INTERNAL IMAGE ANTI-IDIOTYPIC ANTIBODY INDUCED IMMUNE REGULATION OF ANTIBODY RESPONSES TO ALLERGEN *Lol p*IV

BY

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENT

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF IMMUNOLOGY

UNIVERSITY OF MANITOBA

WINNIPEG, MANITOBA

OCTOBER, 1992

ISBN 0-315-81766-6

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

TO MY BELOVED MAM AND DAD

PREFACE

As partial fulfilment of the requirements for the degree of Doctor of Philosophy in Immunology, I present this dissertation to the Graduate Study of the University of Manitoba of Canada.

Nature is always fashionable, so is the immune system. The characteristics by which antibody molecules recognize antigens and are recognized in turn as antigens by other antibody molecules and so on constitutes the principle of Jerne's network theory that controls the immune response to a given antigen stimulation.

In the course of scientific advancement, no scientific truth can be regarded as permanent; even well-established principles are modified by subsequent discoveries. The idiotype network is a very complex system, the intended studies are, therefore, focused on a narrow aspect of the nature of the network: my interest is to investigate whether the anti-idiotypic antibody can modulate, in a murine model system, the antibody responses to allergen *Lol p*IV.

ACKNOWLEDGMENTS

I would like to sincerely thank Dr. Fred T. Kisil for his kind support and supervision over the years.

I would also like to thank my committee members for their suggestions and constructive criticisms of the projects.

I would like to express my gratitude to the Manitoba Health Research Council for the Postdoctoral Fellowship Award granted to me during my research studies from 1991 to 1992.

I am very grateful to the Canadian Society for Immunology for the travel awards granted to me to attend the 6th Spring Meeting of the Canadian Society for Immunology in Mont Gabriel, 1992 and attend the VIIIth International Congress of Immunology, Budapest, 1992.

I thank the American Academy of Allergy and Immunology for the travel awards granted in 1990, 1991 and 1992.

Finally, I thank Dr. E. Rector for performing the FACS analysis.

ABBREVIATIONS

Ab1: the antibody used to induce the anti-idiotypic antibody.

Ab2: anti-idiotypic antibody: antibody produced to Ab1.

Ab3: anti-anti-idiotypic antibody: antibody produced to Ab2.

ABTS: 2,2'-azino-di(3-ethyl-benzthiazoline-6-sulfonic acid.

Ag^+: antigen positive: the specificity possessed by antibodies to antigen Site A of *Lol p*IV.

Anti-Id: anti-idiotypic antibody against idiotope(s).

B1/1: an internal image anti-idiotypic monoclonal antibody generated to mAb91.

BBS: borate buffered saline.

BSA: bovine serum albumin

EDAC: 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide.

ELISA: enzyme-linked immunoassay.

HRP: horseradish peroxidase.

Id: idiotope(s) or idiotype(s).

Id^+: Id possitive (= Id91)

Id91: Id possessed by mAb91.

IdI: a private Id.

IdX: a common or shared Id.

KBG: Kentucky bluegrass pollen allergen.

KLH: keyhole limpet hemocyanin.

*Lol p*IV: Ryegrass (*Lolium perenne*) pollen allergen.

MHC: major histocompatibility complex.

OA: ovalbumin.

SDS: sodium-dodecyl sulfate.

ABSTRACT

Idiotypes are unique antigenic determinants which are attributed to the particular amino acid sequences expressed in the variable regions of an antibody molecule or a T cell receptor. The idiotype network theory of immune regulation proposed by Jerne implies that an antibody response to an antigen is controlled by a series of idiotype-anti-idiotype interactions. The expression of a given idiotype (Id) of an antibody (Ab1) is under the control of the corresponding anti-idiotype (anti-Id or Ab2). In turn, the anti-Id can itself be regulated by another set of antibodies referred to as an anti-anti-idiotype (Ab3). These complex sets of interactions operate via feedback mechanisms to control the formation of antibodies to a given antigenic stimulation. Studies were undertaken to investigate the role of anti-Id in the modulation of the formation of antibodies to allergen *Lol p*IV.

The studies focused on an idiotype of monoclonal antibody mAb91 with a specificity for an epitope of *Lol p*IV. For this purpose, a monoclonal anti-idiotypic antibody or Ab2 (designated as B1/1) was produced against the mAb91 by recognizing the Id91 of mAb91. B1/1 recognized an Id which was present on other monoclonal antibodies with the same specificity as mAb91 and on antibodies in mouse, human and

rabbit antisera to *Lol* pIV. The Id91 was located within or near the antigen combining site of the antibodies. These characteristics served to identify Bl/1 as an "internal image" anti-Id which has the capacity to mimic functionally the antigen.

The effects of the internal image anti-Id Bl/1 on the formation of antibodies in a murine model system, were examined in relation to the dose of Bl/1 administered and the interval of time between their immunization with allergen *Lol* pIV. It was shown that treatment of syngeneic Balb/c mice by a single i.v. injection with Bl/1, at a dose from 100ng to 100μg/mouse, or up to 8 weeks prior to the challenge with the allergen *Lol* pIV, induced (i) suppression of the formation of antibodies to *Lol* pIV and (ii) suppression of the expression of the Id91. By contrast, following the treatment with Bl/1 at the dose of 10ng/mouse, a slight elevation of levels of antibodies to *Lol* pIV was observed.

The further studies have shown that the suppression of the antibody responses to *Lol* pIV could be adoptively transferred with the spleen cells from the Bl/1-treated donors to syngeneic recipients. Moreover, it was found that the B cells (but not T cells) from the Bl/1-treated donors that were able to transfer the suppressive effect. These B cells carried presumably membrane-associated molecules which were shown to

possess the specific characteristic of the anti-Id

The immunogenicity of B1/1 was examined for its ability to induce the formation of Ab3. Interestingly, whereas *Lol* pIV induced the formation of both IgE and IgG1 antibodies, B1/1 induced primarily the formation of antibodies of the IgG1 class. In either case, the antibodies possessed the Id91. Monoclonal Ab3s were generated to (i) determine whether the Id of Ab3s is same or similar as that of mAb91 and (ii) evaluate the fine specificity of Ab3s for their binding to allergen *Lol* pIV. It was shown that three mAb3s possessed the same or similar Id as of mAb91 and one of mAb3s also recognized *Lol* pIV. Therefore, the anti-Id B1/1 may serve as a surrogate antigen for immunotherapeutic purpose.

Collectively, these studies established that an anti-idiotope with a well defined specificity may be involved in regulating through the idiotypic network the formation of antibodies to a major pollen allergen such as *Lol* pIV. These studies may also suggest alternate approaches for the treatment of allergies to grass pollen allergens which use anti-idiotypic antibodies for the specific regulation of the antibody responses rather than using the allergen(s).

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CHAPTER 1.

INTRODUCTION

Historical Review:

The immune system of an individual can make millions of different kinds of antibodies: the complex immunoglobulin molecules recognize and bind to antigen through their antigen combining site or paratope. Each antibody can in turn be the target of other antibodies that recognize its unique antigenic determinant. Early hypotheses and investigations on anti-antibodies have been reviewed by Silverstein (Silverstein, A. 1986). The possibility that an antibody might be produced against the antigen combining region of another antibody was suggested by Ehrlich and Morgenroth (Himmelweit, F. 1956) around the turn of the century. They proposed that autoimmunity to red blood cells was prevented by the formation of an auto-anti-lysin that combined with and inactivated lytic antibodies to red cells. Their hypothesis was actually closely related to the "internal image" concept later elaborated by Niels Jerne, because they postulated that the anti-antibody had combining groups or "side chains" equivalent to side chains on red cells. At that time, virtually nothing was known of course about the chemical nature of an antibody.

Idiotypes were first described as unique antigenic determinants (or epitopes) on human myeloma proteins by Lohss and coworkers (Lohss, F., et al. 1953) and Slater and coworkers in the 1950s (Slater, R.J. et al. 1955), who showed

that human myeloma proteins from any one individual possess unique antigenic determinants that are not present on other myeloma proteins or on normal serum immunoglobulins. At that time it was uncertain whether myeloma proteins were representative of the normal immunoglobulin population, so that the general significance of the observation was open to question. This uncertainty was laid to rest in 1963, when the presence of unique determinants was established for antibodies which had been induced by immunization in humans (Kunkel, H.G. et al. 1963) and rabbits (Oudin, J. and Michel, M. 1963). Oudin and Michel, in their work with rabbit anti-*Salmonella* antibodies, used isologous antisera and referred to the relevant antigenic determinants as ***idiotype*** (from the Greek roots for ***individual*** and ***form***). The term, individual antigenic specificity, was used by Kunkel and his collaborators in reference to the unique epitopes on human antibodies that were identified with heterologous (rabbit) antisera. This distinction in terminology was maintained for several years until the terms, idiotype and idiotypic determinant, gradually came into use for specificities recognized by either isologous or heterologous antibodies. In current usage, ***idiotope*** refers to an individual idiotypic determinant and ***idiotype*** to the collection of idiotopes on an individual antibody molecule or T cell receptors. In practical terms, an idiotope can be defined structurally as the region of the molecule that makes contact with an anti-idiotypic antibody. By means of such

antibody-antibody reactions the immune system interacts with itself.

Networks of interactions seem to modulate the normal immune responses. Numerous studies have shown that these interactions, known as idiotype-anti-idiotype (abbr. Id-anti-Id or Ab1-Ab2) reactions, can be exploited to manipulate the immune system (described in the following sections). The possibility could have profound implications for both the vaccine design and therapy of a spectrum of illnesses ranging from infections through many kinds of cancers to autoimmune diseases.

Concept of the Idiotype Network:

1. Structural Basis of Id:

The antibody variable (V) regions, where idiotopes are found, are formed by the juxtaposition of two polypeptide immunoglobulin chains, i.e. heavy (H) and light (L) chains, encoded by genes located on different chromosomes (Malcolm, S. et al. 1982, McBride, O.W. et al. 1982, and Kirsch, I.R. et al. 1982). Each chain is encoded by discontinuous genetic elements that rearrange to form novel immunoglobulin genes during B cell ontogeny (Tonegawa, S. 1983, Seidman, J.G. et al. 1978a, and 1978b). In the embryonic kappa-light chain gene

complex, for example, there are many kappa-V genes (VK) arranged in tandem. Through rearrangement, one VK is juxtaposed with one of five joining segments (JK) to form a VJ complex that encodes the variable region of the kappa-light chain (Brack, C. et al. 1978, Seidman, J.G. et al. 1980, and Hieter, P.A. et al. 1980). The heavy chain complex is arranged similarly except that it has additional genetic elements, named the diversity segments (D), positioned between the VH genes and six functional JH genes (Sakano, H. et al. 1981, Siebenlist, U. et al. 1981, and Early, P. et al. 1980). At least one D segment is juxtaposed with the single JH before rearranging with a VH gene to form a VDJ complex which encodes the V portion of the antibody H chain (Yancopoulos, G.D. and Alt, F.W. 1986, and Reth, M.G. et al. 1986). Comparisons of the primary amino acid sequences of many different antibody V regions reveal four regions of limited amino acid sequence diversity, designated the framework regions (Kabat, E. et al. 1987). Examination of the first three V region frameworks reveals that the human H and L chains may be classified into subgroups. Each V region subgroup has characteristic framework sequences that serve to distinguish it from other V region subgroups. Initially, human H chains were divided into three subgroups, while kappa- and lambda-light chains were divided into four and six subgroups, respectively. Positioned between the framework regions are three segments of extreme hypervariability in both L and H chain sequences (Kabat, E. et

al. 1987). The diversity of the first and second hypervariable regions partly reflects germline DNA-encoded differences between disparate antibody V genes, a diversity often noted even between V genes of the same subgroup. The third hypervariable region is generated in part by the recombinatorial process joining the antibody L chain V gene with the J segment, in the case of the L chain, or the VH gene with the somatically generated DJ segment of the antibody H chain (Tonegawa, S. 1983, Brack, C. et al. 1978, Sakano, H. et al. 1981, Siebenlist, U. et al. 1981, and Early, P. et al. 1980). Somatic events subsequent to V gene rearrangement, however, have been found to play an important role in increasing the diversity noted within these regions. Affinity labelling and crystallographic experiments have substantiated earlier contentions that the hypervariable regions on both chains fold together to form the antigen-combining site (Poljak, R.J. 1975a, 1975b, Padlan, E.A. and Javies, D.R. 1975, Segal, D.M. et al. 1974, and Alzari, P.M. et al. 1988). Hence, these regions of hypervariability are designated the complementarity-determining regions (CDRs) (Kabat, E. et al. 1987).

Each single idiotypic determinant is called idiotope (Id). Then an idiotype denotes a certain set of idiotopes. The patterns of Ids are determined by the same V regions of antibody polypeptide chain (both heavy and light chains) that

also determine the paratopes. Therefore, each Fab arm of an antibody molecule displays one paratope and a set of Ids. It has been demonstrated that the Id can be located either in the antigen combining site (Sirisinha, S. and Eisen, H.N. 1971, and Brient, B.W. et al. 1971) or in the framework region of an antibody molecule (Mudgett, M. et al. 1978). A more precise localization of Id has been obtained by comparison of amino acid sequences of antibodies of given specificities (Clevinger, B. et al. 1980, and Rudikoff, S. et al. 1983), chemical modification experiments (Dickerman, J. et al. 1981), analysis of spontaneous V region variant (Radbruch, A. et al. 1983), and use of synthetic peptides (Chen, P.P. et al. 1984, and McMillan, S. et al. 1983). These studies have indicated that the majority of Id are associated with the CDRs within the V region of the antibody molecules. In particular, the third CDR of the heavy chains has been implicated as an important site for the expression of Id specificities (Kabat, E.A. 1988). In addition, it was classified serologically to study the nature of Id based on recognition by the antibody against the Id (named anti-idiotype or anti-Id or Ab2). Id can be unique to a given antibody or can be shared by antibodies of a given specificity (Carson, D. and Weigert, M. 1973, and Liegerman, R. et al. 1975). A discovery that had a major impact on the structural basis of Id was that of Wu and Kabat (Wu, T.T. and Kabat, E.A. 1970) of hypervariable regions in immunoglobulin. This immediately suggested an association

between Id and hypervariable regions, the diversity of V regions generated during VDJ and VJ recombination and by somatic mutation would permit the expression of large numbers of potential Ids. It also elucidated the structural basis for the association between specificity and Id and for the very large repertoire of Id and antigen binding specificities. Since the term Id defines a collection of light and/or heavy chain V-region associated structures, which can involve any part of the exposed V regions, certain structures will be shared by different immunoglobulin molecules because of the homologies among their V regions. These Ids can be expressed by antibodies with different antigenic specificities as first described by Oudin and Cazenave (Oudin, J. and Cazenave, P.A. 1971).

If the topography of a given Id as detected by an antibody preparation occurs at a high frequency in a given population of immunoglobulins (or Ig receptors on B cells), the Oudin-Cazenave phenomenon will occur to a greater or lesser extent; for example, some determinants expressed by a particular VH or VL region that does not depend on the restricted association of any particular VH with any particular VL, respectively, may be expressed at a high frequency on immunoglobulins not related to the specificity of the immunizing antibody. These kinds of exclusively VH- or VL-associated Ids are not frequently detected although there are

notable exceptions (Prime, D. and Cazenave, P.A. 1988). Furthermore, there are well-documented examples of particular Ids whose interaction with the corresponding anti-Ids are inhibited at the presence of haptens (and therefore, these Ids are binding site related) and which are expressed only when these V regions are associated with particular heavy chain constant regions. Such examples demonstrate that constant region domains can also influence V region structure and topography (Morahan, G. et al. 1983, Nishinarita, S. et al. 1985, and Kiyotaki, M. et al. 1987). It was demonstrated that the presence of appropriate L chains was required for expression of the Id. Possible explanations for this apparent paradox included the possibilities that the L and H chain genetic loci are linked (Blomberg, B. et al. 1972).

The precise interaction between an Id and an anti-Id has been demonstrated by Poljak and coworkers using x-ray crystallography (Bentley, G.A. et al. 1990). They analyzed a conjugate of two Fab fragments, each derived from a mAb. One was specific for hen egg lysozyme and the other was its monoclonal anti-Id. Nearly all of the contacts were between amino acid side chains from the hypervariable regions of the Id and the anti-Id. A few contacts involved side chains from the framework. All six CDRs of the anti-Id and five of the six CDRs of the Id were involved in making the contacts. A total of 15 side chains of the Id and 13 side chains of the anti-Id

participated in the contact regions. It was of interest that without prior knowledge of their sources, one could not distinguish the Id from the anti-Id. Because this was the only example of its kind so far, it remains to be seen whether amino acid residues from the framework may participate to a larger extent in the structure of some Ids.

2. Classification of Idiotopes:

There are two types of Ids which can be classified serologically based on their recognition by the anti-Id. The first category of Id is referred to as a private or individual Id (IdI) and the second type is denominated as the common or cross-reactive Id (IdX). The third type of Ids is the regulatory Id (ri) which is defined by its functions. The classification of these Ids are summarized in Table I.

The definition that an Id was considered to be an entirely unique marker for a myeloma protein or for antibodies of a given specificity from an individual rabbit, broke down in the late 1960's when IdX were found initially on human cold agglutinins. This work was done by Kunkel and coworkers (Williams, R.C. et al. 1968). They found that many cold agglutinins from different individuals share some although not all Ids. Shortly afterward, IdX were observed in mouse antibodies to phosphocholine, by Cohn and his colleagues

Table I.

Classification of Idiotopes

Characteristics	Classes of Ids[1]		
	IdI	IdX	RI
a. Genetic control:	somatic mutation	germ-line gene	?
b. Frequence of recurrence:	very low	high	very high
c. Presence on antibodies:	not shared by individuals of same species	shared by individuals of same or different species	may not be shared
d. Functions:			
(1) as genetic marker:	yes	yes	yes(?)
(2) to elicit Ab2:	Ab2α	Ab2β	Ab2α
(3) as vaccine:	yes	yes	?
(4) as immune regulator:	?	?	yes

1. IdI: private Id

   IdX: common or shared Id

   RI: regulatory Id

(Cohn, M. et al. 1969) and by Eichmann (Eichmann, K. and Kindt, T.J. 1971) in rabbit antibodies to streptococcal carbohydrates from partially inbred families of rabbits. IdX have since been identified in antibodies of various specificities in inbred strains of mice, rats and even in interspecies (e.g. between human, mouse and rabbit).

An Id that I have identified in the studies reported in this thesis is an IdX present on a monoclonal antibody (mAb), designated as mAb91, specific for Ryegrass (*Lolium perenne*) pollen allergen *Lol p*IV. This IdX is named Id91 whose characteristics will be discussed in Chapter 2.

The availability of IdX opened up many avenues of research. However there are many private Ids (IdI), that have a very low frequency of recurrence. Obviously, there is a dramatic difference between IdX and IdI first described by Kunkel and Oudin. The probable explanation is that IdX are encoded by germ-line genes, which can generally tolerate some somatic mutation without loss of the Id. In contrast to IdX, IdI probably reflect random somatic mutations that have a low probability of being repeated in different mice of the same strain, or even among antibodies of the same specificity. The very private nature of Id in human myeloma proteins (Kunkel, H.G. 1970) suggests that many of them are encoded by genes

that have undergone numerous somatic mutations.

Regulatory idiotope (ri) is an Id that is present on a relatively high proportion of antibodies and/or lymphocytes that is capable of acting as a site for a receptor-specific regulatory system. This concept, initially proposed by Paul and Bona (Paul, W.E. and Bona, C.A. 1982), suggests that a special class of Ids exist with unique regulatory functions before antigenic stimulation. Numerous studies have implicated ri as a model for receptor-based regulation of the immune response, in that a given ri can be associated with cells producing antibodies of different specificities (Bona, C.A. et al. 1984). Therefore, a given antibody molecule can possess paratopic, idiotopic, and ri sites within its V region. During the course of a conventional immune response, it is hypothesized that no ri are recognized on antibody and/or lymphocytes, and the Id network consists of only an antibody response to the conventional (normal) Id. A noninternal image anti-Id (discussed below) may be specific for a ri on Ab1, rather than a conventional Id. Immunization of a host with a noninternal image anti-Id should lead to the expansion of cells bearing complementary receptors.

3. Classification of Anti-Id (Ab2):

There are four kinds of anti-Id or Ab2 that can be

distinguished by serological means. They are Ab2α , Ab2β , Ab2γ , and Ab2ε . Originally, Jerne classified Ab2 into Ab2α and Ab2β (Jerne, N.K. et al. 1982) and an alternative classification was proposed by Bona and Kohler (Bona, C.A. and Kohler, H. 1984) that divides the Ab2β class into Ab2β and Ab2γ subgroups.

(1) Ab2α recognizes an Id that is not in the antigen combining site of Ab1 and usually recognizes an IdI or ri.

(2) Ab2β , also referred to as internal image anti-Id, recognizes an Id that is within the antigen combining site of the antibody. It has the capacity to mimic the antigen used to generate the Ab1 and can substitute for the antigen in inducing an anti-antigen response. In addition, the Ab2β recognizes an IdX on Ab1 preparations from the same and even different species.

(3) The Ab2γ subclass also recognizes an Id that is within the antigen combining site but is not an internal image anti-Id.

(4) Another peculiar Ab2 is Ab2ε , also called epibody, that was described by Bona et al. (Bona, C.A. et al. 1982). The Ab2ε reacts not only with the Id on a monoclonal human anti-IgG autoantibody (a rheumatoid factor) but also with the

Fc of human IgG. The structural basis of such an epitope recognized by an Ab2α is a Ser.Ser.Ser. sequence which is shared by the IgM rheumatoid factor light chain and the Fc portion of IgG (Kipps, J. et al. 1988).

4. Idiotype Cascade:

In the studies that established the concept of Id and anti-Id, experimental animals were challenged with antibody generated in other experimental animals. Do similar interactions occur naturally, within the immune system of the same individual? In the early 1970's Niels Kaj Jerne proposed that they do (Jerne, N.K. 1974), a network of Id and anti-Id regulates the immune response. Jerne suggested that Id are displayed on antibodies and the surface receptors of B and T cells. When, in response to an antigen, B and T cells proliferate and antibody levels rise, the concentration of Id increases. His ideas were based on the observed extreme heterogeneity of V region structures of immunoglobulins and he reasoned that each immunoglobulin molecule not only expressed a paratope for a particular antigen but an Id which could be recognized by other antibody (i.e. the anti-Id). The body is normally tolerant to molecules of its own manufacture: it does not mount an immune response against them. However, Jerne proposed that individual Id ordinarily exist at a level too low for tolerance to develop. Hence the rising concentration

of Id stimulates the proliferation of other sets of lymphocytes bearing anti-Id receptors. The anti-Id will regulate the initial immune response. Jerne suggested that they do so either directly, for example, by binding to the Id-bearing antibodies and inactivating them, or indirectly, by binding to Id on the surface of T cells that regulate other lymphocytes. The effects of anti-Id on the immune response may be either stimulatory or suppressive.

The interactions triggered by an antigen, Jerne proposed, do not end with the anti-Id, since anti-Id themselves bear Id. The huge diversity of antibodies has the result that every determinant in an Id is recognized by some other antibodies. Thus, the anti-Id evoke anti-anti-Id or Ab3, which stimulate the production of anti-anti-anti-Id, or Ab4, and so on. The initial antigen thereby triggers a far-reaching perturbation in complex network. This idiotype cascade has been studied in Bona's laboratory (Bona, C.A. et al. 1981). A mAb that was specific for a bacterial polysaccharide served as the Ab1; Bona injected it into the mice to produce an Ab2, which he isolated and reinjected into the animals. In this way he elicited an Ab3 and then, by repeating the cycle, an Ab4.

A cascade of Id-anti-Id T cells, as counterparts of a cascade of anti-Id, have been described by Nisonoff and his

colleagues (Germain, R.N. et al. 1981) for the antibody response to the azobenzenarsonate (ABA) epitope and in the delayed-type hypersensitivity (DTH) reactions. The biologic function of the idiotype cascade will be discussed in later sections.

Applications of Id and Anti-Id

1. Applications of Id:

Since identification of an Id is approached by using the corresponding anti-Id and study of an anti-Id is targeting its Id, the applications of Id cannot be separated from that of anti-Id and vice versa.

(1) <u>As a marker for B cell clones:</u> An early example was reported by McDonald and Nisonoff (MacDonald, A.B. and Nisonoff, A. 1970) who immunized rabbits against the *p*-azobenzoate hapten and studied the Id in affinity-purified antibodies as a function of time after immunization. In each of three rabbits, Id identified two months after the start of immunization were still present in comparable concentrations up to four months. After four months they were replaced by new and non-related idiotypic specificities associated with antibodies having increased affinity for hapten. In two surviving rabbits the new Id were detectable for at least one

year. The concurrent presence of a third major Id was observed in one of the rabbits by 17 months. The high degree of specificity and ease of utilization of anti-Id reagents has provided a useful tool for following clones of antibody-secreting cells. A related application of Id demonstrated the capacity of secondary B cell clones, once established, to dominate the immune response to a specific antigen at the expense of primary B cells that would otherwise be expressed.

Recently, Id was used as a clonal marker to provide serologic evidence for interchromosomal H chain isotype switching in a transgenic mouse (Gestein, R.M. et al. 1990). These C57BL/6 mice carry a recombined VDJQμ transgene in which VDJ was derived from a Id^+ (Id positive) anti-azobenzoate hybridoma and Qμ was of Balb/c origin. Upon immunization the transgene dominates the antibody response of these mice; i.e. the antibodies produced express the Id whereas the antibodies to azobenzoate from normal C57BL/6 do not express the Id. To ascertain whether isotype switching of the transgene can occur interchromosomally, transgenic mice were mated with Balb/c and the F_1 offspring was investigated to determine whether, after immunization, individual antibody molecules contained VDJ encoded by the transgene together with C_H regions of Balb/c origin. If so, this would indicate an interchromosomal switch, because the two segments are necessarily on different chromosomes in the F_1 mice. It was found that a substantial

fraction of the antibody population expressed an allotypic marker characteristic of Balb/c IgG1, and that nearly all such molecules were removed from immune serum by an adsorbent specific for Id^+ molecules. Thus, interchromosomal switching had occurred.

(2) As marker to study maturation of the antibody response: Such research is illustrated by work done in the laboratory of Milstein (Griffiths, G.M. et al. 1984) on an IdX associated with Balb/c or DBA/2 antibodies to the hapten, 2-phenyloxazolone. By mRNA sequencing of hybridoma products it was found that on day 7 after initial immunization there were virtually no somatic mutations of the germ-line genes that control the Id-bearing antibodies. By day 14 such mutations were frequent and were accompanied by substantial increases in hapten-binding affinity. Contrary to some earlier reports mutations were observed in IgM as well as IgG anti-2-phenyloxazolone antibodies, demonstrating that isotype switching is not a prerequisite for somatic mutation. The use of Id markers extended earlier work on affinity maturation in B cells to the level of clones of cells in which the same germ-line genes are transcribed.

2. Applications of Anti-Id:

a. $Ab2\alpha$, Noninternal Image Anti-Id:

The most significant application of the Ab2α is that it can induce specific immune responses in given hosts, which has been studied in different systems (Sacks, D.R. and Sher, A. 1983, Francotte, M. and Urbain, J. 1984, Huang, J.-H. et al. 1986, Schick, M.R. et al. 1987, and Zhou, E.-M. et al. 1987). Schick and Kennedy had produced the Ab2α in rabbit against a mouse mAb that recognized the hepatitis B surface antigen (HBsAg) *a* determinant (Schick, M.R. et al. 1987). Mice immunized with the Ab2α alone produced an Ab3 response with anti-HBs activity. This anti-HBs response induced by Ab2α was associated with the silent clones not normally induced during the immune response to the nominal antigen, HBsAg. Activation of silent clones has also been shown during the immune response to human immunodeficiency virus (HIV) glycoprotein (Zhou, E.-M. et al. 1987) and bacterial levan (Bona, C.A. et al. 1981). The activation of this silent Id network which is not induced upon immunization with bacterial levan was associated with the expansion of Id-specific helper T-cells (Hiernaux, J. et al. 1981). Alternatively, an Ab2α preparation might bind to specific Id receptors on suppressor cells and release the silent Id from suppression (Slaoui, M. et al. 1986).

In the response to certain antigens in which a particular Id dominates, there exist other antigen specific B cell precursors which are not expressed, or which constitute a

minority of the response. However, after administration of appropriate anti-Id, these particular precursors can be expanded to make a substantial contribution in the response to that antigen. The immune response to HIV envelop glycoprotein was also induced by an Ab2α (Zhou, E.-M. et al. 1987). Balb/c mice and rabbits were immunized with this mAb Ab2α. Mice produced an Ab3 response that recognized the antigen and this Ab3 containing sera also inhibited the Id-anti-Id reaction, indicating that an Id^+ antibody response was induced. This Ab2α induced a murine Ab3 which expressed an Id that is not normally expressed during the murine antibody response to HIV envelop glycoprotein. Thus, again silent Id clones may have been activated when Balb/c mice were immunized with this noninternal image monoclonal anti-Id. In rabbits, Ab2α immunization appeared to activate antigen-negative (Ag^-) Id^+ clones and resulted in an Ab3 response that failed to bind the antigen, but expressed an Id. Therefore, the Ab2α failed to induce an anti-HIV response across species barriers. The failure of this anti-Id to induce an anti-HIV response across species barriers suggests it has a poor potential as a vaccine. On the other hand, the rabbit Ab3 expressed an Id that was similar to that expressed by the chimpanzee Ab1. By comparison, rabbits immunized with the antigen failed to express an Id similar to that shared by the chimpanzee Ab1 and rabbit Ab3. Within a given host, it appears that noninternal image anti-Id may have the capacity to modulate the Id

response. These results suggest the potential use of noninternal image anti-Id or Ab2α for therapeutic strategies.

b. Ab2β , Internal image anti-Id:

This kind of anti-Id recognizes Id associated with the antigen combining site and is capable of mimicking the three-dimensional conformation of the antigen. It is the internal image anti-Id that draw so much attention from immunobiologists to study its biological function and its potential as vaccines and therapeutic agents for the treatment of various diseases.

(1). Internal image of antigen.

The internal image anti-Id to antibody against antigen X (AgX) might in some cases bear a structural resemblance to the antigen. Obviously, AgX and Ab2β have in common the ability to combine with hypervariable regions of anti-X. This concept was advanced by Jerne on the assumption that because of the apparently unlimited potential for immunoglobulin gene diversity, the repertoire of anti-Id must be very large. Some of the anti-Id might interact with the binding site of an antibody through structures that resemble the large universe of naturally occurring nonself external and self antigen epitopes. This idea received its first experimental support in

1978 by Sege and Peterson (Sege, K. and Peterson, P.A. 1978). They prepared anti-Id in rabbits against rat antibodies to bovine insulin and showed that the anti-Id, which was free of anti-insulin antibodies, completely inhibited the binding of insulin to rat epididymal fat cells. This indicated that the anti-Id was capable of reacting with insulin receptors. In addition, the anti-Id stimulated uptake of α-amino-isobutyric acid by the cells, again mimicking the activity of insulin. These results were interpreted as supporting a structural resemblance of at least one segment of the insulin and anti-Id molecules. Another example of mimicry was the anti-Id to antibodies against thyrotropin (TSH) (Briones-Urbina, R. et al. 1987). TSH is composed of two subunits, α and β, which stimulate thyroid hormone synthesis in thyroid follicular cells. Both subunits are required for biological activity with possible conformational modifications unique to the intact hormone necessary for activity. They found that the anti-Id could stimulate TSH action. This cooperative binding effect could even be seen using TSH-receptor protein separated by SDS-PAGE followed by Western blotting to nitrocellulose. This binding by anti-Id could be blocked by previous incubation of the blot with unlabelled TSH thereby attesting to the specificity of the reaction.

It is not universally accepted that the anti-Id, anti-anti-X, used in such experiments necessarily resemble the

structure of the AgX. Obviously such a resemblance would nicely account for the data. However, an alternative explanation is in general possible. For example, consider anti-anti-insulin that is reactive with the insulin receptor. It seems very unlikely that the insulin receptor would share much of its amino acid sequence with an antibody to insulin. However, all that is needed to explain the results is that the receptor share a small, similar, or identical, structural motif with the Ag-binding site of anti-insulin antibody, perhaps a stretch of amino acids sufficient in length to serve as an epitope. An anti-Id prepared against anti-insulin might interact with this stretch of amino acids in the insulin receptor even if the anti-Id bore no structural resemblance to insulin. It is difficult to distinguish experimentally between these two possibilities, i.e., whether anti-anti-X resembles AgX or the receptor resembles anti-X. However, in a few instances there are data suggesting that anti-Id in fact may resemble the Ag structurally. Perhaps the most striking are the results shown by Greene and his colleagues (Bruck, C. et al. 1986). They demonstrated strong resemblances of amino acid sequences between the anti-Id and type 3 reovirus hemagglutinin. About half of a particular 16-amino acid sequence of the hemagglutinin shows very significant homology with a framework segment of V_H, the remainder is homologous to a V_L sequence, most of which is from the second CDR.

The structural resemblance of anti-Id and Ag was also demonstrated in other systems (Mazza, G. et al. 1985, and Taub, R. et al. 1991). For the Ag, a random copolymer of glutamic acid, alanine, and tyrosine, the D_H region of each of several monoclonal anti-Id contained infrequently observed sequences, Tyr-Tyr-Glu or Glu-Glu-Tyr, closely related to immunodominant sequences of the copolymer. For the auto-anti-Id (obtained after immunization of mice with TSH) to TSH, significant resemblances of amino acid sequence to that of TSH were found in the CDR regions of two anti-Id mAb that were capable of binding to the TSH receptor. Synthetic peptides corresponding to the TSH-related anti-Id sequences inhibited both the binding of the corresponding antibodies to TSH receptors on rat thyroid cells and the stimulation of cAMP production by TSH.

Although these examples strongly support the existence of internal images in anti-Id they do not eliminate the possibility that in other instances the alternative mechanism applies, e.g, resemblance of the anti-X to the receptor. Moreover, it was anticipated that only a small percentage of anti-Id molecules would carry an internal image of the antigen.

(2). Internal image anti-Id as potential candidates for vaccines:

The ability of anti-Id to confer protective immunity in the absence of the nominal antigen constitutes the basis for the anti-Id based vaccine potential. This concept was first proposed by Nisonoff and Lamoyi (Nisonoff, A. and Lamoyi, E. 1981) and Roitt and his colleagues (Roitt, I.M. et al. 1981) and then confirmed by several experimental studies. There are several areas of application in which an anti-Id based vaccine would represent a viable alternative to conventional vaccines. The first advantage is that anti-Id preparations are not infectious and may therefore be useful when a conventional attenuated vaccine has the capability of reverting to a virulent form. This could also apply to killed viral vaccine which may contain potentially harmful products as a result of the production methods. Another advantage is in the situation where adequate amounts of antigen are difficult or impossible to obtain. Efficient *in vitro* tissue culture systems have not yet been developed for the growth of many organisms. A third advantage is apparent when immunity to a single epitope of an infectious agent not only is sufficient but advantageous for protection. Some infectious organisms contain antigenic determinants which cross react with host tissue or body components which can lead to the induction of an autoimmnue response. Conversely, an anti-Id vaccine could induce immunity to a single epitope and thereby bypass a possible deleterious autoimmune response. For the fourth advantage, a protein based anti-Id vaccine could be used to induce immunity to a

nonprotein based antigen. The immune response to polysaccharides, such as those secreted by some bacteria, does not normally develop until late in ontogeny. An anti-Id can mimic the three dimensional structure of a carbohydrate determinant and still be, by its protein nature, immunogenic. The fifth advantage is dependent on the selective nature of Id-anti-Id interactions. By selecting those Id from which the most desirable immune response would develop, the possibility to pre-select those antibodies with, say, a higher or even lower affinity, to a specific epitope, arises. Thus, the qualitative , as well as quantitative aspects of an antibody response can be modulated.

Anti-Id immunization has been used to induce a specific immune response to several viral, bacterial, parasitic, and tumor antigens. Viral systems in which anti-Id immunization alone has been used to induce a specific immune response to the antigen include: (i), hepatitis B virus; (ii), rabies virus; (iii), reovirus; (iv), Sendai virus; (v), herpes simplex virus; (vi), tobacco mosaic virus; (vii), Newcastle disease virus; (viii), polio virus; (ix), influenza virus; (x), coxsackie virus; and (xi), human immunodeficiency virus (Uytdehaag, F.G.C.M. et al. 1986, Hiernaux, J.R. 1988, Zhou, E.-M. and Kennedy, R.C. 1989, and Kennedy, R.C. and Attanasio, R. 1990). The anti-Id-based vaccine to hepatitis B virus surface antigen studied in Kennedy's laboratory is about as

close as anyone has come, to my knowledge, to a practical anti-Id vaccine (Kennedy, R.C. et al. 1986). Their internal image anti-Id protected two chimpanzees against hepatitis.

Of concern with a potential vaccine preparation is its cost per dose, along with its safety and efficacy. Anti-Id have been shown to be safe and effective in protecting a small number of chimpanzees against hepatitis, the relevant animal model for human hepatitis B surface antigen immunization and the virus infection. Although the cost of producing a polyclonal anti-Id can be expensive, monoclonal anti-Id represents a means by which potential vaccine candidates can be produced in a cost-effective manner. A disadvantage of a monoclonal anti-Id, when compared to its polyclonal counterpart, is that the monoclonal anti-Id may only possess the internal image of a single epitope that is contained within a multideterminant antigen. If numerous epitopes are required to induce protective immunity against an infectious organism, a pool of different monoclonal anti-Id may be necessary to induce complete immunity.

