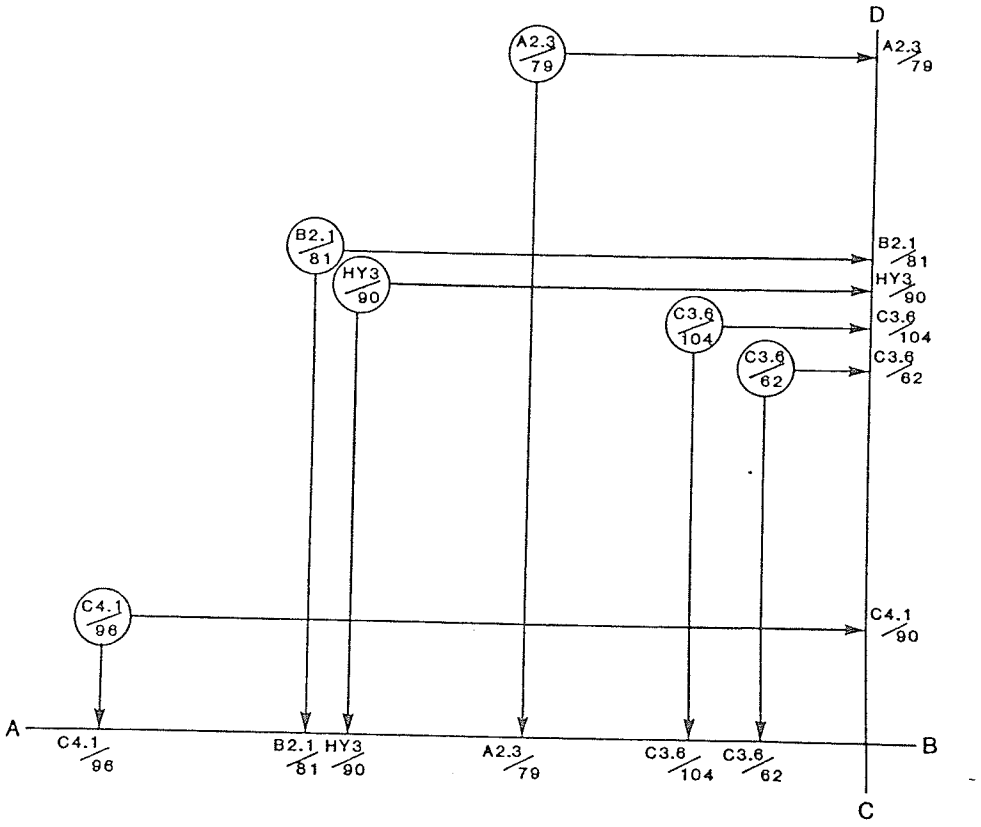


Figure 12: A model of epitope topography on P1 molecule.

steric hindrance from true crossreactivity of antibodies; however, it is likely reflecting, to a certain extent, the physical topography. The relationship between the operational and physical model of epitope topography could be fully established if the antigens were studied by x-ray crystallography and affinity labeling. Nevertheless, the operational one is simple to establish, and is adequate in certain studies on the function of different determinants of biologically active macromolecules, such as antigens, viruses, hormones or receptors.

In order to use mouse Mab to identify human anti-idiotypic antibodies, one prerequisite is that Mabs share a public idiotype with human antibodies. Our Mabs were selected on the basis that their binding to Pl could be inhibited by human anti-Pl antibodies aiming to obtain Mabs binding to the same epitope as human antibodies. This should increase the probability that some of these Mabs would have similar or identical paratopes or combining site idiotypes as human anti-Pl antibodies. The binding of Mabs to Pl was indeed inhibited by human sera containing anti-Pl antibodies with an inhibition ranging from 70 to 100%, as opposed to 13-50% inhibition when normal sera were used. By two-way variance analysis, the difference between inhibitions by atopic and nonatopic human sera was highly significant ($P < 0.01$), and the difference among the inhibitions of different clones of Mabs by anti-Pl positive human sera was not significant. This suggested that the epitopes recognized by Mabs were all

recognized by human antibodies, and that pooled human anti-Pl antibodies were binding to a similar extent to the different epitopes recognized by different Mabs. Different possibilities may be considered to explain the 13-50% inhibition by the nonatopic human sera. Whole serum could nonspecifically interfere with the binding of antibody to antigen, or this interference could be due to the presence of auto-anti-idiotype antibodies in normal serum. Abdou et al (1981) reported that sera of some active systemic Lupus erythematosus patients as well as some normal donors could inhibit the anti-DNA antibody binding to DNA, and it was found later that the inhibition was due to the auto-anti-idiotype antibody. Zouali et al (1983) reported a similar finding that sera of normal donors could inhibit affinity purified anti-DNA antibody binding to DNA, and the inhibitory factor was an IgG auto-anti-idiotype. Therefore, it will be interesting to elucidate the nature of the inhibition caused by normal human serum in the anti-Pl and Pl ligand system, and this topic will be dealt with in the next Chapter.

In order to examine the extent of crossreactivity between Mab and human anti-Pl IgE antibody, Mabs were used to inhibit the binding of Pl by IgE antibodies from 8 different human sera and a serum pool from HDM allergic donors. The mean inhibition ± 1SD was 55.4 ± 21.3%, and the monoclonal antibodies to Pl, even at high concentration, could not completely inhibit the binding of Pl by human IgE anti-Pl (Table 16). This was not unexpected, since the human antibody response to Pl is

"heterogenous", i.e., directed against several epitopes, whereas by definition, a Mab is directed against a single epitope. Variance analysis of the data showed that all the clones except HY3/90 were similar in their ability to inhibit human IgE antibodies. This could be easily explained by the fact that these Mabs were all recognizing the same contiguous antigenic moiety consisting of several overlapping epitopes. The inhibition by pooled Mabs from three different families was comparable to that obtained by each Mab tested individually. This is another line of evidence that these Mabs (although they have some quantitative difference in epitope specificity), were essentially against the same antigenic moiety on Pl. It was noticed that although Mab could not completely inhibit the human anti-Pl IgE, the mean inhibition was more than 50% (Table 16). This suggested that a large portion of human IgE was against the same antigenic moiety as that recognized by Mab. Taking into account that all our anti-Pl Mabs, plus the Mab 5H8C12 prepared by Chapman recognize this region, we could reasonably suggest that this region is the major antigenic site on Pl. The overlapping area among the epitopes could be considered as a major public immunogenic region for both mouse and man. If the above conclusion is correct, then the human anti-Pl response should predominantly involve clones with combining site idiotype against this region, and consequently, the corresponding anti-idiotype could be present at a detectable level.

Eight different human allergic sera were inhibited by Mabs to a different extent. When every sera was compared in turn with the other 7 sera, the difference of inhibition in half of such pairs was statistically significant. This indicated that the proportion of IgE against a certain antigenic moiety of Pl among the total anti-Pl IgE varied significantly among allergic individuals. An interesting finding was that the serum from a patient W78, who had received crude HDM antigen injections for two years, was the only one which differed significantly from all the other 7 sera, and was inhibited most strongly (mean $\pm$ 1SD = 70.2 $\pm$ 36.0%) by Mabs. This could suggest that after the prolonged hyposensitization, the epitope specificity of anti-Pl IgE was changed and that the IgE against the antigenic moiety recognized by Mab became dominant. It will be interesting to know if this ratio change is due to the suppression of IgE production against other epitopes or due to enhancement of the IgE production against the epitopes recognized by Mabs. With this regard, it is known that there is no definite pattern of change of specific anti-Pl IgE level during hypersensitization treatment (Dalaunois et al 1985; Pauli et al 1984).

CHAPTER III

ANALYSIS OF THE INHIBITION BY HUMAN SERA OF THE BINDING OF P1 TO ANTI-P1 ANTIBODIES

Abbreviations used in this Chapter:

AASP	HDM antigen-4B absorbed allergic serum pool
ANSP	HDM antigen-4B absorbed nonallergic serum pool
Anti-IgA-4B	Sepharose CL-4B coupled with anti-human IgA antibodies
Anti-Id	anti-idiotype antibody
Anti-IgM-4B	Sepharose CL-4B coupled with anti-human IgM antibodies
BSA-4B	Sepharose CL-4B coupled with bovine serum albumin
BSA	bovine serum albumin
BBS	borate buffered saline
CB	carbonate buffer
CCAA	colorectal carcinoma associated antigen
Cpm	count per minute
DDW	double distilled water
DNA	deoxyribonucleic acid
FCS	fetal calf serum
HDM	house dust mite
HDM antigen-4B	Sepharose CL-4B coupled with HDM antigen
HMBA	high molecular weight basic allergen
Ig	immunoglobulin
IgA, IgE, IgG, IgM	immunoglobulins A,E,G,M
LPS	lipopolysaccharide
Mab	monoclonal antibody
Mab-Affi-gel	Affi-gel 10 coupled with Mab

P1	the major allergen in house dust mite (D. pteronyssinus)
PBS	phosphate buffered saline
PC	L-α-phosphatidyl choline
RAST inhibition	radioallergosorbent inhibition test
RIA	radioimmunoassay
Rye I	rye grass pollen allergen I
SpA	Staphylococcus protein A
SpA-4B	Sepharose 4B-CL coupled with SpA
TT	tetanus toxoid
UASP	HDM antigen-4B unabsorbed allergic serum pool
UNSP	HDM antigen-4B unabsorbed nonallergic serum pool

Abstract

Sera from 12 nonatopic adults, or from 12 HDM allergic patients absorbed with HDM-4B, or from 12 cord blood were pooled, respectively, and then precipitated twice with 50% ammonium sulfate and fractionated by AcA 34 gel filtration. Two peaks were collected from each pool. Peak I from adult sera could inhibit the binding of radiolabeled antigen P1 to IgE or IgG antibodies from another serum pool of 10 HDM allergic patients, and the inhibition ranged from 20-60%. None of the Peaks II from adult serum pools, nor the Peak I or Peak II from cord sera, had significant inhibitory effect. Peak I from nonatopic adult serum pools could also inhibit the binding of ^{125}I-P1 to anti-P1 Mab. However, Peak I from adult sera did not inhibit the binding of Rye I on anti-Rye I antibodies, indicating the inhibition had certain specificity. Nevertheless, the inhibitory factor of adult Peak I could not be absorbed by immunosorbents such as anti-IgA-4B, anti-IgM-4B, SpA-4B, HDM Ag-4B, or anti-P1 Mab 191-Affi-gel. This suggested that the inhibitory factor in adult serum Peak I was not an immunoglobulin, nor did it have idiotype specificity on anti-P1 antibody. Therefore, it was not anti-idiotype antibody but was some factor(s) with restricted specificity.

Introduction

As a supportive evidence for Jerne's immune network theory (1974), auto-anti-idiotype antibodies have been demonstrated in quite a few instances either in animal or human models (Rodkey et al 1974; Sakto et al 1975; Cosenza et al 1976; Cowdery 1981; Eisenberg et al 1985; Cunningham-Rundles et al 1982; Abdou et al 1981; Zouali et al 1983; Dwyer et al 1984; Bose et al 1984). Various methods have been employed to detect anti-idiotype antibodies. One of the commonly used approach is to examine the inhibitory effect of the test sample on the binding of specific antibodies to antigen. An inhibition in the assay will suggest the existence of anti-idiotype antibodies in the sample provided adequate controls are included, and nonspecific inhibition is excluded. The anti-Id thus detected should carry an internal image of the epitope of the antigen, or bind to the vicinity of the combining site of the idiotype positive antibody, because inhibition means that possibly there is anti-Id competing with antigen for the idiotype positive antibody. Some authors use straight sera as test samples to start the study of the existence of anti-Id. For example, Zouali et al (1982) observed that some normal adult sera, which had no detectable anti-DNA antibodies nor DNA, blocked the binding activity of affinity purified anti-DNA antibodies to DNA; and that these sera had no blocking effect on the binding of tetanus toxoid to anti-tetanus toxoid antibody. Further study showed that the inhibitory effect was caused by Fab fragments of IgG. It was

therefore concluded that some normal individuals have auto-anti-idiotypic antibodies against anti-DNA antibodies. In a similar but less well controlled case, Koprowski et al (1984) investigated auto-anti-idiotypic antibody in patients suffering from gastrointestinal adenocarcinoma and treated by injection of monoclonal antibodies to colorectal carcinoma associated antigen (CCAA). He found that the patients' sera could inhibit the binding of rabbit anti-Id antibodies to anti-CCAA monoclonal antibodies. After further characterization of the inhibitory factor, Koprowski concluded to the existence of auto-anti-idiotypic antibodies in the patients' sera against anti-CCAA antibodies.

When we studied the crossreactivity between human and mouse monoclonal anti-Pl antibodies in Chapter II, we noticed that the straight sera from nonatopic individuals who did not have anti-Pl antibodies could block to a certain extent (up to 50%) the binding of anti-Pl Mab to Pl. Bearing in mind Jerne's network theory that the interaction between idiotype and anti-idiotype constantly exists even without apparent immune response to a certain antigen and that there can be anti-idiotypic antibodies carrying internal images of foreign antigen, and being aware of the approaches employed by Zouali and Koprowski, it was logical to speculate that the inhibition of the binding of Pl to Mab caused by human sera might be due to naturally occurring auto-anti-idiotype antibodies, which bore Pl like internal images.

In the present chapter, the nature of the inhibition by human sera of the binding of house dust mite (HDM) antigen to its specific antibody is investigated. It is concluded that the inhibitory effect is not due to anti-Id antibody or anti-Id carrying molecules, but due to a factor or factors with restricted specificity in a sense that it only inhibits the binding of anti-HDM Ag-4B to HDM Ag, and that it only exists in adult but not in neonatal sera.

Materials and Methods

Purification of crude house dust mite antigen

Crude HDM antigen batch #1 (Beecham Pharmaceutical Co., Great Burgh, UK) was partially purified by gel filtration chromatography. A 2 x 105 cm Sephadex G-100 column was equilibrated with borate-buffered saline (BBS, 0.17M borate, 0.12M NaCl), pH 8.0; 120 mg of crude HDM antigen extract was dissolved in 4 ml of BBS, centrifuged at 27,000 g for 30 min at 4°C to remove undissolved material, and then applied to the column. The sample was eluted with BBS at a flow rate of 14 ml per hr. The optical density was monitored at 280 nm and the eluate was collected in 5 ml fractions.

Coupling of HDM antigen to paper discs (Ceska 1972)

Five grams of paper discs of 0.6 cm diameter (about 500 discs per gram) were prepared from Whatman #541 hardened ashless filter paper

(Whatman Ltd., UK), and washed 3 times with distilled water to remove detached fibres. Discs were suspended in 125 ml distilled water with vigorous stirring. Five grams of CNBr (Fisher, Winnipeg, MB) in 10 ml of acetonitrile (Fisher, Winniepg, MB) were added, the pH was maintained at 10-11 for 15 min by adding 1M NaOH dropwise, and the temperature was maintained at about 20°C by occasionally adding pieces of ice. The discs were then washed with cold 0.05M carbonate buffer, pH 10 (CB) 5 times, and resuspended in 100 ml of CB; 25 mg of crude HDM antigen (batch #1) in 25 ml of CB was then added to the disc suspension. The suspension was stirred vigorously for 30 min at room temperature, and placed in the cold room with gentle shaking overnight. Discs were then washed thoroughly with CB, and the remaining active sites were blocked by overnight inhibition in 150 ml of 0.2% gelatin in CB. The discs were subsequently washed with distilled water, 4M guanadine-HCL, PBS and assay buffer (PBS with 0.1% BSA, 0.5% Tween 20), and finally resuspended in assay buffer.

Radioallergosorbent inhibition test (RAST inhibition) (Ceska 1972)

This test was used to determine the antigenic activity of HDM antigen after fractionation by Sephadex G-100 chromatography. HDM antigen coupled discs were placed in flat bottom 96-well tissue culture plates (Becton-Dickinson, Oxnard, CA); 20 µl of each fraction was added to each well as inhibitors; and 50 µl of 1:10 diluted pooled serum from HDM allergic patients were subsequently added to each well. After overnight incubation at room temperature, discs were

washed 10 times with distilled water, and 100 µl (50,000 cpm) of ^{125}I-labeled mouse anti-human (MAH) IgE (clones CIA/E/7.2 from Dr. A. Saxon, UCLA, Los Angeles, CA) were added to each well. After another overnight incubation at room temperature, discs were washed again 10 times with distilled water, suction dried, transferred to plastic tubes and counted in a gamma counter. Samples were in duplicate. The background was the mean cpm of 4 discs incubated with pooled sera from nonatopic donors diluted 1:10. The percentage inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{Mean cpm of wells with inhibitor - background}}{\text{Mean cpm of wells without inhibitor - background}} \times 100\%$$

Origin of the human sera

Serum samples from HDM allergic or rye grass pollen allergic patients were obtained from Dr. R. Pauwels (Gent, Belgium). The patients were selected on the basis of their RAST positivity (3 to 4 +) to Dermatophagoides pteronyssius or rye grass pollen. Twelve sera from HDM allergic patients were pooled, and subsequently fractionated by 50% saturated ammonium sulphate precipitation and gel filtration. Another 10 sera with high titre of anti-HDM IgE and IgG antibodies determined by RIA #2 and SpA assay were pooled and used for inhibition studies. Nonatopic donors have no history nor symptoms of allergy and are negative in allergen specific IgE and IgG antibodies; 12 such sera were pooled and fractionated; and another 10 such sera were pooled and used as background controls for the radioimmunoassays.

Cord sera, free of IgG anti-HDM were also employed; 12 such cord sera were pooled and fractionated.

Inhibition of the Binding of HDM Antigen to Specific IgE Antibodies

A radioimmunoassay (RIA #1) was employed to test the inhibitory effect of sera, fractionated components of sera or control reagents, on the binding of human HDM specific or Rye I specific IgE antibodies to their corresponding antigens. Mouse anti-human IgE (Mab #89 in ascitic fluid, prepared in this laboratory) was diluted 400 times in 0.01M carbonate buffer, pH 9.0, and was used to coat wells of microtitre plates (Dynatech, Alexandria, VA) at a volume of 250 µl per well, overnight at room temperature in a humid chamber. The solutions were then decanted and plates were blocked with 300 µl per well of Hank's solution containing 10% FCS (Gibco, Burlington, ON) for 2 hrs at room temperature. Plates were washed 10 times with PBS and air dried. Pooled sera from HDM allergic individuals, from rye grass pollen allergic individuals, or from nonatopic donors (who were negative in serum anti-HDM or anti-Rye I IgE and IgG), were diluted 1:10 in PBS and added to the wells at a volume of 250 µl per well. After overnight incubation, plates were washed 10 times with PBS; 200 µl, 150,000 cpm of radioiodinated antigen (HDM antigen purified by G-100 gel filtration, P1, or Rye I) and 50 µl of various inhibitors such as sera, fractionated sera, human myeloma IgG, proteins or different control reagents such as Rye I from Dr. D. Marsh (Johns Hopkins University, Baltimore, MD), lipopolysaccharide (LPS) of E.

coli (Fisher, Winnipeg, MB), L-α-phosphatidylcholine (PC, Sigma, St. Louis, MO), tetanus toxoid (TT, Connaught Lab., Willowdale, ON), crude extract of house dust mite D. pteronyssinus, batch #1 (Beecham Pharmaceutical Co., Great Burgh, UK), high molecular weight basic allergen (HMBA) from Kentucky blue grass (prepared by Dr. A.K.M. Ekramoddoullah, University of Manitoba), or human IgE myeloma protein (PS), were added to the wells. After overnight incubation, plates were washed 10 times with PBS. Wells were then cut and counted in the gamma counter. Samples were in duplicate or triplicate. Background was the mean cpm of 4 Mab anti-IgE coated wells incubated with 1:10 diluted serum pool from nonatopic donors. The percentage inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{Mean cpm of wells with inhibitor - background}}{\text{Mean cpm of wells without inhibitor - background}} \times 100\%$$

Radioimmunoassay for the detection of antigen specific IgE in the sera or serum fractions (referred hereafter as RIA #2)

All the steps were the same as described in RIA #1 except for the last incubation, where no inhibitors but 250 µl of labeled antigen (about 150,000 cpm) in assay buffer was added to each well.