The theoretical and experimental considerations discussed in this section suggest that it should be possible to design vaccines to mimic the immunogenic potential of natural pathogens. An ideal vaccine is one that would elicit a durable immune response at both T and B cell levels, against

nonpolymorphic determinants of the surface structure of the natural pathogen. Given that the identity of the immunodominant determinant(s) of any one antigen may vary from one individual to another in an outbred population, it is important that these vaccines induce immunity to as many different immunodominant determinants as possible. Since internal image anti-Id based vaccines can elicit specific humoral or cellular immunity in a non-MHC restricted manner (Sharpe, A.H. et al. 1984), they may indeed have the potential of being effective in confering immunity in individuals with a diverse genetic background. The anti-Id may also result in efficient generation of protective immunity against parasites or viruses which employ antigenic drift as the natural mechanisms of escape from the host's immunity. The observations that antibodies specific for different parasites or virus subtypes bear a similar Id (Liu, Y.N. et al. 1981) support the concept of employing anti-Id vaccines for immunotherapy. However, at the present time it is difficult to predict which among the various means for formulating new generation vaccines will be most advantageous. The internal image anti-Id vaccines will require improved ways to analyse and modify appropriately the V region of antibody molecules of specific interest. Nevertheless, anti-Id have come a long way in a relatively short time in comparison with other vaccine development. Further studies are needed to determine the practicality and efficacy of anti-Id vaccines as well as to

unterstand in detail the mechanisms of the Id network.

(3) Regulation of immune responses.

Numerous studies have shown that certain immune responses could be up- or down-regulated by the administration of anti-Id. Experimental evidences have indicated that anti-Id acted on T or B cell pathway in regulating the immune responses. In studing the primary and secondary antibody responses to phenylarsonate, Owen and Nisonoff (Owen, F. and Nisonoff, A. 1978) demonostrated that Id-specific suppressor T cells were involved in the regulation events. In another study that anti-Id helper T cells participate in antibody responses (Eichmann, K. et al. 1978, Hetzelberger, D. and Eichmann, K. 1978). Malley's study of the immune regulation of the antibody responses to Timothy grass pollen allergen, found that the anti-Id generated the suppressor T cells which actively suppressed the antibody responses to the allergen (Malley, A. et al. 1981 and 1983).

On the other hand, B cells, which possessed the anti-Id found to be capable of activating or suppressing the upcoming Id^+ antibody responses. For example, B cells specific for the Id associated with NP could enhance the antibody responses to NP (Sherr, D.H. and Dorf, M.E. 1984). The anti-Id B cells have also been shown to regulate the antibody responses to dextran

in both the in vivo and in vitro experiments (Yamamoto, H. et al. 1984 and 1987, Yamamoto, H. and Katz, D.H. 1980, Bitoh, S. et al. 1990).

Studies on Idiotype Network of Antibody Responses to Allergen:

1. Charaterization of Ryegrass (*Lolium perenne*) Pollen Allergens:

The term "allergy" was conceived as describing all states of changed immunologic reactivity in animals, including hypersensitivity and immunity. The agents causing such changes, were the "allergens" ("allos", a deviation from the original state). The recognition of immediate hypersensitivity was brought about by the production of reaginic antibody, IgE isotype. The discovery of reaginic IgE by Ishizaka (Ishizaka, K. et al. 1966, and Ishizaka, K. and Ishizaka, T. 1968) as the principal carrier of reaginic antibody activity in man was of primary importance. Analogous IgE classes were subsequently identified in many other mammalian species (Bloch, K.J. 1967). The IgG class that may also carry reaginic antibody activity has been clearly established in man as well as in many animal species (Parish, W.E. 1973). The state of immediate hypersensitivity, which follows as a consequence of the biosynthesis of IgE antibody, is characterized by the binding

of specific IgE molecules, by their Fc portions, to mast cells and basophils. Following subsequent allergenic exposure of the host, the allergen combines with its specific cell-bound IgE antibody, initiating a series of biochemical reactions which lead to the release of chemical mediators possessing vasodilating and smooth muscle constricting activities. Providing sufficient allergen and high affinity antibody is present, this immunologic reaction will normally lead to symptoms characteristic of allergic disease.

It has been known for years that the allergenic components extracted from pollens of grasses (Gramineae) and ragweeds (Ambrosieae) are two of the main agents causing hay fever in North America. There are at least 14 distinct antigenic components in extracts of most common grass pollens, all of which may be regarded as potential allergens. Three highly purified allergens, Groups I, II, and III have been isolated from the pollen of *Lolium perenne* (perennial rye grass) by Marsh and his colleagues (Johnson, P. and Marsh, D.G. 1965, Johnson, P. and Marsh, D.G. 1966a, 1966b, and Marsh, D.G. 1971). A further partially purified fraction, Group IV, isolated in this laboratory was named high molecular weight basic antigen (HMBA) with a M.W. of 56,800 dalton (Ekromoddoullah, A.K.M. et al. 1983). Subsequently, the HMBA was further examined, following cleavage with CNBr to produce two fragments, CB-1 (17KD) and CB-2 (22KD) by Western

immunoblot analysis (Ekramoddoullah, A.K.M. et al 1986). These two peptides retained the capacity to react with a murine mAb12 and also IgE antibodies present in a pool of sera from grass allergic individuals. The new nomenclature, *Lol pIV* (*Lolium perenne* Group IV), was used for HMBA according to the recommendations by International Union of Immunological Societies (Marsh, D.G. et al. 1987). Furthermore, the antigenic and allergenic sites present on this glycoprotein was examined with two different mAbs, mAb90 to mAb94 generated against *Lol pIV* (Jaggi, K.S. et al. 1989) and mAb12 which was generated in mice against the retentate fraction of Kentucky bluegrass (KBG-R) and recognized also an epitope on *Lol pIV* (Ekramoddoullah, A.K.M. et al. 1986). Two distinct antigenic epitopes of *Lol pIV* (named Site A and B) were defined by these two sources of mAbs, which were also recognized by human IgE antibodies present in the serum of grass-allergic patients. The other two epitopes of *Lol pIV*, Site C and D, were identified by a group of mAbs, mAb96 to mAb100 to Site C and mAb102 to Site D.

2. Anti-Id Regulates the Antibody Responses to Grass Pollen Allergens:

The immediate type hypersensitivity is mediated by IgE antibodies that are produced in response to a wide range of allergens and the formation of IgE antibodies in the course of

an immune response is frequently associated with allergic reactions of the immediate type. The production of IgE antibodies as such is not by itself pathologic, since IgE plays an important role in the defense against parasites and has possibly also other physiological roles. Therefore, attempts at their regulation should be to directly prevent the formation of only those IgE antibodies that are specific for the allergen. Thus, one approach which may be considered to modulate allergen-specific immune responses would be to make use of the regulatory potential of anti-Id.

The leading study, using anti-Id as modulator, on the antibody responses to grass pollen allergen was carried out in Malley's laboratory around the turn of 80's. The specific suppression of antibody responses to Timothy grass pollen allergen has been achieved by the administration of anti-Id antibodies (Malley, A. et al. 1983). The ability of suppression was mediated by specific suppressive T cells and T suppressor factors (Malley, A. and Dresser, D.W. 1983a, and 1983b). The preliminary studies on Ryegrass pollen allergens using anti-Ids have also been carried out by Hebert, J. and by Bose, R. in separate studies. They characterized that the anti-Ids could recognize the IdX shared by human and murine IgG and IgE antibodies to Ryegrass pollen allergen *Lol* pI (Mourad, W. et al. 1988a, 1988b, and Mecheri, S. et al. 1988) and *Lol* pIV (Bose, R. et al. 1988). Moreover, the anti-Ids

could cross-link human IgE antibodies on basophils and induced histamine release (Mecheri, S. et al. 1988). In allergic and non-allergic individuals, auto anti-Ids to anti-*Lol pI* antibodies were detected. The level of the anti-Ids was significantly influenced in allergic individuals by seasonal exposure to pollen and by immunotherapy with extracts of grass pollen, whereas in non-allergic individuals the level of the anti-Ids did not change (Bose, R. et al. 1986).

These studies suggested that the interaction between the Id and anti-Id may play a role in the regulation of antibody responses to Ryegrass pollen allergens. However, the nature of the regulatory function of anti-Id on antibody responses to Ryegrass pollen allergen, especially *Lol pIV*, has not been studied. My interest was to investigate the regulatory function of murine monoclonal anti-Id(s) on the murine antibody responses to Ryegrass pollen allergen *Lol pIV*. The reasons for chosing the allergen and monoclonal antibody model system were three-fold: (i) allergen *Lol pIV* was identified in this laboratory as one of the major allergens of grass pollen; (ii) epitope mapping studies using monoclonal antibodies to 4 different epitopes of *Lol pIV*, identified immunodominant site (Site A) recognized by antibodies in human and mouse antisera; and (iii) experimental evidence that anti-Id could regulate the immune responses cited earlier indicated that it could also be used for the study on antibody responses to *Lol pIV*.

The regulatory function of anti-Id could be studied specifically for the Id and its corresponding epitope of *Lol* pIV. The specific aims of my studies are outlined as follows:

I. To generate and Characterize Monoclonal Anti-Ids to Id91 of mAb91 Specific for *Lol* pIV.

1. Identify and characterize serologically the anti-Id which is representive of an internal image anti-Id.

2. Determine the Id heterogeneity of the mouse, human and rabbit antibody responses to *Lol* pIV utilizing the mAb anti-Id.

II. To investigate the Ability of Anti-Id to Modulate the Antibody Responses to *Lol* pIV in Murine Model.

1. To establish the conditions for the *in vivo* administration of the anti-Id that would either suppress or enhance the antibody responses to *Lol* pIV.

2. To evaluate if the modulating effect observed in the anti-Id-treated animals was transferable by spleen cells to syngeneic recipients.

3. To charaterize the cells which are responsible for the transferred modulating effect.

III. To Generate, Characterize and Perform Functional Studies on Monoclonal Anti-Anti-Id (Ab3).

1. To investigate the immunogeneic properties of the anti-Id and evaluate if the anti-anti-Id and/or anti-*Lol* *p*IV responses are induced in inbred strains of mice.

2. To evaluate the level of the Id expressed on the antibodies (Ab3) following immunization with the anti-Id.

3. Examine the Ab3 anti-*Lol* *p*IV antibodies specificity directed to Site A of *Lol* *p*IV.

CHAPTER 2.

A Murine Internal Image Anti-Idiotypic Monoclonal Antibody Detects A Common Idiotope on Human, Mouse and Rabbit Antibodies to Allergen *Lol p*IV

A version of this chapter has been published in the *Scand. J. Immunol. vol. 34, p. 307.* 1991.

SUMMARY

A syngeneic mouse monoclonal anti-Id designated as B1/1, was generated against mAb91 specific for epitope Site A of Ryegrass pollen allergen *Lol* pIV. This anti-Id recognized an Id91 that was present also on other monoclonal antibodies with the same specificity as mAb91. Observations that (i) the anti-Id inhibited the binding of mAb91 to *Lol* pIV and (ii) the Id-anti-Id interaction could be inhibited by *Lol* pIV indicated that the Id was located within or near the antigen combining site. These properties served to characterize B1/1 as an internal image anti-Id. Evidence that an immune response in different species to *Lol* pIV elicits the formation of antibodies which express a common Id was provided by the observations that (i) the Id-anti-Id interactions could be inhibited by mouse, human and rabbit antisera to *Lol* pIV and (ii) the binding of these antisera to *Lol* pIV could be inhibited by the anti-Id. Interestingly, the internal image anti-Id B1/1 also recognized an Id on a monoclonal antibody which was directed to an epitope of *Lol* pIV, different from that recognized by mAb91.

INTRODUCTION

An Id is defined as an antigenic determinant within the variable regions of immunoglobulins and the antigen receptors of B and T lymphocytes. Idiotopes have also served as structural and functional markers for the variable regions. Upon immunization with an antibody which contains a variety of idiotopes, collectively referred to as idiotypes, the corresponding anti-Id or Ab-2 can be generated. According to Jerne (Jerne, N.K. 1974), anti-Id or Ab-2 can be classified into two major categories, Ab-2α and Ab-2β. The Ab-2α recognizes the Id outside the antigen combining site. On the other hand, the Ab-2β, also known as internal image anti-Id, has the capacity to mimic the three dimensional structure of the antigenic determinants or epitopes and can substitute for the antigen in inducing or suppressing the immune response against those specific epitopes. It has been shown that antibodies which share antigen-binding specificity often share the same idiotopes with one another (Kennedy, R.C. et al. 1983, Mourad, W. et al. 1986, and Mourad, W. et al. 1988).

In this report, the generation and characterization of a mouse monoclonal anti-Id, designated as B1/1, which bears the characteristics of Ab-2β is described. This anti-Id was produced against a mouse monoclonal antibody mAb91 specific for Site A on *Lol p*IV that is one of the major allergens in

Ryegrass (*Lolium perenne*) pollen. Studies in our laboratory using monoclonal antibodies have identified four different epitopes on *Lol pIV* (Jaggi, K.S. et al. 1989, Kisil, F.T. et al. 1989). These epitopes are also recognized by human IgE antibodies of grass pollen allergic patients. The monoclonal anti-Id B1/1 recognized an Id that was found to be common to mouse, human and rabbit anti-*Lol pIV* specific antibodies. The Id was found to be located within or near the antigen combining sites of these antibodies. Furthermore, the anti-Id also recognized the Id on monoclonal antibody mAb12 which recognized Site B of *Lol pIV*. Our studies demonstrated that an immune response to *Lol pIV* in three different species leads to the expression of a common Id on the resulting antibodies which was identified by an internal image anti-Id, B1/1.

MATERIALS AND METHODS

Preparation of Immunoadsorbent.

Sepharose 4B gel (10ml, Pharmacia, Uppsala, Sweden) was washed well in distilled water (dH_2O) and resuspended in 5ml of dH_2O. Three grams of cyanogen bromide (Sigma, St. Louis, MO) were dissolved in 5 ml of dH_2O and immediately mixed with the suspension of the Sepharose 4B gel. The reaction mixture was kept in an ice bath and the pH of the mixture was immediately raised and maintained at pH 11-12 with NaOH for a period of 12 min. The gel was then quickly washed with 200ml of 0.2M borate buffered saline (BBS) pH 8.2. and transferred into a solution containing 30mg of mAb91. This mixture was maintained at 25^0C for 2 hr and at 4^0C overnight. Protein which had not bound was washed out. It was estimated that 97% of the protein was coupled to the gel. Finally, the immunoadsorbent was washed with a solution of 0.1M acetic acid followed by BBS.

Isolation of Allergen, Lol pIV.

A solution of the retentate fraction of Ryegrass (*Lolium perenne*) pollen (Ekramoddoullah, A.K.M. et al. 1983) prepared in BBS (20mg/ml) was incubated with the mAb91-Sepharose 4B immunoadsorbent at 4^0C, overnight. After draining off the

material which had not bound, the column was washed with BBS until the OD_{280} was less than 0.1. A solution of 0.1M Glycine-HCl at pH 3.0 was employed to elute the protein from the immunosorbent. The fractions having an OD_{280}0.1 or higher were collected, dialysed against BBS and concentrated using the Amicon Ultrafiltration Systems (YM 5, Amicon Canada Limited, Oakville, Ontario). The *Lol* pIV-containing fraction may be contaminated by mAb91 which came from the immunoadsorbent eluted by using low pH solution. To eliminate this possibility, the fraction was passed through a Protein A-Sepharose 4B affinity column (Pharmacia, Uppsala, Sweden) which had been equilibrated with BBS. Therefore, any contaminating mAb91 would be absorbed out. The purity and antigencity of *Lol* pIV were tested utilizing sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE 12%) and enzyme-linked immunoassay (ELISA) respectively. The protein concentration of *Lol* pIV was determined by the method of Lowry et al (Lowry, O.H. et al. 1951).

Preparation and Characterization of Monoclonal Anti-Lol pIV Antibodies.

Monoclonal antibodies mAb90 to mAb94 (all IgG1κ isotype) generated against *Lol* pIV have been characterized as recognizing an epitope designated as Site A (Jaggi, K.S. et al. 1989). Monoclonal antibody mAb12 (IgG1) was produced

against the retentate fraction of KBG pollen allergen, and was identified to recognize an epitope referred to as Site B on *Lol p*IV. In the present study, mAb91 was used to induce the production of the syngeneic anti-Id.

Purification and isolation of monoclonal antibodies (IgG1 isotype) from ascitic fluid was performed as recommended by Dr. M.R. Schick, (Stanford University, personal communication). One volume of the ascitic fluid containing the monoclonal antibodies was mixed with four volumes of a solution of 1.5M glycine-HCl and 3M NaCl, pH 8.9. The mixture was passed through a Protein A-Sepharose 4B (Pharmacia, Uppsala, Sweden) affinity column which had been equilibrated with the glycine-HCl-NaCl buffer. This high salt-containing buffer was employed to enhance the binding of mouse IgG1 to Protein A and resulted in the binding of 5mg IgG1 per ml of the affinity gel. The column was washed with the buffer and then eluted with BBS and fractions were collected. The protein concentration was calculated using an extinction coefficient of 13.5 for a 1% preparation at OD_{280nM}.

Generation of Syngeneic Monoclonal Anti-Id.

Female Balb/c mice (Jackson Laboratory, Bar Harbor, ME) were used in this study to generate the anti-Id against mAb91. Mice were given, every two weeks, a total of three i.p.

injections of 25μg of mAb91 that was conjugated to keyhole limpet hemocyanin (KLH) (see below) and then the conjugate was precipitated in alum. One month later, the mice were given the fourth injection. Two mice that had a high titer of serum anti-Id were boosted i.p. with 25μg of mAb91 coupled to KLH, five days prior to using their spleen cells for the fusions. The hybridoma cells were prepared as described previously (Ekramoddoullah, A.K.M. et al. 1984). The hybridoma cell culture fluids were screened for anti-Id using a biotin-avidin ELISA (described below). Hybridomas secreting anti-Id were subcloned by the limiting dilution method to obtain monoclonal antibodies. A monoclonal anti-Id, designated as B1/1 was identified as an IgG2bκ using murine isotype-specific antibody reagents (BioRad Laboratories, Richmond, CA). The monoclonal anti-Id was isolated from ascitic fluid using a combination of salt fractionation and molecular sieve chromatography which have been described in detail elsewhere (Kennedy, R.C. et al. 1983).

ELISA Protocols.

(i) *Antibody Titers to Lol pIV.*

The specific binding of antibodies in the mouse, human and rabbit antisera to *Lol* pIV was determined by a direct ELISA. The solid-phase was prepared by coating the wells of

microtiter plates (Immulon II; Dynatech Laboratories, Inc., Alexandria, VA) with *Lol pIV* (200ng in 50µl BBS) at 4^0C, overnight. For control, ovalbumin (200ng in 50µl BBS) was employed to coat wells. After blocking unbound sites with a solution of 2% gelatin at 37^0C for 30 min, antisera obtained from mice, humans and rabbits were diluted over a wide range and 50µl samples were added to the microtiter wells and incubated at 37^0C for 1 hr. Then, the wells were washed three times with BBS containing 0.2% of Tween-20 (Tween-BBS, v/v). The antibodies which had bound to the solid-phase *Lol pIV* were detected by the addition of the corresponding horseradish peroxidase (HRP)-conjugated goat antibodies to mouse, human and rabbit immunoglobulins (BioRad Laboratories, Richmand, CA) at 37^0C for 1 hr. After washing the wells with Tween-BBS, the substrate, 2,2'-azino-di (3-ethyl- benzthiazoline-6-sulfonic acid) (ABTS) in 0.1M citrate buffer and 0.01% H_2O_2 were used to develop the colorimetric reaction. The reaction was stopped by adding 5% SDS and the optical density at 410 nM of each well was measured on an automatic ELISA plate reader (MR600; Dynatech Laboratory).

(ii) *Detection of Antibodies Specific to Site A of Lol pIV.*

A biotin-avidin competitive inhibition ELISA procedure was employed to establish the relative levels of antibodies in the different antisera, which recognized site A of *Lol pIV*.

For this purpose, biotinylated mAb91 (40ng in 10µl) (see below) was mixed with the various inhibitors e.g. monoclonal antibodies (2µg in 10µl) and antisera (diluted ten-fold; 10µl), and volumes of 10µl of the mixtures were added to the wells which had been coated with *Lol* pIV (200ng/well). After incubating at 37^0C for one hr, the wells were washed and a solution of Streptavidin-conjugated HRP was added and kept at 25^0C for 25 min. The assay was continued as described above.

(iii) *Two-Site ELISA for Evaluating Specificity of Anti-Id.*

The specificity of the anti-Id B1/1 for mAb91 was determined by the biotin-avidin two-site ELISA. Briefly, the wells of microtiter plates were coated with mAb91 (400ng in 50µl BBS). For control, mouse monoclonal anti-ovalbumin (mAb2) and anti-*Poa* pI (mAb60) antibodies were individually employed to coat the wells. After blocking unbound sites with gelatin, 10µl of the mouse antisera or hybridoma cell culture fluids or purified protein preparation containing the anti-Id was added to the wells. The anti-Id which had bound to the solid-phase mAb91 was detected by adding the biotinylated mAb91 (50ng/well). The assay was continued as described above.

(iv) *Detection of a Common Id on Antibodies to Lol pIV.*

Antibodies to *Lol* pIV that possessed an Id in common with

mAb91 were detected in terms of their capacity to compete with mAb91 and inhibit its binding to the anti-Id. For this purpose, solid-phase was prepared by coating individual wells of the ELISA plate with the purified preparation of mAb91 (200 ng in 50µl BBS) at 4^0C, overnight. The wells were blocked with gelatin as described above. A constant amount of the anti-Id B1/1 preparation (200ng/100µl) was mixed with the various inhibitors i.e. monoclonal antibodies or antisera from mouse and rabbit to *Lol* pIV and sera from grass pollen allergic and nonallergic individuals and then volumes of 100µl of the mixtures were added to the wells and kept at 37^0C for 1 hr followed by washing three times with Tween-BBS. HRP labelled goat anti-mouse IgG2b (Cedarlane Laboratories Ltd. Ontario, Canada) was used to determine the extent to which the anti-Id had bound to the solid-phase. These values were compared to the maximum binding of the anti-Id which occurred in the absence of a competing antibody or antiserum. The assays were performed in triplicate.

(v) *Inhibition of the Binding of Antibodies to Lol pIV by the Anti-Id.*

To ascertain whether the anti-Id could bind to antibodies against *Lol* pIV and inhibit their binding to *Lol* pIV, a competitive inhibition assay was performed in triplicate. For this purpose, solid-phase was prepared by coating individual

wells of ELISA plates with *Lol* pIV (200ng in 50µl BBS) at 4^0C overnight. Mixtures of the anti-Id and the monoclonal antibodies or antisera to *Lol* pIV were prepared prior to addition to *Lol* pIV coated assay plates. The anti-Id was used at various amounts (µg/well); the final amounts of the monoclonal antibody per well was 100ng for mAb90 and mAb91; 200ng for mAb12 and mAb98; the antisera were used at the final dilution of: 1/10,000 for mouse antisera #1, #3; 1/5,000 for mouse antisera #2, #4, #5; 1/500 for human serum Hu1; 1/100 for sera Hu2, Hu3, and the serum Hu5; 1/50 for Hu4; 1/5,000 for rabbit antiserum #1; 1/2,500 for rabbit antisera #2, #3. HRP-labelled goat antibodies to mouse IgG1, human and rabbit immunoglobulins were used to detect the levels of the antibodies which bound to the solid-phase. The addition of the substrate and quantitation of the colorimetric reaction was performed as described above.

(vi) *Inhibition of the Binding of Anti-Id to the Id mAb91 by Lol pIV.*

To determine whether the allergen *Lol* pIV could interfere with the binding between the anti-Id B1/1 and the Id of mAb91, a competitive inhibition assay was employed. The assays were performed in triplicate as described in section (iv) except that the inhibitor employed in this assay was *Lol* pIV.

The degree of inhibition in the respective assays was calculated according to the formula: % inhibition = 1 - [OD_{410} with inhibitor minus background OD / OD_{410}without inhibitor minus background OD] x 100.

Preparation of Antibody-KLH Conjugate.

The method for coupling antibodies to KLH has been described in detail elsewhere (Schick, M.R. et al. 1987). Briefly, the purified preparation of mAb91 (5mg/ml) in BBS cooled to 4^0C was mixed with 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide (EDAC) (Sigma Chemical Co., St. Louise, MO) at a molar ratio of 1:10,000. After incubation at 4^0C for 30 seconds, KLH (Calbiochem Corporation, La Jolla, CA) was added to the IgG preparation at a molar ratio of 1:50 and incubated for 2 hrs at 25^0C and at 4^0C, overnight. After dialysing against BBS at 4^0C, overnight, the IgG-KLH conjugate was then precipitated in alum.

Biotinylation of Antibodies.

The method used for biotinylation of antibodies has been described in detail elsewhere (Zhou, E.-M. et al. 1987). Briefly, 2mg of purified monoclonal antibody preparation was incubated with the mixture of 0.5M $NaHCO_3$, pH 9.0 and 0.5mg of biotin-N-hydroxysucinimide ester (Pierce Chemical Co.,

Rockford, IL) at 25^{0}C for 1 hr. A volume of 25µl of a solution of NH_4Cl (1M) was added to stop the reaction and the mixture was dialysed against BBS. Biotinylated monoclonal antibody was mixed with glycerol at a ratio of 1 to 1 (v/v) and stored at -20^{0}C for further use.

RESULTS

Characterization of Antibodies against Lol pIV.

The relative levels of serum antibodies to *Lol* pIV present in allergic and nonallergic human sera, and mouse and rabbit antisera are reported in Table II. Nonallergic human serum, Hu6, contained antibodies of the IgG class to Ryegrass pollen and KBG allergens. However, this serum was found to be devoid of detectable IgE antibodies, in the P-K and RAST assays (Jaggi, K.K. et al. 1989). Normal sera from human, mouse and rabbit lacked antibodies of the IgG and IgE classes to Ryegrass and KBG pollen allergens.

The relative specificities of antibodies of different sources to *Lol* pIV were compared to that of mAb91. The specificities were evaluated in terms of the capacities of the antibodies to inhibit the binding of mAb91 to *Lol* pIV. As shown in Figure 1, neither mAb98 nor mAb12 was able to inhibit the binding of mAb91 to *Lol* pIV. These observations confirmed the results of previous studies in this laboratory which have shown that (i) mAb90 and mAb91; and (ii) mAb12, respectively recognized two distinctly different epitopes on *Lol* pIV (Jaggi, K.S. et al. 1989) while mAb98 recognized the third (Site C) epitope (Kisil, F.T. et al. 1989). Sera from five individuals allergic to Ryegrass and KBG pollen all inhibited,

Table II.

Antibody Titers to *Lol pIV*[1]

Antisera	Specificity	End Point Titer
Mouse #1	Anti-Lol p IV	10,000
Mouse #2	"	5,000
Mouse #3	"	10,000
Mouse #4	"	5,000
Mouse #5	"	5,000
Mouse #6	Normal Serum	0
Allergic Human Serum:		
Hu1	Grass Pollens	500
Hu2	"	100
Hu3	"	100
Hu4	"	50
Hu5	"	100
Nonallergic Human Serum:		
Hu6	"	100
Hu7	Normal Serum	0
Rabbit #1	Anti-Lol p IV	5,000
Rabbit #2	"	2,500
Rabbit #3	"	2,500
Rabbit #4	Normal Sera	0

1. A direct ELISA was utilized to determine the relative total levels of the serum antibodies to Lol p IV. The values under the heading End Point Titer refer to the final dilution of the antiserum which gives an OD_{410} value three times higher than that obtained with normal sera.

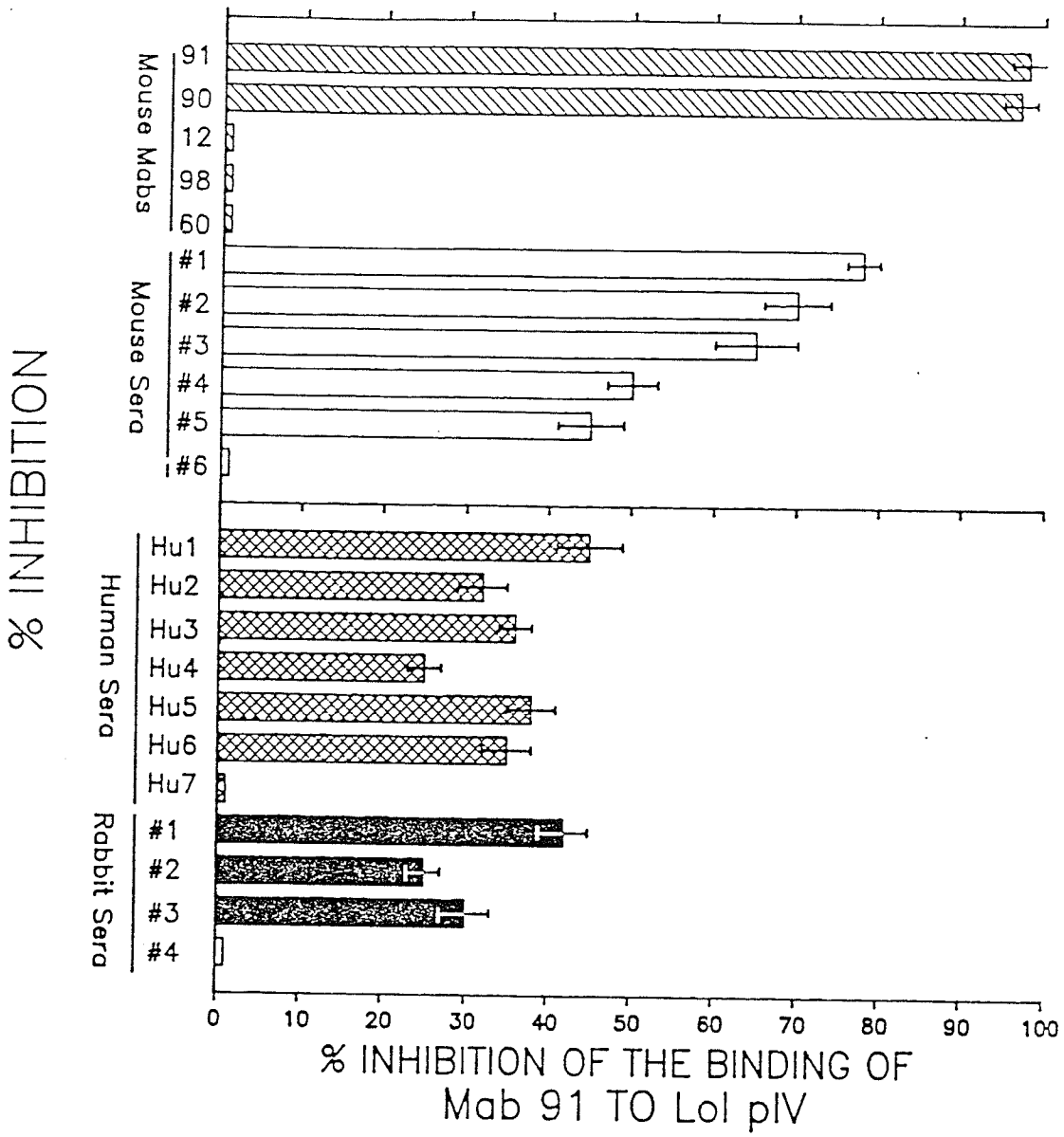


Figure 1. *Determination of Relative Antibody Specificities by Competitive Inhibition Assays:*

Biotinylated mAb91 (40ng in 100µl) was mixed with various inhibitors. Monoclonal antibodies were used at 20µg in 100ul and the anti-*Lol* pIV sera were diluted ten-fold. The maximum binding of the antibodies to solid-phase *Lol* pIV was determined in the absence of inhibitor. The degree of inhibition (%) of the binding of the biotinylated mAb91 to solid-phase *Lol* pIV was calculated from the mean values (±SEM) of ELISA assays performed in triplicate.

to different degrees, the binding of mAb91 to *Lol* pIV. One nonallergic human serum, Hu6, also partially inhibited (to the extent of 35%) the binding of mAb91 to *Lol* pIV. Similarly, five mouse and three rabbit antisera produced against *Lol* pIV also inhibited the binding of mAb91 to *Lol* pIV to an appreciable extent. On the other hand, this binding was not affected by mAb60 (anti-*Poa* pI) and the sera obtained either from normal human or mouse and rabbit obtained prior to their immunization with *Lol* pIV. The results of these inhibition experiments indicated that at least part of the repertoire of the mouse, human and rabbit antibodies to *Lol* pIV was directed to Site A of *Lol* pIV, which was recognized also by mAb91.

Specificity of the Monoclonal Anti-Id B1/1.

The specificity of the mouse monoclonal anti-Id B1/1 was tested by a biotin-avidin two-site ELISA. The results are illustrated in Figure 2. The lowest concentration of anti-Id B1/1 which bound to solid-phase mAb91 was of the order of 500ng/ml. Monoclonal anti-Id B1/1 did not bind to other IgG1 monoclonal antibodies directed to OA (mAb2) or to *Poa* PI (mAb60). These results indicated that B1/1 recognized an Id on an antibody specific for *Lol* pIV.

Monoclonal Anti-Id recognizes an Id Common to Antibodies to Lol pIV.

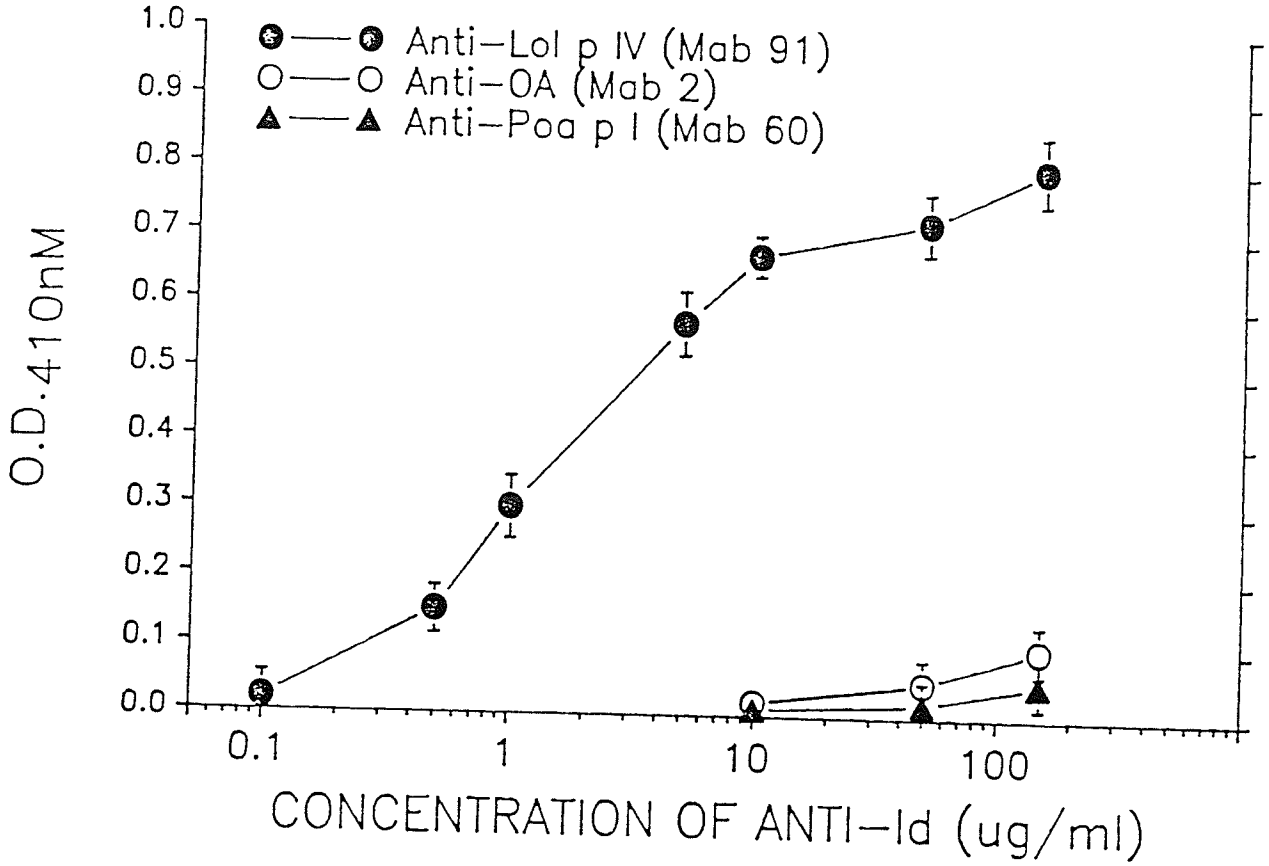


Figure 2. ***Specificity of the Monoclonal Anti-Id B1/1:***

The biotin-avidin two-site ELISA was used to determine the extent to which the anti-Id B1/1 bound to: mAb91 anti-*Lol* pIV (possesses the homologous Id) and control mAb2 anti-OA, and mAb60 anti-*Poa* pI. Each point represents the mean value (±SEM) of ELISA assays performed in triplicate.

To examine the possibility that antibodies to *Lol* pIV present in mouse, human and rabbit antisera share an Id in common with mAb91, a competitive inhibition assay was employed. In this assay, the abilities of antibodies from the different species to inhibit the binding between the Id (mAb91) and anti-Id (B1/1) were evaluated over a range of doses. The results are shown in Figure 3 A-D. The homologous monoclonal antibody, mAb91 and another monoclonal antibody, mAb90, which also recognized Site A of *Lol* pIV, inhibited the Id-anti-Id interaction in a dose-dependent fashion and total inhibition was achieved at an amount of 20μg/well (Figure 3A). By comparison, a ten-fold increase in the amount of mAb12 which recognized Site B of *Lol* pIV (Jaggi, K.S. et al. 1989), was required to inhibit the binding to the extent of 80%. On the other hand, an anti-*Lol* pIV monoclonal antibody, mAb98 specific for Site C, and an anti-*Poa* pI, mAb60, did not inhibit the binding between the Id and anti-Id even at high amounts.

All five murine antisera to *Lol* pIV inhibited the binding between the Id and anti-Id (Figure 3B). The degrees of inhibition ranged from 25-65% at the serum dilution of 1 to 10 and increased to 50-85% when the amount of the antisera was increased 5-fold.

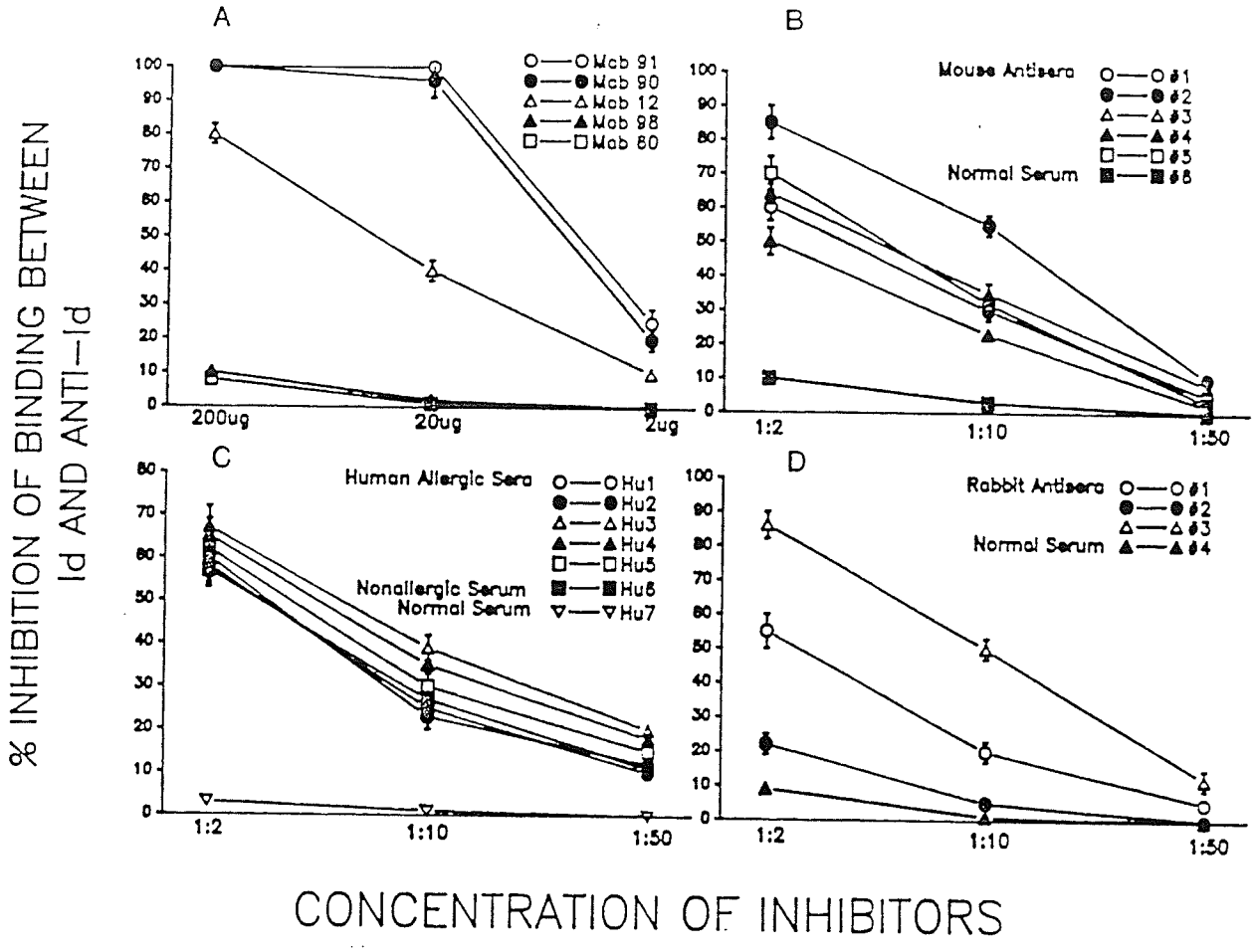


Figure 3. ***Demonstration that Antibodies to Lol pIV and mAb91 Share a Common Id:***

The extent to which antibodies from different sources (i.e. the inhibitors) were capable of inhibiting the binding between the Id of mAb91 (on solid-phase) and anti-Id B1/1 (200ng/100µl) was evaluated. **A:** Monoclonal antibodies were used at various amounts (µg/100µl); **B-D:** Antisera were used at various dilutions. The maximum binding of the anti-Id to the solid-phase mAb91 was determined in the absence of inhibitor. The % inhibition were determined from the mean values (±SEM) of the ELISA assays performed in triplicate.

All five Ryegrass pollen allergic human sera and one nonallergic serum, diluted 10-fold were also able to partially inhibit (25-42%) the murine Id-anti-Id interaction. The degrees of inhibition increased to 55-68% when these sera were used at a 2-fold dilution (Figure 3C).

Of three rabbit antisera to *Lol* pIV, diluted 10-fold, two of them partially inhibited (25% and 55%) the binding between the Id and anti-Id. The degrees of inhibition increased to 55% and 85%, respectively, when the sera were used at a dilution of 1 to 2 (Figure 3D).

The specificity of the inhibitions was demonstrated by the observations that the sera obtained from mice and rabbits prior to immunization and from a normal human individual had no effect on the Id-anti-Id interaction. Moreover, after removing the anti-*Lol* pIV antibodies from the mouse, human and rabbit antisera by affinity absorption with *Lol* pIV conjugated to Sepharose 4B, the capacity of the absorbed antisera to inhibit the Id-anti-Id interaction was markedly reduced (Figure 4). Thus, on the basis of these collective results, the monoclonal anti-Id B1/1 was considered to recognize an Id that was common to antibodies in the mouse, human and rabbit antisera to *Lol* pIV.

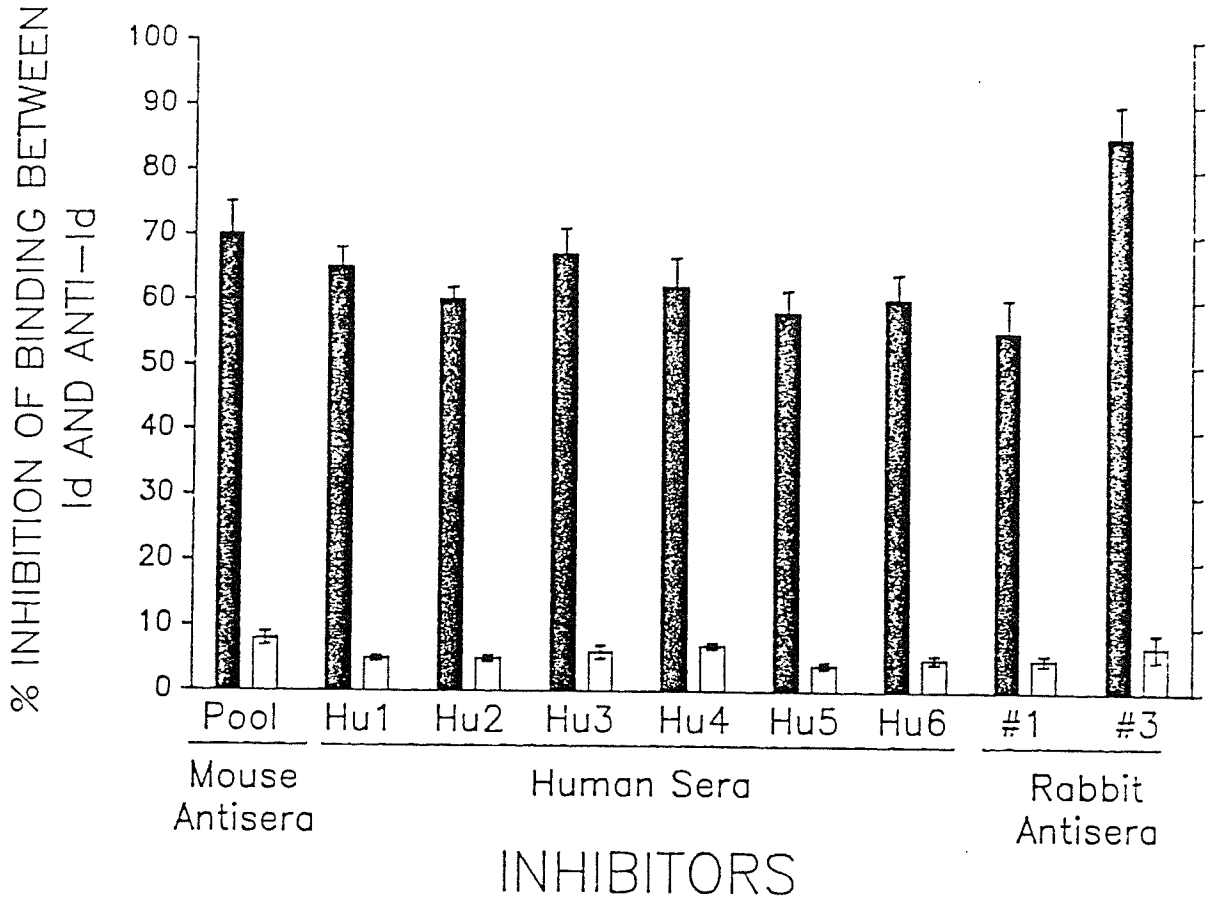


Figure 4. ***Effect of Absorption of Anti-Lol pIV Antibodies on the Ability of Antisera to Inhibit the Id-Anti-Id Interaction*:**

The extent to which the Id-anti-Id interaction was inhibited by antisera, before (solid columns) and after (open columns) removing anti-*Lol* pIV antibodies by affinity absorption on *Lol* pIV-Sepharose 4B. Antisera were diluted 10 fold. The % inhibition was determined from the mean values (±SEM) of the ELISA assays performed in triplicate.

Monoclonal Anti-Id Possesses the Characteristics of an Internal Image Anti-Id.

The anti-Id B1/1 was examined to determine if it possessed the properties of an internal image anti-Id, i.e. whether or not: (i) anti-Id B1/1 could inhibit the binding of antibodies to *Lol pIV* and, (ii) the binding of anti-Id B1/1 to the Id could be inhibited by *Lol pIV*. The degrees of inhibition were found to be dose dependent. In the presence of anti-Id B1/1 (25μg/well), the binding of mAb90 and mAb91 to solid-phase *Lol pIV* was inhibited to the extent of 100% (Figure 5A). Similarly, it was found that the antibodies in murine antisera to *Lol pIV* were inhibited from binding to *Lol pIV* by anti-Id B1/1 to the extent of 60-85% (Figure 5B); the binding of antibodies in human allergic and nonallergic sera to *Lol pIV* was inhibited by 60-80% (Figure 5C) and by 75-90% for the rabbit antisera (Figure 5D). In the presence of *Lol pIV* (final concentration of 100μg/ml), the binding of anti-Id B1/1 to solid-phase mAb91 was inhibited by 50% whereas no inhibition was achieved with ovalbumin even at a concentration of 1 mg/ml (Figure 6). Together, these results indicate that the anti-Id B1/1 possesses the characteristics of an internal image anti-Id which recognizes the Id located within or near the antigen combining sites of the antibodies to *Lol pIV*.

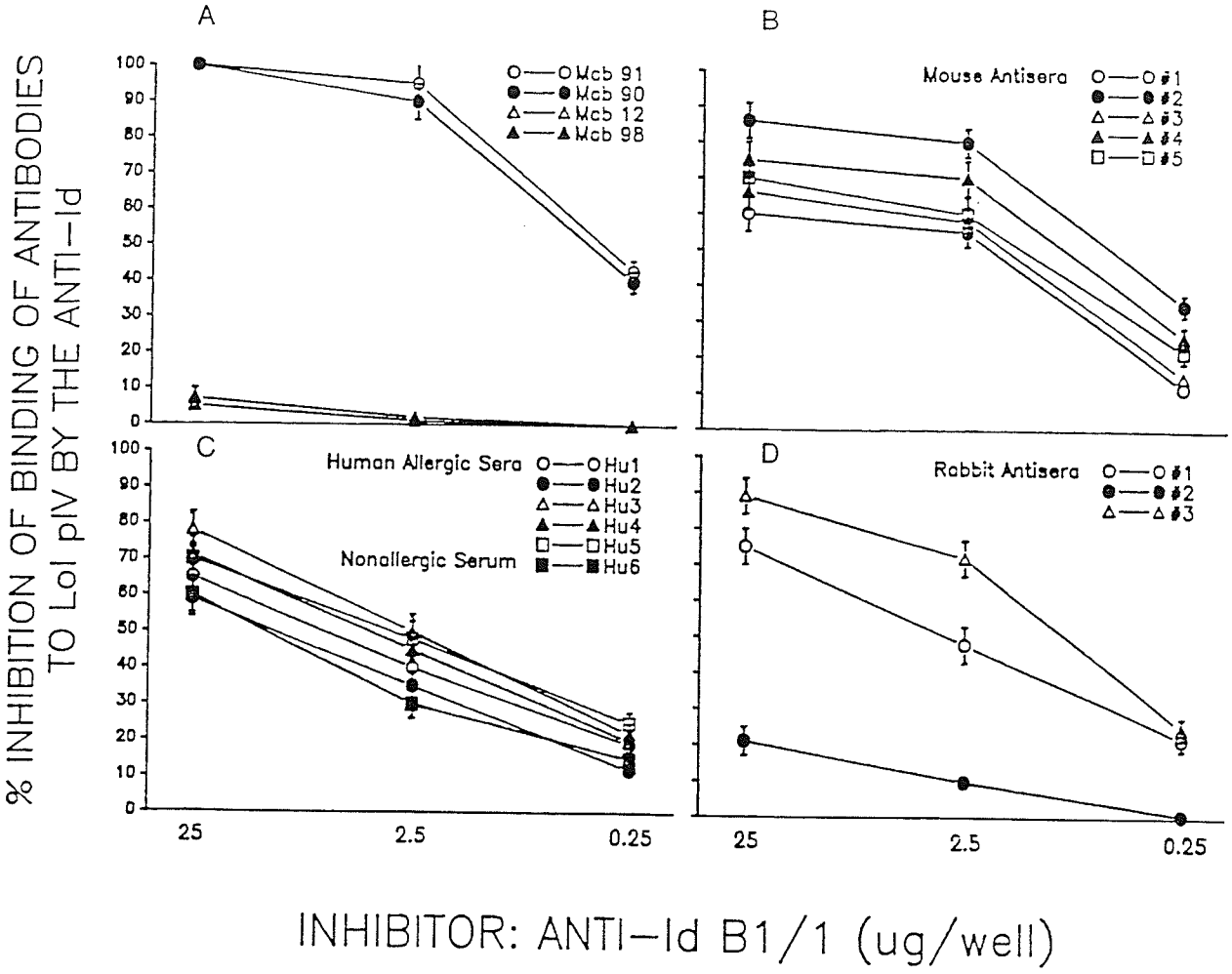


Figure 5. ***Demonstration That the Binding of Antibodies to Lol pIV Is Inhibitable by the Anti-Id B1/1:***

The extent to which the anti-Id B/1 recognized the Id on mouse monoclonal antibodies (*A*) and antibodies present in mouse (*B*), human (*C*) and rabbit (*D*) antisera to *Lol pIV* and interfered with their binding to *Lol pIV* was evaluated. The maximum binding of the antibodies to the solid-phase *Lol pIV* was determined in the absence of the anti-Id B1/1. The % inhibition was determined from the mean value (±SEM) of the ELISA assays performed in triplicate.

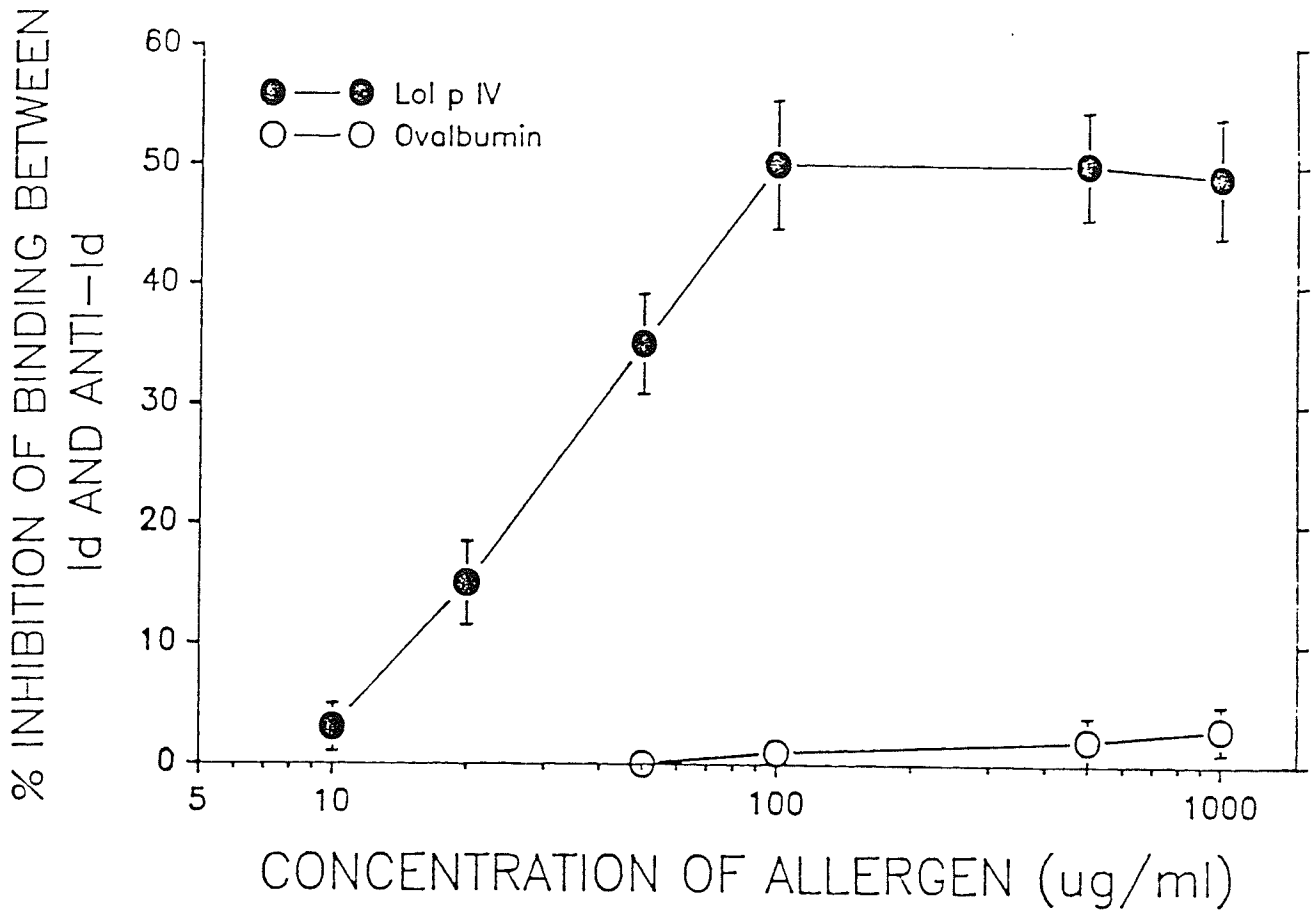


Figure 6. ***Demonstration That the Id-Anti-Id Interaction Is Inhibitable by the Homologous Antigen:***

The extent to which the presence of *Lol p*IV or ovalbumin inhibited the biding of the anti-Id B1/1 (100ng/well) to the solid-phase mAb91 (200ng/well) is illustrated. The maximum binding of the anti-Id to the solid-phase mAb91 was determined from the mean values (±SEM) of the ELISA assays performed in triplicate.

DISCUSSION

The studies described in this Chapter report on the generation and characterization of a mouse monoclonal anti-Id, designated as B1/1, directed to an Id on monoclonal antibody, mAb91. Monoclonal antibody mAb91 recognized an epitope referred to as Site A on the Ryegrass pollen allergen *Lol p*IV (Jaggi, K.S. et al. 1989). The finding that (i) the Id of mAb91 and anti-Id (B1/1) interaction could be inhibited, in a dose dependent manner, by mouse, human and rabbit antisera to *Lol p*IV (Figure 3) and (ii) the anti-Id could inhibit these antibodies from binding to *Lol p*IV (Figure 5), indicated that the Id is common to antibodies that are formed to *Lol p*IV in unrelated species. Moreover, evidence was also obtained which revealed that the interaction between the Id and anti-Id could be inhibited by *Lol p*IV (Figure 6). From these observations, it was inferred that anti-Id B1/1 recognized an Id either within or near the antigen combining sites of mAb91. According to the classification of anti-Id (Jerne, N.K. et al. 1982 and Bona, C.A. et al. 1988), the anti-Id B1/1 has the characteristics of an internal image anti-Id. Although the level of the expression of the Id recognized by B1/1 on the antibodies of various species to *Lol p*IV varied, it was evident that these antibodies all possessed an Id which is the same or similar to that present on mAb91.

The levels of the Id on the antibodies to *Lol* pIV were evaluated in terms of the ability of the antisera to inhibit the binding between the Id (mAb91) and the anti-Id (B1/1). It was clear that in the presence of homologous Id of mAb91 (or mAb90 which had identical specificity to mAb91) the Id-anti-Id interaction was completely inhibited. On the other hand, none of the mouse and rabbit antisera and the human allergic sera at the maximum dose employed (diluted 2-fold) was able to completely inhibit the Id-anti-Id interaction (Figure 3). Since the antibodies to *Lol* pIV in the different sera are of polyclonal origin and therefore are capable of recognizing any of the determinants of *Lol* pIV, eg. Sites A, B, C or D or other as yet unidentified epitopes, it is evident that only that fraction of the entire repertoire of antibodies which is (1) directed to site A of *Lol* pIV and (ii) possesses the Id which is the same or similar to that of mAb91, has the capacity to inhibit the Id-anti-Id interaction. Therefore, it was not surprising to find that the sera were not able to completely inhibit the Id-anti-Id interaction.

Similar observations were made in evaluating the capacity of anti-Id B1/1 to inhibit the binding of antibodies of various sources to *Lol* pIV. It was demonstrated that in the homologous system the binding of mAb91 and mAb90 to *Lol* pIV were completely inhibitable by the anti-Id, whereas antibodies to *Lol* pIV in the various antisera, were capable of being

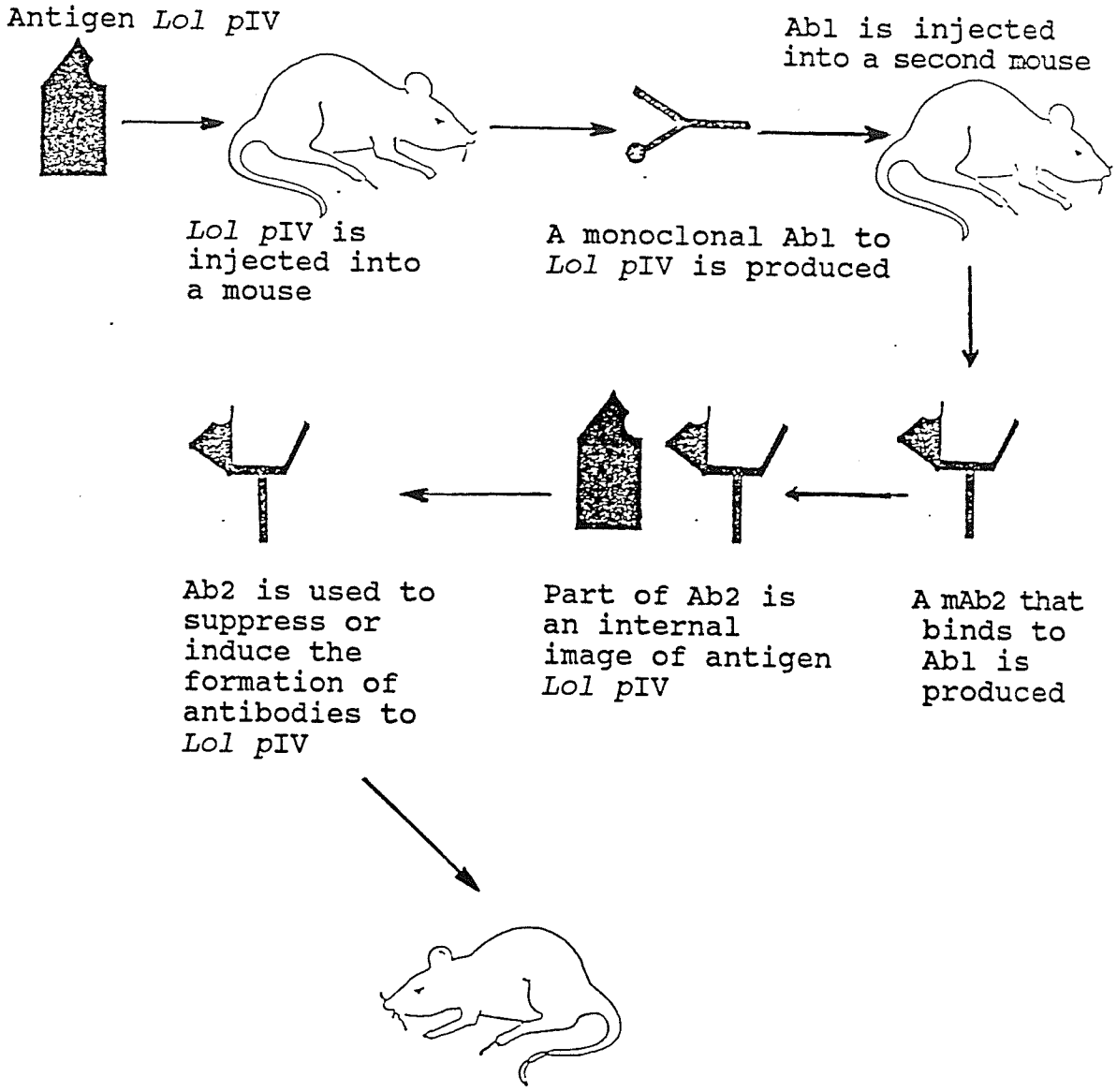
inhibited from binding to *Lol* pIV to a maximum of approximately 70% (Figure 5). In these assays, the polyclonal antibodies which lacked the Id of mAb91, would not be expected to be inhibited from binding to *Lol* pIV by the anti-Id, as was indeed the case.

A previous study in this laboratory (Bose, R. et al. 1988) has shown that a rabbit polyclonal anti-Id antiserum that was rendered specific for the Id of a murine monoclonal anti-*Poa* pIV antibody (which recognized epitope Site B of Kentucky bluegrass and Ryegrass pollen allergen *Lol* pIV) also recognized an Id on human antibodies to *Lol* pIV. Thus, the human antibodies and murine monoclonal antibodies to *Lol* pIV possessed Ids of the same or similar specificity. Other studies have shown that mouse monoclonal and rabbit polyclonal anti-Id produced to antibodies against the Ryegrass pollen group I allergen (Mourad, W. et al. 1986 and 1988) inhibited the binding of the mouse and human antibodies to the allergen. In our investigations, the anti-Id recognized not only the Id on mouse antibodies to *Lol* pIV but also the Id on human and rabbit antibodies to *Lol* pIV. This finding was confirmed by the observations that specific removal of the anti-*Lol* pIV antibodies from the mouse, human and rabbit antisera by affinity absorption, removed also the capacity of these absorbed antisera to inhibit the binding between the Id and anti-Id (Figure 4). The antibodies to *Lol* pIV in one of three

rabbit anti-*Lol* *p*IV antisera appeared to lack the Id recognized by anti-Id B1/1 since the anti-Id had no effect on the binding of the antibodies to *Lol* *p*IV and the antibodies were not able to inhibit the Id-anti-Id interaction (Figure 5D). It was concluded that the expression of an idiotypic specificity can vary from one individual to another.

The observations that in the presence of the anti-Id B1/1, both monoclonal and polyclonal antibodies to *Lol* *p*IV were inhibited from binding to *Lol* *p*IV (Figure 5) and that *Lol* *p*IV inhibited the Id-anti-Id interaction (Figure 6), indicated that B1/1 has the characteristics of an internal image anti-Id. The diagram on the next page illustrates how an internal image anti-Id might mimic *Lol* *p*IV. The reason(s) for the finding that *Lol* *p*IV could inhibit the Id-anti-Id reaction only to the extent of 50%, is(are) not presently known. It may be speculated that the affinity, specificity or precise location of the Id within the antigen combining sites of mAb91 was not identical.

Interestingly, even though mAb91 and mAb12 recognized different epitopes on *Lol* *p*IV (i.e. Site A and B, respectively) (Jaggi, K.S. et al. 1989), mAb12 was also capable of inhibiting the binding between the Id and anti-Id. This fact indicated that mAb12 possessed an Id with a specificity which is the same or similar to that found on



This diagram shows the potential mechanism of how an internal image anti-Id might mimic the allergen *Lol pIV*. A mouse produces an antibody response (Ab1) against *Lol pIV* (precisely the Site A of *Lol pIV*). The Ab1 (here is mAb91) is then used to immunize a second mouse and an anti-Ab1 (Ab2) response can be induced. Based on the concept that *Lol pIV* and Ab2 (here is B1/1) bind the Ab1 at its paratope and idiotope, respectively, and these sites on the Ab1 can be the same or similar, the B1/1 may mimic the structure of *Lol pIV*. Such an Ab2, i.e. B1/1, is referred to as an internal image anti-Id.

mAb91. Observations of a comparable nature have been reported by other investigators who found that monoclonal antibodies which recognized different epitopes of an antigen [e.g. GAT, (Germain, R.N. et al. 1979), hen egg lysozyme (Metzger, D.W. et al. 1980), whale myoglobin (Kohno, Y. et al. 1982), lipopolysaccharide (Hiernaux, J. and Bona, C.A. 1982), hemagglutinin of influenza virus (Liu, Y.N. et al. 1981), and herpes simplex virus (Kennedy, R.C. et al. 1983)] possessed the same or a similar idiotypic specificity. In the present study, mAb91 (anti-Site A) which bears the homologous Id, was clearly superior to mAb12 (anti-Site B) in its ability to inhibit the Id-anti-Id interaction. These observations may be interpreted to indicate that the Id on mAb12 is either not identical or as accessible as the homologous Id for binding the anti-Id. Moreover, mAb12 could not be inhibited by the anti-Id from binding to *Lol* pIV. On the basis of these observations, it is suggested that the Id on mAb12 which is recognized by anti-Id B1/1 is not located within the antigen binding sites. By comparison, the rabbit anti-Id produced against mAb12 partially inhibited the binding of this monoclonal antibody to *Lol* pIV (Bose, R. et al. 1988). This result is not surprising since the rabbit anti-Ids are of polyclonal origin and are therefore capable of recognizing collectively the idiotypes on mAb12 and of inhibiting, at least partially, the binding between this monoclonal antibody and *Lol* pIV.

In summary, this study described the generation and characterization of a monoclonal anti-Id, designated B1/1. This anti-Id possessed the characteristics of an internal image anti-Id or Ab2β which are (i) it recognizes the Id which is located within or near the antigen combining sites of the antibody, (ii) it recognized the Id which is shared by the antibodies from different species. One prevailing concept in immunology developed from the Id network theory (Jerne, N.K. 1974) is that the immune response to a given antigen can be regulated by a series of Id-anti-Id interactions. The expression of a given Id is under the control of an anti-Id and the anti-Id can also be regulated by an anti-anti-Id. This complex set of interactions operates via a feedback mechanism to either enhance or suppress the immune response (i.e. formation of antibodies with a particular Id). It is the Ab2β that has the potential to mimic the antigen to induce the immune responses. The investigations on the immune response to grass pollen allergens (Malley, A. et al. 1982, Mourad, W. et al. 1988, and Bose, R. et al. 1988) indicate the existence of the Id networks. However, the nature by which the Id-anti-Id networks act in controlling the immune responses to Ryegrass pollen allergens is (are) not known at this time. Further studies in murine model systems were undertaken to determine whether the internal image anti-Id B1/1 could modulate the immune response to Ryegrass pollen allergen *Lol p*IV in terms

of level of the formation of antibodies and the expression of Id91.

CHAPTER 3.

REGULATION OF ANTIBODY FORMATION TO RYEGRASS POLLEN ALLERGEN *Lol p*IV BY AN INTERNAL IMAGE ANTI-IDIOTYPIC MONOCLONAL ANTIBODY[1]

A version of this Chapter has been submitted to the *Journal of Immunology*, January 1992.

ABSTRACT

The ability of the anti-Id Bl/1 to modulate the formation of antibodies to allergen *Lol* pIV was investigated in three murine model systems. In the first system, the anti-Id Bl/1 (50μg) conjugated to KLH and precipitated in alum was administered i.p. to treat three different strains of mice (C57BL/6, Balb/c and C3H). In the second and third model systems, a solution of Bl/1 in PBS was used to treat syngeneic Balb/c mice by an i.v. injection. All animals were challenged i.p. with *Lol* pIV in alum. The treatment with either form of Bl/1 administered at doses, ranging from 100ng to 100μg/mouse, resulted in a significant reduction of the levels of the antibodies to *Lol* pIV. In particular the formation of IgE antibodies to *Lol* pIV was greatly reduced. The administration of a single i.v. injection of a solution of Bl/1 8 weeks prior to the challenge with *Lol* pIV was still effective in reducing the level of antibodies to the allergen. Moreover, the level of antibodies to *Lol* pIV that expressed the Id91 ($Id91^+$) was also markedly decreased. By contrast, it was observed that the level of antibodies to *Lol* pIV in mice pre-treated with Bl/1 in PBS at a dose of 10ng/mouse increased (albeit slightly) and the titre of the IgE antibodies to *Lol* pIV increased twofold compared to that in mice treated with control mAb. The level of $Id91^+$ antibodies to *Lol* pIV also increased in the mice treated with Bl/1 at the low dosage. The observed change in

the level of antibodies to *Lol p*IV persisted for a period of 56 days. These experimental models lend themselves for investigating the mechanism(s) by which an anti-Id modulates antibody responses to a grass pollen allergen.

INTRODUCTION

Idiotopes, which can be recognized as unique epitopes, are attributed to particular amino acid sequences expressed in the variable regions of an antibody molecule. This concept was first defined by Kunkel and Oudin in 1963 (Kunkel, H.G. et al. 1963, Oudin, J. and Michel, M. 1963). It paved the way for the Id network theory of immune regulation proposed by Jerne in 1974 (Jerne, N.K. 1974). The theory implies that an antibody response to an antigen is controlled by a series of Id-anti-Id interactions that may either upregulate or downregulate the immune response to the antigen. The expression of a given Id is under the control of the homologous anti-Id and similarly, the level of the anti-Id can be regulated by yet another set of antibodies referred to as an anti-anti-idiotype. This complex set of interactions operates via feedback mechanisms to control the formation of antibodies to a given antigenic stimulation.

While the physiological significance of idiotypic interactions has not been firmly established, modulation of the immune responses via Id-anti-Id interactions has nevertheless been demonstrated. The observations (Bose, R. et al. 1984 and 1986) that levels of anti-Id increased in allergic patients who had received hyposensitization treatment with allergenic extracts, stimulated the interest to

investigate the use of anti-Id for modulating the formation of antibodies to allergens.

A monoclonal anti-Id B1/1 was produced against an Id91 of mAb91 [mAb91 recognized one of the epitopes, referred to as Site A of Ryegrass pollen allergen *Lol pIV* (Jaggi, K.S. et al. 1989)]. Anti-Id B1/1 was characterized as an internal image of the antigen *Lol pIV* that constitutes one of the major groups of allergens in Ryegrass (*Lolium perenne*) pollen. Characteristically, this internal image anti-Id: (i) inhibited the binding between the homologous antibodies and the allergen; (ii) its interaction with the Id91 could be inhibited in the presence of the allergen; and (iii) recognized the Id91 that was common to human, mouse and rabbit antibodies to *Lol pIV* (see Chapter 2). Since administration of the anti-Id to mice with an ongoing IgE antibody response to *Lol pIV* had no detectable effects on the levels of antibodies to the allergen, the initial attempts to develop a clinically relevant model were not successful. On the other hand, the protocol of administering the anti-Id B1/1 to mice prior to their challenge with the allergen served as models to evaluate the regulatory function of the internal image monoclonal anti-Id B1/1 on the formation of specific antibodies to *Lol pIV* and their expression of the Id91. The results have shown that *in vivo* administration of either B1/1 (50μg) conjugated to KLH and precipitated in alum or B1/1 in PBS at a dose of

100ng to 100μg/mouse resulted in a significant reduction in the level of the formation of antibodies to *Lol pIV*, whereas Bl/l in PBS at a dose of 10ng/mouse a slight elevation of their level was observed. In addition, it was found that the level of the $Id91^{+}$ antibodies to *Lol pIV* was markedly reduced in mice treated with a high dose (100ng-100μg) of Bl/l, whereas in mice treated with a low dose (10ng) of Bl/l the elevated level was also observed. The administration of a single i.v. injection of Bl/l in PBS 8 weeks prior to the challenge with *Lol pIV* was effective in reducing the antibody response to the allergen. The reduction or elevation of levels of antibodies to *Lol pIV* persisted for a period of 56 days. The experimental model employed in this study was being used to investigate the role(s) and mechanism(s) by which the anti-Id may regulate the antibody responses to grass pollen allergens.