Inhibition of the Binding of P1 to Monoclonal Anti-P1 Antibodies

A radioimmunoassay (RIA #3) was used to test the inhibitory effect of different fractions of sera on the binding of P1 to monoclonal anti-P1 antibodies. Microtitre plates were coated with

purified anti-P1 Mab at the concentration of 12.5 µg/ml in carbonate buffer. After overnight incubation in a humid chamber, the plates were blocked with 150 µl of Hank's containing 10% FCS, and they were then washed 10 times with PBS. Twenty µl of different fractions of human sera or human myeloma IgG as inhibitors along with 80 µl (about 200,000 cpm) of radioiodinated P1 in assay buffer containing 10% FCS were added to each well. After another overnight incubation, the wells were washed 10 times with PBS, cut individually and counted. Samples were in duplicate and the background was the mean cpm of 4 wells coated with anti-Rye I Mab #18. The percentage inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{Mean cpm of wells with inhibitor - background}}{\text{Mean cpm of wells without inhibitor - background}} \times 100\%$$

Protein A assay (SpA assay)

This assay was employed to test the inhibitory effect of fractionated human sera on the binding of HDM antigen or Rye I by their respective specific IgG antibodies. It was also used to determine the level of IgG anti-HDM antibodies in the sera or in the fractionated serum components. Twenty µl of a suspension of protein A-Sepharose CL 4B bead (SpA 4B, Pharmacia, Uppsala, Sweden) in PBS (1 volume of packed beads: 1 volume of PBS) were mixed in 1.5 ml Eppendorf centrifuge tubes (Brinkmann Instruments, Rexdale, ON) with 25 µl of the following samples depending on the assay: pooled human sera diluted 1:10 from 10 HDM allergic patients with high IgG anti-HDM

titre; pooled sera diluted 1:10 from 10 rye grass pollen allergic patients with high IgG titre anti-Rye I; pooled sera diluted 1:10 from 10 nonatopic donors without detectable allergen specific IgG. The tubes were shaken overnight at room temperature, and 25 µl of human IgG myeloma protein at 6 mg/ml was added to each tube to saturate the binding site of protein A. After 3 hrs shaking at room temperature, the beads were washed with 1 ml assay buffer; 25 µl of various inhibitors were added to each tube along with 25 µl (about 40,000 cpm) of the corresponding radiolabeled allergen. When the assay was used to determine the allergen specific IgG level, no inhibitor but only radiolabeled allergen was added. The tubes were again incubated overnight at room temperature with constant shaking. Beads were subsequently washed 3 times with assay buffer, and tubes were counted in a gamma counter. Samples were in duplicate or triplicate. Background was the mean cpm of 4 tubes incubated with sera from nonatopic donors diluted 1:10. The percentage inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{Mean cpm of samples with inhibitor - background}}{\text{Mean cpm of samples without inhibitor - background}} \times 100\%$$

Radioiodination of proteins

HDM antigen purified by gel filtration, Pl, Rye I, or mouse anti-human IgE Mab was radioiodinated as described in Chapter II.

Preparation of HDM antigen immunosorbent (Axen 1967)

Sepharose 4B-CL was washed in a 15 ml sintered funnel with 10 volumes of distilled water and then washed with 3 volumes of 2M Na_2C0_3. The beads were resuspended in a beaker to 50% volume/volume with 2M $NaC0_3$ and stirred constantly. In the fume hood, 1/10 volume of 1 g/ml CNBr in N,N-dimethyl foramide was added slowly to the slurry, and the stirring was continued for 105 sec. The beads were washed immediately, first with 5 volumes of cold distilled water, and then with 3 volumes of cold coupling buffer (1M NaCl with 0.1M Na_2C0_3, pH 8.5). The activated beads were dried to a soft cake. The partially purified HDM antigen was dissolved in coupling buffer at 1 mg/ml. The activated beads were mixed with an equal volume of antigen in coupling solution. The mixture was rotated at room temperature for 1 hr and then at 4°C overnight. Two volumes of 1M monoethanolamine was added to the mixture, and the tube was rotated again for 4 hr at room temperature to block the remaining active sites of the beads. Finally, the beads were washed thoroughly with 0.1M glycine-HCl, pH 3.0, then with PBS in the sintered funnel and stored in PBS at 4°C. The coupling efficiency was 71%, and therefore 0.7 mg of partially purified HDM antigen was coupled to 1 ml of packed Sepharose 4B CL beads. This immunosorbent is designated as HDM antigen-4B.

Preparation of antibodies coupled Sepharose CL-4B beads (Johnstone et al 1982)

Sepharose CL-4B was washed with 10 volumes of double distilled water (DDW) in the sintered funnel, suspended in equal volume of DDW and stirred vigorously in the fume hood; 60 µl of 50% (weight/volume) CNBr (Fisher, Winnipeg, MB) in acetonitrile per 1 ml of packed gel was added to the gel slurry. The slurry was stirred constantly, and the pH was maintained between 10-11 for 20 min by adding 2M NaOH dropwise. After the activation, the gel was washed immediately with cold 0.05M carbonate buffer, pH 10 (CB), and dried to a soft cake. Ten mg of rabbit anti-human-IgA (Dako, Copenhagen, Denmark) or 11 mg of rabbit anti-human IgM (Dako, Copenhagen, Denmark) in 2 ml of CB was mixed with 1 ml of packed activated gels, and the mixtures were stirred overnight at 4°C. The gels were suction dried, resuspended in 2 ml of CB containing 10% BSA, and stirred for 3 hr at room temperature. The gels were then washed successively with water, 4M guanadine-HCl and PBS. To determine the coupling efficiency, the O.D. and the volume of the antibody solutions before and after coupling were measured. The coupling efficiency of anti-IgA was 93.6%, and that of anti-IgM 97%, therefore, 9.36 mg of anti-IgA or 10.7 mg of anti-IgM was coupled to 1 ml of packed gel. Twenty mg of the gamma globulin fraction from an allergic patient serum containing high titre of IgG anti-Pl antibody was coupled to 2 ml of Sepharose CL-4B beads by using the same procedure.

Affinity chromatography

The HDM antigen specific antibodies in the pooled sera from 12 HDM allergic patients were absorbed on HDM-antigen coupled Sepharose CL-4B (HDM antigen-4B). Twenty ml of pooled sera were mixed with 2 ml (packed volume) of HDM antigen-4B in a tube and the tube was rotated overnight at 4°C. The immunosorbent was recovered by filtering the mixture through a sintered funnel, washed with PBS followed by 0.1M glycine-HCl, pH 3.0 and equilibrated with PBS. The regenerated HDM antigen-4B was added back to the filtrate and the whole cycle was repeated 3 times in total. The final filtrate was subsequently fractionated. For control, 20 ml of pooled sera from 12 nonatopic donors were absorbed with HDM-antigen-4B in parallel with the pool of atopic sera.

Peak I of different serum pools (refer to p.165 and Fig. 3) were absorbed with anti-human IgA coupled-Sepharose 4B (anti-IgA-4B), anti-human IgM coupled Sepharose 4B (anti-IgM-4B), protein A-Sepharose 4B (SpA-4B) and BSA coupled Sepharose 4B (BSA-4B). One volume (0.3 ml) of Peak I was mixed with 1 volume of packed immunosorbent gel in a test tube and the mixture was rotated overnight at 4°C. The gel and the filtrate were separated by a minicolumn made of a Pasteur pipette and the filtrates were collected. The gels were washed with 20 volumes of PBS, and eluted with 1 volume of 0.1M, glycine-HCl, pH 3.0. The eluates were neutralized immediately with 20 µl of 1M Tris-HCl, pH 7.6.

Fractionation of serum pools

Twenty ml of pooled serum from HDM allergic or nonallergic donors, or from cord sera, were diluted with an equal volume of PBS. Forty ml of saturated ammonium sulphate, pH 7.2, was added dropwise to the sample while the sample was being stirred over a period of 4 hrs at room temperature. The mixture was centrifuged at 10,000 g at room temperature for 20 min. The pellet was dissolved in PBS to a volume of 40 ml. Another 40 ml of saturated ammonium sulphate was added dropwise to the solution over a period of 2 hrs while the solution was being stirred. The mixture was centrifuged at 10,000 g at room temperature for 30 min. The pellet was dissolved in 10 ml PBS, and dialysed against PBS in dialysis tubing with mol.wt. cutoff 12-14 kilodaltons (Spectrum Medical Industries Inc., Los Angeles, CA). The solution was then concentrated to a volume of 10 ml in Amicon ultrafilters with YM5 membrane (Amicon, Danvers, MA; mol.wt. cutoff 5,000). This protein preparation was fractionated by filtration through a 2.5 x 90 cm Ultrogel AcA 34 (LKB, Bromma, Sweden) column equilibrated with PBS. The flow rate was 20 ml per hr, and the fraction size was 60 drops (about 3 ml) per tube. The optical density of the fractions was monitored at 280 nm.

Results

Purification of HDM antigen

Crude HDM antigen, batch #1, was purified by filtration through a Sephadex G-100 column and the fractions were tested in the RAST inhibition assay. Fractions 43-70 showed strong inhibition (60-95%); there was no correlation with the protein concentration of the fractions (Fig. 1) and their inhibiting activity. These fractions were pooled, and radiolabeled.

Specificity of RIA #1 and SpA assay in Pl or HDM antigen system

In the RIA #1, the binding of ^{125}I-Pl to specific IgE could be inhibited (89%) by 1 µg/ml of HDM antigen, but not by unrelated antigen such as HMBA or TT used at similar concentration (Table 1). The binding of ^{125}I-HDM antigen to specific IgE could be inhibited (90.8%) by 2 µg/ml of HDM antigen, but not by TT, Rye I, HMBA, LPS etc. at the same concentration (Table 2). It was also observed that a large excess of human IgE PS had no inhibitory effect on RIA #1. In the SpA assay the binding of ^{125}I-HDM antigen by specific IgG antibody could be inhibited (93.3%) by 3.3 µg/ml of HDM antigen, but not by Rye I or human myeloma IgG (2 mg/ml) (Table 2). Therefore, the RIA #1 and the SpA assay were considered to be specific.

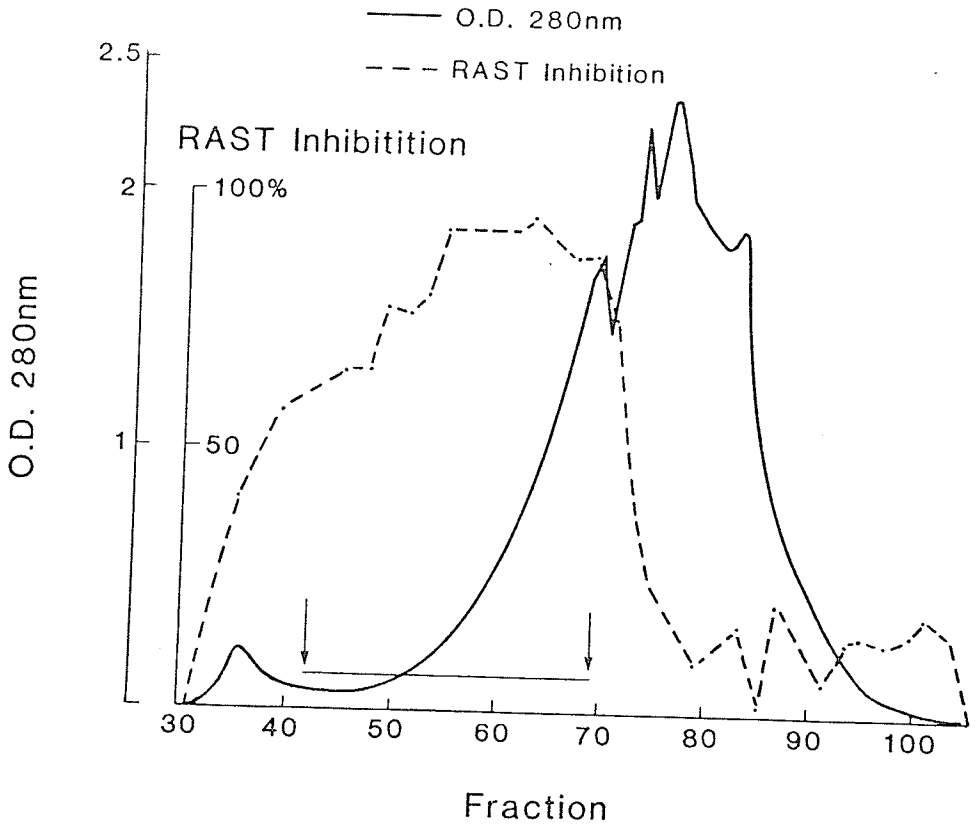


Figure 1: Fractionation of HDM crude extract by gel filtration on Sephadex G-100.

The antigenic activity of the fractions was tested by RAST inhibition. Maximum cpm bound in the absence of inhibitor was 2,100 cpm, the background was 370 cpm.

Fractions between arrows were pooled.

Table 1. Specificity of the RIA #1 for the Detection of IgE Anti-P1 Antibodies

Inhibitor	(final concentration)	cpm Bound	% Inhibition
HMBA	2 µg/ml	25,538	0
TT	5 U/ml	25,375	0
IgE PS	1 µg/ml	23,049	6.5
HDM Ag (Batch #1)	1 µg/ml	2,734	89
	0.25 µg/ml	6,452	72.8
	0.06 µg/ml	26,097	0
PBS		24,662	0
Background		891	

The data are the mean values of duplicate determinations.

Table 2a. Specificity of the RIA #1 for the Detection of IgE Anti-HDM Antibodies

Inhibitor	Final Concentration	Inhibition (%)	cpm ± SD
PBS		0	3,275 ± 542
Human Myeloma IgG	1.2 mg/ml	0	
Crude HDM Antigen	2 µg/ml	90.8	
Rye I	2 µg/ml	0	
PS IgE	400 ng/ml	5.5	
LPS	20 µg/ml	0	
HMBA	2 µg/ml	2	
TT	5 U/ml	2	
Background			309

Table 2b. Specificity of the RIA for the Detection of IgG Anti-HDM Antibodies (SpA Assay)

Inhibitor	Final Concentration	Inhibition (%)	cpm ± SD
PBS			1,376 ± 82
Human Myeloma IgG	2 mg/ml	0	
Crude HDM Antigen	3.3 µg/ml	93.3	
Rye I	3.3 µg/ml		
Background			874

The data are the mean values of duplicate determinations.

Sensitivity of RIA #1 and SpA assay using ^{125}I-HDM antigen

A series of dilutions of crude HDM antigen (batch #1) were used as inhibitors to test the sensitivity of RIA #1 and SpA assay using ^{125}I-HDM as an antigen. The results are shown in Figure 2. The inhibition was dose-dependent; 200 ng/ml of crude HDM antigen gave 90.8% and 93% inhibition in the RIA #1 and the SpA assay, respectively. A 50% inhibition in RIA #1 was caused by 62 ng/ml of crude HDM antigen, and in SpA assay by 64 ng/ml.

Inhibition by human sera of the binding of P1 or Rye I to their corresponding specific IgE antibodies

Nine sera from adult nonatopic donors and 10 cord sera were tested for their inhibitory effect on the binding of P1 to P1 specific IgE as indicated in Materials and Methods (RIA #1). The results are shown in Table 3. The sera from adult nonatopic donors caused 3.4 to 46% inhibition with mean ± 1SD of 23.9 ± 13.2%, while the cord sera caused only 0-14.8% inhibition with mean ± 1SD of 4.0 ± 6.2%. Variance analysis of these results, after angular transformation of the data showed that the difference of inhibition between adult and cord sera was highly significant ($P < 0.01$).

Four sera from nonatopic donors and 3 cord sera were tested for their inhibitory effect on the binding of Rye I to IgE anti-Rye I antibodies. The results, in Table 4 indicate the absence of significant inhibition. Comparing the inhibitory effect of the 4 sera

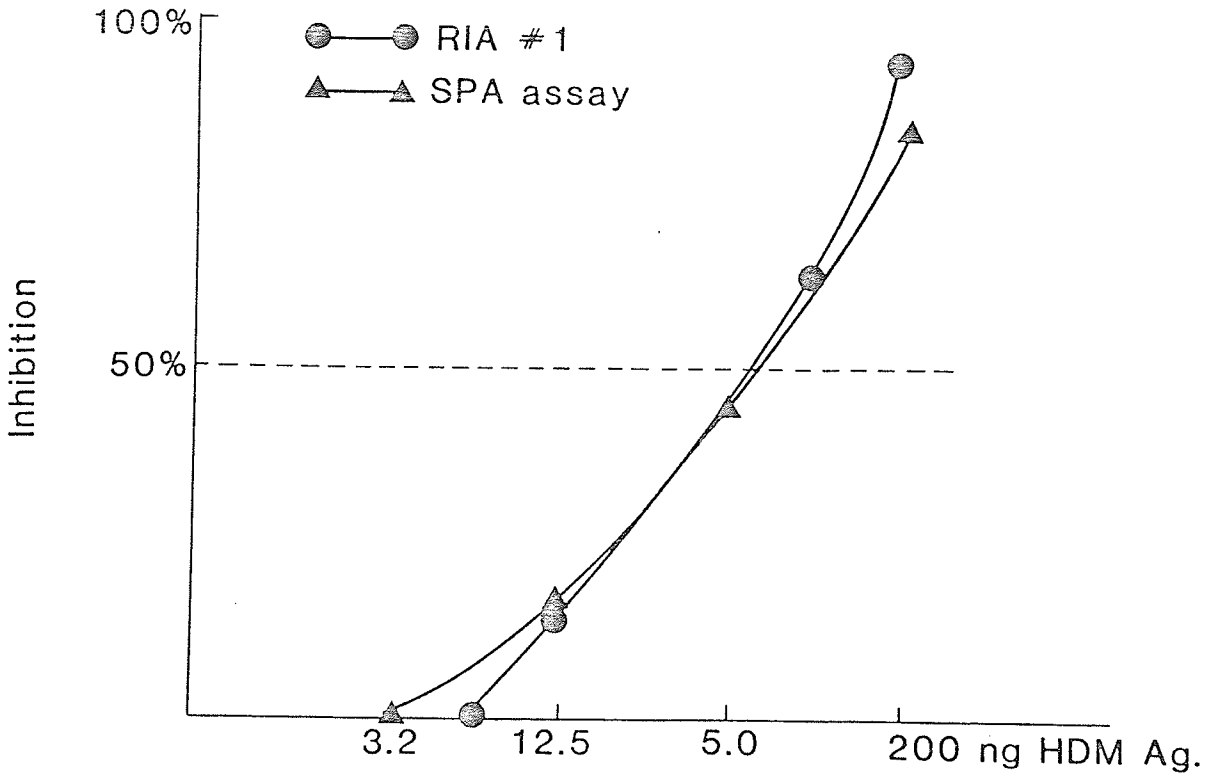


Figure 2: Sensitivity of radioimmunoassay for the detection of the inhibitory effect of human serum fractions on the binding of IgE or IgG to ^{125}I-HDM.

For RIA #1, the maximum binding (3,594 cpm) was determined by using 0.12 mg of human myeloma IgG as an inhibitor, and the background was 309 cpm; for the SpA assay, the maximum binding (2,419 cpm) was determined by using 0.12 mg of human myeloma IgG as an inhibitor, and the background was 874 cpm.

Table 3. Inhibition by Human Sera of the Binding of Pl Specific IgE Antibodies

	Serum	cpm Bound	% Inhibition
	R48	11,218	3.4
	R63	11,145	3.4
	R60	6,826	46.0
	M35	9,366	26.7
Nonatopic	N1	9,342	26.8
	N2	9,507	25.6
	N3	8,646	28.3
	N4	9,159	25.7
	N6	8,989	29.6
	C52	11,738	8.2
	C53	12,528	1.9
	C54	10,910	14.6
	C57	11,020	14.8
Cord	C59	13,096	0
	C60	13,571	0
	C61	13,786	0
	C62	13,334	0
	C64	12,256	0
	C68	13,049	0
Assay buffer		17,358	
FCS		12,761	0
Background		502	

Samples were tested in duplicate.

Table 4. Influence of Human Sera on the Binding of Rye I Specific IgE Antibodies

	Serum[a]	cpm Bound	% Inhibition
Nonatopic	N6	4,988	0
	N1	4,045	8.6
	N2	4,120	6.9
	N3	4,976	0
Cord	C52	3,971	10.3
	C53	4,520	0
	C54	4,792	0
Assay buffer		4,308	3
FCS		4,427	0
Rye I 200 ng[b]		1,018	77
Background		165	

[a]Twenty µl of serum was added to a well.

[b]Amount of Rye I added to a well.

from the nonatopic adults on the binding of Pl-IgE anti-Pl versus RIA #1-IgE anti-Rye I by paired student t test (after square root transformations of the percentages), the difference was statistically significant ($P < 0.05$). These results suggested the presence in adult but not in cord sera of an inhibitory factor(s) specifically inhibiting the binding of IgE anti-Pl to Pl.

Pl specific antibodies in Peak I and Peak II

Pooled human sera from allergic and nonallergic adults or from cord blood were precipitated by 50% saturated ammonium sulphate and then fractionated on a AcA 34 column chromatography as described in Materials and Methods. In some experiments, the pooled sera were first adsorbed on HDM antigen-4B prior to being fractionated. All the profiles were very similar; Figure 3 shows a typical experiment. Two distinct peaks were obtained. Fractions 41 to 51 were pooled and designated as Peak I, and fractions 85 to 105 were pooled and designated as Peak II. Each peak was concentrated by ultrafiltration; the protein concentration was measured according to Lowry (1951), and was adjusted to 6 mg/ml. The presence of HDM IgE and IgG antibodies in Peak I and Peak II was tested with RIA #2 and SpA assay. The results are given in Table 5. It is obvious that neither Peak I nor Peak II, prepared from serum pool of atopic or nonatopic donors, after adsorption with HDM antigen-4B contained IgE or IgG anti-HDM antibody. The Peaks I and II from atopic serum pool without absorption on HDM antigen-4B contained IgE and IgG anti-Pl antibodies with the majority of the antibodies being detected in Peak II.

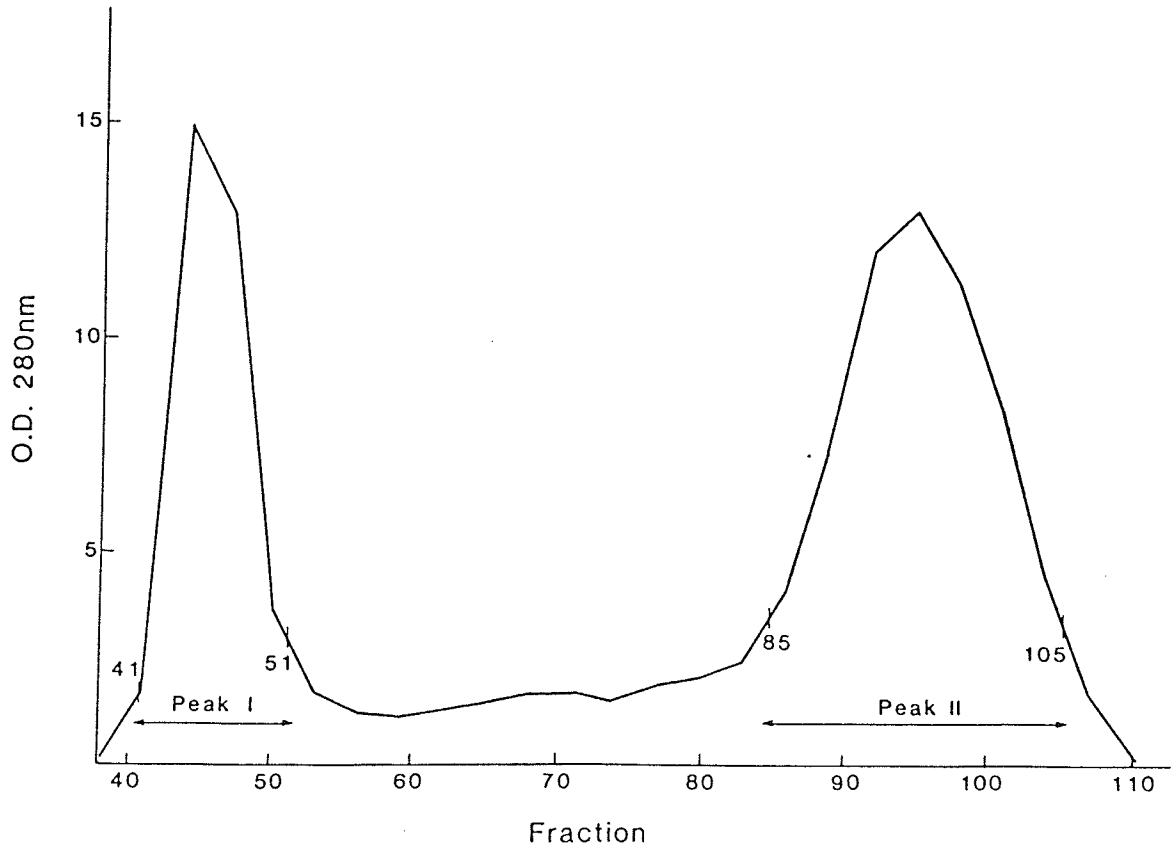


Figure 3: Fractionation of nonatopic adult serum pool by gel filtration on an AcA 34 column.

Table 5. IgE and IgG Anti-HDM Antibodies in Peak I and Peak II from Different Serum Pools

FRACTION TESTED	ORIGIN OF SERUM POOL	IgE ANTIBODIES[a]	IgG ANTIBODIES[a]
Serum pool[b]	Normal donors	280	771
	HDM allergic patients	4,086	3,280
Peak I[c]	Normal donors	236	923
	HDM allergic patients	1,178	348
Peak I from pool absorbed on HDM-Sepharose-4B	Normal donors	97	472
	HDM allergic patients	105	504
Peak II	Normal donors	236	929
	HDM allergic patients	3,756	4,415
Peak II from pool absorbed on Sepharose-4B	Normal donors	36	522
	HDM allergic patients	46	574

[a]Results are expressed in cpm of ^{125}I-HDM Ag bound to IgE or IgG antibodies; mean values of triplicate determinations.

[b]Serum pools were tested at the dilution of 1/20.

[c]Peaks I and II contained 6 mg protein/ml.

Inhibitory effect of Peak I and Peak II on the binding of human HDM specific antibody to ^{125}I-HDM antigen or ^{125}I-Pl

The inhibitory effect of Peak I and Peak II was first tested on the binding of IgE-anti-HDM antibodies to ^{125}I-HDM antigen. Peak I from HDM antigen-4B absorbed allergic serum pool (AASP) and HDM antigen-4B absorbed nonallergic serum pool (ANSP) could inhibit the binding of human IgE antibodies to ^{125}I-HDM antigen by 60% and 54.2%, respectively. The inhibition was dose-dependent (Fig.4). As a control, human myeloma IgG with or without similar absorption with HDM antigen-4B had no inhibitory effect even when used at a concentration of 1.2 mg/ml. Peak II from either AASP or ANSP did not inhibit the binding of HDM antigen specific IgE to ^{125}I-HDM antigen. Similar results were obtained with Peaks I and II from HDM antigen-4B unabsorbed nonallergic serum pool (UNSP). Indeed, as shown in Table 6, Peak I inhibited in a dose-dependent manner the binding of IgE antibodies to ^{125}I-HDM antigen, while Peak II or human myeloma IgG employed at the same concentrations had no inhibitory effect. Identical results were obtained if purified Pl antigen was used instead of HDM antigen (Fig.5). Peak I (at 0.6 mg/ml) inhibited 40% of the binding of specific IgE to ^{125}I-Pl, while Peak II at the same concentration gave only 19% inhibition (Fig.5). This difference was statistically significant as assessed by paired student t test ($P<0.05$).

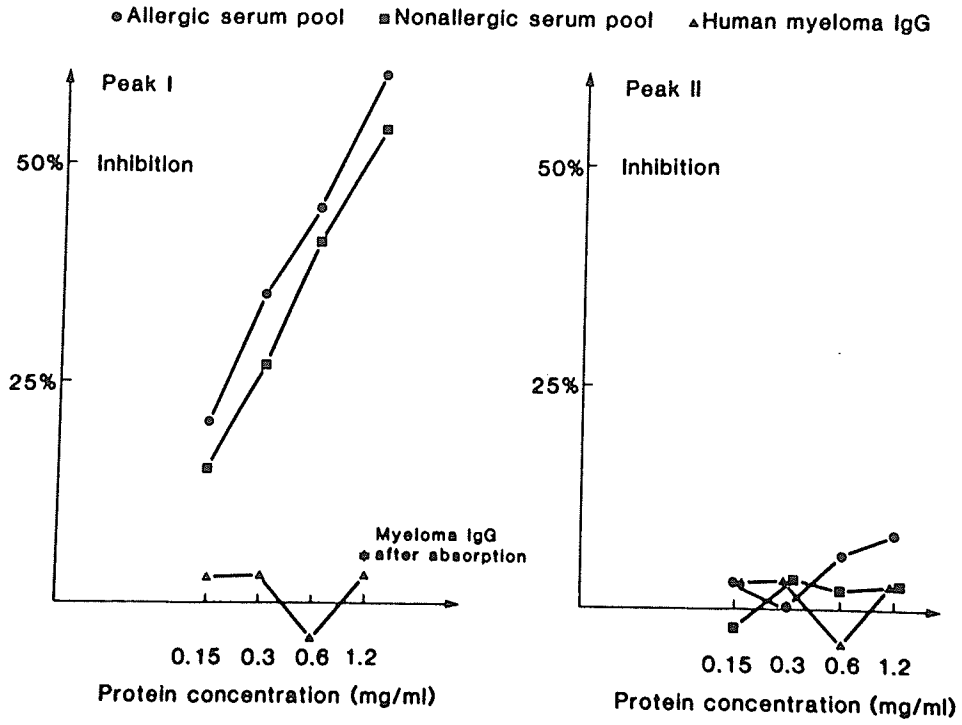


Figure 4: Inhibition of the binding activity of IgE anti-HDM antibodies by Peak I and Peak II of the serum pool of nonallergic or allergic donors.

Serum pools were absorbed on HDM Ag-4B before fractionating into Peak I and II.

The samples were tested in triplicate.

The protein concentrations correspond to final concentrations.

The maximum binding was 4,227 cpm; the background was 238 cpm. Similar results were observed three times.

Table 6. Influence of Peak I and Peak II from the Serum[a] of Nonatopic Donors on the Binding of IgE Antibodies to ^{125}I HDM

Inhibitor		cpm Bound	%Inhibition
Peak I	1.2 mg/ml[b]	2,559[c]	25.8
	0.6 mg/ml	3,306	19.2
	0.3 mg/ml	3,372	15.5
	0.15 mg/ml	3,660	8.3
Peak II	1.2 mg/ml	3,796	4.8
	0.6 mg/ml	3,871	3.0
	0.3 mg/ml	3,921	1.8
	0.15 mg/ml	4,156	0
Human IgG Myeloma	1.2 mg/ml	3,863	3.2
	0.6 mg/ml	4,170	0
	0.3 mg/ml	3,876	2.8
	0.15 mg/ml	3,922	1.7
PBS		3,989	0
Background		238	

[a]The serum pool was not absorbed on HDM-Ag-4B.

[b]Final concentration.

[c]Mean of triplicate determinations.

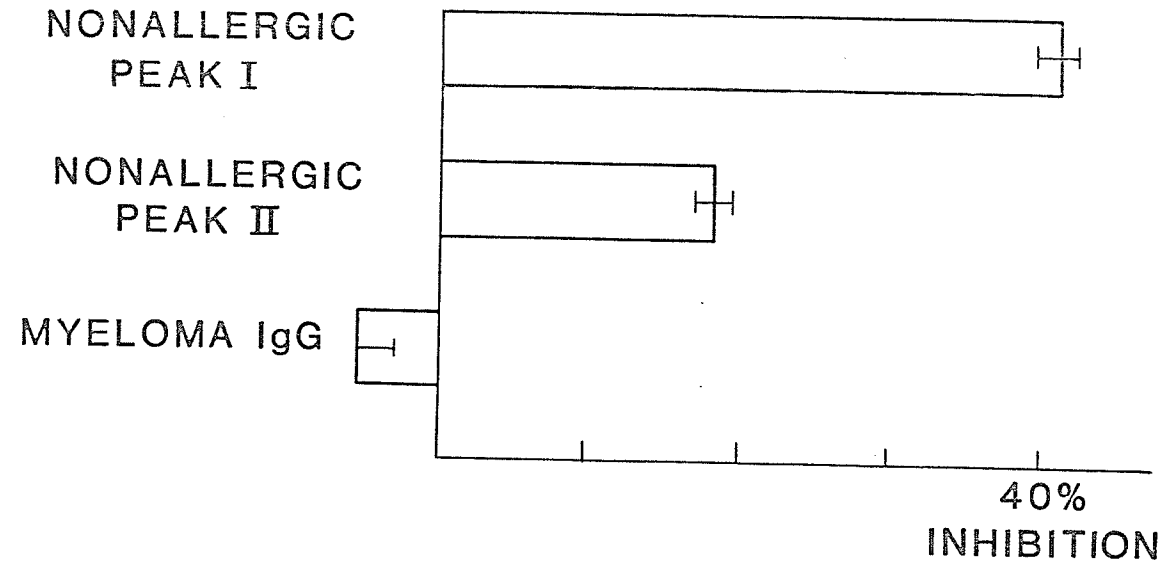


Figure 5: Inhibition of the binding activity of IgE anti-HDM antibodies by Peak I and Peak II of the serum pool of nonallergic donors.

In this experiment, the serum pool had not been absorbed with HDM-Sepharose-4B, and ^{125}I-Pl was employed.

The samples were tested in triplicate; the final concentrations of inhibitors are indicated.

The maximum binding was 7,305 cpm; the background was 1,443 cpm.

The experiments summarized in Table 7 clearly indicated that Peak I from AASP and ANSP could inhibit the binding of specific IgG to ^{125}I-HDM antigen by 51.7% and 56.3%, respectively, and that the inhibition was dose-dependent. By contrast, Peak II from either AASP or ANSP, as well as myeloma IgG (absorbed with HDM antigen-4B) had no inhibitory effect. When ^{125}I-Pl was employed instead of the crude HDM antigen, the inhibitory pattern of Peak I and II (Table 8) was similar. Peaks I and II from cord serum pool were also tested in this assay, but none of the peaks had inhibitory effect.

These results suggested that the serum of both allergic and nonallergic individuals contained factor(s) capable of inhibiting the binding of Pl to IgE and IgG anti-Pl antibodies. These factors were eluted in the void volume of an AcA 34 column, and they were not detected in cord blood sera.

Peak I and Peak II inhibit the binding of Mab to ^{125}I-Pl

Peak I and II from UNSP or cord serum pool were tested for their inhibitory effect on the binding of labeled Pl to anti-Pl Mab as described in Materials and Methods (RIA #3) (Table 9). The binding of ^{125}I-Pl to 6 out of 9 clones of Mab could be inhibited by Peak I from HDM antigen-4B unabsorbed nonatopic serum pool (UNSP), with inhibitions ranging from 20 to 66%. The inhibition was dose-dependent. Peak II from UNSP, Peak I from cord sera or Peak II from cord sera gave little inhibition (0.14%), if any. It was

Table 7. Influence of Peak I and Peak II of Serum Pools[a] on the Binding of IgG Antibodies to ^{125}I-HDM

Origin of the Serum Pool	Inhibitor		cpm Bound Background	%Inhibition
HDM Allergic Donors	Peak I	1.2 mg/ml[b]	1,094[c]	51.7
		0.6 mg/ml	1,484	34.5
	Peak II	1.2 mg/ml	2,104	7.1
		0.6 mg/ml	2,352	0
Nonallergic Donors	Peak I	1.2 mg/ml	990	56.3
		0.6 mg/ml	1,401	38.1
	Peak II	1.2 mg/ml	2,214	2.2
		0.6 mg/ml	2,288	0
Human IgG Myeloma		1.2 mg/ml	2,410	0
		0.6 mg/ml	2,372	0
PBS			2,265	0
Background			853	

[a]Serum pools were absorbed with HDM Ag-4B before fractionating into Peak I and II.

[b]Final concentration.

[c]Mean of triplicate determinations.

Table 8. Inhibitory Effect of Peak I and Peak II on the Binding of Pl Specific IgG to ^{125}I-Pl

Inhibitor (1.2 mg/ml)[a]	cpm Bound-Background	% Inhibition
Peak I - from allergic serum pool absorbed with HDM antigen-4B	7,888[b]	19.0
Peak II - from allergic serum pool absorbed with HDM antigen-4B	11,495	0
Peak I - from nonatopic serum pool	7,119	26.8
Peak II - from nonatopic serum pool	10,557	0
Peak I - from cord blood serum pool	9,950	0
Peak II - from cord blood serum pool	10,180	0
PBS	9,733	0
Background	1,823	

[a]Final concentration.

[b]Mean of triplicate determinations.

Table 9. Inhibitory Effect of Peak I and II from Normal Adult Serum Pool or Cord Blood Serum Pool on the Binding of Several Mab to ^{125}I-Pl

ORIGIN OF SERUM POOLS	INHIBITORS[c]	MONOCLONAL ANTIBODY ON SOLID-PHASE								
		C4.1/96	C4.1/102	C4.1/40	A2.3/79	A2.3/191	HY3/90	C3.6/62	C3.6/104	B2.1/81
	Peak I									
Nonatopic Adults	1.5 mg/ml	31[a]	20	35	11	66	28	3	10	38
	0.38 mg/ml	21	18	12	8	22	7	1	7	14
	0.19 mg/ml	14	15	3	3	11	14	0	0	5
	Peak II									
	1.5 mg/ml	5	9	0	0	0	0	0	0	1
	0.38 mg/ml	9	2	2	2	0	19	0	3	15
	0.19 mg/ml	3	0	0	4	0	3	1	1	2
	Peak I									
Cord Blood	1.5 mg/ml	3	0	14	5	2	9	12	7	0
	0.38 mg/ml	1	3	5	5	0	0	7	6	0
	0.19 mg/ml	0	5	10	3	4	0	3	3	2
	Peak II									
	1.5 mg/ml	4	4	9	0	0	0	0	0	0
	0.38 mg/ml	2	0	5	0	0	12	0	1	9
	0.19 mg/ml	0	0	7	2	0	2	1	3	2
No Inhibitor (PBS)		2,245[b]	2,805	12,851	2,666	10,780	1,042	5,512	5,148	1,678
Human IgG Myeloma	1.5 mg/ml	2,424	3,028	12,568	2,679	10,560	1,263	5,333	5,292	1,690
Background		251	371	344	305	326	241	491	309	230

[a] Percent inhibition.

[b] Cpm of ^{125}I-Pl bound, mean of duplicate determinations.

[c] Concentrations before adding to wells.

concluded that adult sera but not cord sera contained a factor(s) which could inhibit binding of most anti-Pl Mab to ^{125}I-Pl, and that like in the human antibody system, the inhibitory factor(s) was located in Peak I.

Specificity of the inhibition by Peak I

Peaks I from AASP, ANSP, HDM Ag-4B unabsorbed allergic serum pool (UASP), or UNSP which had a inhibitory effect on the binding of HDM antigen or Pl to specific antibodies were tested in Rye I and anti-Rye I system for the specificity of the inhibition. It was shown that none of these Peaks had inhibitory effect on the binding of human IgE or IgG anti-Rye I to ^{125}I-Rye I (Tables 10a and 10b). It was therefore concluded that the inhibitory effect of Peaks from AASP, ANSP, UASP or UNSP at least had some restricted specificity.