MATERIALS AND METHODS

Animals:

Adult female mice of strains C57BL/6, Balb/c and C3H (H-2^b, H-2^d and H-2^k) were purchased from Charles River Breeding Laboratories, Wilmington, MA. Male Long Evans hooded rats were obtained from the local Central Animal Care Services, University of Manitoba, Winnipeg, Canada.

Preparation of Allergen Lol pIV:

*Lol p*IV from Ryegrass pollen was purified by affinity chromatography with a mAb91-immunosorbent. For use in immunization, the allergen was precipitated in alum as described previously (Chapter 2 and Schick, M.R. et al. 1987).

Preparation and Characterization of The Internal Image Anti-Id,B1/1:

The syngeneic monoclonal anti-Id, B1/1 (IgG2b,κ) was generated and purified from ascites as described in Chapter 2. The purified mAb IgG2b MCA specific for influenza virus was obtained from Cedarlane Laboratories LTD., Ontario. This mAb was used for controls. The characterization of B1/1 as an internal image anti-Id or Ab2β was made according to the

classification used by Bona and Kohler (Bona, C.A. and Kohler, H. 1984a and 1984b) and its properties are summarized in Table III.

Table III.

Salient Characteristics* of the Anti-Id, B1/1

A.	It recognized the Id91 on monoclonal antibodies against *Lol p*IV, but not Id of monoclonal antibodies with specificities unrelated to *Lol p*IV.
B.	It bound to the antibodies carrying the Id91 and inhibited their ability to bind the allergen.
C.	Its interaction with the Id91 could be inhibited by the allergen.
D.	It recognized the Id91 which was found to be shared by the human, mouse and rabbit antibodies against *Lol p*IV.

*These characteristics collectively served to identify mAb B1/1 as an internal image anti-Id or Ab2β (Bona, C.A. and Kohler, H. 1984, Bona, C.A. et al. 1984).

Protocols for Treatment with the Anti-Id, B1/1 and Challenge with Lol pIV:

Three model systems were employed to determine the regulatory function of the anti-Id B1/1 on the formation of antibodies to *Lol* pIV.

(1) Treatment with the anti-Id-KLH conjugate: For the first model system, as illustrated in Protocol I (results are shown in Figure 7), groups of five mice of each strain were treated by the i.p. administration of either anti-Id B1/1 (25μg) or the control mAb MCA (25μg) conjugated to KLH and precipitated in alum. Fourteen days later, all mice were first challenged and then boosted two weeks later by an i.p. injection of *Lol* pIV (5μg) in alum. Blood was obtained after the boost at an interval of 14 days.

(2) Treatment with the anti-Id alone at various times: For the second model system, a solution of anti-Id B1/1 (25μg in PBS) was administered i.v. to 8 groups of adult female Balb/c mice (5 mice/group). The intervals of time between the administration of B1/1 and challenge with the allergen ranged from 0 to -70 days, as illustrated in Protocol II (results are shown in Table IV). For controls, 8 groups of age-matched Balb/c mice (5 mice/group) similarly received MCA. In an alternate control, 8 groups (5 mice/group) received PBS at the

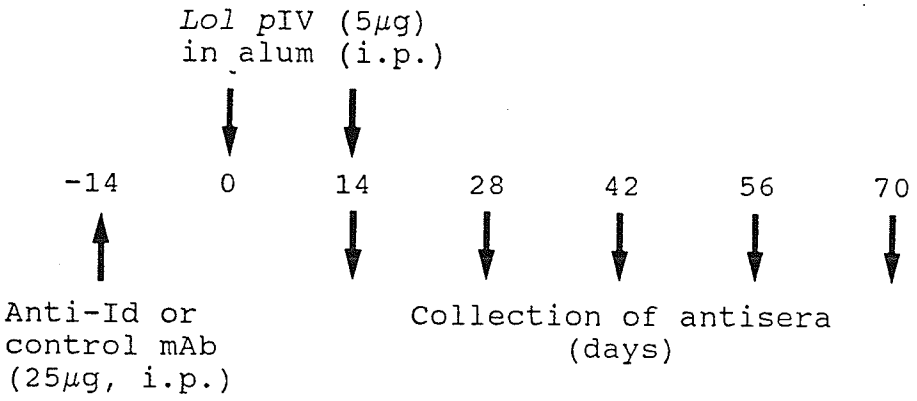
same intervals of times indicated above. All mice were challenged with *Lol* pIV and bled as described for the first model system.

(3) Treatment with the anti-Id alone at various doses: To study the effects of administration of different doses of B1/1 on the formation of antibodies to *Lol* pIV, the third model system was used in which 8 groups of adult female Balb/c mice (5 mice/group) received various doses (10ng to 100μg/mouse) of B1/1 in PBS by an i.v. injection, according to Protocol III (results are shown in Table V). For control, 8 groups of age-matched mice (5 mice/group) received various doses of MCA in PBS. All mice were challenged i.p. with *Lol* pIV and bled as indicated above.

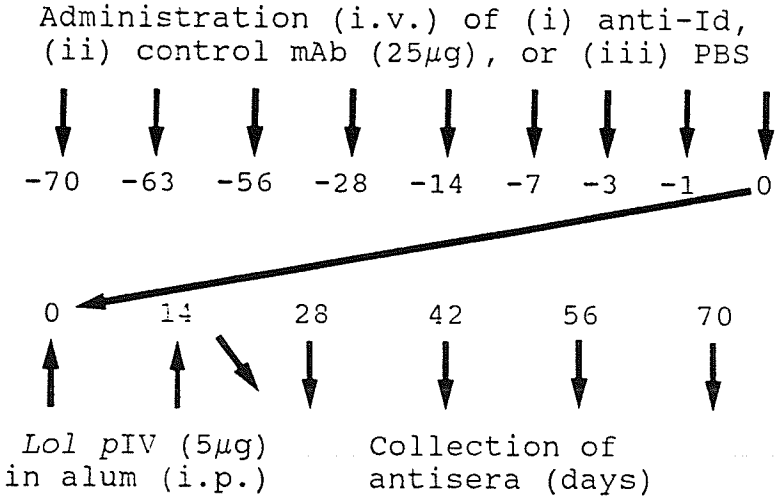
Serum from individual mouse of each group was collected and tested for the level of antibodies to *Lol* pIV and their expression of the Id91. The range of difference of end point titer between the 5 mice was less than 2-fold, therefore, sera from each group were pooled and used for the rest of assays. Sera obtained from the mice before treatment served as negative controls.

DETERMINATION OF THE EFFECTS OF TREATMENT WITH ANTI-ID ON THE ANTIBODY RESPONSES TO ALLERGEN *Lol p*IV.

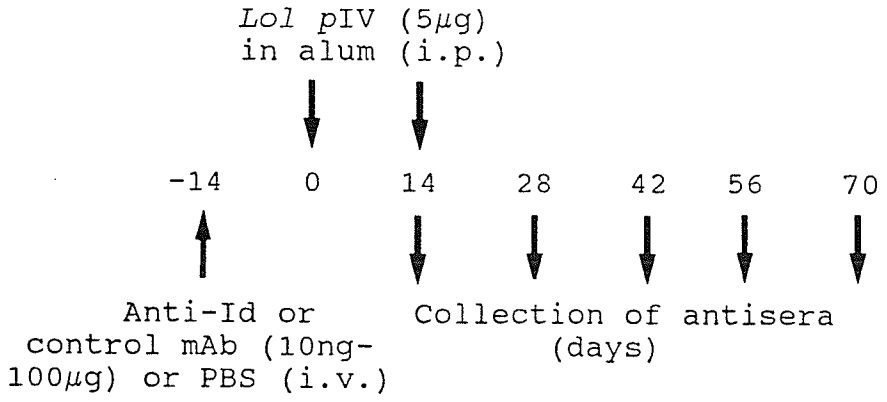
Protocol I. Anti-Id-KLH Conjugate



Protocol II. Solution of Anti-Id At Various Times.



Protocol III. Solution of Anti-Id At Various Doses.



Preparation of B1/1-KLH Conjugate:

The methods for coupling anti-Id B1/1 to KLH has been described in Chapter 2.

Biotinylation of mAb91 and mAb B1/1:

Antibody preparations were biotinylated according to methods previously described (Schick, M.R. et al. 1987, Zhou, E.-M. et al. 1987).

Preparation of Immunoadsorbent:

The method used here is different from that as described in Chapter 2. Freeze-dried cyanogen bromide activated Sepharose 4B (2g, Pharmacia, Uppsala, Sweden) was suspended in and washed with an aqueous solution of 1 mM HCl. The gel was mixed with the ligand, anti-Id B1/1 (35mg), in the coupling buffer (0.1M $NaHCO_3$, pH 8.3, containing 0.5M NaCl). The mixture was maintained at 4^0C overnight. After washing out the free ligand with coupling buffer, any remaining reactive groups on the gel were blocked with 0.1M Tris-HCl buffer at pH 8, for 16 hours at 4^oC. The immunoadsorbent was washed with 0.1M acetic acid and finally with BBS.

Determination of Levels of Antibodies to Lol pIV by ELISA:

The levels of antibodies to *Lol* pIV in the mouse antisera were determined by a direct ELISA. Briefly, the solid-phase was prepared by coating the wells of microtiter plates (Immulon II; Dynatech Laboratories, Inc., Alexandria, VA) with *Lol* pIV 200ng in 50µl of BBS at 4°C overnight. For control, OA was similarly employed to coat the wells. After blocking unbound sites with a solution of gelatin (2%) at 37°C for 30 min, 50µl of the respective pool of antisera diluted in BSA (1% in BBS) was added to the microtiter wells and incubated at 37°C for 1 hr followed by washing three times with 0.2% Tween in BBS (T-BBS). The antibodies to *Lol* pIV which had bound to the solid-phase *Lol* pIV were detected by incubating the wells with goat anti-mouse Ig conjugated to HRP (Cedarlane Laboratories LTD., Ontario) at 37°C for 1 hr. For the detection of the levels of the individual classes of antibodies to *Lol* pIV, the specific goat anti-mouse IgG1, IgG2b and IgM-HRP conjugates (Cedarlane Laboratories LTD., Ontario) were used. After washing the wells with T-BBS three times, the substrate 2,2'-azino-bis(3-ethyl-benzthiazoline sulfonic acid) (ABTS) in 0.1M citrate buffer and 0.01% H_2O_2 (v/v), was used to develop the colorimetric reaction. The reaction was stopped by adding 5% SDS and the optical density of each well was measured at 410 nM on an automatic ELISA plate reader (Dynatech Instruments Inc. Torrance, California).

The assay was performed in triplicate.

Passive Cutaneous Anaphylaxis (PCA):

The PCA procedure was used to determine the levels of serum IgE antibodies to *Lol* pIV. Two-fold serial dilutions of the mouse antisera were made in 0.9% saline and volumes of 50µl were injected intradermally into rats. Twenty four hrs later, 1 ml of *Lol* pIV (0.5mg in saline) mixed with Evans blue dye (at final concentration of 0.25%) was injected i.v. and 20 min later the animals were sacrificed. The size of the reactions (extravasation of the dye) which appeared on the undersurface of the skin was measured. The highest dilution of antisera which gave the reaction size of 3mm in diameter was considered as the end point titre. Each sample was tested in duplicate.

Inhibition Assays:

(1) *Detection of antibodies to Site A of Lol pIV*: The levels of antibodies specifically directed to an epitope of *Lol* pIV, designated as Site A, were evaluated in terms of the capacity of the mouse antisera to inhibit the binding of the biotinylated mAb91 to *Lol* pIV. Microtiter plates were coated with *Lol* pIV in BBS at 200ng/well, at 4°C overnight. After blocking unbound sites with a solution of gelatin (2%),

biotinylated mAb91 at a concentration of 5μg/ml was mixed with the individual pool of mouse antisera diluted in BSA (1% in BBS), placed into the wells and incubated at 37°C for 1 hr. The levels of the biotinylated mAb91 which bound to the solid-phase *Lol pIV* were determined by adding streptavidin-conjugated HRP in BSA. After incubating at 25°C for 25 min, the substrate was used as described above. The assay was performed in triplicate.

(2) *Detection of antibodies that possess the Id91*: To determine the levels of the $Id91^+$ antibodies to *Lol pIV* in mouse antisera, a competitive ELISA was performed. The capacity of these antibodies to interfere with the Id-anti-Id interaction was examined in a manner similar to the inhibition procedure described above, with the following modifications; the solid-phase was prepared by coating each well of the microtiter plate with a solution of mAb91 (200ng) in BBS at 4°C overnight. After blocking any unbound sites with gelatin, biotinylated Bl/1 (8μg/ml) pre-mixed with individual pool of antisera to *Lol pIV* diluted in BSA was added to the wells and incubated at 37°C for 1 hr. After washing the plates with T-BBS three times, the extent to which the biotinylated Bl/1 had bound to the solid-phase mAb91 was determined by adding streptavidin-conjugated HRP in BSA. The addition of substrate and quantitation of the colorimetric reaction were performed as described above. The extent of the inhibition was

calculated according to the formula: Inhibition (%) = 1 - [$OD_{410}$with inhibitor - background / OD_{410}without inhibitor - background] X 100.

RESULTS

EFFECTS OF ANTI-Id B1/1 ON THE ANTIBODY FORMATION TO Lol pIV:

(I) *Levels of Antibodies to Lol pIV:*

The effects of treatment with the anti-Id B1/1-KLH in alum in three strains of mice (C57BL/6, Balb/c and C3H) on the antibody responses to *Lol* pIV were studied in one model system. Similarly, the effects of treatment of Balb/c mice with B1/1 in PBS at (i) different intervals of time and (ii) different doses, prior to their challenge with *Lol* pIV were also investigated. Treatment of the three strains of mice with B1/1 (25μg)-KLH conjugate precipitated in alum (Figure 7) 14 days prior to the challenge with *Lol* pIV or treatment of Balb/c mice with B1/1 (25μg) in PBS at various times (from day 0 to day -70) (Table IV), or at various doses (100ng to 100μg) (Table V), prior to their challenge with *Lol* pIV, resulted in a significant reduction in the levels of antibodies to *Lol* pIV compared to the control groups. By contrast, slightly elevation of the levels of antibodies to *Lol* pIV in the antisera from the mice that had received B1/1 at a dose of 10ng was observed (Table V) compared to the groups that received a control mAb preparation. The changes in the levels of antibodies to *Lol* pIV and the expression of the Id 91 persisted over a period of 56 days.

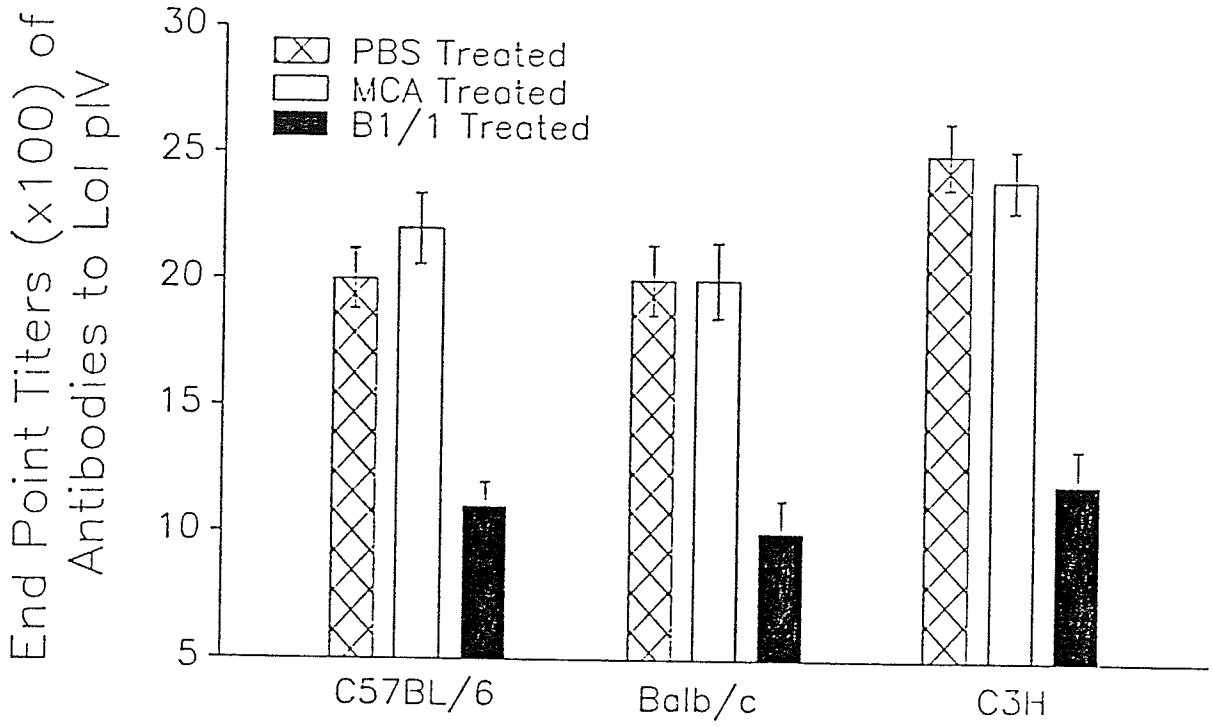


Figure 7. ***Effect of Treatment with B1/1-KLH on the Formation of Antibodies to Lol pIV.***

A direct ELISA was used to evaluate the levels of antibodies to *Lol* pIV in three strains of mice treated with PBS, control mAb MCA-KLH or anti-Id B1/1-KLH at a dose of 50μg/mouse. The end point titer refers to the final dilution of the antiserum which gave an OD_{410} value three times higher than that obtained with normal sera. The values represent mean ± SEM of an ELISA performed in triplicate.

Table IV.

Effect of Different Intervals of Time between in vivo Treatment with Anti-Id and Challenge with *Lol* pIV on the Formation of Antibodies to *Lol* pIV

Interval between Treatment with Anti-Id and Challenge with Allergen[1] (Days)	Level of antibodies to *Lol* pIV[2] following treatment with *Lol* pIV		
	PBS	MCA	B1/1
70	0.50	0.48	0.50
63	0.52	0.50	0.53
56	0.50	0.53	0.20
42	0.50	0.48	0.25
28	0.49	0.48	0.20
14	0.50	0.51	0.25
7	0.45	0.52	0.24
3	0.51	0.50	0.22
1	0.55	0.50	0.27
0	0.50	0.52	0.25

1. Eight groups of mice (5 mice/group) received either PBS or 25μg of MCA (antibody against influenza virus) or B1/1 (anti-Id) at different intervals prior to their challenge with 5μg of *Lol* pIV in alum. All mice were boosted with *Lol* pIV 14 days later.

2. The individual pool of antisera obtained on day 42 were diluted 1000-fold and the levels of antibodies to *Lol* IV were evaluated by an ELISA with *Lol* pIV as the solid-phase. The values represent the mean of OD_{410} of an ELISA performed in triplicate.

Table V.

Effect of in vivo Treatment with Anti-Id at Various Doses on the Formation of Antibodies to *Lol* pIV[1]

Dose of Antibody (μg/mouse)	Level of antibodies to *Lol* pIV[2] following treatment with *Lol* pIV	
	MCA	B1/1
100	0.53	0.25
50	0.48	0.26
25	0.50	0.22
10	0.52	0.21
1	0.49	0.24
0.1	0.50	0.21
0.01	0.52	0.68

1. Eight groups of mice received various doses of either MCA (antibody against influenza virus) or B1/1 (anti-Id). All mice were challenged 2 and 4 weeks later with *Lol* pIV (5μg/mouse in alum).

2. See footnote of Table IV.

The results are shown in Figure 8.

On the basis of these results, it is concluded that (i) anti-Id B1/1 could induce the reduction of formation of antibodies to *Lol* pIV and the expression of Id91 in three different strains of mice; (ii) a single treatment with soluble B1/1 at 25μg 8 weeks prior to the challenge with *Lol* pIV was effective in reducing the level of the antibodies to *Lol* pIV; and (iii) high doses of B1/1 induced a reduction, whereas an elevation of the level of the antibodies to *Lol* pIV was observed from the animals that received the low dose of anti-Id. In order to conserve materials, the subsequent studies of the effects of administering the anti-Id B1/1 on the levels of the antibodies to *Lol* pIV employed B1/1 at a dose of 25μg (referred to a high dose) or 10ng (referred to as low dose) per mouse, administered 14 days prior to their challenge with *Lol* pIV. The mice were challenged again 14 days later and the antisera obtained 42 days after the first challenge were used for the studies.

(II) *Level of IgE Antibodies to Lol pIV:*

The level of serum IgE antibodies in the control group of mice treated with the mAb to influenza virus was the same as the group that had been treated with PBS. In comparison with the control groups, the IgE antibody titre to *Lol* pIV was 160

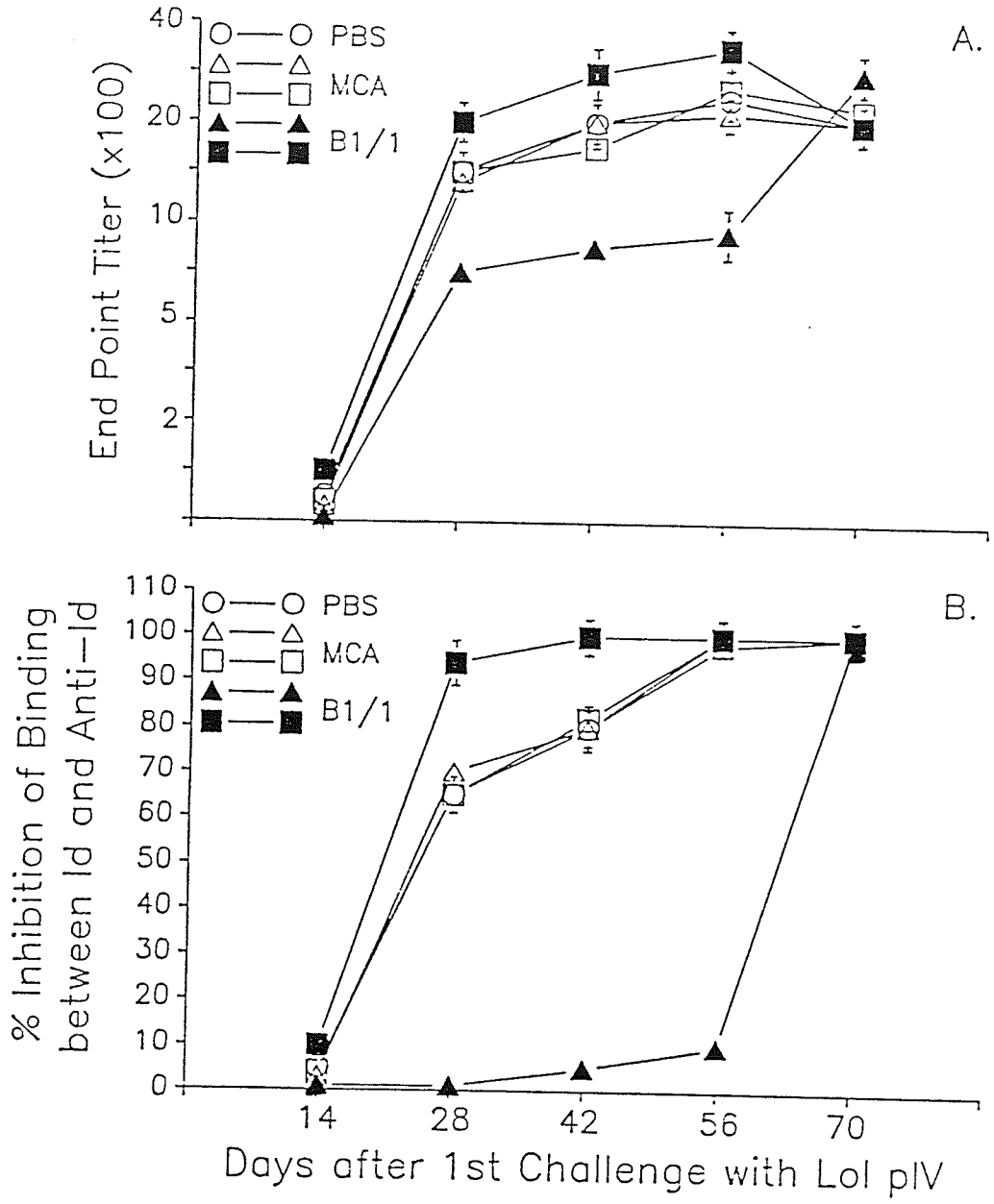


Figure 8. ***Kinetics of the formation of antibodies to Lol pIV and expression of the Id91.***

Balb/c mice were treated with either soluble B1/1, MCA or PBS two weeks prior to their challenge with *Lol pIV* in alum on day 0 and 14. Triangles: 25 µg/mouse and squares: 10ng/mouse of an antibody preparation. *A.* End point titre of antibodies to *Lo pIV*. *B.* The level of Id91 expressed on antibodies to *Lol pIV*. The values represent mean ± SEM from an ELISA performed in triplicate.

to 320-fold lower in the mice treated with B1/1-KLH (Figure 9A) and with B1/1 in PBS at a dose of 25μg/mouse (Figure 9B). On the other hand, the IgE antibody titre was 2-fold higher for the mice treated with B1/1 in PBS at a dose of 10ng. However, it is recognized that IgE antibody titers which by a 2-fold dilution were not considered as significant different from another.

(III) *Level of IgG1, IgG2b and IgM Antibodies to Lol pIV:*

The levels of antibodies that formed on immunization with *Lol p*IV were highest in the IgG1 class and were approximately double the levels associated with the IgG2b and IgM isotypes. Prior treatment with B1/1 (25μg)-KLH in alum (Figure 10A and 10B) or with B1/1 in PBS at a dose of 25μg (Figure 10C) resulted in a significant reduction in the formation of antibodies of the IgG1 and IgG2b isotypes. On the other hand, the administration of B1/1 at a dose of 10ng, an elevation of the level of antibodies of the IgG1 class to *Lol p*IV was observed, whereas, the levels of antibodies of the IgG2b and IgM classes did not change (Figure 10D).

(IV) *Levels of Antibodies Directed to Site A of Lol pIV:*

A competitive ELISA was employed to determine whether the administration of the anti-Id B1/1 had any effect on the

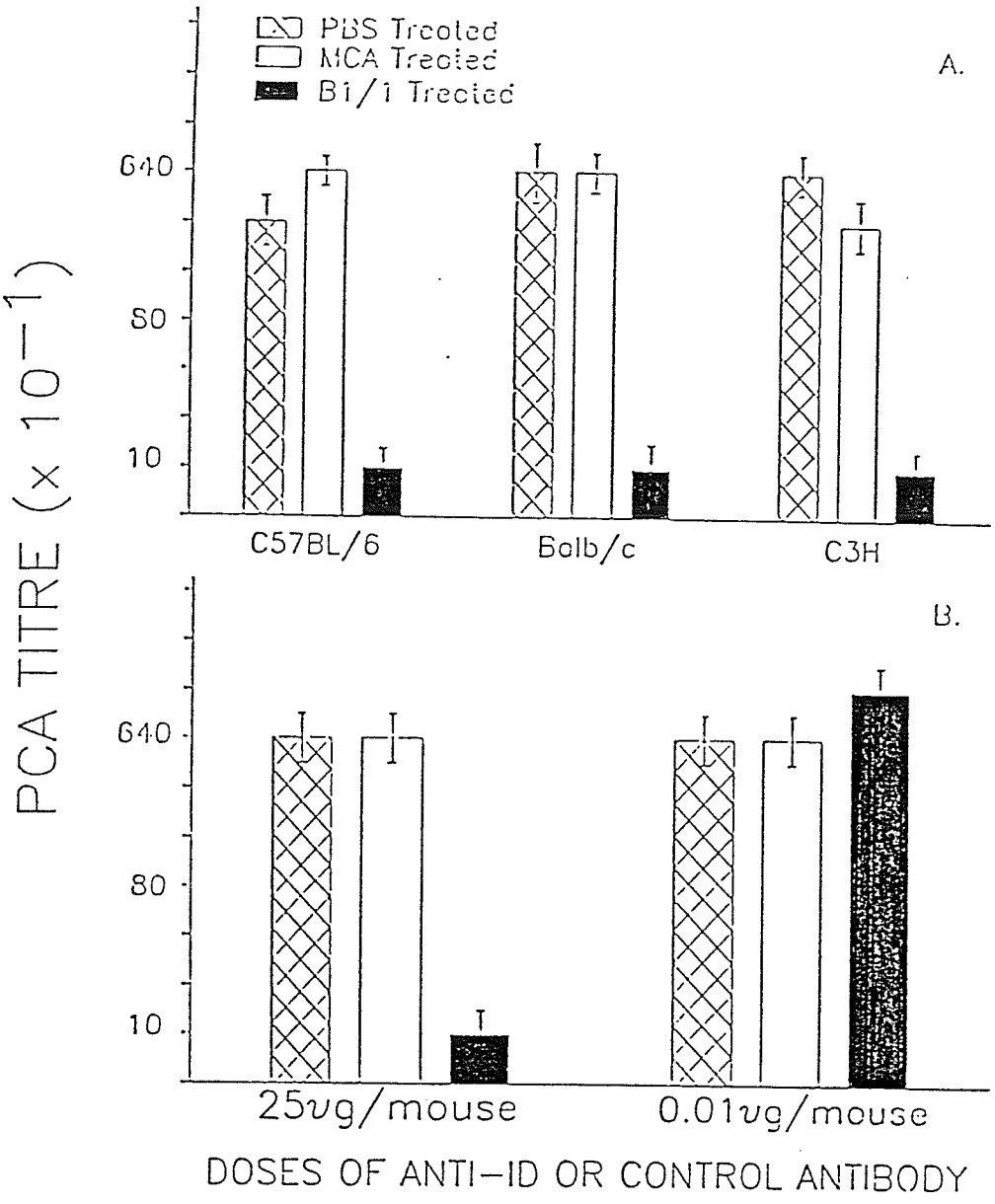


Figure 9. ***Effect of in vivo treatment with the anti-Id on the formation of IgE antibodies to Lol pIV.***

A PCA assay was used to detect IgE anti-*Lol* pIV antibodies in the antisera obtained from *A.* three strains of mice treated with PBS, MCA-KLH or B1/1-KLH and *B.* Balb/c mice treated with PBS, MCA or B1/1 in PBS. The values represent mean ± SEM of PCA titre performed in duplicate.

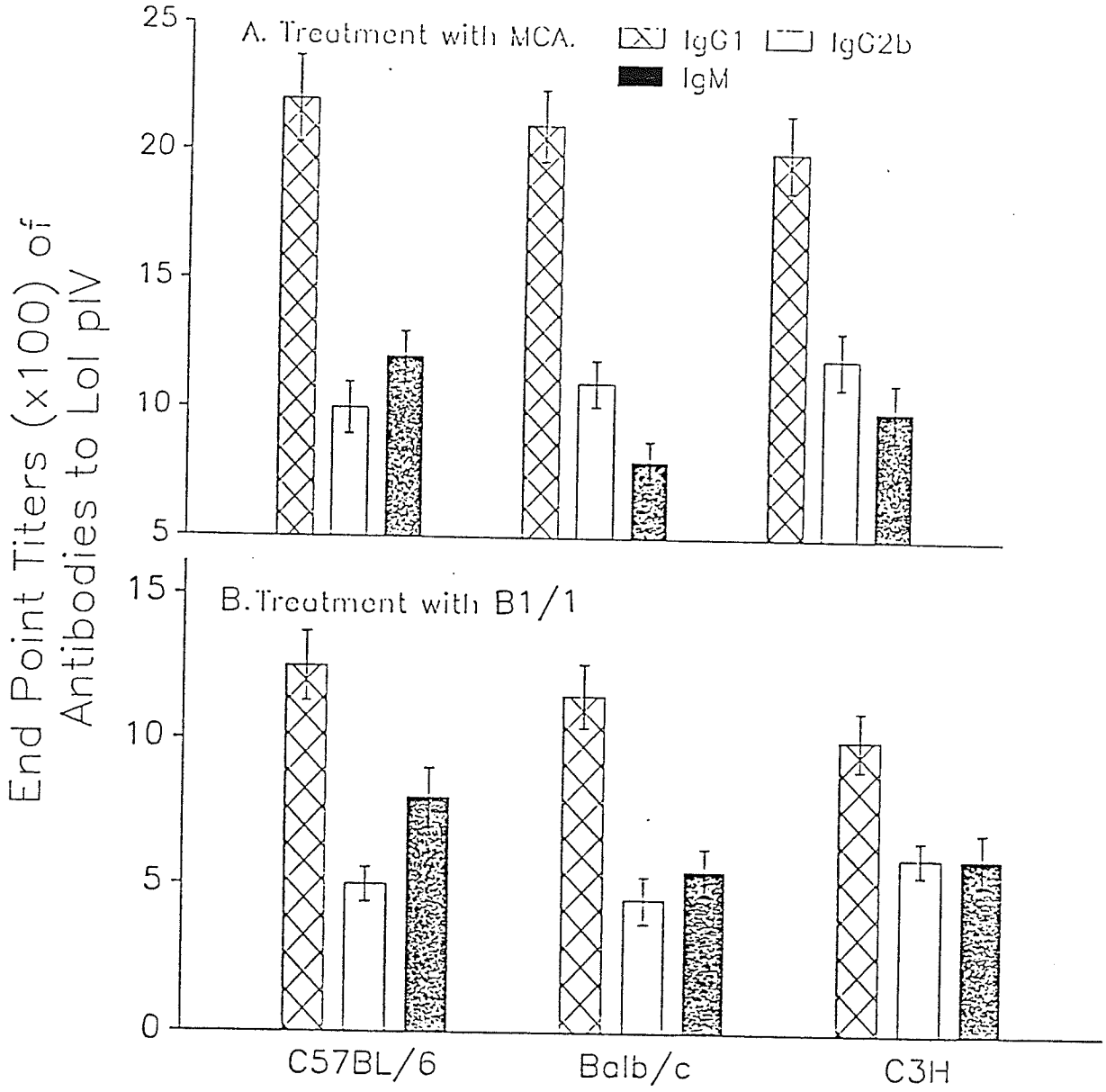


Figure 10. ***Effect of in vivo treatment with the anti-Id on the formation of antibodies to Lol pIV.***

A direct ELISA was employed to detect IgG1, IgG2b and IgM antibodies to *Lol* pIV. *A.* the antisera were obtained from MCA-KLH or *B.* B1/1-KLH treated mice.