Nonimmunogloibulin nature of the inhibitory factor(s)

Peaks I of AASP and ANSP, which contained inhibiting activity, were absorbed on anti-IgM-4B, anti-IgA-4B, anti-IgM-4B followed by anti-IgA-4B, SpA-4B. Absorptions with BSA-4B served as a control. The filtrates and eluates were tested for their inhibiting activity on IgE anti-HDM. The results in Table 11 indicated that after the removal of either IgM, IgA, IgM plus IgA, or most of IgG subclasses, Peak I could still give 38.6-49.3% inhibition which was not different from that (46-46.3%) of Peak I absorbed with BSA-4B. The eluates, which contained IgM, IgA, or most subclasses of IgG, had no inhibitory

Table 10a. Influence on Peak I on the Binding of Rye I Specific IgE to ^{125}I-Rye I

Inhibitor	Final Concentration	Inhibition (%)	cpm ± SD
PBS		0	4,232 ± 87
Human myeloma IgG	1.2 mg/ml	0	
Rye I	2 µg/ml	77.6	
Allergic HDM antigen-4B absorbed Peak I	1.2 mg/ml	0	
Nonallergic HDM antigen-4B absorbed Peak I	1.2 mg/ml	0	
Allergic nonabsorbed Peak I	1.2 mg/ml	0	
Nonallergic nonabsorbed Peak I	1.2 mg/ml	0	

Table 10b. Influence of Peak I on the Binding of Rye I Specific IgG to ^{125}I-Rye I

Inhibitor	Final Concentration	Inhibition (%)	cpm ± SD
PBS		0	10,108 ± 112
Human myeloma IgG	2 mg/ml	2.8	
Rye I	3.3 µg/ml	75.0	
Allergic HDM antigen-4B absorbed Peak I	2 mg/ml	3.3	
Nonallergic HDM antigen-4B absorbed Peak I	2 mg/ml	10.6	
Allergic nonabsorbed Peak I	2 mg/ml	0	
Nonallergic nonabsorbed Peak I	2 mg/ml	2.3	

Table 11. Influence of Absorption of Peak I with Different Immunosorbents on its Inhibiting Activity on IgE Anti-Pl Antibodies

A. Peak I from Serum Pool of Allergic Donors[a]

Inhibitor	cpm Bound ± 1SD	% Inhibition ± 1SD
Peak I	1,947 ± 21	51.7 ± 0.5
Absorbed on anti-IgM-4B	2,017 ± 133	43.7 ± 3.4
Absorbed on anti-IgA-4B	1,964 ± 64	45.2 ± 1.6
Absorbed on anti-IgM-4B and anti-IgA-4B	2,101 ± 54	41.3 ± 1.4
Absorbed on SpA-4B	1,890 ± 18	46.4 ± 3.2
Absorbed on BSA-4B	1,923 ± 128	46.3 ± 3.3
Eluate from anti-IgM-4B	3,596 ± 46	0
Eluate from anti-IgA-4B	3,498 ± 63	2.3 ± 1.6
Eluate from SpA-4B	3,086 ± 126	2.5 ± 3.2
Eluate from BSA-4B	3,727 ± 69	0

[a]The serum pool was absorbed with HDM Ag-4B.

Table 11 cont'd.....

B. Peak I from Serum Pool of Normal Donors

Inhibitor	cpm Bound ± 1SD	% Inhibition ± 1SD
Peak I	1,844 ± 41	56.3 ± 1.0
Absorbed on anti-IgM-4B	1,854 ± 50	48.2 ± 1.2
Absorbed on anti-IgA-4B	1,952 ± 90	45.5 ± 2.3
Absorbed on anti-IgM-4B and anti-IgA-4B	2,237 ± 84	38.6 ± 2.2
Absorbed on SpA-4B	1,777 ± 30	49.3 ± 0.7
Absorbed on BSA-4B	1,935 ± 29	46 ± 0.7
Eluate from anti-IgM-4B	3,624 ± 122	0
Eluate from anti-IgA-4B	3,685 ± 170	0
Eluate from SpA-4B	3,248 ± 116	0
Eluate from BSA-4B	3,666 ± 118	0

Peak I was employed at the concentration of 6 mg/ml (before absorption), and all the absorptions were carried out in same conditions, hence the dilution factor was the same.

The maximum binding was determined by using 6 mg/ml of human IgG myeloma as an inhibitor.

effect, neither had the eluates from BSA-4B. Peak I from UNSP was also absorbed with various immunosorbents and the inhibitory effect of filtrates and eluates are shown in Figure 6. The results were similar to those of Peak I from AASP and ANSP. After the removal of IgM plus IgA by absorption on anti-IgM-4B followed by anti-IgA-4B, or most subclasses of IgG by SpA-4B, Peak I from UNSP still gave 20-26% inhibition, which was in the same range (20%) caused by Peak I absorbed with BSA-4B. None of the eluates containing either IgM, IgA, or IgG gave any inhibition. These results indicated that the inhibitory factor(s), which could inhibit the binding of HDM antigen to its specific antibodies, in the Peak I of adult sera was not an immunoglobulin.

Further characterization of the inhibitory factor(s) present in Peak I of adult sera

In order to further examine the characteristics of inhibitory factor(s) in the adult sera, the Peak I from UNSP was absorbed with Sepharose CL-4B coupled with the gamma globulin fraction of serum containing high levels of anti-HDM antibodies.

It was found that after absorption Peak I still inhibited the binding of IgE anti-HDM to HDM by 35%. This inhibition was comparable to that of the nonabsorbed Peak I (41.5 ± 16%; Table 12). To exclude the possibility that the capacity of anti-HDM antibody-4B was not high enough, P1 specific Mab A2.3/191 was coupled to Affi-gel 10 (refer to Chapter IV), and was used to absorb the Peak I from UNSP.

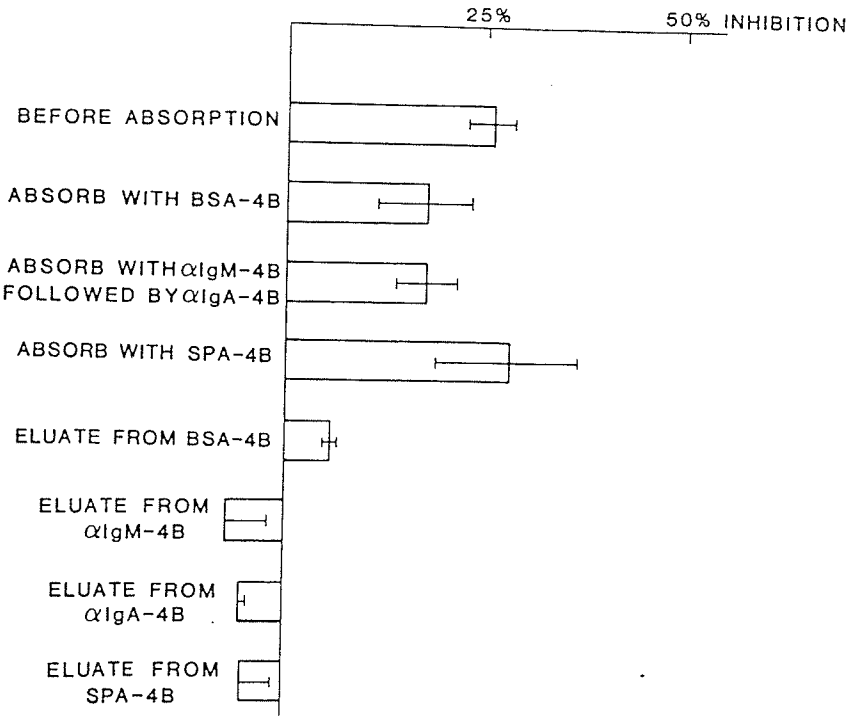


Figure 6: Influence of absorption of Peak I with different immunosorbents on its inhibiting activity on the binding of ^{125}I-HDM to IgE antibodies.

Peak I was derived from a serum pool of nonallergic donors.

The samples were tested in triplicate.

The protein concentration of Peak I before absorption was 6 mg/ml.

The maximum binding (3,900 cpm) was determined by using 6 mg/ml human myeloma IgG as an inhibitor; the background was 237 cpm.

Table 12. Influence of Absorption of Peak I with Anti-HDM Antibodies on its Inhibiting Activity on IgE anti-Pl Antibodies

Inhibitor (final concentration) 0.6 mg/ml	I-Pl Bound to IgE Antibodies	% Inhibition mean ± 1SD
Human IgG Myeloma	6,818 ± 240[a]	8.8 ± 3
Peak I	4,873 ± 123	41.5 ± 1.6
Peak I absorbed with HDM Ag-4B	4,626 ± 104	45.3 ± 1.4
Peak I absorbed with anti-HDM Ab-4B	5,261 ± 58	35 ± 0.1
Background	1,443 ± 159	
0% Inhibition (PBS as an inhibitor)	7,350 ± 228	

[a]Mean ± 1SD.

The samples were tested in duplicate. The immunosorbents were prepared as described in Materials and Methods. Peak I was from a serum pool of nonallergic donors.

The filtrate and eluate were tested for their ability to block the binding of ^{125}I-P1 to A2.3/191 anti-P1 Mab. The results in Table 13, show that Peak I after absorption with anti-P1 Mab 191-Affi-gel gave similar inhibition (36.4%) as before absorption (32.8%), while the eluate from 191-Affi-gel had little inhibitory effect (7.2%). Peak I was also absorbed with HDM antigen-4B to examine if the inhibitor could bind to the antigen; as shown in Table 12, the inhibitory effect could not be removed. Therefore, it could not be concluded that the inhibitory effect of the factor(s) in the adult serum on the binding of HDM antigen or P1 to their specific antibodies was idiotype or epitope-specific.

Discussion

In the previous chapter, it was shown that a serum pool from nonatopic donors could cause up to 50% inhibition of the binding of P1 specific Mab to P1 (Table 14, Chapter II). This led to the present study with a view to examine the possibility that this inhibition was due to the presence of anti-idiotypic antibody against P1 specific antibody. When individual sera were tested, it was found that most of the nonatopic adult sera (7 out of 9) had inhibitory effect on the binding of human P1 specific IgE to P1, while none of the 10 cord sera tested had any substantial inhibitory effect. The difference was statistically highly significant. This suggested that the factor(s)

Table 13. Influence of Absorption of Peak I with Anti-Pl Mab on its Inhibiting Activity on the Binding of ^{125}I-Pl to Anti-Pl Mab

Inhibitor	% Inhibition (mean ± 1SD)
HDM antigen, batch #1 (1 mg/ml)	100.0 ± 0
Human IgG myeloma (1.5 mg/ml)	2.5 ± 0.8
Peak I (1.5 mg/ml)[a]	32.8 ± 2
Peak I absorbed with 191-Affi[b]	36.4 ± 1.3
Eluate from 191-Affi	7.2 ± 0.7
0% Inhibition (PBS as an inhibitor)	5,182 ± 415 cpm[c]
Background	202 ± 25 cpm

[a]Peak I was from a pool of normal serum.

[b]191-Affi: Affi-gel 10 coupled with Pl specific Mab clone A2.3/191.

[c]Cpm of ^{125}I-Pl bound to Mab A2.3/191 coupled to solid-phase.

interfering with the binding of anti-P1 to P1 in the adult sera did not exist in the cord sera. In order to examine the specificity of the inhibition, the individual sera were tested in an unrelated antibody and antigen system. It was shown that the nonatopic adult sera did not interfere with the binding of human anti-Rye I IgE to Rye I, indicating that the factor(s) in the sera had certain specificity in its inhibitory effect.

It would be interesting to know if the inhibitory factor was also existing in adult sera of HDM allergic individuals, and to understand the biochemical nature of the factor(s). The HDM allergic adult sera were pooled and absorbed with HDM antigen-4B immunosorbents to remove HDM antigen specific antibody, which otherwise would cause interference in the RIA by competing with the anti-HDM antibody on the solid phase to bind radiolabeled antigen. After absorptions, the specific antibodies were efficiently removed. Indeed, IgE or IgG anti-HDM antibodies could not be detected in Peak I or Peak II obtained from the absorbed serum pool (Table 5). The serum pools were fractionated into Peak I and II by AcA 34 gel filtration chromatography. Peak I was eluted in the void volume, so the molecular weight of the protein molecules in Peak I was more than 350 KD. Ouchterlony analysis showed that Peak I contained mainly IgM, plus some IgA and IgG, while Peak II contained mainly IgG (data not shown). The IgG in Peak I was probably made of aggregates.

Peak I from both allergic and nonallergic serum pool absorbed with HDM antigen-4B inhibited the binding of specific IgE or IgG antibody to ^{125}I-HDM or to ^{125}I-Pl. The inhibition ranged from 20 to 60%. The variation was, most likely, due to assay conditions such as freshness of labeled antigen, because when freshly labeled antigen was used, the inhibition was higher. Therefore, it was appropriate to consider the inhibition in relative, rather than absolute terms, i.e., within a given assay, the Peak II always had no or substantially lower inhibitory effect than Peak I (Fig.4, Table 7). Several lines of evidence could exclude the possibility that the inhibition of Peak I from AASP or AUSP was due to the HDM antigen which had leaked from the HDM antigen-4B during the absorption. First, it is known that Pl, the major antigen of HDM, is of low molecular weight (about 24,000, Chapman 1984). In our study, when crude HDM antigen was fractionated on a Sephadex G-100 column, the fractions inhibiting the binding of human IgE to HDM antigen were way beyond the void volume of the G-100 column. However, in the fractionated HDM antigen-4B absorbed sera, the inhibitory effect was located in the void volume of Ultrogel AcA 34, which had a higher upper fractionation limit (about 350,000) than that of G-100 (about 100,000). Second, the human IgG myeloma had no inhibitory effect even after parallel absorption on HDM antigen-4B. To further exlude the possibility that antigen leaking into the serum during absorption was the cause of inhibition, a nonatopic adult serum pool was directly fractionated into Peak I and II without absorption on HDM antigen-4B, and Peak I had still inhibitory effect.

It was observed that Peak II from AASP, ANSP or UNSP had no or substantially lower inhibitory effect than Peak I, and Peak I or II from cord serum pool had no inhibitory effect. These facts led to two conclusions. First, the inhibitory effect of Peak I was not due to the interference of HDM antigen specific antibodies. Indeed, inhibition was still observed after absorption with HDM antigen-4B, and moreover, the inhibiting activity was not in Peak II where most of the antibodies were found in the case of nonadsorbed allergic sera (Table 5). Second, it shed some doubts on the postulation that the inhibitory activity of Peak I was caused by auto-anti-idiotypic antibodies, since most auto-anti-idiotypic antibodies reported so far in the human system were of the IgG class (Abdou et al 1981; Zouali et al 1983; Dwyer et al 1986; Bose et al 1984). Therefore, if the inhibition was caused by an anti-Id, most likely the major inhibitory effect should be present in Peak II, and there should not be a significant difference between adult and cord sera, because, human IgG can cross the placenta. Of course, more studies were carried out later to formally refute the possibility that inhibitory effect of Peak I was due to auto-anti-Id.

It is worthy to note that similar results were obtained by employing either purified Pl or HDM antigen as labeled antigen; the only difference was the intensity of the signal whereas the inhibition patterns were identical.

Peak I and II from UNSP were tested for their inhibitory effect on the binding of Pl specific Mab to Pl. Six out of 9 clones (from 4 families), could be inhibited by Peak I with inhibitions ranging from 20 to 66%. Clones of family IV, i.e., C3.6/62 and C3.6/104, apparently were not inhibited (inhibition ranging from 3.8 - 10.8%). This indicated a certain specificity of the inhibition by Peak I. If the inhibition was indeed due to auto-anti-idiotypic antibodies, then possibly the anti-Id carried internal images recognized by Mab families other than family IV. Family IV was located at one extreme of the topographic map (Fig.9, Chapter II), and the center of the epitope it recognized was remote from that reacting with the other three families. If the major repertoire of internal images was at the location where faimily I, II and III were located in the map, then it was possible that family IV would not bind the internal image.

It must be pointed out that no complete inhibition could be achieved by using Peak I in various assays, and the maximum inhibition was 66% (Table 9). Since the inhibition seemed to be dose-dependent, and the inhibition curve had not reached the plateau of the maximum inhibitor concentration employed, it could have been possible to increase the maximum inhibition by increasing the concentration of Peak I. However, if the inhibition was caused by anti-Id, higher

concentrations of Peak I might still not be able to give complete inhibition of binding of human antibody to HDM antigen, because the repertoire of anti-Id might not contain all the epitopes of the labeled HDM antigen or Pl.

None of the Peak I from AASP, ANSP, UASP or UNSP had inhibitory effect on the binding of IgG or IgE anti-Rye I to Rye I. These results showed that there was certain specificity in the inhibitory activity of Peak I. In order to further characterize the nature of the inhibitory factor(s), Peak I from AASP and ANSP were absorbed on anti-IgA-4B, anti-IgM-4B and SpA-4B. The inhibitory factor(s) could not be removed by absorption of the immunoglobulin. The absorption was adequate since after absorption, Peak I would no longer form precipitation bands with anti-IgM, anti-IgA or anti-IgG antibodies in the Ouchterlony assays (data not shown). However, these data do not exlude the possibility that the inhibiting activity of adsorbed Peak I might be due to IgE, IgD or IgG3. Because this possibility seems unlikely, it is suggested that the inhibitory factor was not a classical anti-idiotype antibody.

There are two possible mechanisms to account for the specific inhibition of the binding of anti-Pl antibody to Pl by Peak I. One possibility is that the inhibitor may bind to Pl and compete with the anti-Pl antibody used in the assay. The inhibitor might be Ig or non-Ig, because some non-Ig in the serum do bind certain antigens.

Clark (1982) reported that an ubiquitous non-immunoglobulin protein, which could bind azobenzen arsonate determinants, existed in lymphoid cells and liver cells. It is also known that C-reactive protein in the serum could bind to pneumococcal C polysaccharide (Volanakis 1981). However, this mechanism was excluded by the fact that Peak I from AASP and ANSP still gave inhibition after absorption on HDM antigen-4B. An alternative mechanism explaining the specific inhibition is that the inhibitor carries determinants similar to those of Pl, and compete with ^{125}I-Pl. Anti-idiotypes carrying internal image of Pl are such inhibitors. In this study, as discussed above, the inhibitor was unlikely to be anti-idiotype antibody. However, molecules other than Ig such as T cell factors or detached T cell receptors (Greene et al 1981) could still possibly carry anti-idiotypic determinants. To examine such a possibility, Peak I was absorbed with anti-HDM antibody coupled Sepharose-4B gel to remove internal image carrying molecules. However, the inhibitory effect remained after the absorption. The anti-HDM antibody used coupled to Sepharose-4B gel was a gamma globulin fraction of the serum from a HDM antigen desensitized patient. Although this protein fraction contained high titer of HDM antigen specific antibody, conceivably the large percentage of the protein was antibodies not specific to HDM antigen. Consequently, the capacity of the immunosorbent might not be adequate and the absorption of Peak I might not be sufficient. To circumvent this problem, Mab A2.3/191 against Pl was coupled to Affi-gel 10, and Peak I was absorbed on this immunosorbent. Peak I

still inhibited to the same extent the binding of Mab A2.3/191 to P1 after the absorption. Hence, the inhibition was not caused by internal image bearing anti-idiotype positive molecules in the Peak I.

Based on the observations that: 1) the inhibitory factor(s) acted in HDM antigen or P1 assay system but not in Rye I assay system, 2) the inhibitory factor(s) existed only in adult but not in cord sera, and 3) it inhibited the binding of different clones of anti-P1 Mab to P1 at a different extent, it was suggested that the factor(s) had certain specificity. On the other hand, the factor(s) did not bind to HDM antigen nor anti-HDM antibody, indicating that the factor(s) was not epitope or idiotype specific. The nature of the inhibitory factors with such restricted specificity is still unclear. The binding force between antibody and antigen is noncovalent, and is due to hydrogen bond, ion bond, hydrophobic bond or Van de Wales' forces. One of these forces might play a major role in a HDM antigen and antibody system, but not in another antigen and antibody system. It is possible that certain serum macromolecules, or aggregated molecules, which are present with a higher concentration in adult than in cord blood serum, interfere with the major binding force between HDM antigen and its specific antibody but not the major force between Rye I and its specific antibody. To study this assumption and further characterize the inhibitory factor(s) in Peak I of adult serum is obviously out of the scope of the present study, since our primary

interest is to investigate the presence of auto-anti-idiotypic antibody in HDM allergic individuals.

The inhibition assays similar to those employed in the present work are commonly employed in the study of idiotype and anti-idiotype antibodies. Some authors (Zouali et al 1982; Koprowski et al 1984; Chalopin et al 1984) used such inhibition assays as a first step to identify anti-idiotypic antibodies in human sera. This is a logical first step, but the present study clearly illustrated the limitations of this approach. The existence of anti-idiotype can only be concluded if the inhibitory factor(s) can be isolated by specific immunosorbents and if the specificity is thoroughly studied with the appropriate controls.