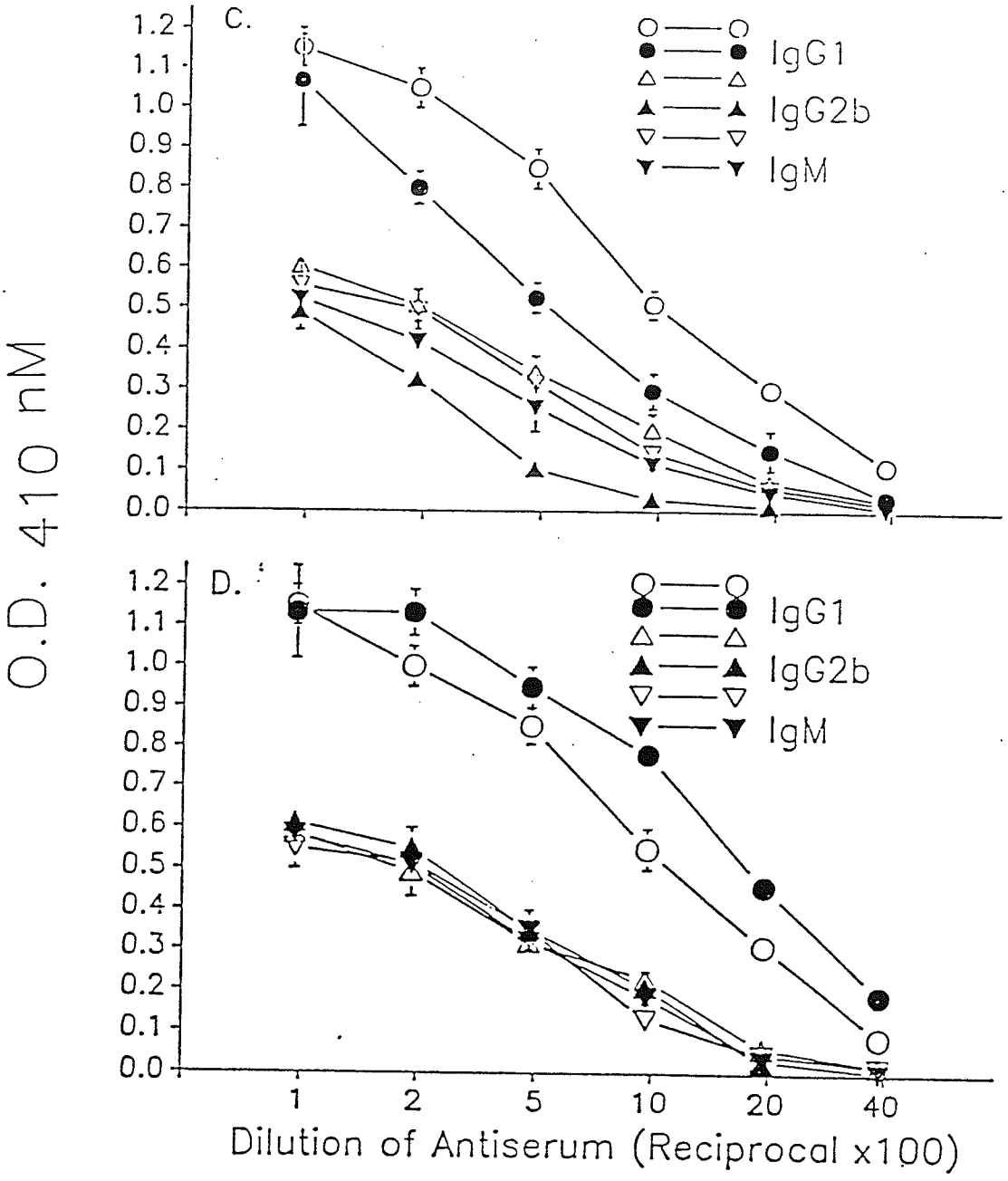


Figure 10. ***C.*** **and** ***D.*** **the antisera were obtained from the mice treated with MCA (open symbols) or Bl/l in PBS (solid symbols). These antibody preparations were administered at a dose of 25 µg/mouse** ***C.*****, and 10ng/mouse** ***D.*** **OD values represent mean ± SEM from an ELISA performed in triplicate.**

formation of antibodies with a specificity either identical or similar to that of mAb91 which was directed to Site A of *Lol* pIV (Jaggi, K.S. et al. 1989). In this assay, the capacity of antisera to inhibit the binding of mAb91 to solid-phase *Lol* pIV was evaluated. The results presented in Figure 11A and 11B indicated that the antisera (diluted 20-fold), obtained from the mice treated with either Bl/1-KLH in alum or with Bl/1 in PBS at a dose of 25µg, gave an inhibition value of the order of less than 30%, whereas the antisera from the control groups had inhibition values in the range of 75 to 90%. Therefore, as a corollary of these observations it was concluded that the formation of a major portion of the antibodies to Site A of *Lol* pIV had been reduced in the anti-Id treated animals. On the other hand, the antisera from mice treated with Bl/1 at the low dose (10ng) inhibited completely the binding between mAb91 and *Lol* pIV (Figure 11B). On this basis, it was concluded that treatment with the low dose of Bl/1 resulted in an elevation in the formation of those antibodies directed to Site A of *Lol* pIV. Collectively, these results indicated that the formation of antibodies with specificities related to mAb91 was indeed modulated by treatment with the anti-Id Bl/1.

EFFECTS OF TREATMENT WITH B1/1 ON THE LEVEL OF Id91$^+$ ANTIBODIES:

A competitive ELISA procedure was used to investigate

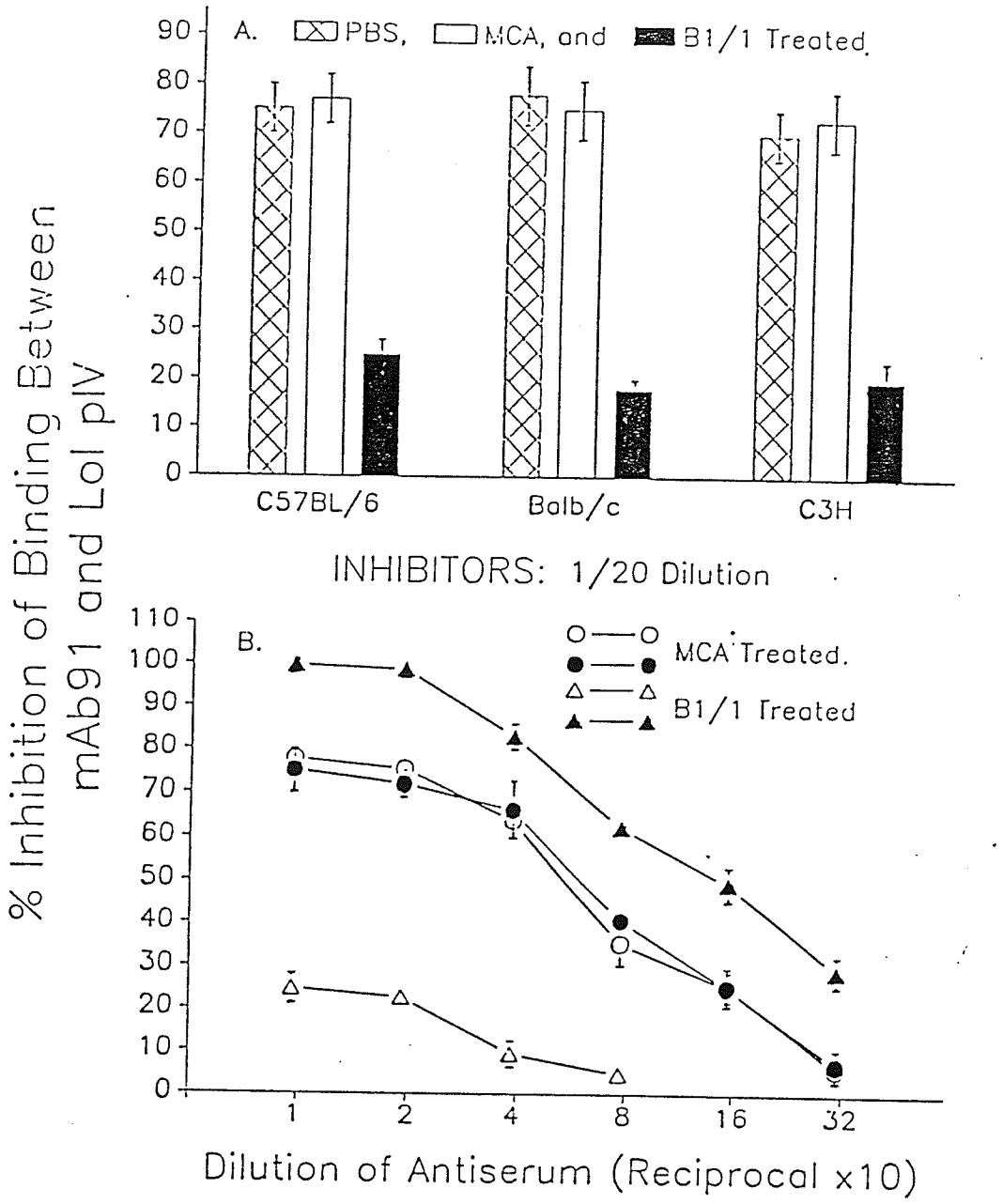


Figure 11. ***Effect of in vivo treatment with the anti-Id on the formation of antibodies to an epitope of Site A of Lol pIV.***

The levels of serum antibodies to Site A of *Lol* pIV were evaluated in terms of the degree to which the antisera were able to compete with mAb91 (anti-Site A) for binding to *Lol* pIV. *A.* antisera from three strains of mice treated with PBS, MCA-KLH or B1/1-KLH, and *B.* antisera from Balb/c mice treated with MCA or B1/1 in PBS at a dose of 25μg/mouse (open symbols) or 10ng/mouse (solid symbols). The values represent mean ± SEM from an ELISA performed in triplicate.

whether the treatment with the anti-Id Bl/1 affected the levels of the $Id91^+$ antibodies to *Lol* pIV. In this assay, the capacities of antisera from the different groups of mice to inhibit the interaction between mAb91 and Bl/1 were evaluated. The antisera from mice treated with either the control antibody preparation or PBS followed by challenge with *Lol* pIV inhibited strongly the interaction between the Id and anti-Id (Figure 12). Treatment of the animals with either Bl/1-KLH in alum or with Bl/1 in PBS at a dose of 25μg, followed by the challenge with *Lol* pIV, produced antisera that were at best, only poorly capable of inhibiting (< 25%) the interaction between the mAb91 and anti-Id. By comparison, the antibodies from the mice treated with the low dose (10ng) of Bl/1 expressed higher levels of the antibodies carrying the Id91 (Figure 12B, inhibition values increased approximately 30% relative to that of the control groups). These results indicated that administration of the anti-Id Bl/1 regulated the levels of formation of antibodies to *Lol* pIV that possessed the Id91.

THE PROPORTION OF Id^+Ag^+ AND Id^-Ag^+ ANTIBODIES TO Lol pIV:

To establish what proportion of the antibodies to Site A of *Lol* pIV (i.e. Ag^+) possessed also the Id91 (i.e. Id^+), the antisera from mice treated with a low dose (10ng/mouse) of Bl/1 or a control antibaody preparation and challenged with

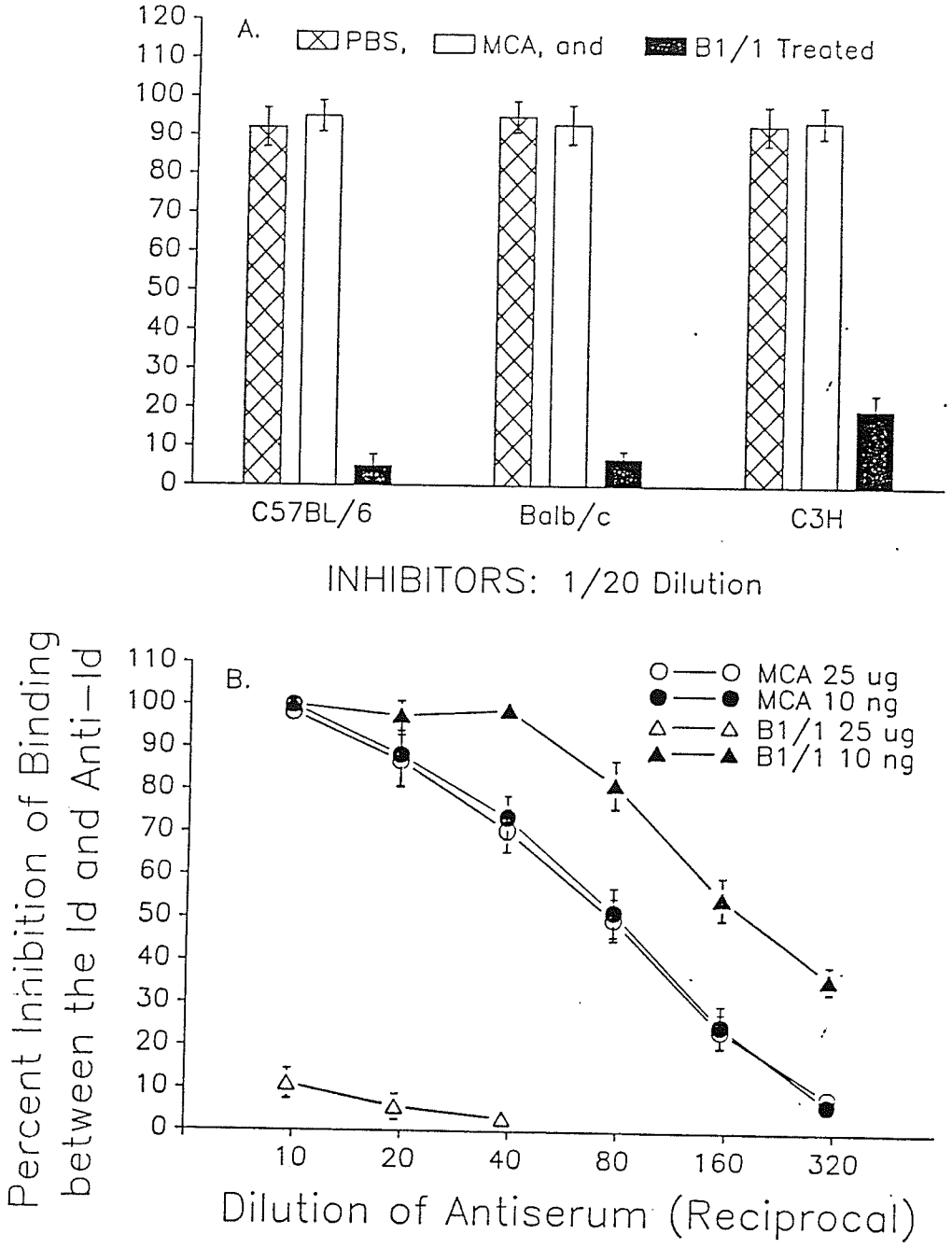


Figure 12. Effect of in vivo treatment with the anti-Id on the formation of Id91$^+$ antibodies.

The level of Id91$^+$ antibodies in antisera was evaluated in terms of the degree to which the antisera were able to compete with B1/1 for binding to mAb91 in a competitive ELISA. *A.* antisera obtained from three strains of mice treated with 25μg of antibody preparation of MCA-KLH or B1/1-KLH, or PBS; *B.* antisera from Balb/c mice treated with B1/1 in PBS at 25μg/mouse (open symbols) or 10ng/mouse (solid symbols). The values represent mean ± SEM from an ELISA performed in triplicate.

Lol pIV were passed through a reversed-immunoadsorbent prepared with the anti-Id B1/1. The absorption was repeated using recycled adsorbent until the effluent was devoid of detectable Id^+ antibodies. The absorbed antisera were concentrated to their original volume. Samples of the absorbed antisera were evaluated by the relevant assay in order to establish (i) the levels of the Id^+ antibodies (in terms of their capacity to inhibit the interaction between mAb91 and B1/1); (ii) the levels of total antibodies to *Lol* pIV (by direct binding to solid-phase *Lol* pIV) and IgE antibodies to *Lol* pIV (by PCA) and (iii) the levels of Ag^+ antibodies (in terms of their capacity to inhibit the binding of mAb91 to *Lol* pIV). The results shown in Figure 13A and 13B, revealed: that (i) before the absorption, the antisera could inhibit completely the binding between the Id and anti-Id, whereas after removing the Id^+ antibodies from the antisera, the remaining Id^- antibodies were not able to inhibit this interaction (Figure 13A); (ii) the levels of antibodies which bound to the solid-phase *Lol* pIV decreased after the absorption of Id^+ antibodies (Figure 13B).

It was of interest to establish to what extent the Id^+ antibodies were also Ag^+. From the results shown in Figure 13C, (i) it was clear that absorption of the Id^+ antibodies markedly reduced the inhibitory capacity of the antisera in the assay. On the basis of this observation, it was deduced

that a major portion of the Id^+ antibodies were directed to Site A of *Lol* pIV. (ii) Since the PCA titers of the antisera from the control group and the group treated with the low dose of the anti-Id dropped from levels of 640 and 1280, respectively, down to 10 (Figure 13D) after passage through the anti-Id immunoadsorbent, it was considered that practically the entire repertoire of the antibodies of the IgE class to *Lol* pIV was Id^+.

In the group of mice treated with the low dose (10ng) of mAb B1/1, the level of antibodies to Lol pIV was slightly higher compared to that of the control group (Figure 13B). Since removal of Id^+ antibodies from the antisera of either the treated group or the control group reduced the levels of antibodies to *Lol* pIV that were comparable to one another, the higher level of antibodies to *Lol* pIV in the treated group was attributed to an elevation in the level of Id^+ antibodies.

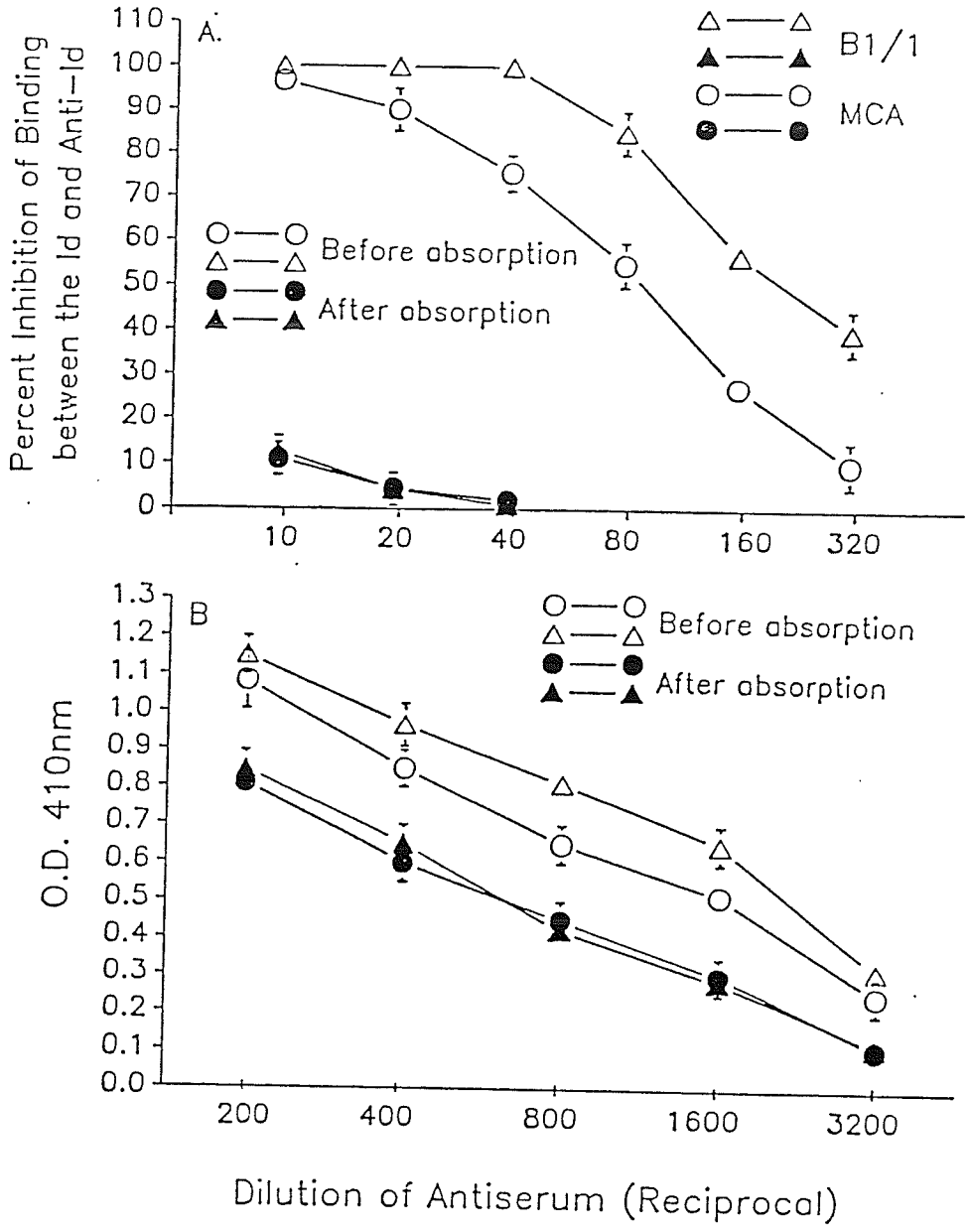


Figure 13. The proportion of the Id^+Ag^+ and Id^-Ag^+ antibodies in antisera to Lol pIV.

Antisera from mice treated with 10ng of B1/1 or MCA in PBS were absorbed by passage through B1/1-Sepharose 4B immunoadsorbent. A series of ELISA assays were used to evaluate: *A.* Levels of Id^+ antibodies detected by its capacity of inhibiting the Id-anti-Id interaction. *B.* Levels of antibodies to *Lol* pIV detected by its direct binding to solid-phase *Lol* pIV.

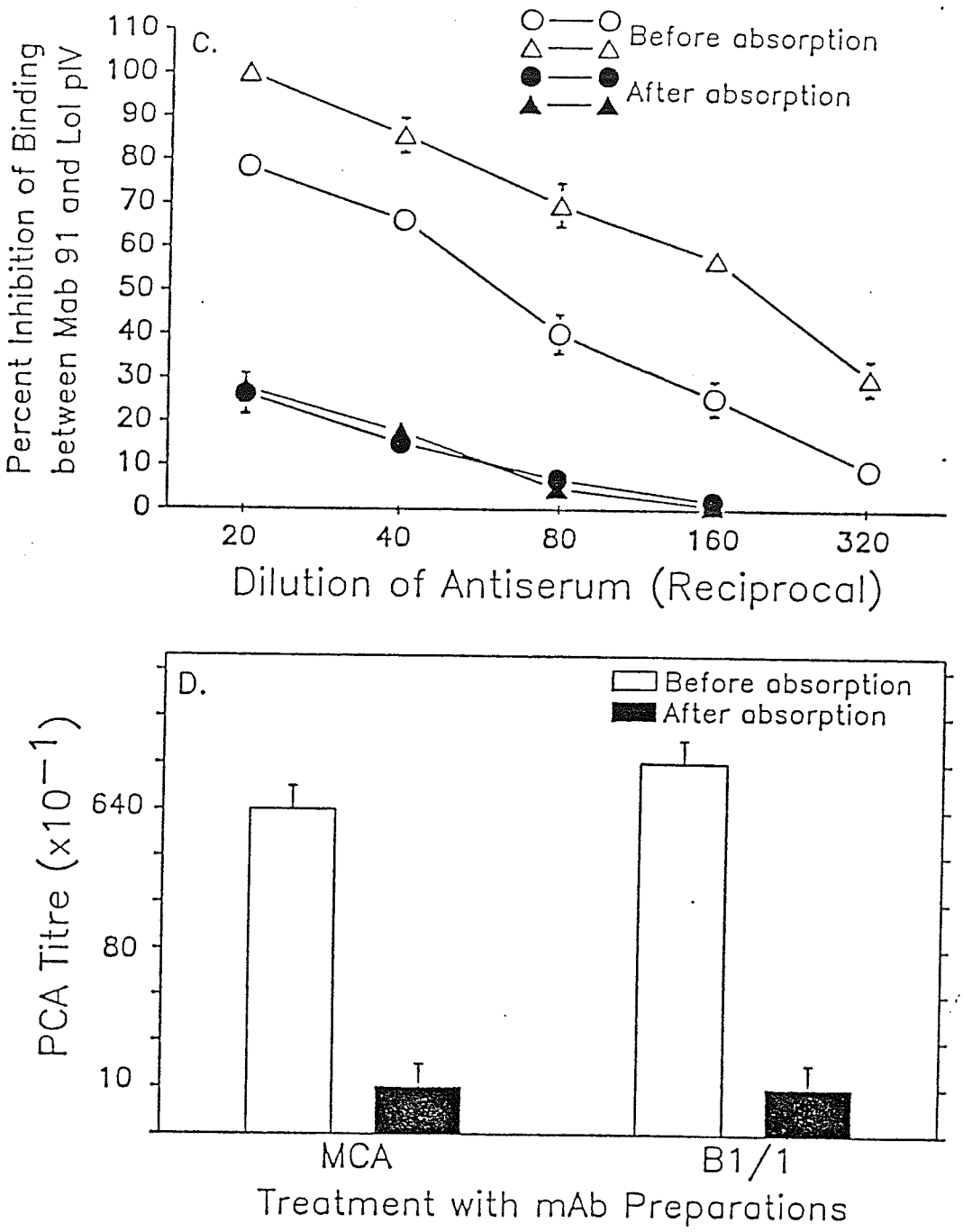


Figure 13. ***C.*** **Levels of Site A specific antibodies detected by its capacity of inhibiting mAb91 from binding to solid-phase** ***Lol*** **pIV.** ***D.*** **Levels of anti-*Lol* pIV IgE antibodies detected by a PCA assay. The values represent the mean ± SEM from an ELISA performed in triplicate.**

DISCUSSION

Anti-Ids which could potentially serve as vaccines for infectious diseases and as therapeutic anti-tumor agents have been studied over the past few years (Zhou, E.-M. et al. 1987, Zhou, E.-M. and Kennedy, R.C. 1989, Kennedy, R.C. et al. 1987, Oosterlaken, T.A.M. et al. 1991, Su, S. et al. 1992). However, potential therapeutic approaches using anti-Id to regulate the antibody responses to allergens, such as Ryegrass pollen allergen *Lol p*IV has not yet been explored to the same extent.

This study demonstrated that it is possible to modulate the formation of antibodies to the allergen *Lol p*IV by a single injection of mice with the internal image monoclonal anti-Id B1/1. There were three model systems used in this study, one was the treatment of three different strains of mice with B1/1 at 25μg conjugated to KLH and precipitated in alum by an i.p. injection 14 days prior to their challenge with *Lol p*IV (Figure 7). The reason for the use of B1/1-KLH conjugate was to compare with B1/1 alone for their effect on modulating the formation of antibodies to *Lol p*IV. As described below, the second was the treatment of syngeneic Balb/c mice with B1/1 in PBS at a dose of 25μg/mouse at various times (up to 8 weeks) (Table IV), and the third at various doses, (10ng to 100μg/mouse) (Table V), prior to their challenge with *Lol p*IV. The treatment of mice in the first two

systems was effective in reducing the level of (i) antibodies to *Lol pIV* (Figure 7, Table IV and V), (ii) the Id^+ antibodies (Figure 8 and 11), and (iii) the reduction of these responses persisted over a period of 56 days (Figure 8). On the other hand, at a dose of 10ng/mouse B1/1 in PBS resulted in an elevation in both the levels of antibodies to *Lol pIV* and antibodies possessed the Id91 (Figure 8). These results clearly indicated that anti-Id B1/1-KLH could suppress the antibody responses to *Lol pIV*, i.e. the formation of antibodies to *Lol pIV* and the expression of Id91. The possibility that KLH had some influence on the suppressive effect was ruled out by the observation that B1/1 in PBS had similar effect as the B1/1-KLH conjugate. On the basis of these observations, the standard protocol adopted for all further experiments consisted of administering B1/1 in PBS at either a (high) dose of 25μg/mouse or a (low) dose of 10ng/mouse.

A major criterion for evaluating the effectiveness of any therapy for pollen allergies is that a reduction in the formation of IgE antibodies to the offending allergens should occur. It must be true that allergen *Lol pIV* consists of many antigenic and/or allergenic epitopes. IgG, IgM and IgE antibodies against *Lol pIV* have been observed from an antibody response to *Lol pIV*. The reasons for determining each isotype of antibodies to *Lol pIV* were to delineate the effect of anti-

Id treatment on antibody responses to this allergen, i.e., the anti-Id modulation through the Id network effects on IgE and/or other isotypes of the antibodies to *Lol pIV*. In the present study, it was found that the level of IgE antibodies to *Lol pIV* were dramatically reduced (Figure 9) in the mice that had received treatment with Bl/1 at high dose (25 or 50μg). By comparison, the formation of other classes of antibodies (IgG1 and IgG2b) to *Lol pIV* were only reduced to the maximum extent of 50% of the control values (Figure 10). This suggests that the IgE antibody response may be more susceptible to modulation by Bl/1 compared to other classes of antibodies. Other studies have also demonstrated the suppressive effect of anti-Id. For example, the formation of IgE and IgG antibodies to benzylpenicilloyl (BPO) can be specifically suppressed in Balb/c mice for a period of 2-3 weeks by a single i.v. injection of anti-BPO anti-Id (Blaser, K. et al. 1980). In another murine system, the anti-Id to antigen specific T helper factor and IgE antibody has been used to specifically suppress the IgE antibody response to an allergen of timothy grass pollen (Malley, A. and Dresser, D.W. 1982b and 1983b). These studies collectively indicated that anti-Id could be used to suppress the formation of IgE antibodies to allergens.

The suppression of antibody formation by anti-Id has been studied in other Ag-antibody systems. For example, an anti-Id

has been used to suppress the formation of murine antibodies to a defined peptide of tobacco mosaic virus protein (Noton, F.L. et al. 1985). In this case, administration of the anti-Id suppressed the formation of Id^+ antibodies to the peptide, however, the level of the antibodies to the virus protein was not affected (Norton, F.L. and Benjamini, E. 1987). By contrast, in the present study, not only was the level of antibodies to the Site A of *Lol* pIV affected (Figure 11), but the levels of the entire repertoire of antibodies to *Lol* pIV were also altered (Figure 8A).

The proportion of the levels of Id^+Ag^+ and Id^-Ag^+ antibodies was evaluated (Figure 13). The data have indicated that a major portion of antibodies to *Lol* pIV possessed Id91 (Figure 13B). Moreover, a major portion of antibodies to Site A was also Id^+ (Figures 13C). Similarly, most, if not all, IgE antibodies to *Lol* pIV were Id^+ (Figure 13D) and this antibody class appeared to be most susceptible to suppression by Bl/1.

The observation that the formation of antibodies to Site A of *Lol* pIV was not completely suppressed (Figure 11) cannot be attributed simply to an inadequate amount of the anti-Id administered since doses of Bl/1 which differed a thousand-fold (100ng to 100μg) had similar effects on the formation of antibodies to *Lol* pIV (Table IV). To account for this observation, it may be speculated that at least two

families of B cell clones which recognize the same epitope (Site A) were generated by challenge with *Lol* pIV. One of these clones produced an antibody which possessed the Id91 and is referred to as Id^+Ag^+, whereas the antibody produced by the other clone lacked the Id91 and is referred to as Id^-Ag^+. It is suggested that administration of the Bl/1, at the high dose, suppressed the Id^+Ag^+ clone and resulted in a reduction in the level of antibodies to *Lol* pIV. On the other hand, the Id^-Ag^+ clone was not affected and produced antibodies by challenge with *Lol* pIV. The observation that the formation of Id^+Ag^+ antibodies to *Lol* pIV was elevated by treatment of the mice with the low dose of Bl/1 may be accounted for by the preferential activation of the Id^+Ag^+ clones. Clearly, the direction of the modulating properties of the anti-Id Bl/1 (i.e. up- or down-regulation) was dose-dependent.

In a separate study, Malley, A. and Dresser, D.W. (Malley, A. and Dresser, D.W. 1982b), using anti-Id to modulate the immune response to the hapten NP, have shown that administration of 10μg of the anti-Id to antibodies against NP, suppressed the formation of the Id^+ antibodies, whereas the total antibody level to NP was not affected. They proposed that the Id^+ clones were suppressed but the Id^- clones were still secreting the Id^- antibodies to NP. Another study (Bitoh, S. et al. 1990) has shown that cloned anti-Id producing B cells possessed the capacity of modulating the in

vitro Id^+ anti-dextran antibody response. This effect was dose dependent; thus, at a high dose (1000 cells), suppression was induced, whereas at a low dose (10 to 100 cells), the Id^+ antibody response was enhanced.

In a recent report (Hebert, J. et al. 1991), it was claimed that passive transfer of nonatopic beekeeper's plasma containing the anti-Id to antibodies against bee venom, protected sensitive patients from a subsequent systemic reaction to bee venom. Observations of this nature suggest that anti-Id may have a therapeutic potential for the treatment of allergy.

In summary, this study demonstrated that in murine model systems the antibody responses to allergen *Lol pIV* could be effectively modulated by the use of the internal image monoclonal anti-Id B1/1. At high doses, the B1/1-induced suppression of the responses can be achieved in three different strains of mice, C57BL/6, Balb/c and C3H (their haplotyes are ($H\text{-}2^b$, $H\text{-}2^d$ and $H\text{-}2^k$, respectively) suggesting there is no restriction of the modulating properties of the B1/1 at the level of the MHC.

The questions which may be asked following this aspect of the study include: (i) identification of cell type(s) which can transfer the suppressing effects and (ii) whether the

treatment with the anti-Id B1/1 could have induced, in turn, the anti-anti-Id (Ab3) responses, which may also have played some regulatory role(s) on the antibody responses to allergen *Lol p*IV. These questions were addressed in the next two chapters.

CHAPTER 4.

ANTIBODY RESPONSES TO ALLERGEN *Lol pIV* ARE SUPPRESSED FOLLOWING ADOPTIVE TRANSFER OF ANTI-ID-B-LYMPHOCYTES FROM THE MICE TREATED WITH THE INTERNAL IMAGE ANTI-ID

ABSTRACT

The suppressive function of an internal image anti-Id on the formation of antibodies to allergen *Lol* pIV was investigated in a Balb/c mouse model system. In the previous studies described in Chapter 2 and 3, the anti-Id, Bl/1 was generated against the Id91 of mAb91 to *Lol* pIV. The administration of Bl/1 in PBS, at doses ranging from 100ng to 100μg/mouse, to syngeneic mice resulted in the suppression of the formation of anti-*Lol* pIV antibodies that possessed the Id91. In the present study, spleen cells obtained from the mice 2 weeks after the treatment with Bl/1 (25μg/mouse) were adoptively transferred i.v. into the syngeneic recipients which were challenged i.p. with *Lol* pIV in alum 2 hours after the transfer. The recipients were boosted with *Lol* pIV 14 days later. The serum levels of antibodies to *Lol* pIV and antibodies that possessed the Id91 were evaluated by ELISA. The results have shown that the transfer of splenic B cells (but not of T cells) from Bl/1-treated donors induced a significant suppression of not only the level of IgE and IgG antibodies to *Lol* pIV, but also the level of antibodies possessing the Id91. Treatment of the B cells with mAb91 plus complement abrogated their ability to transfer the suppression. This study indicates that the treatment with the anti-Id, Bl/1, generated B cells that were characterized, serologically, as possessing the anti-Id-like antibodies on

their surface and were responsible for transferring the suppression of the formation of antibodies to allergen *Lol pIV* and the expression of Id91.

Introduction

The idiotype network theory proposed by Jerne (Jerne, N.K. 1974) implies that an antibody response to an antigen is controlled by a series of Id-anti-Id interactions that may either suppress or enhance the immune response to the antigen. Since the antibody molecules on B lymphocytes can serve as surface receptors which enable the cells to bind antigen, one might envisage that anti-Id antibody molecules could substitute for antigen in the induction of an immune response. In the case of immunological paralysis, such a functional equivalence of antigen and anti-Id has already been demonstrated. It has been shown that B lymphocytes have the function of regulating the formation of antibodies (Inada, T. et al. 1982, Okumura, K. et al. 1982, and Hayakawa, K. et al. 1983). B lymphocytes carrying the anti-Id on their membrane could maintain the Id network in the immune system (Sherr, J.H. and Dorf, M.E. 1984) and/or to induce autoantibody formation (Hayakawa, K. et al. 1983). The study described in this Chapter focused on investigating (i) whether the suppressive effect of the anti-Id-induced suppression of the formation of antibodies to *Lol* pIV can be transferred by the lymphocytes from the anti-Id-treated mice; and (ii) if this was indeed the case, to identify if these lymphocytes were B and/or T cells.

The surprising results reported in this Chapter describes that the suppression of antibody responses to *Lol* pIV could be adoptively transferred into syngeneic mice by the B lymphocytes obtained from the anti-Id, B1/1-treated mice. These B lymphocytes appeared to possess the anti-Id-like antibodies on their surface.

Materials and Methods

Antibodies:

Monoclonal antibodies mAb91 and a control IgG1κ mAb2, specific for OA, contributed by Dr. Sehon, A.H. of the MRC Group were used as Ab1. Monoclonal anti-Id B1/1 (IgG2bκ) has been generated and characterized as an internal image anti-Id as described in chapter 2. A monoclonal antibody MCA (IgG2bκ), specific for influenza virus, was purchased from Cedarlane Laboratories Ltd., Ontario.

Purification and isolation of monoclonal antibodies from ascitic fluid was performed as described previously (Chapter 2 and Kennedy, R.C. et al. 1983).

Treatment of Syngeneic Balb/c Mice with Anti-Id B1/1:

Adult female Balb/c mice received affinity purified anti-Id B1/1 at a dose of 25μg/mouse by an i.v. injection. For control, mice similarly received MCA (25μg/mouse). Fourteen days later, the mice were sacrificed and their spleens were removed for preparation of suspensions of cells.

To determine whether the anti-Id B1/1 which had been administered i.v. may have been carried over with the spleen

cells on their adoptive transfer, the anti-Id B1/1 was radiolabelled with ^{125}I by using chloramine T as described by Greenwood, F.C. et al. (Greenwood, F.C. et al. 1963) and free iodide was removed by Sephadex G-25 (Pharmacia, Uppsala, Sweden) gel filtration. The radio-activity of ^{125}I-labelled anti-Id B1/1 was 5.3×10^4 cpm/μg and the total of 1.325×10^6 cpm/25μg was injected i.v. into the donor mice. Fourteen days later, the mice were sacrificed and single splenic B cell suspensions were prepared as described below for the detection of radio-activity of B cells.

Culture Media:

Dulbecco's minimal essential medium (DMEM, Sigma, St. Louis, MO) supplemented with streptomycin (50 μg/ml), penicillin (50 IU/ml), 4 mM L-glutamine, 1% (v/v) nonessential amino acid, and 10% fetal calf serum (FCS) (Sigma, St. Louis, MO).

Preparation of Spleen Cells:

Spleen cell suspension was prepared by removing the spleens from the mice treated with anti-Id B1/1. The spleens were gently homogenized in a glass homogenizer and a single cell suspension was washed three times with the DMEM medium. The red blood cells were eliminated by the treatment of the

cell suspension with 0.85% NH_4Cl and the treated cells were washed with DMEM. The cell viability as determined by trypan blue exclusion, was more than 95%. The spleen cell suspension was then used for either the direct adoptive transfer or the separation and isolation of Ig positive (B cells) and Ig negative (T cells) cells (described below).

Separation of Splenic B and T Cells:

To isolate separately the splenic B and T cells for the purpose of adoptive transfer, first of all, splenic B cells were prepared by using the panning procedure described by Wysocki, and Sato (Wysocki, L.J. and Sato, V.L. 1978). Briefly, bacteriological Petri dishes (100 x 15 mm) (CanLab Ltd., Ontario) were coated with affinity isolated goat anti-mouse Ig (Dakotatts Laboratory, Glostrup, Denmark) at 5μg/ml in 10 ml coating buffer (0.05 M Tris-HCl, pH 9.5) for 40 min at room temperature. The dishes were washed with 1% FCS in PBS and the remaining binding sites were blocked with 1% FCS in PBS for 15 min at room temperature. Splenocytes (3×10^7) suspended in PBS (containing 5% FCS) were incubated on these dishes for 70 min at 4^0C with an occasional redistribution of the cells. The nonadherent cells (containing T cells) were collected by decanting the fluid and the bound cells were recovered by flushing the dish with PBS containing 1% FCS. Secondly, the recovered B cells and nonadherent spleen cells

were then treated, respectively, with rabbit anti-mouse T-cell serum (anti-Thy-1) and rat anti-mouse B cell antibody J11d.2 (ADCC) along with Low-Tox-M rabbit complement (CedarLane Laboratories Ltd., Ontario) using the procedure recommended by the manufacturer. Briefly, 1 x 10^7 cells in 1 ml of DMEM were mixed with the corresponding antibody preparation (at a final dilution of 1:40 for anti-Thy-1 and 1:10 for J11d.2, respectively) for 60 min at 4^0C. The cell mixture was centrifuged to pellet the cells and the supernatant containing the unbound antibodies was discarded. After resuspending the cell pellet to the original volume in DMEM the complement at a final dilution of 1:10 was added into the cell suspension and incubated for 60 min at 37^0C. The lysed cells were removed by centrifugation and the viability of remaining cells was determined by the trypan blue exclusion method.

Charaterization of Isolated Splenic B and T Cells:

The isolated splenic B and T cells were placed into individual tubes at 1 x 10^7/tube in 5% FCS in DMEM and were allowed to interact, for 40 min on ice, with either with goat anti-mouse Ig-FITC (Becton Dickinson Canada Inc. Mississauga, ON), at 20µl/10^6 cells, or with monoclonal antibody to Thy1.2-FITC (Cedarlane Laboratories LTD. Hornby, ON), at 1µg/10^6 cells. After washing the cells with 5% FCS in DMEM three times, each fraction of cells was analyzed by a fluorescence-

activated cell sorter as determined by Dr. E. Rector of this Department. The purity of isolated B cells was more than 99% and that of T cells was more than 85%.

Preparation of Supernatant from Lysed Cells:

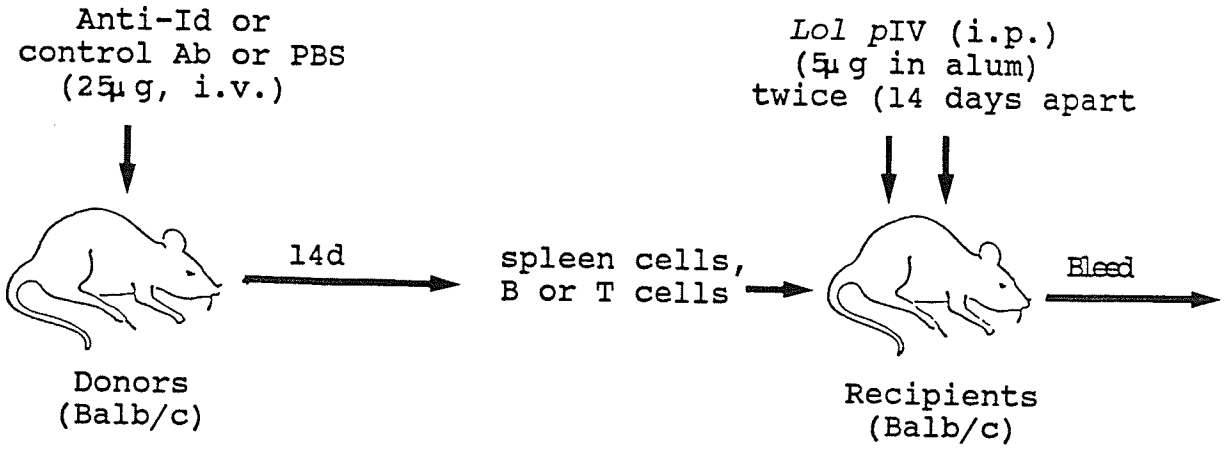
To determine whether the soluble cell-free extracts could induce the suppression of the antibody formation, spleen cells or B lymphocytes isolated as described above were resuspended in DMEM at $2x10^7$ cells/ml. The cell suspensions were frozen at -60^0C and thawed at 37^0C, three times and then centrifuged at 12,000 x G for 20 min to remove cell debris. The supernatant was recovered and 0.5 ml, i.e. the amount corresponding to the equivalent of 1×10^7 cells, was injected i.v. into each recipient.

Treatment of Splenic B Cells with4 mAb91 or Anti-Id B1/1:

To characterize the nature of the highly purified splenic B cells isolated from the anti-Id B1/1-treated mice, the B cells were further treated in vitro with either mAb91 or anti-Id B1/1. Monoclonal mAb2 or MCA served as controls. The procedure for the treatment was as follow: 1×10^7 B cells in 1 ml of DMEM were mixed with mAb91, B1/1, mAb2, or MCA at the amount of 5µg for 1×10^7 cells for 60 min at 4^0C. The remaining procedures were same as that described above. After

the treatment of the B cells, they were used for the adoptive transfer experiment.

PROTOCOL OF ADOPTIVE TRANSFER



Adoptive Transfer:

According to the protocol, three groups of donor Balb/c mice (5 mice/group) received, by an i.v. injection, either the anti-Id B1/1, MCA or PBS. Nine groups of syngeneic recipient mice received either the B, T, Id-treated or anti-Id-treated B cells. All recipients were challenged approximate 2 hours after the adoptive transfer,and boosted i.p. 14 days later with *Lol* pIV (5μg/mouse) and OA (2μg/mouse) in alum. Antiserum from each recipient was collected on day 21 after the boost and the antibody responses to *Lol* pIV and OA were tested by an

ELISA. Sera obtained from the recipients before the treatment served as negative controls.

ELISA Protocols:

(i) *Detecting Antibody Titers to Lol pIV and OA.* The antibodies to *Lol* pIV and OA present in the recipient antisera were detected by a direct ELISA which has been used routinely through the entire study. Briefly, ELISA plates (Immulon II, Dynatech Laboratories Inc., Alexandria, Va., USA) were coated with *Lol* pIV (200ng/well) or OA (100ng/well), individually, at 4^0C, overnight. After blocking the unbound sites with 2% gelatin in BBS, antisera obtained from the recipients were added to the wells and incubated at 37^0C for 1 h. The antibodies which had bound to *Lol* pIV or OA were detected by the horseradish peroxidase (HRP)-conjugated goat antibodies to mouse Ig (BioRad Laboratories, Richmond, Calif., USA) at 37^0C for 1 h. The colorimetric reaction was developed by adding the substrate, ABTS and H_2O_2. The reaction was stopped by adding 5% SDS and the OD_{410} nM of each well was measured on an automatic ELISA plate reader (MR600. Dynatech Laboratory). The assay was performed in triplicate.

(ii) *Detection of Antibodies Specific to Site A of Lol pIV.* A competitive inhibition ELISA procedure was employed to establish the relative levels of antibodies in the recipients'

antisera, which recognized Site A of *Lol pIV*. Briefly, biotinylated mAb91 at a concentration of 5μg/ml was mixed with the various antisera inhibitor at final dilution of 1:10. The mixture was added to the wells which had been coated with *Lol pIV*. After incubation at 37^{0}C for 1 hr, the plates were washed with T-BBS. Streptavidin-conjugated HRP was used, at 25^{0}C for 25 min, to determine the extent to which the biotinylated mAb91 had been bound to the solid-phase *Lol pIV*. The assay was continued as described above. The extent of the inhibition was calculated according to the formula: Inhibition (%) = 1 - [$OD_{410}$with inhibitor - background / OD_{410}without inhibitor - background] x 100

(iii) ***Detection of Id91$^+$ Antibodies to Lol pIV***. Two ELISA procedures were used to determine the levels of the Id91$^+$ antibodies in the antisera.

First, a two-side ELISA was used to determine the direct binding of Id$^+$ antibodies to monoclonal anti-Id B1/1. The wells of ELISA plates were coated with B1/1 (400ng in 50μl of BBS) at 4^{0}C overnight. After blocking the unbounded sites with gelatin (2% in BBS), the solid-phase B1/1 was allowed to interact with the Id$^+$ antibodies present in the recipients' antisera at 37^{0}C for 1 hr followed by washing the plates with T-BBS. Biotinylated B1/1, at a concentration of 8μg/ml was added into the wells and incubated at 37^{0}C for 45 min. The

assay was continued as described above.

Secondly, the capacity of these antibodies to interfere with the Id-anti-Id interaction was examined in a manner similar to the inhibition procedure described above, with the following modifications; the mAb91 (200ng in 50µl of BBS) was used to coat the wells. Biotinylated anti-Id B1/1 (8µg/ml) was pre-mixed with recipients' antisera and the mixture was added to the wells. The remaining steps were performed as described above.

Passive Cutaneous Anaphylaxis (PCA):

The levels of IgE antibodies to *Lol p*IV and OA were determined by the PCA procedure as described in Chapter 3. Briefly, the antisera were serially diluted two-fold in saline and volumes of 50µl of each dilution injected intradermally into rats. Twenty four hrs later, *Lol p*IV or OA mixed with Evans blue dye was injected i.v. and 20 min later the size of the reactions was measured on the undersurface of the skin. The highest dilution of antisera which gave the reaction size of 3 mm in diameter was considered as the end point titre. Each sample was tested in duplicate.

RESULTS

Transfer of Suppression of Antibody Responses to Lol pIV by Cells from Anti-Id B1/1-treated Donors.

The possibility that the suppression of the antibody formation which had been induced by the treatment of mice with the anti-Id B1/1 could be transferred along with spleen cells into syngeneic recipients was examined. For this purpose, donor mice were treated with 25μg of anti-Id B1/1 in PBS by an i.v. injection. For control, the identical isotype of a monoclonal antibody preparation (MCA) to influenza virus, i.e. with the specificity unrelated to that of anti-Id B1/1, was used. The donor mice were sacrificed 14 days later and the suspensions of spleen cells, or enriched preparations of B or T cells were adoptively transferred i.v. into syngeneic recipients which were challenged i.p. 2 hrs later with a mixture of 5μg *Lol* pIV and 2μg OA precipitated in alum. They were boosted two weeks later with the same antigenic mixture and their blood was collected after an additional 21 days. The level of the recipients' antibodies to *Lol* pIV and OA were evaluated by ELISA procedure.

(i) *The levels of Antibodies to Lol pIV and OA:* A direct ELISA was employed to evaluate the levels of the IgG1 antibodies to *Lol* pIV and OA. As shown in Table VI, transfer

of spleen cells from the donor mice that received MCA as a control mAb (instead of anti-Id) had no demonstrable effect on the levels of antibodies to *Lol* pIV (Group 2). Neither did the spleen cells from the mice which had received PBS (Group 1). By contrast, transfer of spleen cells from the donor mice that received anti-Id B1/1 resulted in 72-77% reduction of the level of antibodies of the IgG1 class in terms of lower ELISA titers(control Group 1 vs. experimental Group 5, i.e. 2000 vs. 500) and 98-99% reduction of IgE class in the PCA titers (1280 vs. 10) (Group 5).

Further studies which involved the separation of the spleen cells into B and T lymphocytes indicated that the suppressive activity of the spleen cells from the anti-Id-treated donor mice was associated with the B lymphocytes, i.e. the transfer of 1×10^7 B cells resulted in the reduction of level of IgG1 antibodies to the extent of 78-82% and the reduction for IgE antibodies in titers of the order of 98-99% (Group 7). The possibility of non-specific activation of B cells by positive selection can be ruled out since MCA-treated B cells, after positive selection, had no effect on the suppression. On the other hand, transfer of splenic T lymphocytes from the anti-Id B1/1-treated donor mice have no effect on the level of either IgG1 or IgE antibodies of the recipients (Group 6). Furthermore, the results shown in Table VI indicated that the magnitude of the effect of transfer of

suppression of antibody responses was cell dose dependent (Groups 7-9).

It is evident from the results in Table VI that transfer of the suppression of the formation of antibodies to *Lol pIV* was antigen specific since the transfer of cells from the anti-Id-treated mice had no effect on the levels of IgG1 and IgE antibodies to OA.

The possibility that the reduction of the levels of antibodies to *Lol pIV* in the recipient mice may have been due to the carry-over of the anti-Id B1/1 with the transferred spleen cells was virtually excluded by the negative results of the experiment in which the ^{125}I-labelledanti-Id B1/1 was used. For the estimation of the amount of anti-Id B1/1 associated with the cells transferred, the recipients were treated with ^{125}I-labelledanti-Id B1/1 14 days prior to the use of their spleen cells for the detection of radio-activity. The radio-activity associated with the 1 x 10^7 spleen cells as well as with the B or T cells was measured. The amounts of anti-Id B1/1 associated with the cells were less than 1ng. By comparison, the minimal amount of 100ng of anti-Id B1/1 was required to induce the suppression (refer to Chapter 3).

The results of these experiments indicated that the suppression of antibody responses to allergen *Lol pIV* could be

Table VI.

Transfer of Suppression of Formation of Antibodies to Allergen *Lol* pIV by Spleen Cells from B1/1-Treated Mice

Group[a] #	Treatment of donors[b]	# of cells transferred	Antibodies to: *Lol* pIV[c]		OA[c]	
			IgG1	IgE	IgG1	IgE
1	PBS	$5x10^7$ Spleen cells	2000	1280	10000	5000
2	MCA	$5x10^7$ Spleen cells	2200	1280	9000	4000
3	MCA	$1x10^7$ T cells	2000	640	10000	5000
4	MCA	$1x10^7$ B cells	1800	640	8000	5000
5	B1/1	$5x10^7$ Spleen cells	500	10	10000	4000
6	B1/1	$1x10^7$ T cells	2000	1280	9000	4000
7	B1/1	$1x10^7$ B cells	400	10	8000	5000
8	B1/1	$1x10^6$ B cells	1000	80	10000	4000
9	B1/1	$1x10^5$ B cells	1500	640	9000	5000

[a] Each group consisted of 5 mice. The recipients received by i.v. the donor's cells and 2hr later immunized with an i.p. injection of 10μg of *Lol* pIV and 2μg of OA in alum and boosted 14 days later. The antisera were collected after 21 days of the 2nd injection.

[b] Donor mice were treated once with PBS, MCA or B1/1 at 25μg/mouse 14 days prior to the transfer of their cells to recipients.

[c] Direct ELISA was employed to detect IgG1 and PCA to detect IgE anti-*Lol* pIV and anti-OA antibodies, respectively, in antisera from recipients. The values represented the end point titre.

adoptively transferred by the spleen cells from anti-Id-treated donor mice and the cells responsible for the transferred suppression were B lymphocytes.

(ii) *The Levels of Antibodies Directed to Site A of Lol pIV:* Site A of *Lol* pIV is the epitope that recognized by mAb91. A competitive ELISA was used to determine whether the transferable suppression of antibody responses to *Lol* pIV was specific for Site A. In this assay, the capacity of antibodies to inhibit the binding of mAb91 to solid-phase *Lol* pIV was evaluated. The results presented in Figure 14 indicated that the antisera, obtained from the recipients transferred with spleen cells or B lymphocytes from anti-Id B1/1-treated mice, gave an inhibition value of less than 30% at 1:10 dilution. By contrast, the antisera from the recipients that were transferred with spleen cells from control antibody MCA-treated donor mice or T cells from B1/1-treated donor mice had inhibition values of 70-75%. Therefore, these results collectively indicated that the majority of antibodies to *Lol* pIV which had been suppressed were directed to Site A.

(iii) *The levels of Antibodies Possessing the Id91:* ELISA procedures were employed to evaluate the levels of $Id91^+$ antibodies from antisera of the adoptive transfer experiments. (a) A two-site ELISA was used to evaluate the levels of $Id91^+$ antibodies that react with anti-Id by determining their

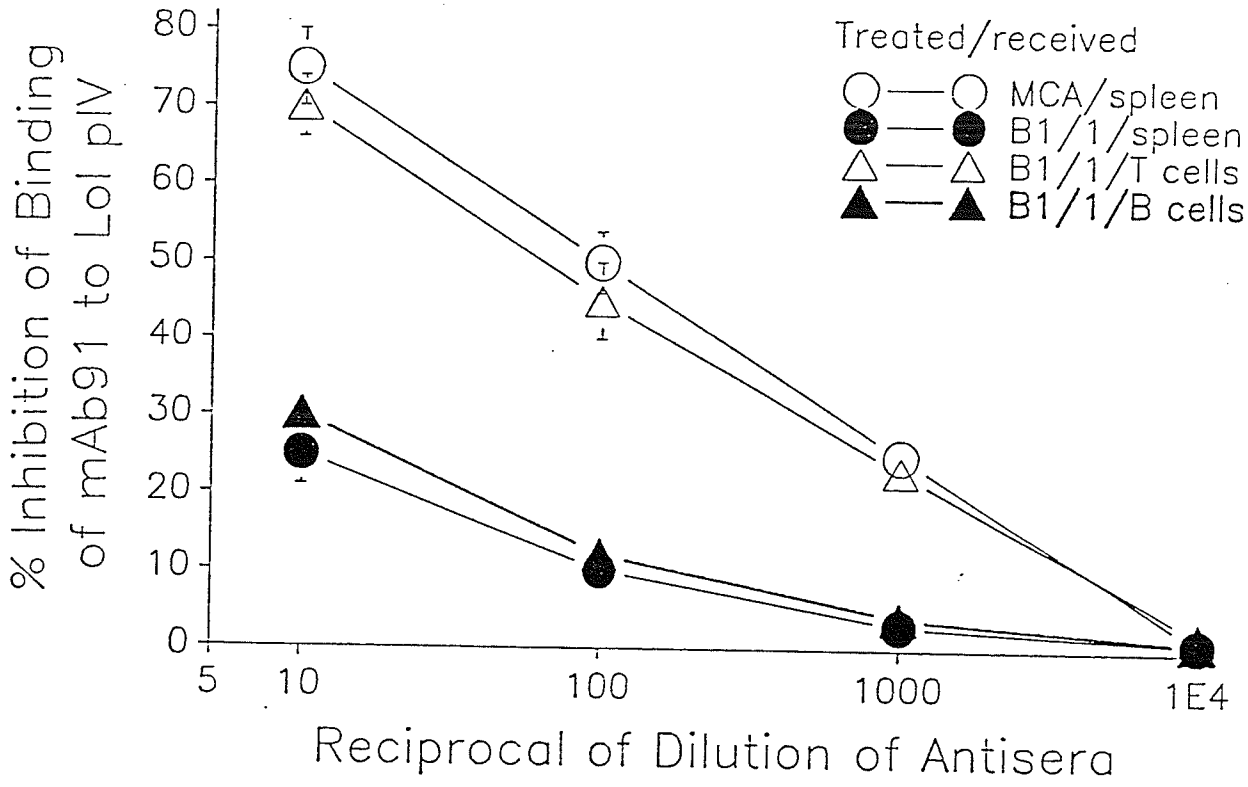


Figure 14. ***Transfer of Suppression of Formation of Antibodies to the Epitope Site A of Lol pIV.***

The levels of sera antibodies to Site A of *Lol* pIV were evaluated in terms of the degree to which the antisera were able to compete with mAb91 (anti-Site A) for binding to *Lol* pIV. The number of cells transferred was $1x10^7$/recipient. The values represent mean ± SEM from an ELISA performed in triplicate.

capacity for directly binding to anti-Id B1/1. As indicated in Table VII (column 4), end point titer of 2500 was obtained for the antisera from the recipients that had received cells from PBS-treated (Group 1) or the control antibody MCA-treated donor mice (Groups 2-4). Similar results were also observed for the antisera from recipients that had received T lymphocytes from B1/1-treated mice. Significant reduction of the end point titers reflected decreased level of antibodies that may possess the Id91 was observed for the antisera from the recipients received either spleen cells or B lymphocytes from B1/1-treated donor mice (end point titer of 200 for Groups 5 and 7). (b) A competitive ELISA procedure was used to determine that transfer of the B lymphocytes of donor mice that had been treated with anti-Id B1/1 resulted in the reduction of the levels of antibodies possessing the Id91. In this assay, the capacities of antisera from the different groups of recipient mice to inhibit the interaction between the $Id91^{+}$ mAb91 and anti-Id B1/1 were evaluated. As shown in Table VII (column on far right), the Id-anti-Id interaction could be inhibited significantly by the antisera from recipients that received spleen cells from donors which have been treated with either PBS (75%, Group 1) or control monoclonal antibody MCA (78%, Group 2). Similarly, the antisera from recipients that received either the T or B cells from donors which were treated with control antibody MCA could inhibit the interaction between the Id and anti-Id (76% and

75% for Group 3 and 4). It was the transfer of spleen cells or the B cell fraction, but not T cells (78%), from the anti-Id B1/1-treated donors that resulted in the formation of antibodies which had a lower capacity to inhibit (8-10%) the Id-anti-Id interaction (Groups 5, 6 and 7). It is also demonstrated that the suppressive effect of the B lymphocytes was cell dose dependent as shown in Table VII (Groups 7, 8 and 9).

These results collectively indicated that by transfer of B lymphocytes from the anti-Id treated mice, the suppressive effect of the formation of $Id91^+$ antibodies could be effected.

Inability to Transfer the Suppression by Cell-free Extract from Lysed B Lymphocytes.

To study the characteristics of the factors of B lymphocytes responsible for the suppression of antibody responses to allergen *Lol* pIV, cell-free extracts were prepared from 1 x 10^7 B cells isolated from spleens of B1/1-treated or, for control, MCA-treated donor mice. The supernatant was injected i.v. into normal syngeneic recipients that were challenged 2 hrs later with *Lol* pIV (5μg) and OA (2μg) precipitated in alum. All recipients were boosted with the allergens 14 days later and blood was collected after additional 21 days. A competitive ELISA and a direct binding

Table VII.
Transfer of Suppression of Id91 Expression by Splenic Cells from B1/1-Treated Mice

Groups[a] #	Treatment of donors[b]	# of cells transferred	End Point Titers[c]	% Inhibition of Id-anti-Id Interaction[d]
1	PBS	$5x10^7$ Spleen cells	2300	75
2	MCA	$5x10^7$ Spleen cells	2500	78
3	MCA	$1x10^7$ T cells	2500	76
4	MCA	$1x10^7$ B cells	2400	75
5	B1/1	$5x10^7$ Spleen cells	200	10
6	B1/1	$1x10^7$ T cells	2400	78
7	B1/1	$1x10^7$ B cells	200	8
8	B1/1	$1x10^6$ B cells	1000	33
9	B1/1	$1x10^5$ B cells	2200	73

[a] Each group consisted of 5 mice. The donor cells were transferred into recipients that were injected 2 hrs later with 10μg of *Lol pIV* and 2 μg of OA in alum and boosted 14 days later. The antisera were collected 21 days.

[b] Donor mice were treated once with PBS, MCA or B1/1 at 25μg/mouse 14 days prior to the transfer of their cells to recipients.

[c] The $Id91^+$ Anti-*Lol pIV* antibodies were detected by two-site ELISA. The data were expressed as the reciprocal of dilution of antisera which gave an OD_{410} value three times higher than that obtained from pre-immune sera diluted 10-fold. These values were referred to as the end point titer.

[d] The $Id91^+$ antibodies from the recipients were further evaluated for their capacity to inhibit the Id-anti-Id interaction by a competitive ELISA. The values represent the mean ± SEM from an ELISA performed in triplicate.

ELISA were used to evaluate (a) the levels of the Id^+ antibodies and (b) the antibodies to *Lol* pIV and OA. As is evident from the results in Table VIII, the B lymphocytes from Bl/1-treated donor mice could transfer the suppression of the antibody responses to allergen *Lol* pIV (Group 3). However, the supernatant of lysed B lymphocytes from Bl/1-treated donor mice was devoid of the capacity to affect the antibody responses to *Lol* pIV (Group 5). Similar results were obtained with the supernatant of B cells of MCA-treated donor mice (Group 4). These results indicated a requirement for B lymphocytes to be intact in order to be able to effectively transfer the suppression of antibody responses to allergen *Lol* pIV.

To further characterize the population of B cells which were found to mediate the suppression, the B cell preparations from (a) control antibody (MCA)-treated and (b) anti-Id (Bl/1)-treated donor mice were treated in vitro with either (a) the $Id91^+$ mAb91 and complement or (b) the anti-Id Bl/1 and complement prior to their transfer to syngeneic recipients. To evaluate the levels of Id^+ antibodies and the antibodies to *Lol* pIV, two ELISA procedures (inhibition of Id-anti-Id interaction and direct binding to allergens) were used to evaluate the effect of treatment on the transferable suppression of antibody responses to allergen *Lol* pIV.

Table VIII.

Effects of Transfer of Cell-free Supernatant Prepared from B Cells of B1/1-treated Mice to Syngeneic Recipients on the Formation of Antibodies to *Lol* pIV

Groups #	Transfer to Recipients[a] (treated/ received)	% Inhibition of Id-anti-Id Interaction[b]	Antibodies to: *Lol* pIV[c]		OA[c]	
			IgG1	IgE	IgG1	IgE
1	Medium	78	2200	1280	10000	4000
2	MCA/B cells	80	2000	640	8000	5000
3	B1/1/B cells	9	400	10	10000	4000
4	MCA/Sup	75	2000	1280	9000	4000
5	B1/1/Sup	76	2200	1280	10000	5000

[a] Recipients were injected i.v. with either intact B cells ($1x10^7$) or the supernatant (Sup) of lysed B cells ($1x10^7$) from MCA- or B1/1-treated donors. All recipients were challenged i.p. twice at 14 days apart with 10μg of *Lol* pIV and 2μg of OA in alum. Their blood was collected at 21 days after the 2nd challenge.

[b] The antisera from the recipients (inhibitors) were used at a dilution of 1:100. The data represented mean of triplicate assays.

[c] Direct ELISA and PCA were employed to detect IgG1 and IgE anti-*Lol* pIV and anti-OA antibodies, respectively, in antisera from recipients. The values represented the end point titre.

As can be seen from the results in Table IX, treatment of the B cells with either medium or complement alone had no demonstrable effect on the abrogation of transfer of suppression of antibody responses, since there was no enhancement of the degree of inhibition of Id-anti-Id interaction and of the direct binding to allergens. By contrast, the ability of B cells to transfer the suppression could be successfully abrogated by their prior treatment with the $Id91^{+}$ mAb91 plus complement (Group 8). On the other hand, treatment of B cells with the anti-Id B1/1 plus complement had no demonstrable effect on the ability of these treated cells to transfer the suppression of formation of antibodies to *Lol* pIV and the expression of Id91 (Group 4).

The results of these experiments indicated that the B cells from anti-Id B1/1-treated donor mice possessed the equivalent of an anti-Id B1/1 on their surface.

Table IX.

Abrogation of Transfer of Suppression of Antibody Responses by Treatment of B Cells with mAb91 and Complement

Groups #	Treatment of Donors	Treatment of Donor B Cells	% Inhibition of Id-anti-Id Interaction[a]	Antibodies to: *Lol pIV*[b]		OA[b]	
				IgG1	IgE	IgG1	IgE
1	PBS	medium	80	2000	1280	10000	5000
2	MCA	mAb91 + C'	78	2200	640	9000	4000
3	B1/1	medium	10	400	10	8000	4000
4	B1/1	B1/1 + C'	15	400	12	10000	5000
5	B1/1	mAb2 + C'	12	200	10	8000	5000
6	B1/1	mAb91	10	200	15	9000	4000
7	B1/1	C'	14	400	12	8000	4000
8	B1/1	mAb91 + C'	75	2000	1280	10000	5000

[a] The inhibitors were used at 1:100 dilution. The data represent the mean of assays performed in triplicate.

[b] Direct ELISA and PCA were employed to detect IgG1 and IgE anti-*Lol pIV* and anti-OA antibodies, respectively, in antisera from recipients. The values represented the end point titre.

DISCUSSION

The study described in this Chapter is an extension of the investigations on the regulation by an internal image monoclonal anti-Id B1/1 to Id91 of mAb91 on the immune responses to allergen *Lol* pIV. It demonstrated that the antibody responses to *Lol* pIV could be down-regulated followed an adoptive transfer of splenic B lymphocytes obtained from an donor treated with the anti-Id B1/1. The generation and characterization of the internal image anti-Id B1/1 was described in Chapter 2. The anti-Id could be used to regulate the antibody responses to *Lol* pIV as described in Chapter 3.

In this study it was demonstrated that spleen cells from anti-Id B1/1-treated donor mice could, on their transfer to syngeneic recipients, result in the reduction of antibody responses to *Lol* pIV. The reduced responses included: the level of antibodies to *Lol* pIV, level of antibodies to Site A of *Lol* pIV and level of the expression of Id91. The spleen cells responsible for the transferable suppression were considered to be B lymphocytes on the basis of the following criteria: (i) they were Ig^+ spleen cells, and (ii) $Thy\text{-}1^+$ spleen cells in the absence of B cells were not able to transfer the suppression in the adoptive transfer experiments. The studies by Malley, A. et al. on the regulation of immune responses to timothy grass pollen allergen by using anti-Id

have indicated that the anti-Id induced T suppressor cells were responsible for the suppression of the responses (Malley, A. et al. 1981, Malley, A. and Dresser, D.W. 1983a and 1983b). Thus, it would appear that administration of anti-Id may result in the involvement of multiple pathways, e.g. B cells, T suppressor cells and/or helper T cells in modulating the antibody responses to allergens. The tolerance of antibody responses mediated by both B and T suppressor cells was observed in other systems (Lee, W.Y. et al. 1981, Sehon, A.H. and Lang, G.M. 1985).

Furthermore, the B lymphocytes responsible for the transferable suppression were shown to possess the membrane-bound antibody molecules which were characterized as the anti-Id-like antibodies since their treatment with $Id91^+$ mAb91, plus complement, abrogated their capacity to transfer the suppression of antibody responses to *Lol* pIV. That Id-specific helper B cell population can augment the antibody responses to NP has been demonstrated by Sherr, D.H. and Dorf, M.E. (Sherr, D.H. and Dorf, M.E. 1984). Similarly, Yamamoto, H. and his colleagues have employed an adoptive transfer technique by transferring a common Id-primed splenic anti-Id B cells, which resulted in a striking enhancement of the Id^+ antibody responses upon simultaneous immunization of recipients with the antigen dextran (Inada, T. et al. 1982, Yamamoto, H. et al. 1984 and 1987). Together, these studies indicated that

anti-Id B cells could regulate the immune responses and maintain the Id network in immune systems. Even though in vivo administration of anti-Id could induce or reduce the formation of idiotypic antibodies, the direct effect of the anti-Id on Id B lymphocytes has remained unclear. Some experimental data based on in vitro studies have indicated that the anti-Id could directly bind to the Id on B cells and suppressed their production of antibodies (Yamamoto, H. and Katz, D.H. 1980, Bitoh, S. et al. 1990). However, the sequence of events which followed in animals after their treatment with anti-Id B1/1, in our study, was not clear. Nevertheless, it was demonstrated that B lymphocytes that were able to modulate the antibody responses possessed on their membrane the antibody molecules which was anti-Id-like antibodies.

Unlike intact B cells, the cell-free extract from the preparation of splenic B lymphocytes had no capacity to transfer the suppression. The reason for this is not known at the present time. According to Jerne's theory (Jerne, N.K. 1974), it may be speculated that the anti-Id B cells recognize the common Id, Id91, present on membrane-bound antibodies to *Lol pIV*, control the antibody responses and maintain its repertoire. However, this B-B cell interaction, if it exists in vivo, can not exclude the participation of T lymphocytes between this interaction.

CHAPTER 5.

IMMUNIZATION WITH THE INTERNAL IMAGE ANTI-ID INDUCES IgG, BUT NOT IgE, ANTIBODIES TO *Lol p*IV

ABSTRACT

As described in Chapter 2, a murine monoclonal antibody, mAb91 (Ab1), directed to an epitope of Ryegrass pollen allergen *Lol pIV* (Ag) was used to generate syngeneic monoclonal anti-Id or Ab2. One of the Ab2, designated as B1/1, which was characterized as an internal image Ab2 recognized the Id91 that was common to human, mouse and rabbit antibodies to *Lol pIV*. As continuation of the study, the immunogenicity of the anti-Id B1/1 was examined in terms of the generation of anti-anti-Id, i.e. Ab3. Immunization of syngeneic mice with a conjugate of B1/1 and KLH, in alum, elicited Ab3 which were of the IgG1 isotype. Three monoclonal Ab3, designated as CA-1, CB-1 and CB-2 were examined for their ability to bind to *Lol pIV* (i.e. Ag^+) and for their expression of the Id91 (i.e. Id^+). Compared to Ab1 that was Ag^+Id^+, CA-1 was Ag^+Id^+, whereas CB-1 and CB-2 were Ag^-Id^+. Those animals that had been immunized with B1/1 had reduced levels of IgE antibodies to *Lol pIV* followed by the challenge with *Lol pIV*. This study indicates that an internal image Ab2 could be used to modulate the formation of IgE antibodies to *Lol pIV* and generate the Ab3 which possess the salient characteristics of Ab1.

INTRODUCTION

The Id network theory of immunoregulation postulated by Jerne in 1974 (Jerne, N.K. 1974) suggested that Id networks, via Id and anti-Id interactions, may be involved in regulating the immune response to a given antigen (Jerne, N.K. 1974 and 1984). The ability of anti-Id to modulate the immune responses in vivo has been documented in numerous antibody systems (reviewed by Uytdehaag, F.G.C.M. et al. 1986 and Zhou, E.-M. et al. 1987a). Perhaps one of the most significant aspects of modulating the immune responses is the use of anti-Id as potential candidates for vaccines. Jerne has classified Ab2 into two categories, Ab2α and Ab2β (Jerne, N.K. 1984). It is the Ab2β, also referred to as internal image anti-Id, that has the capacity to mimic the nominal antigen and can substitute for it in inducing an anti-anti-idiotypic antibody (Ab3) response that has the antibody specificity for the nominal antigen. Many experimental data have shown that immunization with the anti-Id alone could induce a specific antibody response to virus antigens (Kennedy, R.C. and Dreesman, G.R. 1984, Zhou, E.-M. et al. 1987b and 1990, Fung, M.S.C. et al. 1990, Brodeur, B.R. et al. 1991), bacterial antigens (Stein, K.E. and Soderstrom, T. 1984, McNamara, M.K. et al. 1984) and allergens (Nagpal, S. et al. 1989, Hebert, J. et al. 1990).

As demonstrated in Chapter 2, monoclonal anti-Ids were

generated against a syngeneic Balb/c mouse monoclonal antibody, mAb91 (which possesses the Id91, i.e. Id^+) specific for allergen *Lol* pIV (Ag^+). An anti-Id designated as B1/1 was characterized as an internal image anti-Id or Ab2β. Its ability to regulate the formation of antibodies to allergen *Lol* pIV and expression of the Id91 have been studied as described in Chapter 3. In this Chapter, we describe the generation and characterization of Ab3s induced by immunization of syngeneic mice with the anti-Id B1/1. The Ab3s present in the antisera were of the IgG isotype i.e. no IgE Ab3s were found. Three monoclonal Ab3s were generated and characterized with respect to their antigen-binding properties (Ag^+) and expression of the Id91 (Id^+). The Ag^+ Ab3 recognized an epitope (Site A) that was also recognized by human and mouse IgE antibodies. This study revealed that the internal image anti-Id B1/1 could induce the formation of IgG Ab3 and modulate the IgE antibody response to allergen *Lol* pIV.

MATERIALS AND METHODS

Animals:

Female Balb/c mice 6-8 week old were purchased from Jackson Laboratory, Bar Harbor, Maine, USA. Long Evans Hooded rats, male, 200-300g were obtained from the animal care service of the University of Manitoba Health Sciences Center. All animals were kept in a clean environment.

Preparation of B1/1 for Immunization:

The monoclonal anti-Id B1/1 (IgG2bκ) generated in syngeneic mice as described in Chapter 2 was purified from ascites fluid using Sepharose 4B Protein A affinity column (Pharmacia Chemicals, Uppsala, Sweden) in a solution of BBS at 0.2 M, pH 8.2. B1/1 at a concentration of 5mg/ml was coupled to KLH (Calbiochem Cop., La Jolla, CA) and the conjugate was precipitated in alum as described in Chapter 2.

Generation of Syngeneic Anti-anti-Id (Ab3):

Female Balb/c mice were immunized with a conjugate of B1/1-KLH in alum at a dose of 25μg of B1/1 per mouse injected i.p. Mice were given a total of 4 injections at internals of two weeks. Fourteen days after the 4th injection, blood was

collected and the Ab3 titers were detected by a combination of ELISA and PCA procedures (described below). The mice which had higher titers of anti-anti-Id were divided into two groups, one was used for the further immunization with allergen *Lol* pIV (described below) and another was rested for a month and were boosted i.v. with 5μg of B1/1 in PBS 3 days prior to using their spleen cells for fusion. The hybridoma cells were prepared as described previously (Ekramoddoullah, A.K.M. et al. 1984). A two-site ELISA procedure (see below) was used to screen for antibodies in the hybridoma cell culture fluids. Monoclonal Ab3s, designated as CA-1, CB-1 and CB-2, were identified as IgG1κ, IgG2bκ and IgG1κ, respectively using the murine isotype-specific antibody reagents (BioRad Laboratories, Richmond, CA). The monoclonal Ab3s were isolated from ascites fluid by Sepharose 4B Protein A affinity column (Pharmacia Chemicals, Uppsala, Sweden) as described in Chapter 2.