CHAPTER IV

DEMONSTRATION OF ANTI-IDIOTYPIC ANTIBODY AGAINST ANTIBODIES SPECIFIC FOR HDM-ANTIGEN P1

Abbreviations used in this Chapter:

AB	assay buffer
AchR	acetylcholine receptor
102-Affi(C4.1/102-Affi)	Affi-gel 10 coupled with anti-P1 Mab C4.1/102
Ag B	timothy grass pollen antigen B
Anti-Id	anti-idiotype antibody
APL-Affi	Affi-gel 10 coupled with Mab anti-prolactin
BPO	benzylpenicilloyl
Cpm	count per minute
CRI	crossreactive idiotype
DNA	deoxyribonucleic acid
FCS	fetal calf serum
HBS Ag	hepatitis B surface antigen
HDM	house dust mite
HPLC	high performance liquid chromatography
Ig	immunoglobulin
IgA,E,G,M	immunoglobulin A,E,G,M
Mab	monoclonal antibody
Mab-Affi-gel	Affi-gel 10 coupled with Mab
NMS	normal mouse serum
OA	ovalbumin
P1	the major allergen of house dust mite (D. pteronyssinus)
PC	L-α-phosphatidyl choline

RIA	radioimmunoassay
Rye I	rye grass pollen allergen I
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SpA-4B	Sepharose CL-4B coupled with staphylococcus protein A
SpA + 102	purification on 102-Affi of the eluate from SpA-4B
SRIA	sandwich radioimmunoassay
TNP	trinitrophenyl
TT	tetanus toxoid

Abstract

Four Mab from 4 different families were employed in a solid-phase sandwich RIA to identify human anti-Id specific to anti-Pl antibody. In this assay, the same Mab was used to coat the solid-phase as the labeled second antibody. One of the 4 Mabs (C4.1/102) could detect human anti-Id in 6/101 sera from HDM allergic patients, 1/26 normal sera (free of anti-Pl) and in 0/86 cord blood sera. The reactivity of positive sera with Mab C4.1/102 was inhibited in a dose-dependent fashion by HDM antigen and by free Mab C4.1/102 but not by unrelated antigens or Mabs, nor by normal mouse serum. Moreover, the anti-Id could be absorbed on either Mab C4.1/102-Affi-gel or on protein A Sepharose (from which they were recovered by acid elution), but not on unrelated Mab-Affi-gel. Analysis of the affinity purified anti-Id by Ouchterlony and SDS-PAGE confirmed that it was an IgG. It is concluded that HDM sensitive patients may have anti-Id to a public Id which is also expressed on mouse anti-Pl, it is also suggested that some anti-Id express the internal image of a Pl epitope.

Introduction

Since Jerne formulated the immune network theory (1974), the existence of idiotype and auto-anti-idiotypic antibodies (auto-anti-Id) has been documented in many experimental models (as reviewed in Chapter I). Rodkey (1974) first demonstrated auto-anti-Id in rabbits immunized with their own anti-trimethyl-ammonium antibodies. Sakato et al (1975) demonstrated the anti-Id by immunization of syngenic mice with myeloma protein. More importantly, Cowdery (1981) reported the occurrence of auto-anti-Id during the normal immune response of mice to TNP-Ficoll. Cohen et al (1982) and Eisenberg et al (1985) reported that the F1 hybrids of NZB and normal mice spontaneously developed auto-anti-Id that specifically recognized the idiotype of anti-erythrocyte antibodies. In the human system, a few studies have documented the existence of auto-anti-Id. Geha (1982) demonstrated auto-anti-Id in individuals hyperimmunized with tetanus toxoid. In several pathological conditions, spontaneously occurring auto-anti-Id have also been reported. Cunningham-Rundles (1982) found that patients with selective IgA deficiency would develop anti-casein antibodies and subsequently auto-anti-Id-casein antibodies. Abdou et al (1981) and Zouali et al (1983) showed that auto-anti-Id against anti-DNA antibodies appeared in systemic lupus erythematosus patients and in some normal individuals. Dwyer et al (1983) discovered naturally occurring anti-Id against anti-acetyl-

choline receptor antibody in myasthenia gravis patients. Bose et al (1984) documented auto-anti-Id to anti-Rye I antibodies in rye grass pollen allergic patients. More recently, Troisi et al (1985) detected auto-anti-Id against anti-HBS antigen (hepatitis B surface antigen) antibodies in hepatitis B patients. The immunoregulatory effect of the anti-Id has been demonstrated in some cases, indicating that the immune network is functional. Bottomly et al (1978) reported that in mice, anti-Id could suppress, via T suppressor cells, the antibody response to phosphorylcholine. Eichman (1974) documented that guinea pig IgG_2 subclass anti-Id had a suppressive effect on the production of anti-CHO-A (group A streptococci carbohydrate) antibodies carrying A5A idiotype in A/J mice, while the guinea pig IgG_1 subclass anti-Id had a slightly enhancing effect. In a recent study, Geha (1984) reported that rabbit anti-Id antibodies could induce in vitro human specific T suppressor cells capable of inhibiting T cell proliferation upon stimulation with tetanus toxoid (TT). Saxon et al (1984) reported that in TT hyperimmunized human individuals, the occurrence of auto-anti-Id was correlated with the development of skin anergy to TT and with the disappearance of the in vitro peripheral blood mononuclear cell blastogenic response to soluble TT.

The role of Id-anti-Id interaction in the regulation of IgE antibody response has been documented in one animal model. Blaser et

al (1983) reported that anti-Id antibodies could modulate the production of IgE antibody against benzylpenicilloyl (BPO) or phosphorylcholine in Balb/c mice. Very little is known about the Id-anti-Id interactions in relation to human allergic disease. Bose et al (1984) reported the presence of auto-anti-Id in rye grass pollen allergic patients. In this study, we investigated the presence of auto-anti-Id specific to antibody against HDM major allergen P1 in human sera. It was found that 5.9% of the sera from HDM allergic individuals, 3.8% of the sera from nonatopic adults and 0% of the cord sera, contained anti-Id reacting with mouse Mab to P1. The auto-anti-Id from one allergic serum (W77) was fully characterized. It could be absorbed on and eluted from anti-P1 Mab coupled immunosorbent. Its binding to anti-P1 Mab could be specifically inhibited by HDM antigen and autologous Mab, but not by other unrelated antigen, or unrelated Mab.

Materials and Methods

Sandwich radioimmunoassay for the detection of auto-anti-Id in human sera (referred as SRIA #1)

Flexible polyvinyl chloride microtitre plates (Dynatech Laboratories Inc., Alexandria, VA) were soaked in 75% acetic acid for about 2-3 hrs and then rinsed with distilled water. One hundred µl of PBS containing 12.5 µg/ml Mab were added to each well and the

plates were incubated overnight at room temperature. After washing, wells were supplemented with 150 µl of Hank's solution containing 10% fetal calf serum. After 2 hr incubation at room temperature, wells were washed 10 times with PBS. Tests were performed in duplicate by reacting 100 µl of test sample with Mab-coated well, for 24 hr at room temperature in a humid chamber. Plates were washed 10 times with PBS, and 100 µl of ^{125}I-labeled Mab (identical to that used for coating the plates; diluted in assay buffer containing 5% FCS and 5% normal Balb/c serum) was added to the wells. After overnight incubation at room temperature, the plates were washed 10 times with PBS, and the wells were counted in a gamma counter. The background was determined by using 12 wells coated with anti-Rye I Mab; one-third of these wells were incubated with allergic sera, one-third with nonallergic sera and one third with cord sera. The mean radioactivity bound to these 12 wells was considered as the background.

Sandwich radioimmunoassay to test the specificity of anti-Id (referred as SRIA #2)

This assay was performed exactly as the previous one except for the last step, where 20 µl of inhibitor (in PBS) was added to the well along with 80 µl ^{125}I-labeled Mab (150,000-200,000 cpm in assay buffer containing 5% FCS and 5% normal Balb/c mouse serum). After overnight incubation, plates were washed, wells were cut and

counted individually in a gamma counter. The background was calculated as described for SRIA #1.

Fractionation of serum protein by HPLC ion exchange chromatography

Serum W77 from a HDM allergic patient was precipitated twice by 50% saturated ammonium sulphate (refer to Chapter III), and then dialysed against 0.05M Tris-HCl, pH 8.0 in Spectrapor dialysis tubing. One ml of sample containing 24 mg protein (determined by the Lowry method) was applied to a DEAE ion exchange column (Ultrapac, DEAE SK 545, 7.5 x 150 mm, LKB, Bromma, Sweden). Starting buffer designated as buffer A was 0.05M Tris-HCl (pH 8.0), and the ending buffer designated as buffer B was 1M NaCl in buffer A. The gradient schedule was expressed in terms of % buffer B, and the turn points were given as follows:

Time (minutes)	% B
0.7	0
22	50
24	100
27	100
32	0
40	0

The flow rate was 1 ml/min, and 1 ml fractions were collected.

Double immunodiffusion (Ouchterlony assay) (Johnston et al 1982)

Agarose (0.8%) w/v (Marine Colloids Inc., Rockland, MA) in PBS was melted in 100°C water bath, and cooled to about 60°C. Five ml of the solution was dispensed into each of 60 x 15 mm Falcon 3002 Petri dishes (Becton-Dickinson, Oxnard, CA) and allowed to solidify at room temperature. Wells of 0.5 cm in diameter and 0.5 cm apart were punched. Twenty µl of samples or antisera were applied to each well and Petri dishes were incubated in a humid chamber at room temperature for 1-2 days until precipitation bands were clearly shown. The Petri dishes were then washed with 3 changes of PBS over a period of 48 hr to remove unprecipitated protein, and subsequently washed with 3 changes of distilled water over a period of 24 hr to remove salt. Dishes were dried on a Thermolite gel drier, stained with 0.01% acid fuchsin, destained with 0.4% (v/v) acetic acid, and dried on gel drier again. The antisera used in the assays were as follows: rabbit anti-human µ chain specific (Dako, Copenhagen, Denmark), rabbit anti-human γ chain specific (Dako, Copenhagen, Denmark), rabbit anti-human α chain specific (Dako, Copenhagen, Denmark), and goat anti-human total serum (Bionetics, Kensington, MD).

SDS-acrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was run on a Bio-Rad protein slab gel apparatus (Bio-Rad, Mississauga, ON) according to operation instructions from the manufacturer. Reagents were all purchased from Bio-Rad.

In order to prepare one separation slab gel (1.5 mm thick), the following solution was made: 13.2 ml of 30% Bis-acrylamide (which contained 29.2% w/v acrylamide and 0.8% w/v N'N'-bis methylene acrylamide); 16 ml of double distilled water, 10 ml of 1.5M Tris-HCl (pH 8.8), and 4.4 ml of 10% (w/v) SDS. The solution was degassed and then supplemented with 0.4 ml of 4% (w/v) ammonium persulphate and 10 µl of TEMED. The solution was poured into the glass sandwich until the liquid level was 4 cm from the top and then gently overlaid with double distilled water to give a smooth interface after polymerization. After 30-40 min, when the gel was polymerized, the overlaying water was poured off and the gel surface was rinsed once with water. The stack monomer solution (which consisted of 1 ml 30% Bis-acrylamide solution, 6.3 ml double distilled water, 2.5 ml 0.5M Tris-Hcl, ph 6.8, 0.1 ml 4% w/v ammonium persulphate, 0.1 ml 10% SDS, and 2.5 µl TEMED) was poured into the glass sandwich to about 1 cm from the top, and the comb was inserted. Gel was allowed to polymerize for 3-4 hrs and the comb was gently removed. Each sample (10-60 µl) was mixed with 50 µl of sample buffer (Laemmli 1970; i.e., 0.0625M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% ß-mercaptoethanol, and 0.001% bromophenol blue), and boiled in a water bath for 3 min. Samples were then applied to wells, and the wells were filled up with electrode buffer (of 0.025M Tris, 0.192M glycine and 0.1% SDS). The gel was run at 30 mA of constant current for 3-5 hrs until the tracing dye reached the bottom of the slab. The gel was then stained for 1-4 hr with a solution containing 10% acetic

acid, 25% isopropanol and 0.1% Coomassie blue R250, and destained with 3-4 changes of destaining solution containing 8% acetic acid and 25% ethanol until background was clear. Gels were then soaked in a solution consisting of 7% acetic acid, 4% glycerol and 12% isopropanol to improve the texture of the gels to prevent cracking during drying. Gels were finally photographed and dried on a Bio-Rad gel drier.

Coupling of monoclonal antibodies to Affi-gel 10

Purified anti-Pl Mab C4.1/102 and anti-prolactin Mab (prepared in this laboratory) were coupled to Affi-gel 10 (Bio-Rad, Mississauga, ON), following the instructions from the manufacturer. Affi-gel 10 was first washed with 3 volumes of isopropyl alcohol, followed by 3 volumes of cold (4°C) deionized water. Ten mg of Mab were dissolved in 3 ml of 0.1M MOPS (Fisher, Winnipeg, MB), and mixed with 1 ml of packed Affi-gel 10. The gel suspension was rotated overnight at 4°C, and rotated for another 2 hrs after 0.1 ml 1M ethanolamine HCl (pH 8.0) per ml of packed gel was added. The gel was then washed in a Buchner funnel successively with 20 ml of PBS, 20 ml of 0.1M glycine-HCl, pH 3.0, and 20 ml of PBS. The coupling efficiency was about 70%.

Affinity chromatography

Serum W77 from a HDM allergic patient was precipitated by ammonium sulfate (50% saturation) and fractionated by affinity chromatography with protein A Sepharose CL-4B (SpA-4B, Pharmacia, Uppsala, Sweden), C4.1/102 Affi-gel (102-Affi), or anti-prolactin-

Affi-gel (APL-Affi), in order to characterize the nature of auto-anti-idiotypic antibodies. The immunoabsorbent gels were packed in 0.7 x 1.0 cm Bio-Rad econo-column, washed with 5-bed volumes of 0.1M glycine-HCl, pH 3.0, and equilibrated with 20 bed volumes of PBS. The sample (24 mg protein/ml, about 2 bed volume) was applied at a flow rate of 1 ml/hr; and the columns were washed with 20-30 bed volumes of PBS at a flow rate of 20 ml/hr. The absorbed components were eluted with 0.4 ml of 0.1M glycine-HCl, pH 3.0 at a flow rate of 5 ml/hr. The eluates were neutralized immediately with 20 µl of 1M Tris-HCl, pH 7.0. Eluates from SpA-4B column was rechromatographed on 102-Affi column by the same protocol, and the final eluate was designated as eluate from "SpA + 102".

Results

Standardization of SRIA for the detection of auto-anti-Id

In order to eliminate false positive reactions caused by the presence in some sera of antibodies against mouse Ig, serum from normal Balb/c mice, (the same strain employed to prepare the Mab), was added to the assay buffer used to dilute the labeled Mab. This large excess of normal mouse Ig should inhibit by competition, the binding of the putative nonspecific human anti-mouse Ig antibodies to radio-labeled Mab. To decide the adequate concentration of mouse serum to be used in the assay buffer, C4.1/102-coated wells were reacted with a human serum known to have anti-mouse Ig antibodies. After washing,

the wells were incubated with ^{125}I-C4.1/102 diluted in assay buffer containing different amounts of mouse serum. The results are shown in Figure 1. When ^{125}I-C4.1/102 was diluted in assay buffer containing 10% FCS but no NMS, the bound radioactivity was 1,230 cpm as compared to 141 cpm when the assay buffer contained 5 or 10% mouse serum. It was therefore decided to supplement assay buffer with 5% mouse serum and 5% fetal calf serum.

Screening of auto-anti-Id in human sera

Three groups of sera, including 101 sera from HDM allergic adults, 26 sera from nonallergic donors and 86 cord blood sera, were screened for auto-anti-Id to anti-Pl antibodies by means of the SRIA. Each serum was tested for its reactivity with a representative antibody from each of the 4 families of anti-Pl Mab. The sera from the nonallergic donors did not contain anti-Pl IgG antibodies; they were screened as described in Chapter III. HDM allergic patients had positive skin and RAST tests to HDM, as determined by Dr. R. Pauwels (Gent University, Belgium). Cord sera were selected on the basis of their lack of anti-Pl antibodies. All the samples were tested in duplicate. The results are shown in Table 1. A serum was arbitrarily graded as (+), if the cpm were twice the background, it was graded as (++), if cpm were 3 times the background, and so on. According to this criteria, 6 out of 101 allergic sera (5.3%), 1 out of 26 nonallergic sera (3.8%), and 0 out of 86 cord sera contained auto-anti-Id binding to C4.1/102 Mab. The cpm of the positive samples

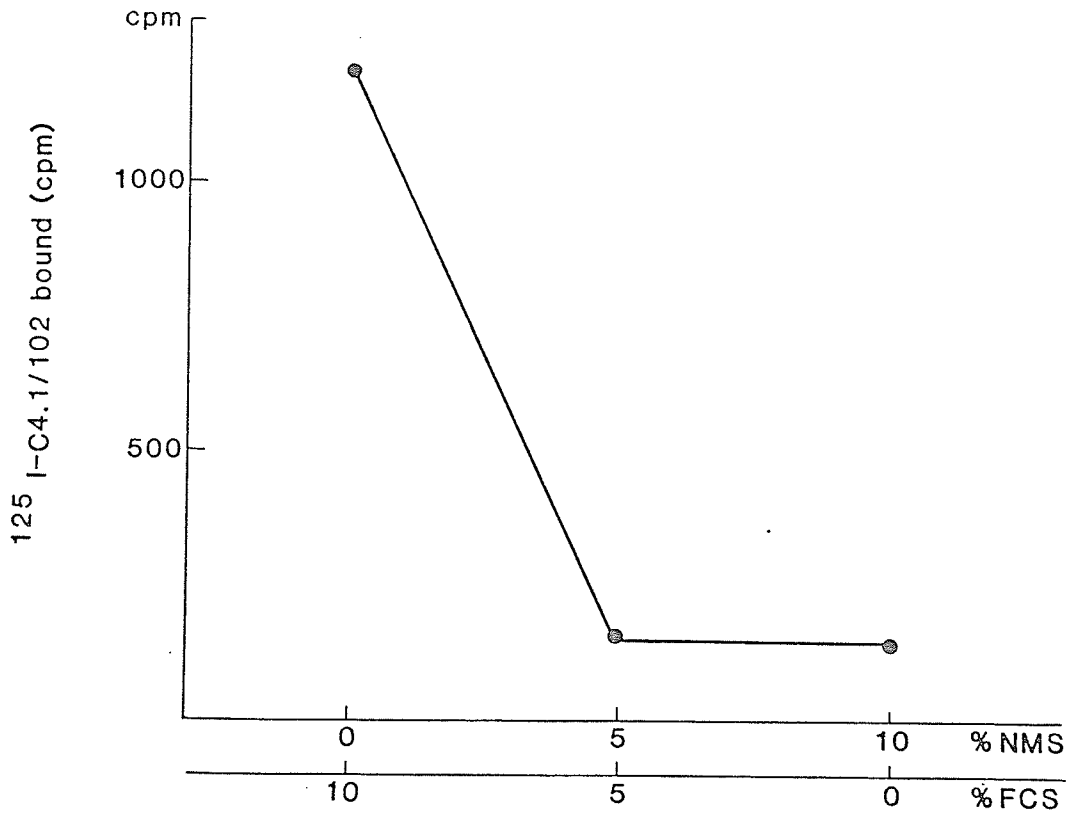


Figure 1: Neutralization of human anti-mouse IgG by normal mouse serum (NMS).

Background (137 cpm) was the mean cpm of 8 wells coated with normal human sera and incubated with ^{125}I-C4.1/102 diluted in assay buffer containing 10% FCS.

Samples were tested in duplicate.

Table 1a. Screening of Auto-Anti-Idiotypic Antibodies by SRIA

	Serum Sample	C4.1/102 SRIA cpm ± SD	C4.1/102 SRIA Positiveness[a]	A2.3/191 SRIA Positiveness	C3.6/104 SRIA Positiveness	B2.1/81 SRIA Positiveness
Atopics	W99	950 ± 113	++++++	(-)	(-)	(-)
	W77	522 ± 19	++	(-)	(-)	(-)
	W143	427 ± 131	++	(-)	(-)	(-)
	W31	680 ± 34	++++	(-)	(-)	(-)
	R96	288 ± 24	+	(-)	(-)	(-)
	R114	349 ± 24	+	(-)	(-)	(-)
	Other 28 Samples		(-)	(-)	(-)	(-)
	Other 67 Samples		(-)	ND	ND	ND
Non-atopics	MN19	912 ± 107	++++++	(-)	(-)	(-)
	Other 25 Samples		(-)	(-)	(-)	(-)
Cord Blood	27 Samples		(-)	(-)	(-)	(-)
	Other 59 Samples		(-)	(-)	ND	ND

[a]The sample with cpm doubled the background were graded as (+), tripled the background were graded as (++), and so on. The background ranged from 134 cpm (Mab C4.1/102) to 357 cpm (Mab A2.3/191). These experiments were repeated with concordant results.

Table 1b. Summary of Table 1a.