Immunization Procedures:

For the generation of antibodies to allergen *Lol* pIV, a group of adult Balb/c mice received two injections of *Lol* pIV (purified as described in Chapter 2) at 5μg/mouse in alum, at an interval of 14 days.

To investigate the influence of immunization with anti-Id

Bl/1 on the formation of IgE antibodies to *Lol* pIV, the mice which had been immunized 4 times with anti-Id Bl/1 (as described above), were challenged twice with *Lol* pIV in alum at an interval of 14 days. Blood was collected from each of the group of mice and the titer of IgG and IgE antibodies to *Lol* pIV was evaluated by a direct ELISA and a PCA, respectively, as described below.

ELISA Procedures:

(i) *Two-Site ELISA:* Ab3 present in either antisera, cell culture fluids, or in ascites fluids were detected by means of a two-site ELISA. Amounts of 400ng of Bl/1 in 50µl BBS were adsorbed to wells of microtiter ELISA plates (Corning Laboratory Sci. Com. Corning, New York) at 4^0C overnight. ELISA plates similarly coated with MCA, a monoclonal antibody preparation with a specificity for influenza virus (Cedarlane Laboratories, LTD., Ontario) served for control. After blocking unbound sites of the wells with 2% gelatin, 50µl of Ab3-containing preparation was added to the Bl/1- or MCA-coated wells and allowed to bind to the solid-phase at 37^0C for 1 hr. The plates were washed three times with BBS containing 0.2% T-BBS. Ab3 which had bound to the solid-phase Ab2 was determined using Bl/1 conjugated to biotin (Pierce, Rockford, IL) at 37^0C for 45 min. After washing the wells with T-BBS, a solution of Streptavidin-conjugated HRP was added and

kept at 25^{0}C for 25 min followed by washing the wells with T-BBS. The substrate, ABTS H_2O_2 were used to develop the colorimetric reaction. The reaction was stopped by adding 5% of SDS and the optical density at 410 nM of each well was measured on an automatic ELISA plate reader (MR600, Dynatech Instruments Inc. Torrance, CA). The assay was performed in triplicate.

(ii) *A Direct ELISA:* The levels of antibodies (Ab1 or Ab3) to *Lol pIV* in the mouse antisera were determined by a direct ELISA. Briefly, the solid-phase was prepared by coating the wells of microtiter plates with *Lol pIV* at 200ng in 50µl of BBS. Plates similarly coated with OA served as control. After blocking unbound sites with a solution of gelatin, the antisera diluted in BSA was allowed to interact with the solid- phase *Lol pIV*. A solution of goat anti-mouse Ig conjugated to HRP (Cedarlane Laboratories LTD., Ontario) was used to detect the extent to which the antibodies binding to solid-phase *Lol pIV*. The addition of substrate and quantitation of the colorimetric reaction were performed as described above.

(iii) *Inhibition Assays:* To determine the levels of the Ab3 antibodies which possessed the Id91, two competitive ELISA were performed in which the capacity of (i) Ab3 to inhibit the Ab1-Ab2 interaction and (ii) Ab1 to inhibit the Ab2-Ab3

interaction, was examined. The solid-phase was prepared by coating each well of the microtiter plates with a solution of (i) mAb91 or (ii) CA-1 (200ng) in 50µl of BBS at 4^0C overnight. After blocking any unbound sites with gelatin, biotinylated anti-Id B1/1 (8µg/ml) pre-mixed with (i) Ab3- or (ii) Ab1-containing preparation (as inhibitors) diluted in BSA was added to the wells and incubated at 37^0C for 1 hr. After washing the plates with T-BBS, the extent to which the biotinylated B1/1 had bound to the solid-phase was determined by adding streptavidin-conjugated HRP in BSA. The addition of the assay was performed as described above.

The specificity of the binding of monoclonal Ab3 to solid-phase *Lol* pIV was established using the Ab1, *Lol* pIV, homologous Ab3, and Ab2 as an inhibitor to block the interaction between the biotinylated CA-1 and the solid-phase *Lol* pIV. Briefly, microtiter plates were coated with *Lol* pIV (200ng) in 50µl of BBS at 4^0C overnight. After blocking unbound sites with gelatin, biotinylated CA-1 (5µg/ml) was pre-mixed with the inhibitors and placed into the wells and incubated at 37^0C for 45 min. The addition of enzyme, substrate and quantitation of the colorimetric reaction were performed as described above. The extent of the inhibition was calculated according to the formula: Inhibition (%) = 1 - [$OD_{410}$with inhibitor - background / OD_{410}without inhibitor - background] x 100.

Passive Cutaneous Anaphylaxis (PCA):

The levels of IgE antibodies present in Ab1 or Ab3 containing antisera to *Lol* pIV were determined by the PCA procedure. Mouse antisera were diluted two-fold in saline were injected intradermally into rats. The allergen challenge was carried out 24 hrs later by an i.v. injection of *Lol* pIV (0.5mg) mixed with Evans blue dye. Twenty min later, the animals were sacrificed and the size of the reactions (extravasation of the dye) which appeared on the undersurface of the skin was measured. The highest dilution of the antisera which gave the reaction size of 3mm in diameter was considered as the end point titer. Each assay was performed in duplicate. The PCA inhibition assay was used to determine whether the Ab3 recognizes the IgE-binding epitope of *Lol* pIV. For this purpose, mouse antisera containing IgE antibodies to *Lol* pIV at a dilution of 1 to 1600 in saline were used to inject rats. *Lol* pIV (1mg) in Evans blue dye was mixed with the preparation of Ab3 and the mixture was injected i.v. into the rats. The assay was performed continuing as described above.

RESULTS

In vivo Administration of Anti-Id B1/1 Induces the Ab3 Responses.

On the basis of studies presented in previous Chapters where treatment with the internal image anti-Id B1/1 was found to modulate the antibody responses to allergen *Lol* pIV, the possibility that the anti-Id B1/1 had also the capacity to induce the anti-anti-Id, i.e. Ab3 responses was explored. For this study, syngeneic Balb/c mice were used to examine whether the Ab2 could induce the formation of Ab3 with characteristics of the anti-*Lol* pIV antibodies (Ag^+) that possess also the Id91 (Id^+), i.e. Ag^+Id^+. One group of five mice each was immunized by four i.p. injections of monoclonal Ab2 conjugated to KLH and precipitated in alum. A second group received the IgG2b monoclonal antibody preparation, MCA, (Cedarlane Laboratory LTD., Ontario) which served as a negative control. A third group of mice was immunized with two injections of *Lol* pIV in alum which served as the positive control. Antisera obtained 14 days after the final immunization were tested for (i) the levels of anti-anti-Id, (ii) antibodies to *Lol* pIV, and (iii) the level of antibodies which possess the Id91 (Figure 15). The group of mice that was immunized with anti-Id B1/1 produced the anti-anti-Id antibodies (Ab3) (Figure 15A) that not only bound the anti-Id but also interacted with

allergen *Lol* pIV (Figure 15B) and possessed the Id91 (Figure 15C). The immunizations with MCA (control group) did not produce any detectable Ab3 responses to *Lol* pIV. These results indicated that immunizations with Ab2 could induce the formation of Ab3 antibodies that were Ag^+ and Id^+.

Isolation of Monoclonal Anti-anti-Id.

To study the fine specificity of Ab3 in terms of their antigen-binding property or expressing the Id91, monoclonal Ab3s were generated, of which 3 bound to Ab2 and were designated as CA-1, CB-1 and CB-2. As shown in Figure 16, three monoclonal Ab3s, along with Ab1 (mAb91), specifically bound to Ab2 but did not bind to a control mAb MCA.

Specificity of Binding of Ab3 to Allergen Lol pIV.

The ability of mAb3s to bind antigen was evaluated by a direct ELISA employing solid-phase *Lol* pIV. It was demonstrated that one of Ab3s, CA-1, had the capacity of binding to *Lol* pIV similar to that of Ab1 (Figure 17). By contrast, the other two monoclonal Ab3s, CB-1 and CB-2, had no ability to bind to *Lol* pIV.

The specificity of the binding of CA-1 to *Lol* pIV was assessed by the capacity of *Lol* pIV to inhibit the binding of

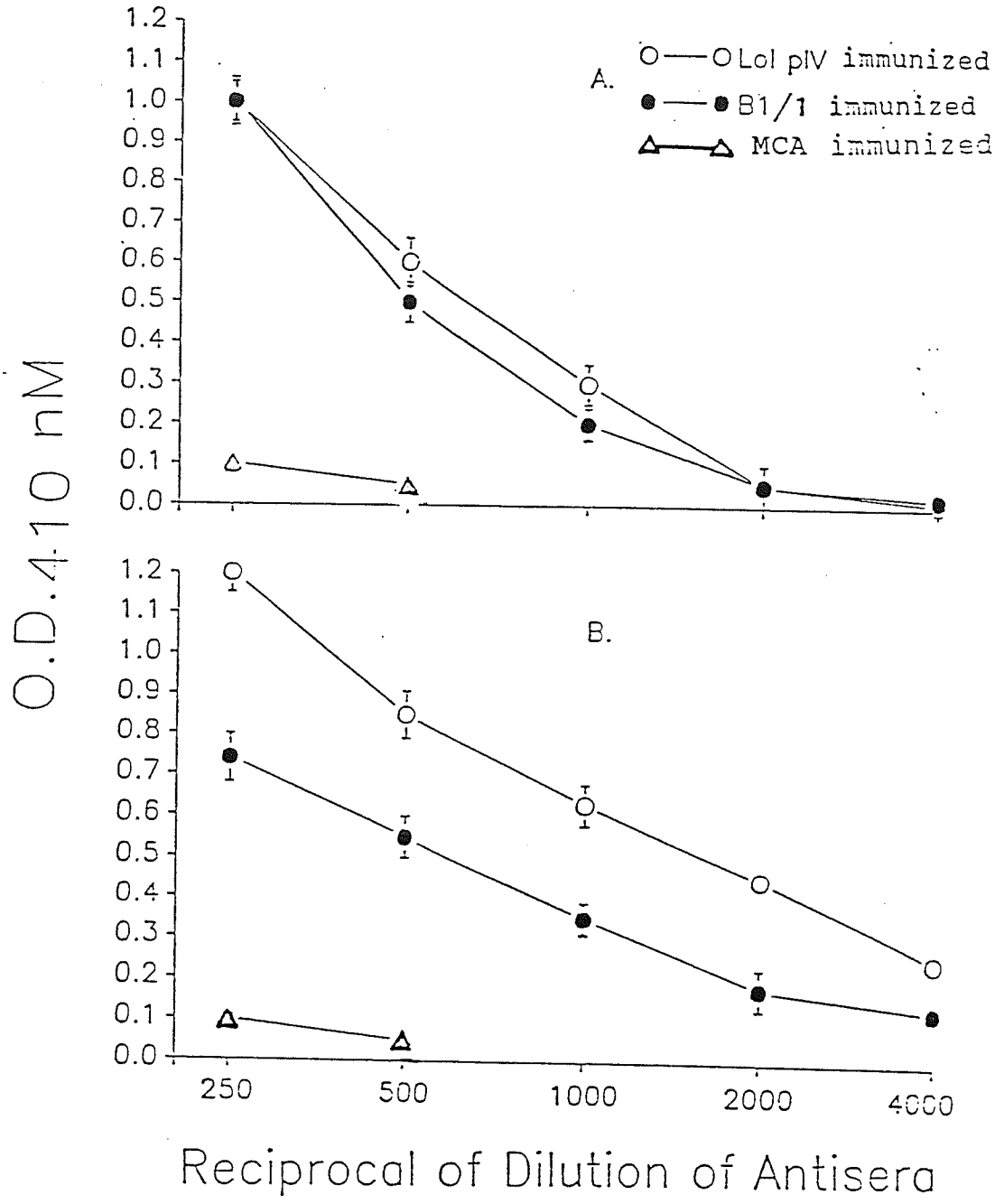
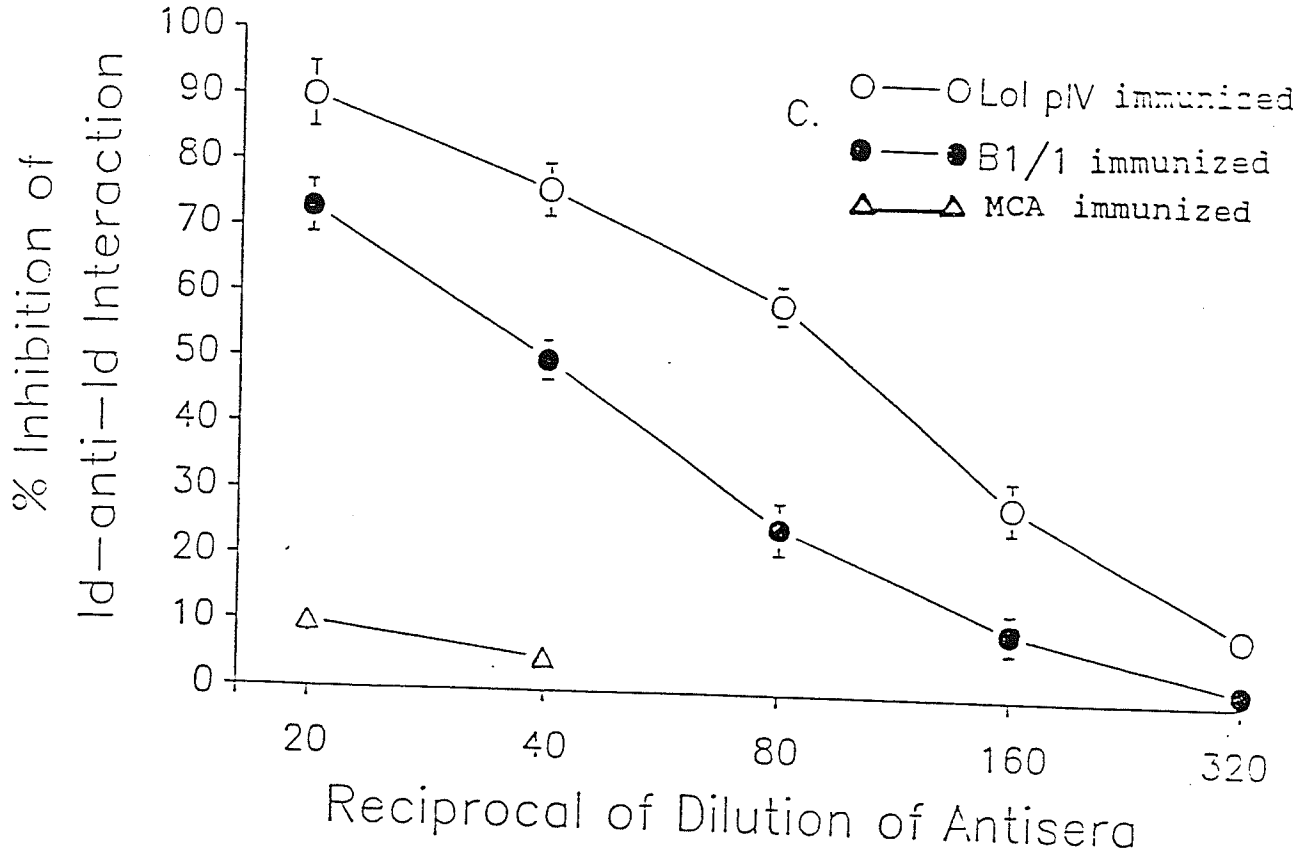


Figure 15. ***Immunization with the Ab2 Induces the Production of Ab3 Antibodies.***

The antisera were collected on day 14 after the fourth immunization of the mice with 25μg of B1/1 or MCA conjugated to KLH and precipitated in alum or those from the mice immunized with 5μg of *Lol* pIV in alum. *A*: a two-site ELISA was used to detect the Ab3 with Ab2 on solid-phase, *B*: direct binding ELISA with *Lol* pIV on solid-phase to detect antibodies to *Lol* pIV, and



***C*: detection of Id91. The levels of Id91 present on antibodies in the antisera of animals were evaluated by their capacity of inhibiting Ab1-Ab2 interaction. The values represent the mean ± SEM of an ELISA performed in triplicate.**

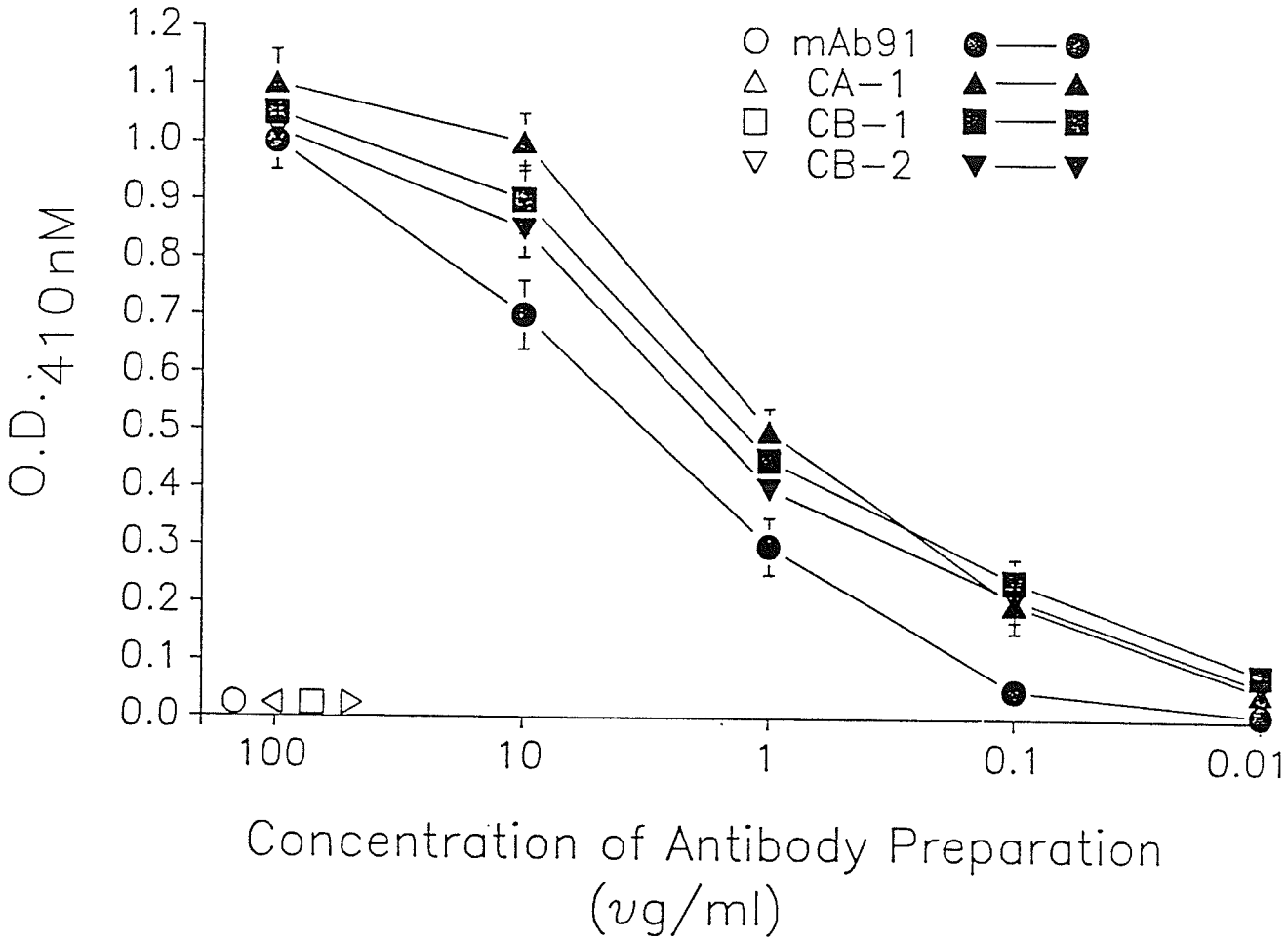


Figure 16. ***Generation of Monoclonal Anti-anti-Id (Ab3).***

A two-site ELISA was employed to detect the mAb3s capable of binding to solid-phase B1/1 (solid symbols) and MCA (open symbols) with the use of biotinylated B1/1 (8μg/ml). The values represent the mean ± SEM of ELISA performed in triplicate.

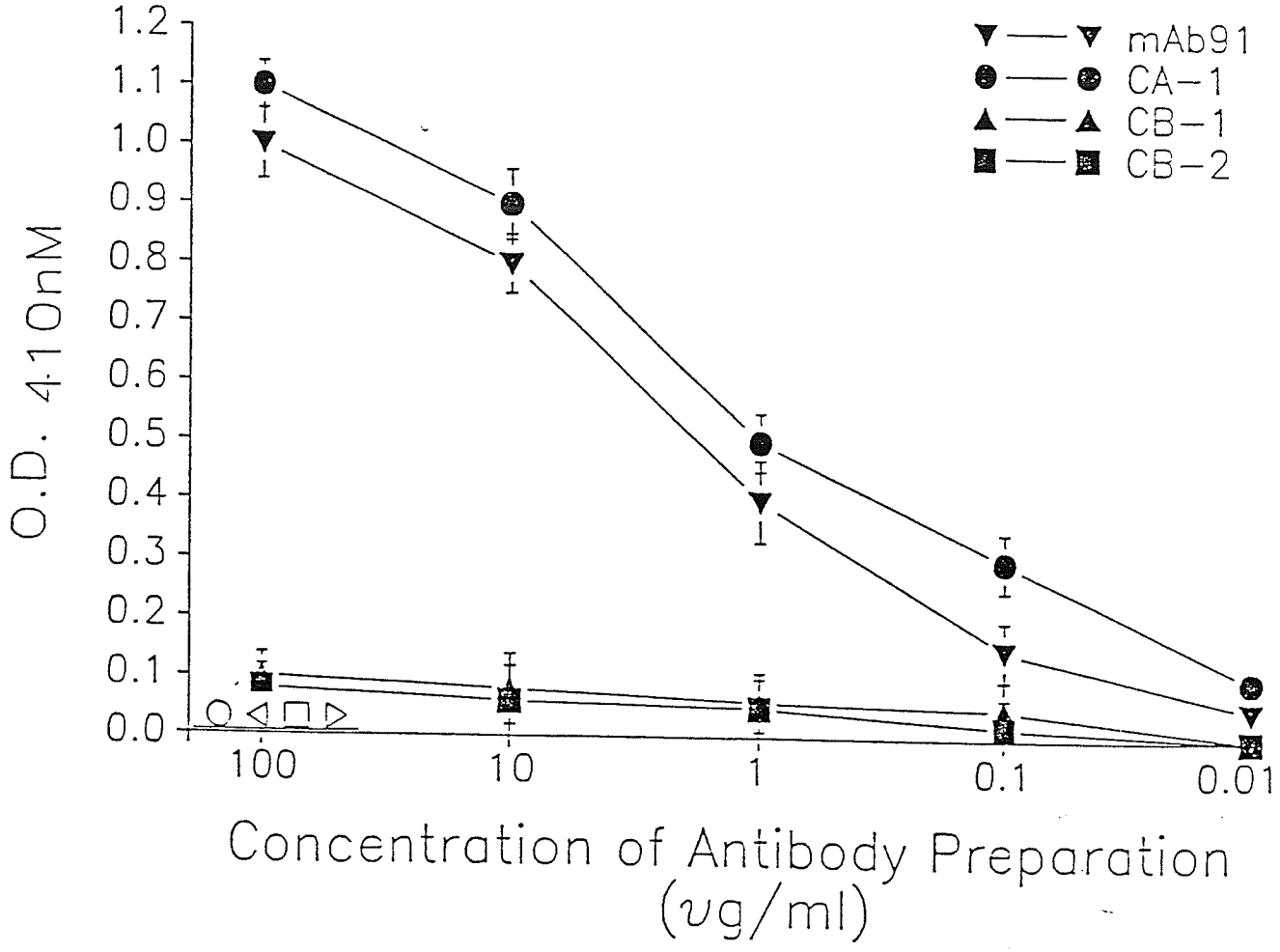


Figure 17. ***Demonstration that mAb3, CA-1, binds to Lol pIV.***

A direct ELISA was used to detect the ability of mAb3s to bind *Lol* pIV on solid-phase. Open symbols represented the binding to solid-phase OA. The values represent the mean ± of an ELISA performed in triplicate.

CA-1 to solid-phase *Lol* pIV (Table X). In this inhibition experiment, CA-1 was tested for its binding to *Lol* pIV in the presence of *Lol* pIV. For control, allergen OA was used in place of *Lol* pIV as the inhibitor. The binding of CA-1 to *Lol* pIV was inhibited 80% by the *Lol* pIV at a final concentration of 1mg/ml, whereas at the same concentration of OA no inhibition was detected. The competitive ELISA was employed to establish if the Ab3, CA-1 bound to Site A of *Lol* pIV. To test this, the capacity of CA-1 to bind to solid-phase *Lol* pIV in the presence of mAb91 (specific for Site A of *Lol* pIV) was evaluated. In this inhibition assay, mAb91, at a concentration of 1mg/ml, could completely inhibit the binding between CA-1 and *Lol* pIV (Table X). By contrast, a monoclonal antibody preparation, mAb60, specific for *Lol* pI, had no inhibitory effect in this assay.

Furthermore, the binding of CA-1 to *Lol* pIV could be successfully blocked by the homologous CA-1. To localize the Id of Ab3, CA-1, the Ab2 was also used to inhibit the interaction between CA-1 and *Lol* pIV. As shown in Table X (column on far right), B1/1, at a concentration of 1mg/ml, could completely inhibit the interaction. Collectively, these data demonstrated that (i) the Ab3, CA-1, was specific for the epitope Site A of allergen *Lol* pIV and (ii) the Id carried on Ab3 CA-1 was located within or near the antigen binding sites.

Table X.

CHARACTERIZATION OF FINE SPECIFICITY OF THE Ab3, CA-1[a]

Concentration of Inhibitors (μg/ml)	% Inhibition of CA-1-*Lol pIV* Interaction in the presence of						
	mAb91	mAb60	*Lol pIV*	OA	CA-1	CB-1	B1/1
1000	100[b]	5	80	2	100	4	100
100	70	0	50	0	90	0	67
10	38	0	20	0	50	0	45
1	20	0	10	0	30	0	25
0.1	8	0	5	0	13	0	10

[a] Specificity of the Id^+Ag^+Ab3, CA-1, to bind to *Lol pIV* was examined by an inhibition ELISA in which various inhibitors was used to inhibit the interaction between CA-1 and solid-phase *Lol pIV*.

[b] The number represented mean ± SEM of an ELISA performed in triplicate.

Monoclonal Ab3s Possessed the Id Characteristic of mAb91.

The Ab3s induced by immunization with Ab2 may or may not have possessed the Id^+ characteristic of Ab1 (mAb91). To resolve this issue, two similar competitive ELISA assays were performed.

One assay employed Ab3s to inhibit the Ab1-Ab2 interaction (Figure 18A). The results have shown that all three monoclonal Ab3s, including the Id-carrying mAb91 (positive control), had the capacity of inhibiting the interaction in a dose dependent manner. The control antibody mAb2 which lacked the Id91, could not inhibit the reaction.

The other assay used Ab3s to inhibit the interaction between the Ab3 (CA-1) and the Ab2 (B1/1) (Figure 18B). It was clearly shown in the results that all three monoclonal Ab3s as well as Ab1 could effectively inhibit the interaction, in which the homologous CA-1 produced the highest inhibition values compared to the other two Ab3s.

These inhibition assays indicated that (i) the Id possessed by the three monoclonal Ab3s was the same or similar specificity to the Id91 carried by Ab1 mAb91 and (ii) all three Ab3s carried the same or similar Id in terms of the binding with anti-Id B1/1.

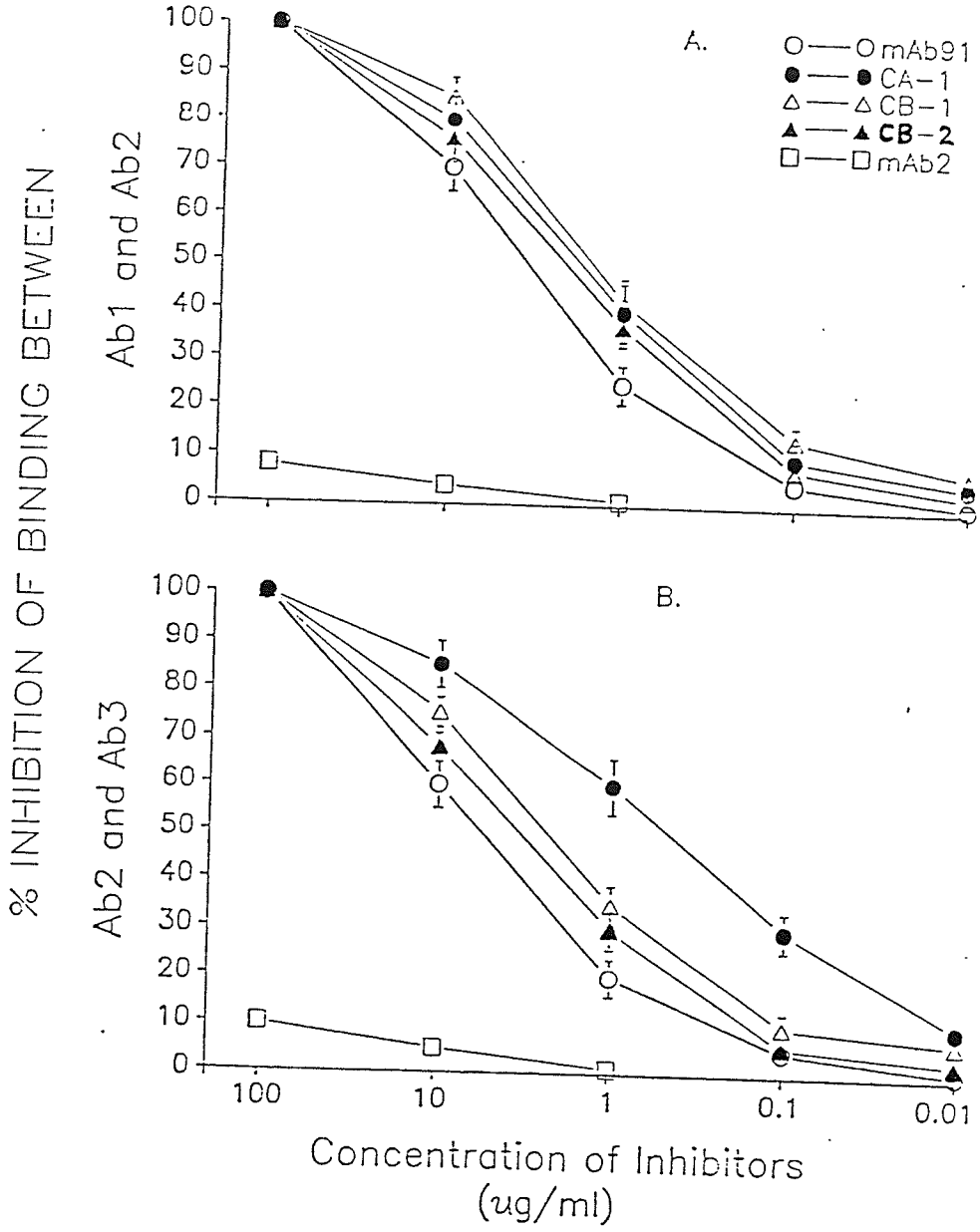


Figure 18. ***Demonstration that the mAb3s Possesses the Id91.***

A competitive ELISA was employed to detect whether the mAb3s carried Id91. *A.* mAb3s, CA-1, CB-1 and CB-2, were examined for their ability to inhibit the interaction between the Id (mAb91) and anti-Id (B1/1). Biotinylated B1/1 (8μg/ml) was mixed with the inhibitors and allowed to bind the solid-phase mAb91; and *B.* the three mAb3s competed with biotinylated Ab2, B1/1, for binding to mAb3, CA-1 on the solid-phase. The values represented the mean ± SEM of the assay performed in triplicate.

Treatment with Anti-Id B1/1 Induced IgG1 Ab3s.

Studies described in Chapter 3 have shown that immunization of mice with *Lol* pIV could induce the formation of not only the IgG1 but also IgE antibodies to *Lol* pIV. In order to compare whether the immunization of mice with anti-Id B1/1 alone (i.e. in the absence of *Lol* pIV) also induced an IgE, in addition to IgG, antibody response to *Lol* pIV, the antisera from mice immunized with *Lol* pIV or B1/1 were tested by PCA for the presence of IgE antibodies to *Lol* pIV. As shown in Figure 19A, the IgG1 antibodies to *Lol* pIV could be detected in both antisera. Their specificity was confirmed by the negative binding to OA. However, the IgE antibodies to *Lol* pIV were only detected in antisera from the mice immunized with *Lol* pIV (Figure 19B). The antisera from the mice immunized with the Ab2 had almost no detectable IgE antibodies to *Lol* pIV (the titer of 1:10). These results indicated that the treatment with the anti-Id B1/1 could induce the production of Ab3s of the IgG class, but no IgE, Ab3 antibodies to allergen *Lol* pIV. Furthermore, since the immunization with the anti-Id alone could not induce the IgE antibodies to *Lol* pIV, its influence on the following formation of IgE antibodies upon challenge with *Lol* pIV was studied. As shown in Figure 19B, the group of mice that were immunized with the anti-Id B1/1 and challenged with *Lol* pIV 14 days after the final immunization with B1/1, had a PCA titer

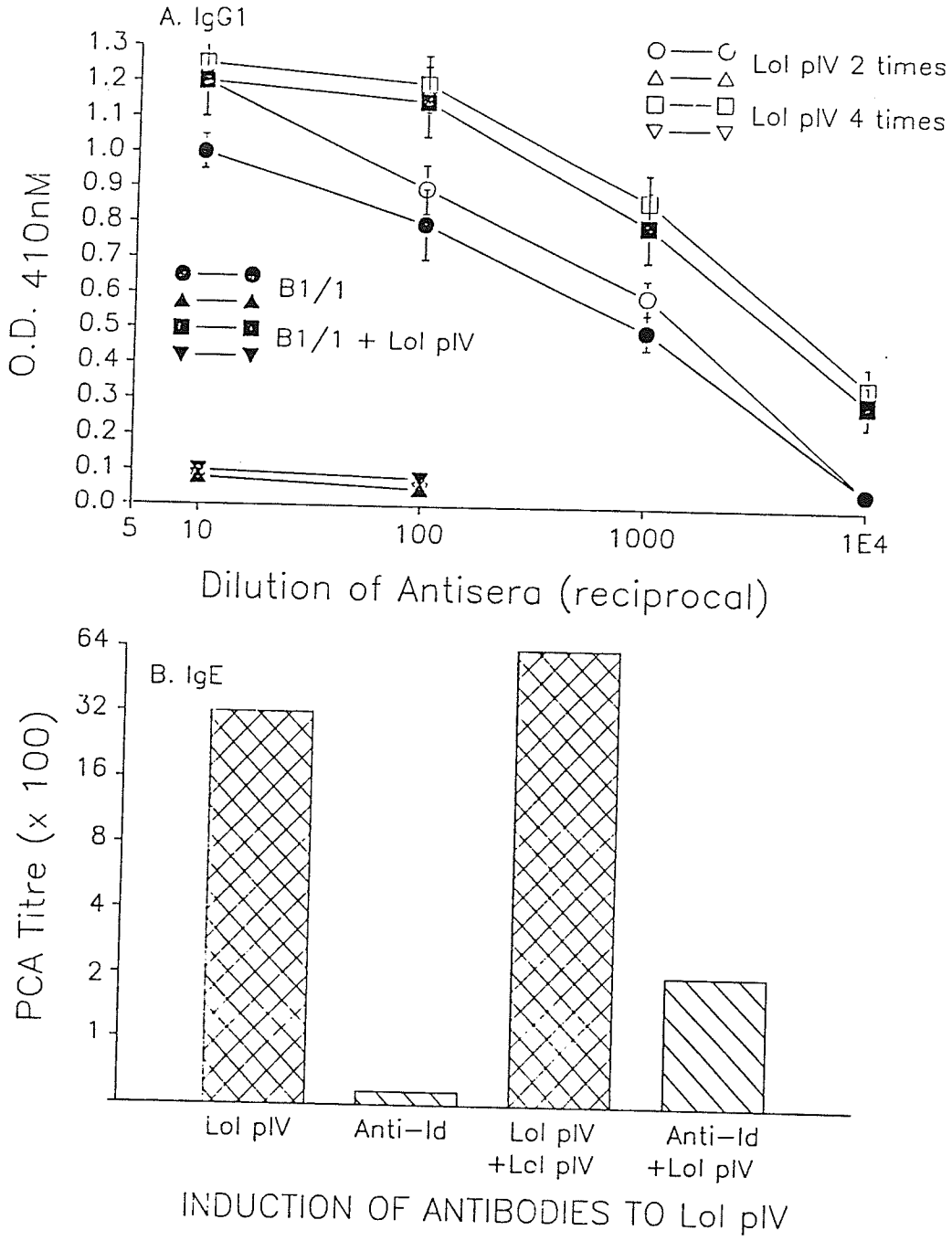


Figure 19. ***Immunization with Ab2 Produces IgG, but not IgE, Ab3 Antibodies to Lol pIV.***

The Ab3 antibodies induced by immunization with Ab2 B1/1 were detected using *A.* a direct ELISA for IgG1 isotype. Triangles represent the values obtained from the antisera bound to OA. *B.* PCA for evaluating titer of IgE antibodies to *Lol pIV*. The values represent the mean ± SEM of an assay performed in triplicate (ELISA) or duplicate (PCA).

of 200. This level of IgE antibodies to *Lol* pIV was still far below the level of that observed with the antisera from the group of mice immunized directly with *Lol* pIV (PCA titer of 3200). The highest PCA titer (1 to 6400) was obtained in the mice after they were boosted with *Lol* pIV. These findings indicated that immunization with the anti-Id B1/1, which produced high levels of IgG1 antibodies, could down-regulate the subsequent formation of IgE antibodies to *Lol* pIV.