	Number of Sera Tested	Samples positive in anti-Id by C4.1/102 SRIA	%
Atopics	101	6	5.9
Nonatopics	26	1	3.8
Cord Blood	86	0	0

were 2-7 times the background. These positive sera were compared to several anti-Id negative sera (28 allergic sera, 25 nonallergic sera and 27 cord sera) for their binding to A2.3/191, C3.6/104 and B2.1/81 Mab. None of these sera reacted with any of the latter 3 anti-Pl Mab.

Optimization of the C4.1/102 SRIA #1 and #2

A few anti-Id positive sera were obtained after the panel screening with 3 groups of sera, and it was possible then to optimize the assay conditions using anti-Id positive sera. To decide the optimal total cpm of ^{125}I-C4.1/102 added per well, anti-Id positive serum W77 was incubated in C4.1/102 coated plates, and 100 µl of ^{125}I-C4.1/102 of different cpm were added to each well. Results are shown in Figure 2. The specifically bound radioactivity increased as the total cpm increased, so did the background. At 200,000 cpm/well of total radioactivity, the difference between background and ^{125}I-C4.1/102 bound was most prominent, yet the specific binding had not reached the plateau of the dilution curve. This total radioactivity was therefore used throughout the subsequent assays. Different concentrations of C4.1/102 for coating the plates were also tried with anti-Id positive serum W77 as a test sample, and with 200,000 cpm ^{125}I-C4.1/102 added in the last incubation. The results are given in Figure 3. When the concentration of C4.1/102 employed for coating increased from 0.3 µg/ml to 5 µg/ml, the specific bound radioactivity increased from 165 to 591 cpm. As the concentration of C4.1/102 for coating was further increased to 20 µg/ml, the specific

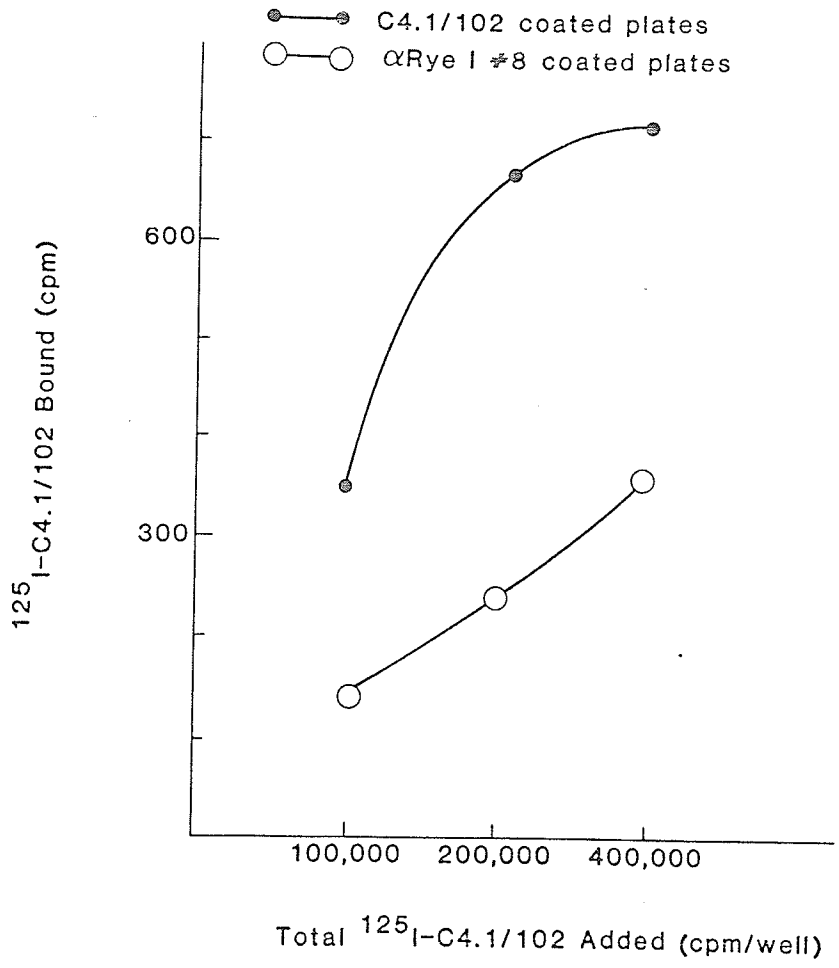


Figure 2: Titration of ^{125}I-C4.1/102 in SRIA.

Serum W77 was used to incubate in wells.

Background was the mean cpm of wells coated with unrelated Mab (Rye I Mab #8) and then incubated with W77.

Samples were tested in duplicate.

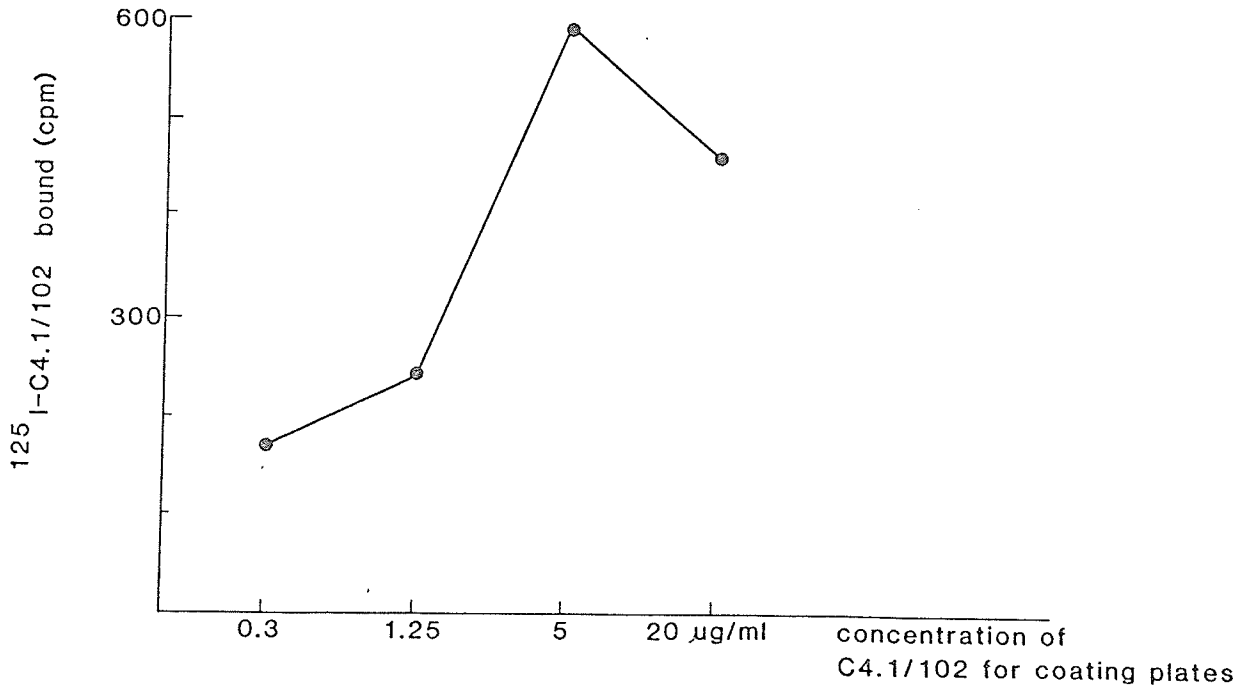


Figure 3: Titration of Mab C4.1/102 for coating the plates in SRIA.
W77 was used to incubate in C4.1/102 coated wells.
Samples were tested in duplicate.

bound radioactivity decreased slightly to 469 cpm. Therefore, 5 µg/ml of C4.1/102 was used to coat the plates in the subsequent assays. The screening was repeated by using the above defined conditions, and the results were concordant with those obtained in the first assay.

Characterization of auto-anti-Id in W77

The auto-anti-Id positive serum W77 was fractionated with HPLC ion exchange chromatography and the fractions were tested for their binding activity to the anti-Pl Mab C4.1/102 by SRIA. The fraction 19 was positive (Fig.4) and was then analysed in 10% SDS-PAGE (Fig.5). The major bands were at the position of γ and L chains, although some minor bands at the position of α and µ chains were also present. This fraction was also examined in immunodiffusion assay (Fig.6), and it formed precipitation with rabbit anti-human α, γ and µ chain antibodies. It was concluded that the auto-anti-Id positive fraction contained mainly IgG, although IgM, IgA and other serum components were also present in relatively low concentrations.

W77 was absorbed on protein A Sepharose-4B, or C4.1/102-Affi-gel (102-Affi), or sequentially on both of them (SpA + 102-Affi) to further characterize the auto-anti-Id. The filtrates and the eluates were tested for their binding to C4.1/102. The auto-anti-Id was found (Fig.7) in the eluates but not in the filtrates of SpA-4B, 102-Affi, or SpA + 102-Affi, and in the filtrate but not in the eluate of the

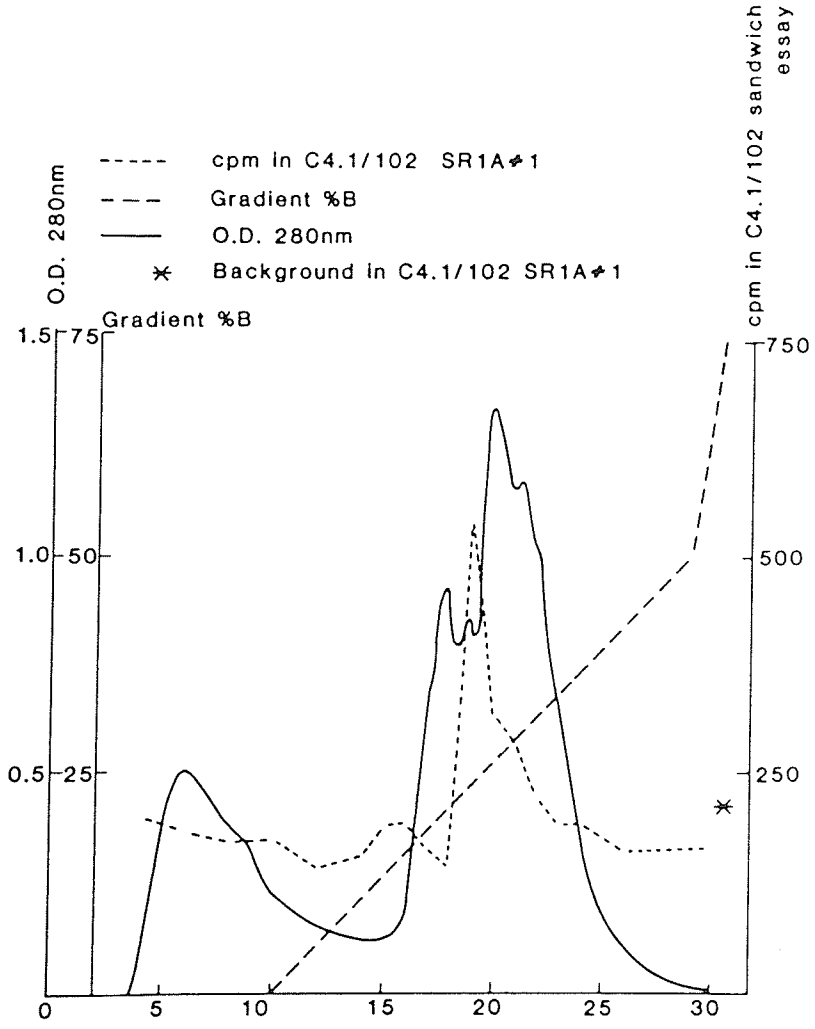


Figure 4: Fractionation of serum W77 by ion exchange chromatography.

The figure shows the anti-Id activity of each fraction, as defined by its binding to Mab C4.1/102-coated wells.

The background was 212 cpm.

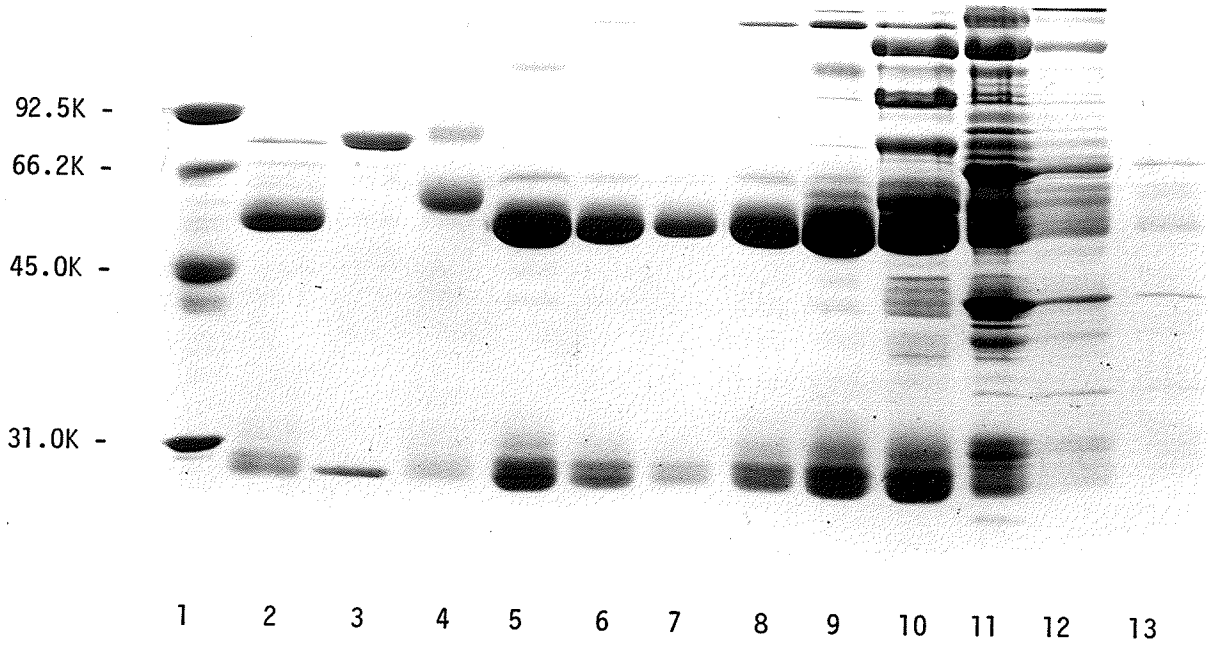


Figure 5: 10% SDS-PAGE analysis of the fractions of serum W77 obtained by ion exchange chromatography.

Lane 1	mol.wt. standard
Lane 2	human IgG
Lane 3	human IgM
Lane 4	human IgA
Lane 5	fraction 6
Lane 6	fraction 9
Lane 7	fraction 13
Lane 8	fraction 16
Lane 9	fraction 18
Lane 10	fraction 19
Lane 11	fraction 21
Lane 12	fraction 24
Lane 13	fraction 26

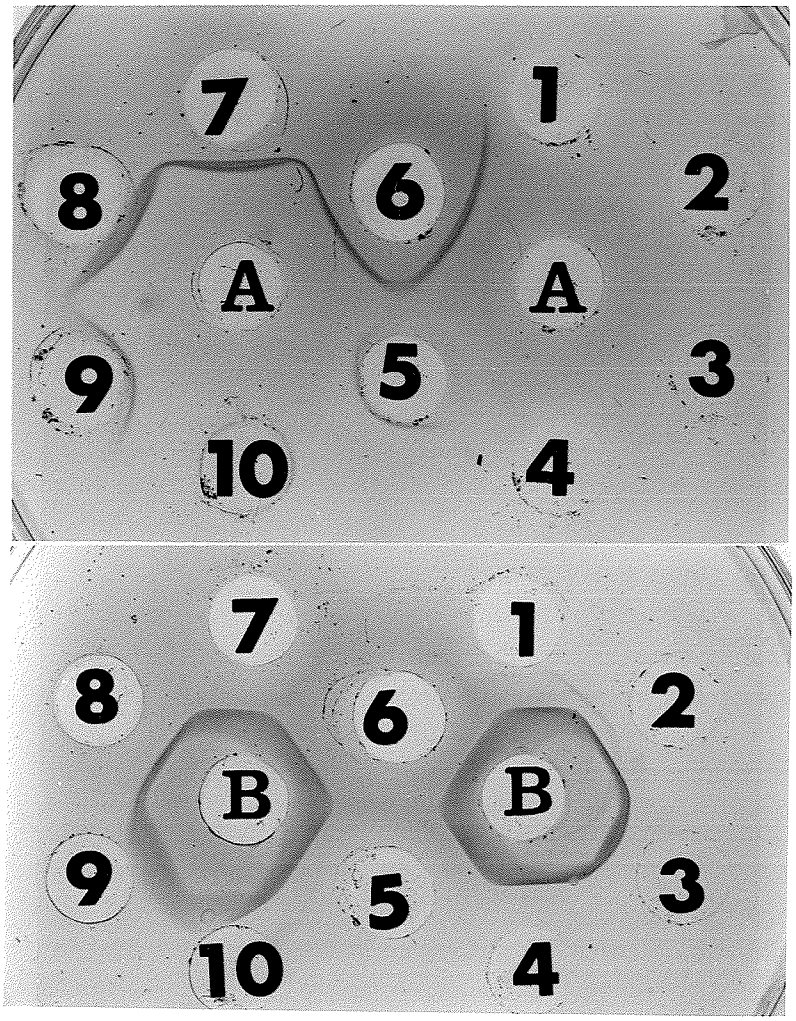
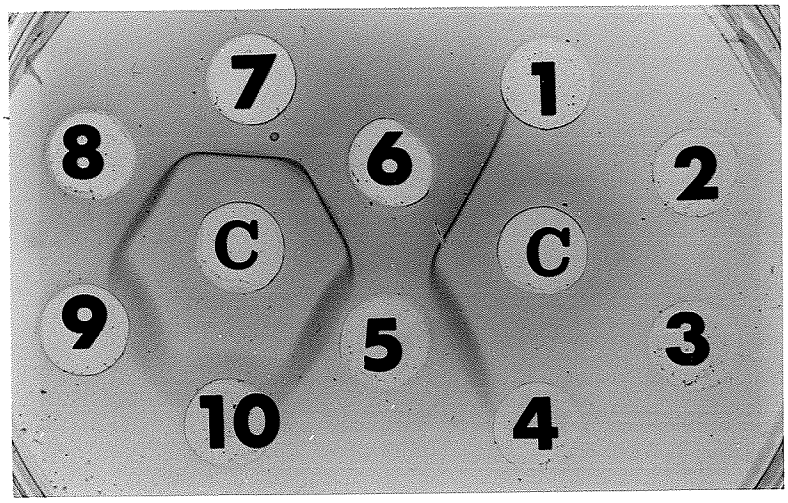


Figure 6: Ouchterlony analysis of ion exchange fractions of serum W77.

Well A	rabbit anti-human μ chain
Well B	rabbit anti-human γ chain
Well C	rabbit anti-human α chain
Well 1	fraction 6
Well 2	fraction 9
Well 3	fraction 13
Well 4	fraction 16
Well 5	fraction 18
Well 6	unfractionated W77
Well 7	fraction 19
Well 8	fraction 21
Well 9	fraction 24
Well 10	fraction 26

cont'd.....



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Well A	rabbit anti-human μ chain
Well B	rabbit anti-human γ chain
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Well 1	fraction 6
Well 2	fraction 9
Well 3	fraction 13
Well 4	fraction 16
Well 5	fraction 18
Well 6	unfractionated W77
Well 7	fraction 19
Well 8	fraction 21
Well 9	fraction 24
Well 10	fraction 26

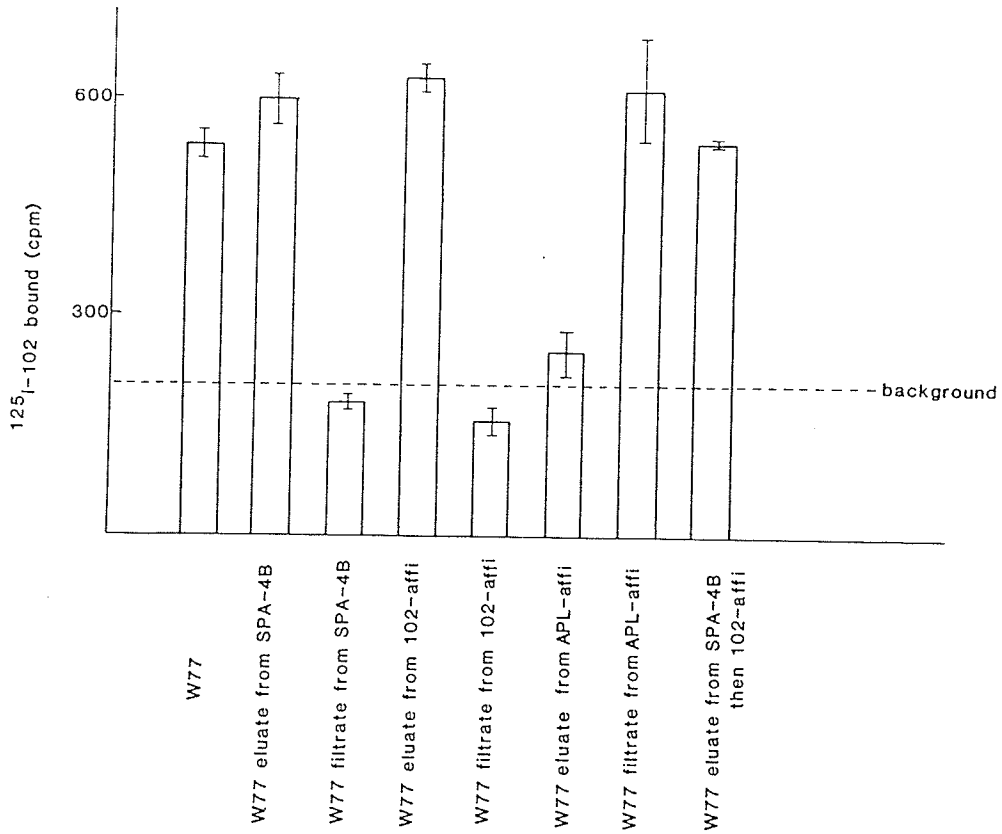

Figure 7: Analysis of auto-anti-Id by affinity chromatography and C4.1/102 SRIA #1.

The filtrates and eluates were tested in SRIA #1.

Samples were tested in duplicate.

This experiment was repeated with concordant results.

Background was 208 cpm.

control immunosorbent (anti-prolactin-Affi-gel). The auto-anti-Id positive eluate of SpA + 102 was examined in 10% SDS-PAGE, and only bands at the position of and L chains were present (Fig.8). The immunodiffusion assay further proved that the eluate from SpA + 102 only contained IgG but no IgM nor IgA (Fig.9). It was concluded that serum W77 contained IgG auto-anti-Id against anti-Pl antibodies.

The specificity of the auto-anti-Id antibody

The specificity of the auto-anti-Id antibody was first tested with unfractionated serum W77 (in SRIA #2) using Mab C4.1/102 for coating the wells and for the radiolabeled preparation. The results are given in Figure 10. Twenty µg of HDM antigen could inhibit the ^{125}I-C4.1/102 binding to auto-anti-Id antibodies, while the same amount of several unrelated antigens, e.g., lipopolysaccharide, or L-α-phosphatidyl choline, had no inhibitory effect. A relative small amount (2 µg) of Mab C4.1/102, which was the same Mab used for coating and labeling, but not unrelated anti-Rye I Mab #8, could inhibit the binding of ^{125}I-C4.1/102 to auto-anti-Id. The specificity of the auto-anti-Id against anti-Pl Mab from different families was examined by another set of SRIA #2, where the eluate of W77 serum from SpA-Sepharose-4B was reacted with solid-phase coated with C4.1/102, and where anti-Pl Mab C4.1/102, C3.6/104 and A2.3/79 from 3 different families were used as inhibitors. The results are shown in Figure 11. Only the clone C4.1/102, which was the same Mab used for coating and radiolabeling in the assay, could inhibit (58%

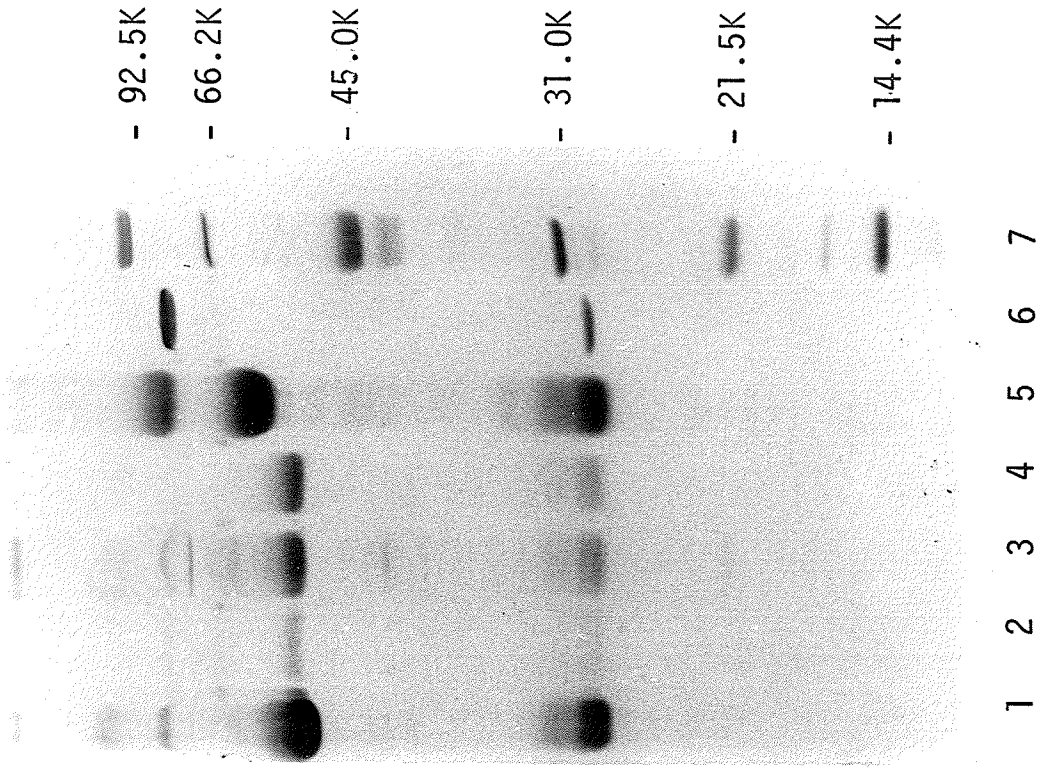

Figure 8: 10% SDS-PAGE analysis of affinity purified auto-anti-Id from serum W77.

Lane 1	eluate from SpA-4B
Lane 2	eluate from SpA-4B absorbed on and eluted from Mab 102-Affi-gel
Lane 3	eluate from Mab 102-Affi-gel
Lane 4	human IgG
Lane 5	human IgA
Lane 6	human IgM
Lane 7	mol.wt. standard

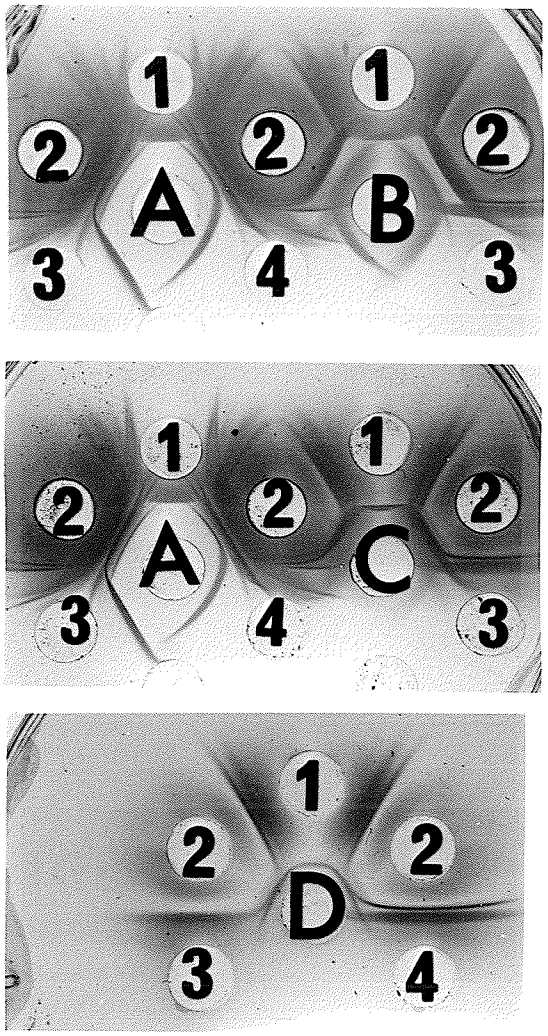

Figure 9: Ouchterlony analysis of affinity purified auto-anti-Id of serum W77.

Well A	control human serum
Well B	eluate from SpA-4B
Well C	eluate from 102-Affi-gel
Well D	eluate from SpA-4B absorbed and eluted from 102-Affi-gel
Well 1	rabbit anti-human IgG
Well 2	goat anti-human total serum
Well 3	rabbit anti-human IgA
Well 4	rabbit anti-human IgM

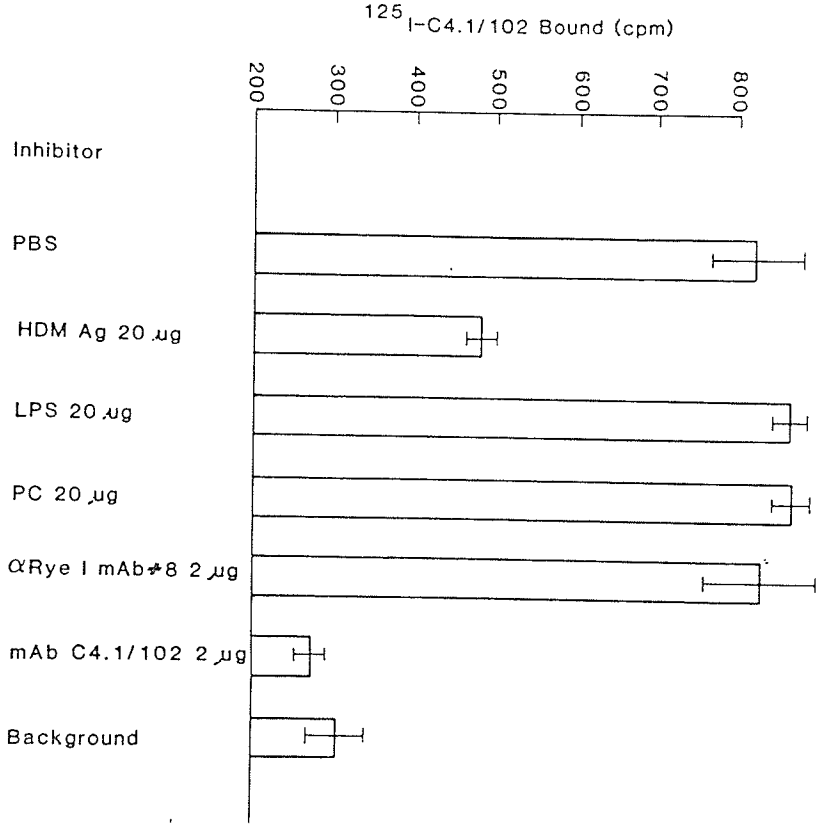

Figure 10: Specificity of auto-anti-Id in serum W77.

Background was the mean cpm of 8 wells coated with unrelated Mab and incubated with serum W77. The experiment was repeated with identical results.

Samples were tested in duplicate.

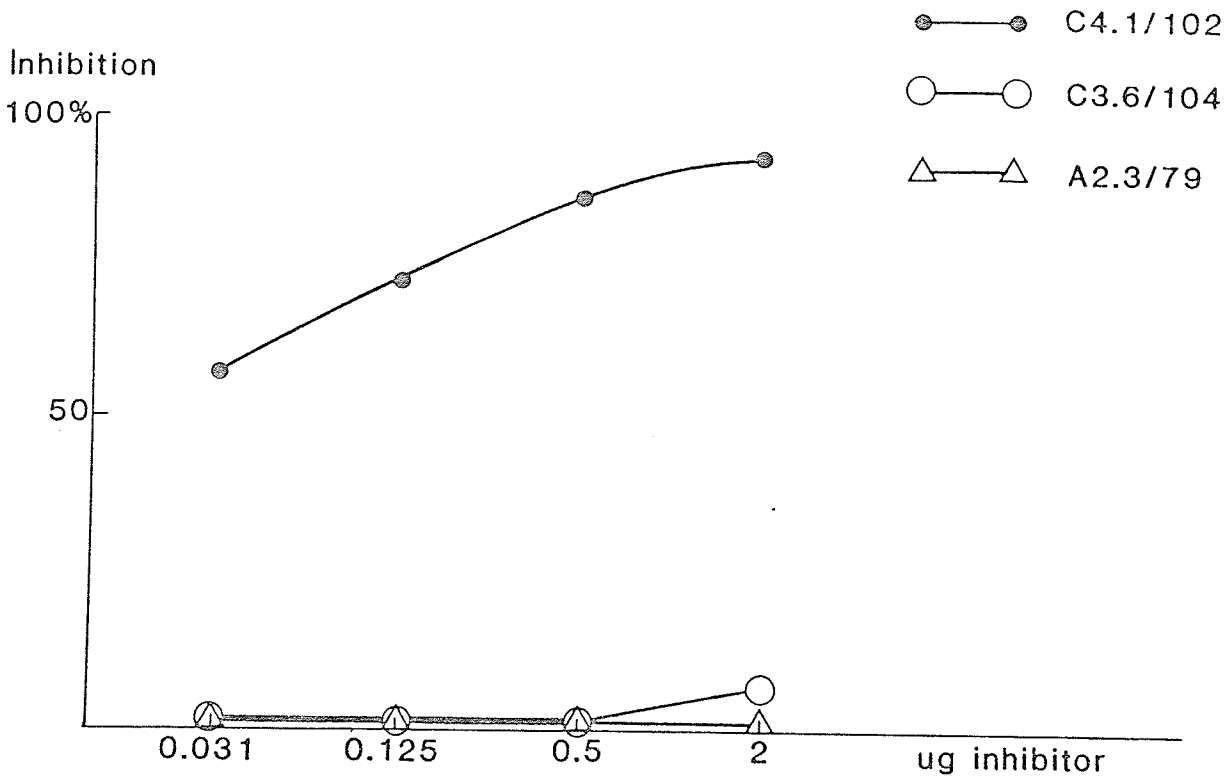

Figure 11: Inhibition of the binding of auto-anti-Id from serum W77 to anti-Pl Mab C4.1/102 by anti-Pl Mabs of different families.

The experiment was performed with the IgG fraction of serum W77. Background was 110 cpm and maximum binding 472 cpm.

inhibition with 0.031 μg, and 93% with 2 μg of cold Mab) the binding of auto-anti-Id to ^{125}I-C4.1/102. The other 2 anti-Pl Mab, i.e., C3.6/104 and A2.3/79, from other families could not inhibit the binding of auto-anti-Id W77 to ^{125}I-C4.1/102, at least at the concentration range employed.

These results indicated that the auto-anti-Id present in serum W77 were idiotope specific. This was further confirmed in a similar experiment employing auto-anti-Id purified by sequential affinity-chromatography on SpA-Sepharose 4B and 101-Affi-gel. The results are shown in Figure 12. Two hundred μg of normal human IgG, and of L-α-phosphatidyl choline had no inhibitory effect, while 200 μg and 20 μg HDM extract caused a dose-dependent inhibition. The anti-Pl Mab C4.1/102, but not the unrelated anti-Rye I Mab #18, could inhibit the binding of ^{125}I-C4.1/102 to auto-anti-Id antibodies. In summary, the auto-anti-Id antibody in serum W77 could only bind to anti-Pl Mab C4.1/102, but not to anti-Pl Mab from other families, nor to unrelated Mab, and their binding could be inhibited by HDM antigen containing Pl, but not by unrelated antigen. It was thus concluded that this auto-anti-Id antibody carried an internal image of one of the determinants on Pl, and that it was indeed an auto-anti-idiotypic antibody.

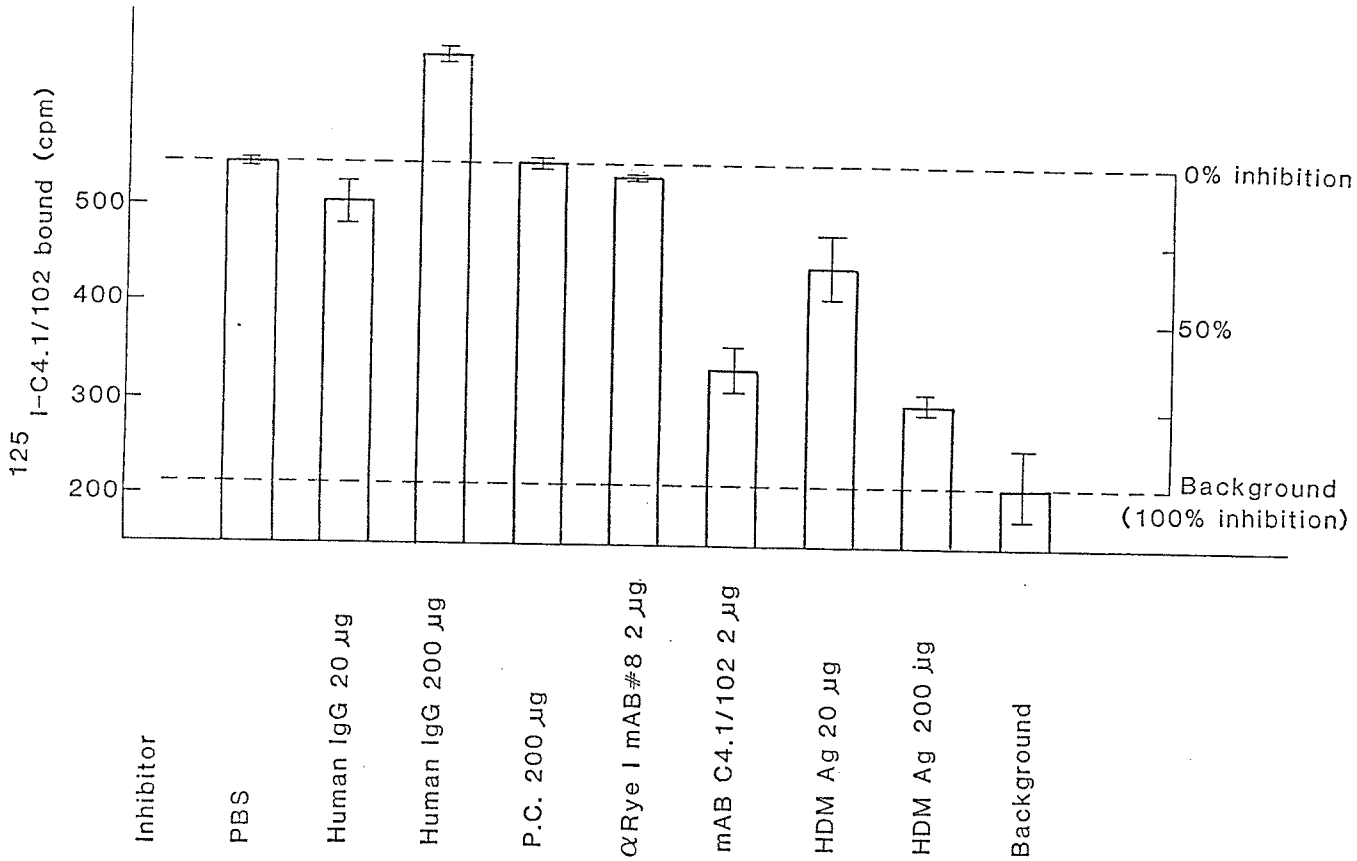

Figure 12: Specificity of purified auto-anti-Id of serum W77.

The auto-anti-Id of serum W77 was purified by sequential affinity chromatography on SpA-4B and Mab C4.1/102-Affi-gel. The experiment was repeated with similar results.

The background was 220 cpm.

Samples were tested in duplicate.