IgG Ab3s Recognized the IgE-Binding Epitope of Lol pIV.

The possibility that IgG Ab3 recognize the IgE-binding epitope was tested by using a competitive assay of PCA. For this purpose, mouse anti-*Lol* pIV IgE antibody-containing antisera were used to locally sensitize sites on the skin of rats. The antisera containing IgG Ab3 were mixed with *Lol* pIV prior to their injection into the rats. As shown in Figure 20, the IgG Ab3s could effectively block the IgE antibodies from binding to *Lol* pIV, since the PCA titer of IgE antibodies to *Lol* pIV dropped from 3200 to 300 at the presence of the inhibitor. There was no blocking effect with the control antibody preparations. These results indicated that IgG Ab3 recognized the epitope which was also recognized by IgE antibodies.

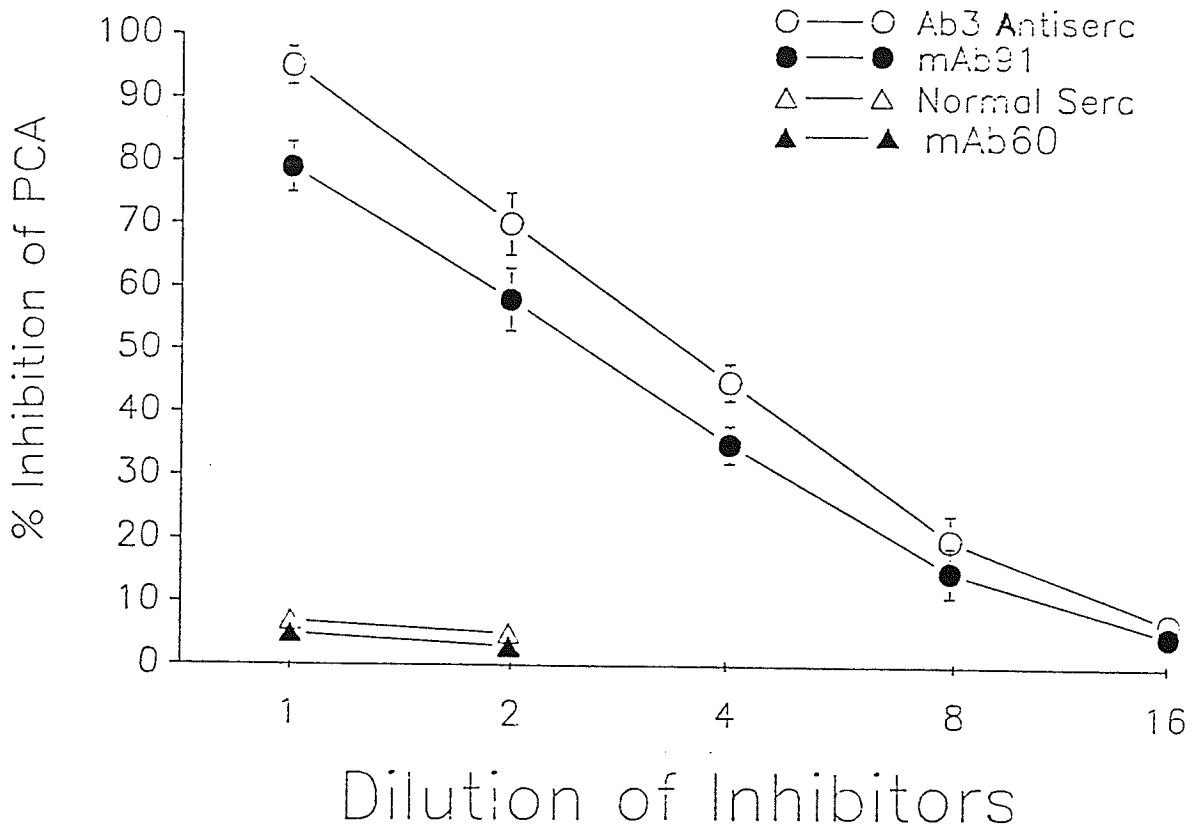


Figure 20. *Ab3 Antibodies Recognize IgE-Binding Epitope.*

A competitive PCA assay was employed to detect whether the Ab3s also recognized an IgE-binding epitope. Mouse IgE antisera to *Lol* pIV at a dilution of 1/1600 were used to sensitize rats by the i.d. injection. One milligram of *Lol* pIV in Evans blue dye was mixed with individual inhibitor: Ab3 antisera or normal sera at a dilution of 1 to 100 and 1mg of mAb91 or mAb60. The values represent the mean ± SEM of a PCA performed in duplicate.

DISCUSSION

Several studies have described different types of Ab2 produced after immunization with an Ab1 and in turn the Ab2 could be used to generate an Ab3 responses (Jerne, N.K. et al. 1982, Bona, C.A. and Kohler, H. 1984, Kennedy, R.C. et al. 1986). The generation and characterization of a mouse monoclonal anti-Id designated B1/1 had been described in Chapter 2. The specificity of B1/1 was shown to recognize an Id91 associated with mAb91 and anti-*Lol* pIV antibodies from human, mouse and rabbit. The anti-Id B1/1 was characterized as an internal image anti-Id or Ab2β (see Chapter 2) and its immune regulatory functions have been studied in Chapters 3 and 4. In this Chapter, it was demonstrated that *in vivo* treatment with B1/1-KLH conjugate can induce antibody responses (Ab3) specific to allergen *Lol* pIV in syngeneic Balb/c mouse.

Numerous investigations have been documented in studying the immune regulatory function of anti-Id which served as agents for inducing the specific immune responses to infectious agents (Sacks, D.H. and Sher, A. 1983, Fracotte, M. and Urbain, J. 1984, Huang, J.-H. et al. 1986, Kennedy, R.C. et al. 1986, Zhou, E.-M. et al. 1987 and 1990), to parasites (Sacks, D.H. et al. 1982 and 1985, Sacks, D.H. and Sher, A. 1983, Bhogal, B. et al. 1987), to bacteria (Stein, K.E. and

Soderstrom, T. 1984, McNamara, M.K. et al. 1984 and 1987, Monafo, W. et al. 1987, Kaufmann, S. et al. 1985, Praputpittaya, K. and Ivanyl J. 1987). Although considerable evidence has accumulated on the development of a protective immune response by anti-Ids, studies on immune responses to allergens induced by anti-Ids are few but nevertheless support the concept of idiotype network. Nagpal et al. have demonstrated that mouse anti-Id raised against human antibodies specific for a major heat-stable shrimp allergen were capable of inducing the Ab3 responses in mice that resemble the Id in their ability to combine with the allergen (Nagpal, S. et al. 1989). More recently, Hebert and his colleagues have studied the immune responses to allergen *Lol pI* in terms of the Id network interaction (Hebert, J. et al. 1990). They have produced monoclonal Ab2 and Ab3 in the same fusion which supported the concept of Id network in which Ab1 induces Ab2 which in turn induced Ab3 response. Their Ab3 specific for the allergen was characterized as Ag^+ and Id^+.

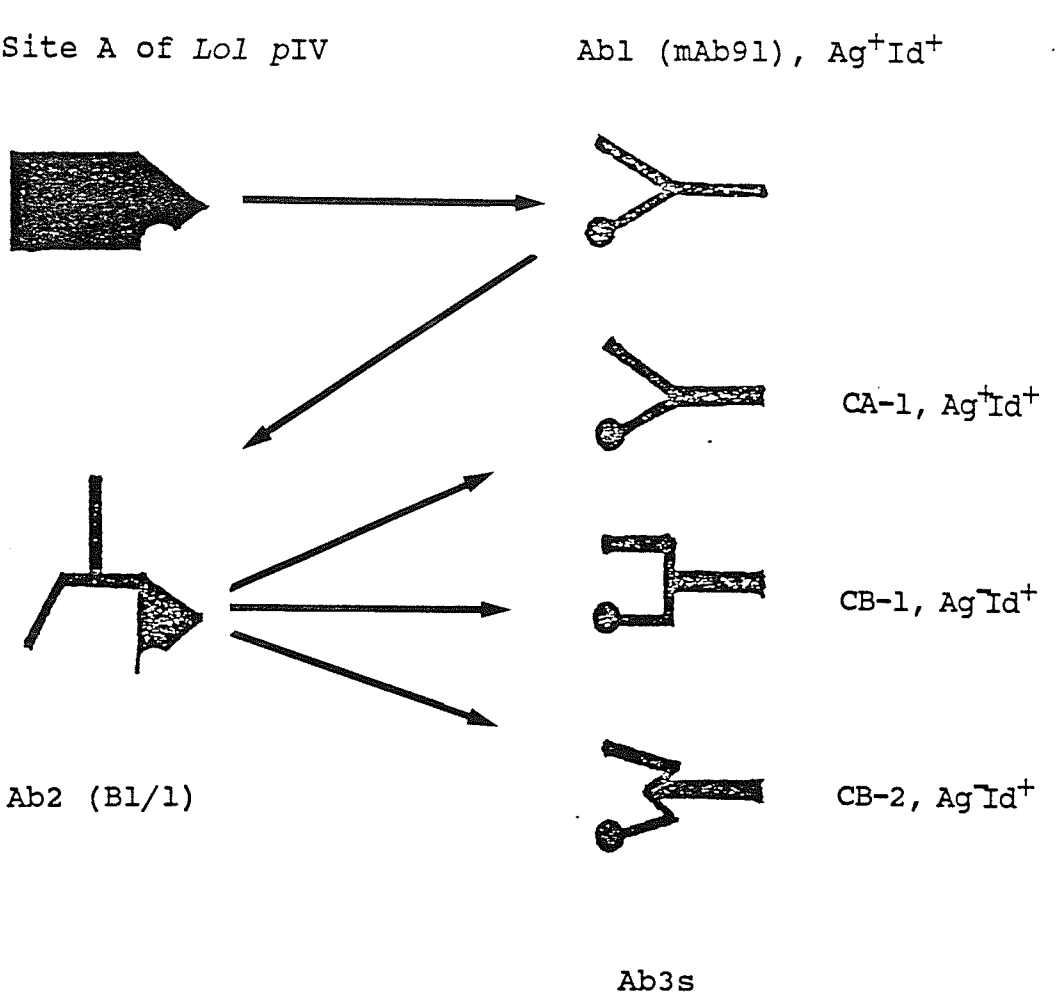
In this Chapter, it was reported that repeated immunization of Balb/c mice with a syngeneic internal image monoclonal anti-Id B1/1 induced the formation of Ab3 antibodies, some of them were specific for allergen *Lol pIV* and possessed the Id91 (Figure 15). It was not surprising that the titer of antibodies to *Lol pIV* from the mice immunized with B1/1 was relatively lower compared to that from the mice

immunized with *Lol pIV* (Figure 15B). This observation was not unexpected since the Ag^+ Ab3s may only constitute a small portion of the entire repertoire of the Ab3 pool. A similar situation might be also true for the Id^+ Ab3s (Figure 15C). To study further the specificity of Ab3s for their antigen binding and expression of the Id91, monoclonal Ab3s were generated and characterized. From 10 monoclonal Ab3s, three were selected based on the characteristics of either Ag^+ and/or Id^+. They were designated as CA-1, CB-1 and CB-2, and had the similar profile for the binding to Ab2 (Figure 16) compared with Ab1. However, only one out of the three mAb3s had the characteristics of being Ag^+, i.e. this mAb3 bound *Lol pIV*. This Ag^+ Ab3 (CA-1) had similar profile of antigen binding as Ab1. The specificity of antigen binding of CA-1 was confirmed by the use of homologous *Lol pIV* to inhibit the binding (Table X).

Previous studies have demonstrated that more than one epitope is present on allergen *Lol pIV* (Jaggi, K.S. et al. 1989) and the Ab1, mAb91, recognized one of epitopes referred to Site A. Therefore the question was asked whether the Ag^+ Ab3, CA-1, recognized the same epitope as the Ab1 did. The fact that Ab1 could successfully inhibit the binding of CA-1 to *Lol pIV* implied that CA-1 recognized Site A (Table X) even though it could not be excluded that steric hindrance may cause the inhibition of the binding.

It was first described from a theoretical point (Jerne, N.K. 1974) and then experimentally demonstrated (Bona, C.A. et al. 1981b) that Ab3 induced by Ab2 recognized the Id on Ab2, or conversely, the Ab2 induced the formation of Ab3 by the recognition of its Id. Therefore, identification of the Id carried by Ab3s is important for the study of the Id network of the immune responses to allergen *Lol p*IV. For this purpose, a series of assays were performed in which the interaction between the Ab1 and Ab2 or between the Ab2 and Ab3 was examined by the use of Ab3s in an attempt to inhibit their interactions. All three mAb3s could inhibit the Ab1-Ab2 interaction, and the interaction between the Ab2 and Ab3 (CA-1) (Figure 18), which suggested that the Id possessed by Ab1 and Ab3s was same or similar, i.e. the Id91 and most likely the Ab2 recognized the Id on Ab3 to induce the formation of Ab3. This Id91 was also demonstrated to be located within or near the antigen combining sites since the Ab2 could inhibit the Ab3 CA-1 from binding to *Lol p*IV (Table X). The location of the Id91 of Ab1 had been previously identified in Chapter 2 to be within or near the antigen combining site of Ab1.

The diagram on the next page illustrates the possible structure of the mAb3s in terms of their ability to bind antigen and to possess the Id91.



The diagram illustrates schematically the possible structures of mAb3s in terms of their characteristics of antigen-binding (Ag^+) and possessing the Id91 (Id^+) based on the data presented in this chapter.

The outcome of antibody responses in two groups of mice induced by allergen *Lol* pIV on one hand and by anti-Id B1/1 on the other has been compared in this study. Immunization with either allergen *Lol* pIV or anti-Id B1/1 induced the production of IgG1 isotype of antibodies to *Lol* pIV, but only the *Lol* pIV could induce the production of antibodies of the IgE isotype (Figure 19). Two immunizations with *Lol* pIV has been shown to induce high titer of IgE antibodies. By comparison, the group immunized with anti-Id B1/1 and then challenged with *Lol* pIV only developed low levels of IgE antibodies (PCA titer of 1/200 compare to that of 1/3200). These results suggested that the anti-Id B1/1 immunized mice, which produced IgG antibodies to allergen *Lol* pIV, were not able to produce high levels of IgE antibodies after the challenge with *Lol* pIV. To account for these results, the mechanism behind these findings may be speculated that the anti-Id B1/1 selectively suppressed Th2 cells and the level of IL-4 secreted by these cells was not sufficient to induce the high level of the formation of IgE antibodies. While the required levels of IL-4 for inducing the production of IgG1 is much lower compared to that for IgE (reviewed by Finkelman, F.D. 1990).

Since two out of three mAb3s were of the IgG1 isotype, i.e. identical to the isotype of the Ab1, the possibility arose that the isotype of Ab1 could influence the isotype of Ab3 produced. Although this idea is highly speculative, a

report by Velge-Roussel et al. (Velge-Roussel, F. et al. 1989) supports this possibility. They demonstrated that the rat IgE Ab1 to *Schistosoma mansoni* induced an Ab2 response which in turn induced Ab3 production. The Ab3s were composed not only of antibodies of the IgG isotype but also the IgE isotype which was the same as Ab1. Other reports by Nagpal et al. and Hebert et al. showed a similar phenomenon (Nagpal, S. et al. 1989, Hebert, J. et al. 1990). Whether the isotype selection of the Ab3 could be influenced by the Ab1 has yet to be proved.

The results presented in this Chapter indicated that the internal image monoclonal anti-Id B1/1 specific for Id91 of mAb91 to allergen *Lol p*IV has the ability to act as a surrogate allergen and may have the potential to replace native allergens in the treatment and the immunodiagnosis of allergic diseases. This study of immune regulation to allergen *Lol p*IV by Id-anti-Id interaction in a murine model system may support the concept that similar events may occur in humans in which the Id network may play a role in the regulation of human IgE responses to allergens.

CHAPTER 6.

GENERAL DISCUSSION

A network theory of the immune system, i.e. the interaction between Id-anti-Id proposed by Jerne in 1974 have generated many interests on the study of understanding the nature of Id networks especially in the use of anti-Id to modulate specific aspects of an immune response. The studies by many biologists, immunologists, virologists and other experts in other fields on Id network during the past 20 years support Jerne's theory and clearly demonstrated at least one fact, i.e., a given immune response is indeed modulated by the Id-anti-Id interactions. Despite the successful modulation of immune responses to infectious diseases and tumors by anti-Ids (Kennedy, R.C. et al. 1986a and Meeker, T. et al. 1985), the study of the use of anti-Id to modulate the immune response to allergens, e.g. grass pollen allergens has not progressed to the same levels.

The objective of my studies was to investigate the modulating functions of the anti-Id on the antibody responses to allergen *Lol p*IV in murine model systems.

In the first aspect of the study, a monoclonal internal image anti-Id, or Ab2β, designated as B1/1, was generated by immunizing syngeneic Balb/c mice with a monoclonal antibody, mAb91, specific for an epitope, Site A, of Ryegrass (*Lolium perenne*) pollen allergen, named *Lol p*IV. The reason for using *Lol p*IV was two-fold: (i) *Lol p*IV is one of major allergens in

grass pollens, which is recognized in a majority of grass allergic individuals and (ii) *Lol pIV* was discovered and first characterized (57KD, glycoprotein) in this laboratory (Ekromoddoullah, A.K.M. et al. 1983 and 1986). Subsequently, a common Id shared by human and murine antibodies to *Lol pIV* had been identified by polyclonal anti-Ids (Bose, R. et al. 1988). The question was addressed: can anti-Id be used to regulate the formation of antibody responses to allergen *Lol pIV* in murine model systems? It was anticipated that, in parallel with this study, the amino acid sequence of the region of the molecule which encompassed the epitope Site A would have been elucidated. This information would have enabled the synthesis of the relevant peptides for their use in studies on the nature of the internal image anti-Id (Ab2β), i.e. whether the Ab2β mimic the allergen at its primary structure level. Unfortunately, this information is not available. Therefore, the present study was undertaken using the whole molecule of *Lol pIV*.

Anti-idiotypic reagents have been serologically classified according to the location of the Id on the antibody molecule they recognized (Jerne, N.K. et al. 1982). This classification has been modified to be consistent with the serological specificities that have been recognized relative to the Id-anti-Id reactivity (Bona, C.A. and Kohler, H. 1984). The reasons for selecting the Ab2β were based on its

characteristics which are (i) it recognizes an Id that is located within or near the antigen combining sites; (ii) the Id is commonly expressed on antibodies from different species and (iii) it can substitute the antigen for the eliciting the antibody responses.

To produce the anti-Id, one of mAbs to *Lol pIV*, designated mAb91 was selected in my study with the following characteristics: (i) it was IgG1 isotype with κ light chain; (ii) it recognized an epitope, designated as Site A, of *Lol pIV*. Site A was one of at least four epitopes on *Lol pIV* identified by using monoclonal antibodies generated to *Lol pIV* (Jaggi, K.S. et al. 1989, Kisil, F.T. et al. 1989). Monoclonal antibodies mAb90 to mAb94 (all IgG1κ) generated against *Lol pIV* have been characterized as recognizing Site A, mAb12 (IgG1κ) was produced against the retentate fraction of KBG pollen allergen and was identified to recognize Site B on *Lol pIV* (other mAbs e.g. mAb95 recognized Site C and mAb102 recognized Site D). The epitope Site A was also recognized by human and murine IgE and IgG, as well as rabbit IgG, antibodies elicited to *Lol pIV*. These facts served to indicate that Site A constitutes one of the predominant epitopes of *Lol pIV* to which antibodies are produced in the antibody responses to *Lol pIV*.

For the study, syngeneic Balb/c mice served as the host

for the generation of Ab2. Therefore, according to prior experiences (Zhou, E.-M. et al. 1987, Schick, M.R. and Kennedy, R.C. 1989), the mAb91 was conjugated to KLH and then precipitated in alum in order to increase the immunogenicity of the Id. It was found that four to eight biweekly injections of 25 to 50μg of an Ab1-KLH conjugate preparation resulted in maximum Ab2 titers when the mice were immunized intraperitoneally. The advantage of employing a syngeneic system was that the Ab1 induced the anti-Id response but not to isotypic or allotypic specificities. This restriction in the specificity of the anti-Id facilitated screening the Ab2.

The frequency of the formation of hybridoma cells producing the internal image anti-Id was expected to be low and the stringent assays were required to screen the cells secreting anti-Id of the Ab2β type. This aspect of the study succeeded in producing an Ab2β. Of about 50 clones producing Ab2, only 2 were characterized as possessing the characteristics of Ab2β. During their propagation, only one cell clone survived and retained Ab2β specificity to the Id of mAb91. This internal image Ab2 was designated as B1/1. This Ab2β B1/1 recognized the Id91 on mAb90 and mAb91 to Site A. Interestingly, the anti-Id B1/1 also recognized an Id on mAb12 whose specificity was directed to epitope Site B of *Lol p*IV. This observation revealed that: (i) the Id on mAb91 and mAb12, recognized by B1/1 was the same or similar, since the

interaction of mAb91 and B1/1 could be inhibited by the presence of mAb12 as shown in Figure 3. However, the locations of Id91 on mAb91 and mAb12 appeared to be different from one another. This conclusion was based on the finding that B1/1 could completely block the interaction between mAb91 and *Lol* pIV but not between mAb12 and *Lol* pIV (Figure 5). This phenomenon has also observed in other Id-anti-Id systems as described in Chapter 2; (ii) although mAb12 was produced against the corresponding allergen of KBG pollen, it recognized the identical or cross-reactive epitope also present on *Lol* pIV allergen. Thus, Ryegrass and KBG pollen group IV allergens not only share the antigenic or allergenic determinants but the corresponding antibodies could share the same or similar Id. Collectively, these observations serve to demonstrate the complexity of an Id network in the antibody responses to a given antigen.

The Id91 present on murine mAbs to Site A of *Lol* pIV was also carried by human IgE and IgG and rabbit IgG antibodies to *Lol* pIV. These facts further indicated that Id91 represents a common or shared Id (IdX) and may represent a dominant Id possessed by antibodies produced to *Lol* pIV. If it is true, the epitope Site A of *Lol* pIV may be one of the dominant epitopes of *Lol* pIV. The murine model systems was selected to investigate this possibility and to study the possible regulatory functions of the internal image anti-Id B1/1 on the

antibody responses to allergen *Lol pIV*.

Experimental data obtained by Bose et al. (Bose, R. et al. 1984 and 1986) of this department, and by others, e.g. Hebert et al. demonstrated that: (i) a common (shared or public) Id was detected on anti-*Lol pI* (Rye) antibodies from allergic and non-allergenic individuals as well as murine antibodies (Mourad, W. and Hebert, J. 1986, Mourad, W. et al. 1988a and 1988b), and (ii) this common Id was recognized by auto-anti-Id present in the serum of Ryegrass allergic patients (Bose, R. et al. 1984 and 1986). Similarly, a common Id on antibodies to the house dust mite, *Dermatophagoides pteronyssinus*, was also detected by the auto-anti-Id in the serum of individuals allergic to this allergen (Saint-Remy, J.M.R. et al. 1988). The levels of auto-anti-Id to the Id of anti-*Lol pI* antibodies in allergic patients was reported to be influenced by seasonal exposure to the pollen and by immunotherapy with extracts of grass pollen (Bose, R. et al. 1986). These studies suggested that: (i) the immune responses to allergens (Ryegrass pollen group I and house dust mite, for example) induced the formation of antibodies which possessed a common Id and (ii) the immune responses, in turn, also induced the formation of an auto-anti-Id that recognized the common Id. In later studies, a common Id present on both human and murine antibodies to Site B of *Lol pIV* was demonstrated in this laboratory (Bose, R. et al. 1988). Collectively, these

data suggested that Id-anti-Id interactions could play a role in the regulation of antibody responses to allergen *Lol* pIV.

To investigate the possibility that anti-Id could play a role in modulating the antibody responses, the monoclonal internal image anti-Id, B1/1, was used to modulate the antibody responses to allergen *Lol* pIV in mouse model systems. The initial experiments attempting to suppress an ongoing IgE antibody response to *Lol* pIV by the treatment of mice with B1/1 were not successful. This failure may be due to the fact that even though the anti-Id B1/1 may target Th2 cells to suppress their secretion of IL-4 on which the primary IgE antibody responses were dependent. However, since the secondary responses may not need IL-4, the ongoing IgE antibody responses could not be suppressed by the anti-Id B1/1. Therefore, alternative protocols in which B1/1 conjugated to KLH was used to treat three different strains of mice, or B1/1 alone to treat syngeneic mice, prior to their challenge with *Lol* pIV served as models to evaluate the regulatory function of B1/1 on the formation of antibodies to *Lol* pIV and their expression of Id91.

In the first model system, we selected three different strains of mice (C57BL/6, Balb/c and C3H) that have different MHC backgrounds (H-$2^{\underline{b}}$, H-$2^{\underline{d}}$ and H-$2^{\underline{k}}$, respectively) to evaluate whether the effects of B1/1 on the antibody responses were MHC

associated. The experimental data revealed that the treatment with internal image anti-Id B1/1 reduced the antibody responses to *Lol* pIV in three different strains of mice, i.e. the effects did not appear to be MHC associated. The conjugates of the B1/1 and KLH or a control mAb, MCA and KLH precipitated in alum was used for the treatment of the mice. The first thought for the use of antibodies conjugated to a carrier protein and an adjuvant was to increase its ability to modulate the antibodies responses. While later experiment using the anti-Id alone resulted in the similar effect on modulating the antibody responses to *Lol* pIV, the carrier protein and adjuvant were not necessary for the anti-Id B1/1 to express its suppressive effect on the antibody responses to *Lol* pIV. Therefore, on the basis of these results a syngeneic Balb/c mice model system, used in all further experiments, consisted of the protocol in which a solution of B1/1 was used in the absence of carrier protein or adjuvant.

The reduction of the levels of IgE and IgG antibodies to *Lol* pIV was observed in the group of mice which had been treated with B1/1 at a dose of 25μg at an interval not greater than 8 weeks prior to their challenge with *Lol* pIV (Table IV of Chapter 3). Considering that the half life of murine IgG2b is about 2.6 days, this relative long-lasting effect following the administration of B1/1 may not be attributed to the continuous presence of B1/1. Rather, it may be suggested that

the activation or suppression of the formation of antibodies to *Lol* pIV was regulated by long-lasting cells.

Given the fact that the anti-Id B1/1 was demonstrated to be capable of up- or down-regulating the antibody responses to allergen *Lol* pIV, there was a greater interest in the suppression of allergic response instead of the enhancement. The expanded investigation proceeded along two directions. One objective was to identify the cell type(s) responsible for the suppression. Another was to investigate whether the B1/1 could function as a potential surrogate allergen for inducing the antibody responses to *Lol* pIV.

Although different hypotheses have been proposed in attempts to explain specific immune suppression, including the anti-Id induced suppression, the hypothesis have been subjected to much well-justified criticism. However, despite our lack of knowledge of the specific suppressive mechanisms, the phenomenon of suppressing antibody responses is a reality. The literature on autoimmunity, allergy, and transplantation immunology provide some of the least ambiguous evidence which demonstrate that specific suppression of immune responses can be induced by anti-Id. In autoimmune diseases, for example, the T cell proliferation in experimental allergic encephalomyelitis (EAE) could by suppressed by the treatment of the T cells with the anti-Id prepared against a shared Id

on rat T cell receptors specific for myelin basic protein (MBP) (Owhashi, M. and Heber-Katz, E. 1988). Clinical signs of EAE were eliminated in MBP-primed rats following the administration of the soluble anti-Id. The regulation, by the anti-Id, of IgE antibody responses to timothy grass pollen allergen had been studied by Malley and his colleagues (Malley, A. et al. 1982 and 1983) who demonstrated that the suppression of the IgE antibody response was due to the activation of Id-specific suppressor T cells.

In yet other studies of anti-Id regulation of antibody responses, the Id-anti-Id interaction at the level of B cells have been demonstrated in vitro with the use of a cloned B cell line (Bitoh, S. et al. 1990) and in the present study (Chapter 4) indirectly by means of the adoptive transfer of B cells from the anti-Id-treated animals. In the research on transplantation immunology, adoptive transfer of suppression by lymphoid cells with specific unresponsiveness to allografts, has been demonstrated in many experimental models (Kilshaw, P.J. and Brent, L. 1977, Dorsch, S. and Roser, B. 1982, and Barber, W.H. et al. 1985). From these and other studies, it is possible to draw at least two important conclusions. First, the suppression induced by the anti-Id is specific. Second, T cells as well as B cells may be individually or collectively responsible for the observed modulating effects.

The B cells were demonstrated to be responsible for the adoptive transfer of the suppression of antibody responses to *Lol p*IV. These B cells expressed anti-Id-like antibodies on their surface, since their capability of transferring the suppression could be abolished following the treatment with mAb91 plus complement (Table IX of Chapter 4).

The result was most interesting because it suggested that the treatment with anti-Id B1/1 may have involved at least one other set of cells which participated in the activation/proliferation of anti-Id B cells.

It was important to question the possibility that the adoptive transfer of the suppression was simply due to the carry-over of B1/1 on the surface of B cells. The results indicated that the level of ^{125}I-labelled B1/1 associated with B cells was simply too low to induce the suppression. It is obvious that administration of the anti-Id may follow a complex pathways and involve network of interacting cells, i.e. APC, suppressor T cells and/or helper T cells, which resulted in the generation of anti-Id B cells. Even though this complex network was not examined, the possible participation of particular subsets of T lymphocytes in the activation/generation of anti-Id B cells in anti-Id B1/1-treated animals could be studied by (i) *in vivo* injection of immunosuppressive drugs, e.g., cylosporin A or/and

cyclophosphamide along with the treatment with the anti-Id B1/1. It is known that cylosporin A inhibits T helper cell responses by inhibiting IL-2 production, thereby preventing the proliferation of activated T cells which express IL-2 receptors; whereas cyclophosphamide inhibits T suppressor cell function and polyclonal B cell activation (this may be a disadvantage of using cyclophosphamide); (ii) *in vitro* study of the direct effect of B1/1 on B cells with or without the participation of T cells, in terms of the induction of anti-Id B cells.

In another approach, anti-Id B cell clones may be generated for the *in vitro* studies. It has been observed that the collaboration between B and T cells occurs through the recognition of Id, i.e. recognition of the Id on B cells to which maturing T cells were exposed determined the final T cell idiotype repertoire (Hayglass, K.T. et al. 1986).

Numerous studies have demonstrated the potential use of internal image anti-Id as surrogate antigens for inducing the specific antibody responses which could protect the animals from infection. The anti-Id induced protection to infection by hepatitis B virus was successfully achieved in chimpanzee (Kennedy, R.C. et al. 1986). In allergy research, anti-Id have been proposed to substitute the allergen for use in the diagnosis of allergic responses to specific allergens (Nagpal,

S. et al. 1989).

The initial purpose to generate anti-anti-Id (Ab3) was to compare the immunogenicity of the anti-Id B1/1 with that of allergen *Lol pIV* in terms of their ability to induce the anti-*Lol pIV* antibodies. Moreover, an evaluation of the relationships of the fine specificities of Ab3 and Ab1 was expected to provide a greater understanding of the effectiveness of using anti-Id as surrogate antigens. The results presented in Chapter 5 indicated that it was possible to generate Ab3 that was similar to Ab1, i.e. (ii) binding to allergen (Ag^+) and (ii) possessing the Id91 (Id^+) (Ag^+Id^+) and therefore it was functionally and idiotypically indistinguishable from the Ab1.

As the studies progressed, some interesting and fascinating discoveries were made. It was found that Ab3 (induced by the anti-Id B1/1) to *Lol pIV*, in contrast to the Ab1 (induced by *Lol pIV*), were mostly of the IgG isotype. The formation of IgE antibodies to *Lol pIV* following the immunization with the anti-Id was at a minimal level (< 1/10 of PCA titers). These observations indicated that even though the anti-Id B1/1 was an internal image of *Lol pIV*, in terms of its functional characteristics, its immunogenic properties responsible for inducing either IgG or IgE antibody responses appeared to be different.

The potential of B1/1 as a candidate for use as a vaccine in down-regulating the IgE responses to *Lol* pIV was examined. Thus, following the treatment with anti-Id B1/1, mice were challenged with *Lol* pIV. After two injections of *Lol* pIV (this was the standard protocol used for the generation of IgE antibodies to *Lol* pIV), the PCA titer was less than 200, whereas the antisera obtained from mice immunized only with *Lol* pIV (i.e. without pre-treatment with B1/1) was 3,200. This result clearly indicated that the animals pre-treated with the Ab2β produced lower levels of IgE antibodies to *Lol* pIV after their challenge with *Lol* pIV in alum. To account for this reduced level of specific IgE antibodies, one mechanism considered was that the anti-Id caused the suppression of Th2 cells. Th2 cells are those that secrete IL-4 and IL-5 (Mosmann, T.R. et al. 1986). It was summarized by Finkelman and his colleagues (Finkelman, F.D. et al. 1990) that IL-4 is required for the generation of both primary polyclonal and secondary antigen-specific IgE responses in vivo. Most, but not all, polyclonal IgE production during a secondary immune response is IL-4 dependent. Memory B cells that have already switched to IgE at the DNA level may no longer require stimulation with IL-4 to be induced to secrete IgE. The generation of a secondary IgE response is not dependent upon the presence of IL-4. However, if IL-4 is not present during primary immunization, it is required during secondary

immunization for the generation of an IgE response. IL-4 does not appear to be required for the generation of in vivo IgG1 responses and IFN-γ appears to suppress polyclonal IgG1 responses more than antigen-specific IgG1 responses. These observations fit well with the findings of the present study that (i) the internal image anti-Id B1/1, Ab2β, could not suppress an ongoing IgE response to *Lol* pIV; (ii) pre-treatment once with the Ab2β could suppress the IgE, but not IgG1, responses and (iii) on the basis that the generation of IgG1 antibody responses requires less IL-4 compared to that required for IgE antibody responses (Snapper, C.M. et al. 1988), it may follow that any down-regulation of Th2 cells for their decreased expression of IL-4 causes the decreased formation of IgE antibodies, while influence on the formation of IgG1 antibodies may not be significantly observed. Similarly, multiple immunization with the Ab2β induced primarily IgG1 Ab3 responses to *Lol* pIV.

The peculiarity of the observed isotype of Ab3 (majority of IgG1) may have some relationship with the isotype of Ab1 (IgG1) used to generate the Ab2 which was in turn used to generate the Ab3. What was their relationship? Does the isotype of Ab1, but not that of Ab2, determine the isotype of Ab3? This possibility was also expected by Nagpal, S. et al. (Nagpal, S. et al. 1989) in their study on the induction of allergen specific IgE and IgG responses by anti-Id. The

speculation of influence of Id on Ab3 isotype selection remains to be proved.

In summary, the studies have established a murine model system for the investigation of the suppressive function of the internal image anti-Id on the antibody responses to allergen *Lol* pIV. The following conclusions were made: (i) an internal image monoclonal anti-Id was prepared and served to identify a common Id present on anti-*Lol* pIV antibodies in mouse, human and rabbit antisera; (ii) this anti-Id could modulate (suppress or enhance) specifically the antibody responses if it was administered to the mice prior to their challenge with *Lol* pIV; (iii) the anti-Id induced suppression of anti-*Lol* pIV antibody responses could be adoptively transferred by the B lymphocytes which possessed the characteristics of anti-Id B1/1; and (iv) the anti-Id induced Ab3s were primarily of the IgG1 isotype. By contrast, only very low levels of IgE antibodies to allergen *Lol* pIV were induced by immunization with the Ab2β.

On the basis of the investigations reported in this thesis, two major contributions have been added to the understanding of regulatory function of the anti-Id on the antibody responses to allergen *Lol* pIV. One is the demonstration that the anti-Id induced suppression of antibody

responses to allergen *Lol p*IV could be adoptively transferred into syngeneic mice by the B lymphocytes from anti-Id-treated donor mice. The B lymphocytes were characterized as possessing the anti-Id-like antibodies on the surface. The second point is the finding that the anti-Id induced anti-anti-Id, i.e. Ab3 responses are of the IgG isotype. In particular, IgE antibodies to *Lol p*IV was in very low level. This study takes us one step closer to the development of anti-Id for their use to suppress the IgE antibody responses to grass pollen allergen(s).

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