Discussion

In the present study, human auto-anti-Id antibody against P1 specific antibody was detected by means of a sandwich radioimmunoassay, employing a P1 specific Mab both for coating the plates and as the labeled second antibody. The assay was performed in the presence of a large excess of normal mouse IgG, in order to avoid false positive results due to the presence of anti-mouse IgG in some human sera. There are several arguments supporting the validity of this assay to detect auto-anti-Id. First, the anti-idiotype antibody was at least bivalent and had at least two identical combining sites which could bind to the idiotype. When the sera were incubated in the wells coated with anti-P1 Mab, the anti-Id would bind to the Id of the Mab on the well, and possibly still had a free arm which would later bind to the idiotype on the radioiodinated Mab in the solution. The Mab on the solid phase should be in limited quantity, otherwise both arms of the anti-Id would be occupied by Id of Mab on the solid phase, and could not bind radiolabeled Mab. This was proven to be true according to Figure 3. Five µg/ml of C4.1/102 for coating gave the highest signal, and when the concentration further increased, the signal decreased. Second, crossreactive idiotypes (CRI) between species had been documented in several cases. Karol et al (1977) reported that the anti-human sickle cell hemoglobulin antibody from a goat and from a sheep shared a CRI. CRI was also detected between murine and human anti-phosphoryl choline antibodies (Reisen 1979; Bach et al 1981),

between murine and human anti-Timothy allergen antibodies (Malley et al 1983a), between murine and human anti-DNA auto-antibody (Morgen et al 1985), and between murine and human anti-acetylcholine receptor (AChR) antibody (Dwyer et al 1983). In the last case, Mab against AChR was successfully employed to isolate human auto-anti-idiotypic antibody against anti-AChR antibody. In the present study, the anti-Pl Mab could competitively inhibit the binding of human anti-Pl antibody to Pl, and the human anti-Pl antibody would competitively inhibit the binding of Pl by Mab (Chapter II). This suggested that the anti-Pl Mab and human antibody were recognizing the same epitope on the Pl molecule, and therefore had a strong possibility of sharing crossreactive combining site idiotypes. It was understood that the human anti-Id detectable by the sandwich RIA could be against idiotypes of mouse anti-Pl antibody in or remote from the combining site. However, if the binding of human anti-Id with mouse Id could be inhibited by HDM antigen, the human anti-Id could be assumed to bind the combining site idiotype of mouse Mab, and logically was against the combining site idiotype of human anti-Pl antibody as well. The human anti-Id could therefore be considered as auto-anti-idiotype antibody. In a short summary, the rationale of using the Mab SRIA to detect human auto-anti-Id was that anti-Id was bivalent or multivalent, and that anti-Pl Mab and anti-Pl human antibody, most likely, carried crossreactive combining site idiotype. There were several advantages to use Mab over using polyclonal human anti-Pl

antibodies in SRIA. Firstly, sufficient quantity and purity could be achieved with Mab. Secondly, Mab was monospecific, and it could distinguish anti-Id antibodies against different Id.

Six in 101 sera from HDM allergic patients, 1 in 26 nonatopic donors and 0 in 86 cord sera (which were negative in anti-HDM IgG antibodies), were shown to contain anti-idiotypic antibody against one clone of P1 specific Mabs. The possibility of false positive results caused by anti-mouse Ig in human sera was excluded by the fact that a large excess of normal Balb/c mouse Ig was added to ^{125}I-C4.1/102 (which was also from Balb/c), and by the fact that those positive sera only bound to Mab C4.1/102 but not to the other 3 anti-P1 Mab (A2.3/191, C3.6/104 and B2.1/81) from different families (Table I). To our knowledge, the auto-anti-Id reported so far are all of the IgG class (Abdou et al 1981; Geha 1982; Cunningham-Rundles 1982; Dwyer et al 1983; Bose et al 1984; Troisi et al 1985), and in this study, the auto-anti-Id from serum W77 was also found to be an IgG. The cord sera selected were all negative in anti-P1 IgG antibodies. It was therefore reasonable to consider these cord sera as equivalent to nonatopic adult sera, and to group them together. Under these conditions, the frequency of auto-anti-Id positive sera in HDM allergic donors was 5.9% (6/101), and that in nonatopic donors was

0.89% (1/112). According to the four-fold table exact method analysis of the frequencies of the two groups of sera, the one-tailed P value was 0.0445, indicating that the difference was not statistically significant with the present sample size. However, there seemed to be a tendency that the HDM allergic sera had a higher frequency of detectable anti-Id compared to nonatopic sera. The difference might become significant statistically if the sample size increased. Because of the huge repertoire of antibodies, the level of anti-Id was supposed to be very low under natural conditions. According to Jerne's theory (1974), when the immune response against a certain antigen is launched, the elevated specific antibody could then stimulate the production of anti-Id antibodies. This seemed to be true because most auto-anti-Id were observed in occasions where Id was elevated, such as in individuals hyperimmunized with TT (Geha 1982), or desensitized with rye grass pollen (Bose et al 1984). Therefore, it was conceivable that the HDM allergic individuals, who had an elevated anti-Pl antibody, would have a higher possibility to develop detectable auto-anti-Id.

The auto-anti-Id in serum W77 from an HDM allergic patient was characterized in detail. When this serum was fractionated by ion exchange chromatography, the anti-idiotype antibody was located in the fraction containing mainly IgG plus a small amount of IgM and IgA as shown by SDS-PAGE and Ouchterlony analysis. The auto-anti-Id activity of W77 serum could be absorbed on and eluted from SpA-4B and C4.1/102

Affi-gel. The anti-Id sequentially purified by these two immunosorbents was proven to be an IgG as shown by SDS-PAGE and Ouchterlony analysis. This is in accordance with most of the already mentioned observations. The specificity of the auto-anti-Id from W77 serum was thoroughly studied. It could be absorbed by and eluted from C4.1/102 Affi-gel, but not by other unrelated Mab such as anti-prolactin antibody coupled to Affi-gel (Fig.7). This further proved that the anti-Id was not against constant region or framework determinants of mouse Ig but against idiotype, because anti-prolactin Mab was also raised from Balb/c mice, and it only differed from anti-Pl Mab in its hypervariable region. The inhibition test showed (Fig.10) that the binding of auto-anti-Id to Mab C4.1/102 could be specifically blocked by 20 µg of crude HDM antigen, but not by the same amount of unrelated antigens, such as lipopolysaccharide or L-α-phosphoryl choline. This strongly suggested that the anti-Id was recognizing the combining site idiotype of the Mab, and that the anti-Id was the internal image of an epitope on Pl. Since the crossinhibition study in the second Chapter suggested that anti-Pl Mabs and human anti-Pl antibodies might share a crossreactive combining site idiotype, the human anti-Id against Mab combining site idiotype was then logically also against the combining site idiotype of some of the human anti-Pl antibodies, and could be considered as true auto-anti-Id antibodies. The binding of anti-Id with radiolabeled anti-Pl Mab C4.1/102 could be inhibited by autologous Mab C4.1/102, but not by unrelated anti-Rye I Mab #8, indicating again

that the binding was idiotype specific. Most interestingly, the binding could not be inhibited by anti-P1 Mab from families other than C4.1/102. According to the crossinhibition study in Chapter II, the anti-P1 Mabs of the 4 families were all crossreactive to a certain extent, but not completely. A model was then proposed that the epitopes recognized by different families of Mab were overlapping; that Mabs of different families all recognized a public region on P1, and each family recognized a different additional private region as well; and that the public region plus a private region would constitute a certain epitope. According to this model, it was quite likely that the anti-Id was binding to the idiotope on the part which was complementary to the private region of the epitope of P1, and that therefore clones from other families, which were crossreactive with C4.1/102 to the public region, could not inhibit the binding of anti-Id with C4.1/102.

It was observed that only 5.9% (6 out of 101) of the sera from allergic individuals were shown by the C4.1/102 SRIA to contain auto-anti-Id. Besides, the positive signals in the C4.1/102 SRIA were relatively low. The positive sera only bound 500-900 cpm of ^{125}I-C4.1/102 versus 100-150 cpm of background, indicating that the level of auto-anti-Id was quite low. There are two possible explanations for the low frequency and low level of auto-anti-Id detected in this study. First, the auto-anti-Id we detected was only an internal image of a private region of an epitope on the P1

molecule, and only C4.1/102 but no other Mabs recognized this region. It was likely that in human, only a small fraction of anti-Pl antibody of a given individual recognized this private region. Second, although the natural exposure to HDM antigen could elicit high titres of IgE anti-Pl antibody in allergic individuals, the absolute concentration of anti-Pl antibody of all classes of antibodies is still very low (when compared to other artificial or pathological conditions reviewed above where auto-anti-Id were found), and can not elicit a great auto-anti-Id response. Consequently, in most cases, the auto-anti-Id level was so low that it was not detectable with the present method.

This study suggests that spontaneously occurring auto-anti-Id exists in HDM allergic individuals as a result of idiotype-anti-idiotype interactions. This provides an additional support to Jerne's immune network theory with a model of human allergic disease, about which few studies have been carried out. The auto-anti-Id was detected in 6 out of 101 allergic and 1 out of 112 nonatopic individuals, and is therefore directed against a crossreactive idiotype antibody to Pl in these individuals. This crossreactive idiotype does not seem to be a dominant one due to the low frequency of the corresponding auto-anti-Id. The significance of these observations is obvious in view of the previous reports that the primary and secondary IgE response in animal models could be modulated

through the manipulation of idiotype-anti-idiotype interactions. The present observation of the existence of auto-anti-Id is the first step that might lead to further study of the regulatory effects of anti-Id in HDM allergy in human.

CHAPTER V

GENERAL DISCUSSION

Abbreviations used in this Chapter:

Anti-Id	anti-idiotype antibody
BPO	benzylpenicilloyl
BSA	bovine serum albumin
CRI	crossreactive idiotype
HDM	house dust mite
IgE	immunoglobulin E
KLH	keyhole limpet hemocynine
Mab	monoclonal antibody
OA	ovalbumin
Pl	the major allergen of house dust mite (D. pteronyssinus)
Rye I	rye grass pollen allergen I
TNP	trinitrophenyl

According to Jerne (1974), idiotype and anti-idiotype interactions are in a dynamic equilibrium and the intruding of foreign antigen will tilt the balance and push the immune system to a new state of equilibrium. One implication of this theory is that anti-idiotypes play a regulatory role in the immune response. This model has now been supported by a large body of evidence which has been reviewed in Chapter I. Anti-Id, auto-anti-Id, and spontaneously occurring auto-anti-Id have been documented in animal as well as in human models (Oudin et al 1963; Cowdery 1981; Eisenberg et al 1985; Geha 1982; Bose et al 1984).

In several studies, the anti-Id was indeed shown to have regulatory, and mainly suppressive effect (Eichmann 1974; Hahn 1984; Saxon et al 1984). Atopy is a category of disease in which there is an abnormal production of IgE antibodies to minute amounts of airborne antigens. To my knowledge, only three studies have analysed idiotype and anti-idiotype interactions in relation to IgE antibody production or to allergic diseases. Blaser et al (1982a) documented that anti-Id could suppress the in vivo production of IgE to BPO and PC in mice. Malley et al (1982) reported that the in vivo production of IgE against Timothy grass pollen antigen B could be suppressed by administration of anti-Id in mice. Bose et al (1984) reported the existence of auto-anti-Id against anti-Rye I antibody in the serum of rye grass pollen sensitive patients.

The purpose of the present work was to examine the possibility that the serum of HDM allergic patients contain auto-anti-Id specific to public idiotype(s) on IgE/IgG antibodies to HDM. In the first part of the study, we prepared Mabs against Pl, the major allergen of HDM. Nine anti-Pl Mab were tested in crossinhibition assays for their epitope specificity, and 4 families of Mabs were defined according to the extent of their crossreactivity. These 4 families are crossreactive but not identical. Based on these results, we postulated that each Mab recognizes a public region on Pl, which is also recognized by Mab from other families, as well as a private region which is recognized only by the members of the same family. According to this view, the public region plus a private region would constitute an epitope on a Pl molecule. Based on the results of crossinhibition assay of the Mabs, the topography of the epitopes was depicted in a linear map, and was postulated in a two-dimensional map. The crossinhibition study between Mab and human anti-Pl antibody showed that Mab could inhibit to a considerable extent the binding of Pl to human IgE antibody, and that human anti-Pl antibodies could inhibit to an even greater extent the binding of Pl to Mab. This strongly suggests that anti-Pl Mabs and at least a considerable portion of human anti-Pl antibody are recognizing the same epitopes on the Pl molecule. This further suggested that these Mab and human anti-Pl were likely to have identical combining site idiotypes.

During the course of the present study, it was found that the commonly employed inhibition assays for detecting anti-Id carrying internal images have many pitfalls. During the process of absorption of idiotype from the sample, the antigen might leak into the sample. Besides, some macromolecules in serum might also interfere with the binding of antibody to antigen. All these might cause false positive inhibitions. Adequate control should be designed to eliminate those possibilities. A binding assay rather than an inhibition assay will be more convincing in demonstrating the existence of anti-Id.

In the last part of the study, anti-Pl Mabs were used as probes to detect human anti-idiotypic antibodies. We found that 5.9% of the atopic sera and 0.89% of the nonatopic sera contained auto-anti-Id. One such auto-anti-Id was fully characterized. Its binding to Mab could be specifically inhibited by HDM antigen, indicating that this anti-Id expresses the internal image of a Pl epitope. Moreover, the binding of auto-anti-Id to Mab C4.1/102 could be inhibited only by the autologous Mab, but not by anti-Pl Mab from other families, suggesting the anti-Id is mimicing the private region of the epitope recognized by anti-Pl Mab C4.1/102. This demonstration of the existence of auto-anti-Id in HDM allergic individuals provides further support to the immune network theory, and might be viewed as the first step of projects aiming to analyze the possible role of anti-Id in the regulation of HDM-specific IgE antibody production.

In the present study, the anti-Ids binding to C4.1/102 Mab were detected only in 5.9% of the HDM atopic individuals. Even if we assume that all these anti-Ids are spontaneously occurring auto-anti-Ids, they do not seem to be against a dominant crossreactive idiotype (CRI) of human anti-Pl. So, a continuous search for such CRI in human anti-Pl antibody is necessary, because when we try to use anti-Id to regulate an immune response, it will be desirable and more practical to have one particular anti-Id which is against a dominant CRI and consequently might be capable of regulating anti-Pl IgE response in many individuals.

According to our observation, there are several epitopes on the Pl molecule, which share a common or "public" region but differ from each other at a private region, while Chapman et al (1985) considered that there are at least two distinct epitopes on the Pl molecule. In any case, the human IgE response to Pl is diverse in terms of epitope specificity. Therefore, it seems that a large panel of anti-Id would be needed to modulate the whole array of anti-Pl IgE antibodies assuming that anti-Id can indeed be used to downregulate the IgE antibody production. Krieger et al (1983) showed that the immunization of mice with Mab against a segment of BSA would lead to the production of anti-Id as well as anti-BSA antibody against another segment of BSA. As suggested by Bona et al (1984), a possible explanation would be that the anti-Id recognized a regulatory idiotype which is expressed on antibodies with different specificity, and that

such anti-Id is capable of modulating the production of all the antibodies which carry this regulatory idiotype even if they are of different specificity. Therefore, it might be a better idea to look for regulatory Id on the anti-Pl antibody, and to try to modulate the production of a panel of anti-Pl antibodies with a single anti-Id against regulatory Id. Obviously, the regulatory Id is not in the combining site of anti-Pl antibody since it is supposed to exist in antibodies with different epitope specificity. So, an in vitro or in vivo functional assay is to be designed to screen for such a regulatory Id.

Blaser et al (1982a) reported that when anti-Id against antibodies specific for carrier protein OA was administered, the production of IgE against BPO or PC, which was conjugated to the carrier was suppressed. By the same token, it will be very useful to screen out from the Pl molecule a possible epitope(s) which functions as a carrier epitope, and try to modulate antibody response to all other Pl epitopes, by the administration of anti-Id against antibodies specific to this functional carrier epitope.

Theoretically, there are two basic approaches to modulate IgE anti-Pl response in human by means of anti-Id: (i) the passive administration of anti-Id and (ii) the induction of in vivo anti-Id production. Although in most studies reported so far, anti-Id downregulate the immune response (Blaser et al 1983; Kennedy et al

1984a; Geha 1984; Saxon et al 1984), in some cases, anti-Ids were reported to have an enhancing effect (Kennedy et al 1984b; Ertl et al 1984a,b; Sharp et al 1984). The effect, i.e., enhancing or suppressing, seems to depend on the dosage and/or the subclass of anti-Id in some instances (Eichmann 1974; Kelsoe et al 1980). In such a situation where the mechanism of anti-Id regulation is still not totally clear, the effect of anti-Id should be closely monitored no matter whether "passive" or "active" immunotherapy with anti-Id is considered.

Passive administration of anti-Id refers to the direct administration of anti-Id preparation to persons with HDM allergy or in high risk of HDM allergy. The anti-Id could be prepared from anti-Id positive human serum. This approach is not very practical due to the low level of spontaneous auto-anti-Id. Human anti-Id monoclonal antibodies may be ideal, except the technique of preparation of human monoclonal antibodies still needs to be perfected. Alternatively, mouse Mab anti-Id can be prepared in large quantities and high purity from Mab anti-Pl antibody. However, the human recipients of mouse Mab will possibly develop anti-mouse antibodies (Miller et al 1982; Lowder et al 1984), and the effect of anti-Id will be greatly interferred. The most immunogenic part of a xenogenic Ig is the Fc portion, so we can simply use $F(ab')_2$ of the Mab anti-Id to modulate the anti-Pl immune response in human, since Geha (1984) and Saxon et al (1984) reported that $F(ab')_2$ of anti-Id

is effective in tetanus toxoid system. There is some controversy about the Fc dependence of the anti-Id function. Eichmann (1974) and Pawlak et al (1973) reported that the anti-Id function was Fc dependent. To circumvent this problem, a chimeric antibody, which is composed of V region of mouse anti-Id against Pl specific antibody, and C region of human Ig, can be constructed by genetic engineering, and then administered to humans. Chimeric antibodies of this nature have already been successfully prepared by Neuberger et al (1985) and Boulianne et al (1985) by recombinant DNA technique. The former constructed chimeric antibodies composed of Fab of mouse antibody against 4-hydroxy-3-nitro-phenacetyl, and Fc of human IgE; and the latter constructed a chimeric IgM composed of V region of mouse Ig against trinitrophenyl (TNP) and C_κ and C_μ of human Ig.

A potential risk of administering anti-Id to HDM allergic individuals is that if the anti-Id mimics a Pl epitope, it might trigger the release of histamine and other mediators in the HDM allergic individuals. This can be circumvented by administration of anti-Id directly to neonates or indirectly to neonates via maternal routes when the neonates are considered as high risk individuals. This approach is safe because the newborn has no IgE anti-Pl, therefore, anti-Id can not trigger allergic systems or anaphylaxia, even though it may mimic the epitopes of allergen. This approach is worth a trial, because it has been proven to be effective in suppression of specific idiotype antibodies in several systems.

Treatment of Balb/c neonates with a Mab anti-Id against T15 idiotype could readily result in T15 Id specific unresponsiveness (Pollock et al 1982). Furthermore, injection of a Mab anti-T15 idiotype antibody into the pregnant mice prior to birth permanently stifled the T15 component of the humoral anti-PC response in the progeny (Pollock et al 1984). Rothstein et al (1984) reported that in the arsonate system, administration of anti-Id to maternal A/J mice could induce idiotope specific suppression in the offspring.

The actively induced anti-Id can also possibly regulate the specific IgE response. Blaser et al (1982) reported that Balb/c mice, which received PC specific myeloma protein T15 and later produced anti-Id to T15, would develop a markedly reduced IgE response to PC upon immunization against PC-KLH with a procedure known to induce high titre of anti-PC IgE. Therefore, the anti-P1 antibody which carries the idiotype (ideally the regulatory idiotype or crossreactive idiotype), could be administered to the HDM allergic individuals or to the pregnant women whose infants belong to the high risk group, with a view to inducing auto-anti-Id which will in turn downregulate anti-P1 IgE response.

It is understood that many basic mechanisms of Id-anti-Id regulation need to be clarified first and that a very basic question: "To what extent is the network regulating the immune response?" is to be examined before we can conclude that the manipulation of the Id-anti-Id network is an effective way to prevent or control the HDM allergy.

Contributions to knowledge

In the present study, we report the production and the characterization of Mabs against P1, the major clinically important allergen of HDM. These Mabs react with epitope(s) close or identical to those recognized by human IgE antibodies. Crossinhibition studies aiming to determine the fine specificity of the Mabs led to the formulation of an original hypothesis, i.e., an epitope may be comprised of a "public" and of a "private" region. Most importantly, this study is the first to document the spontaneous occurrence of human auto-anti-idiotypes specific to anti-P1 antibodies. The data also indicate the existence of crossreactive idiotypes on both mouse and human anti-P1 antibodies. The identification of such auto-anti-Id in HDM allergic patients suggests that it might be possible to manipulate the immune network with a view of downregulating the IgE antibody response to HDM antigen in man.

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