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THE LEVAN-ABPC48 IDIOTYPE SYSTEM:

FUNCTIONAL AND STRUCTURAL ASPECTS

by

FRANCISCO ANTONIO BONILLA

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Doctor of Philosophy

Dr. Constantin A. Bona
Chairman of Examining Committee

10/21/88
Date

Dr. Terry A. Krulwich
Executive Officer

Date

Members of the Examining Committee:

Dr. J. Donald Capra

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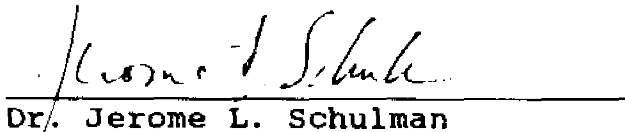

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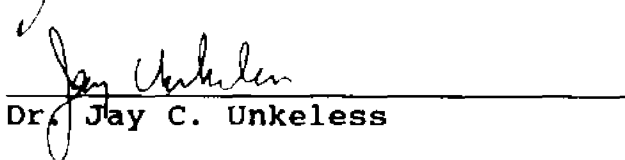

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ABSTRACT

THE LEVAN-ABPC48 IDIOTYPE SYSTEM:

FUNCTIONAL AND STRUCTURAL ASPECTS

by

Francisco Antonio Bonilla

Advisor: Professor Constantin A. Bona

The BALB/c myeloma protein ABPC48 (A48) binds bacterial levan (BL) polysaccharide. Its idiotype, designated the A48Id, is expressed by <10% of normal BALB/c anti-BL antibodies, and 90% or more of those from mice treated with syngeneic polyclonal anti-A48Id.

Hybridomas secreting A48Id$^+$ antibodies were prepared from BALB/c mice injected at birth with syngeneic anti-A48Id, or adults hyperimmunized with anti-A48Id-KLH conjugates, both groups challenged with BL subsequent to anti-Id. Monoclonal antibodies (mAbs) derived from newborn mice bind levan, and interact weakly with the monoclonal anti-A48Id antibody IDA10. MAbs from adults have antigen binding different from A48, and bind IDA10 strongly. Molecular analyses show that 60% of mAbs from young mice, and 80-100% of mAbs from adults express V_HX24 and V_K10 genes, as does A48. These findings suggest antigen-driven clonal expansion in young mice versus Id-specific expansion in adults.

Another group of hybridomas was obtained from BALB/c mice either untreated or treated at birth with A48 or IDA10. Spleen cells were cultured in vitro with B cell mitogens, fused, and hybridomas selected for expression of V_HX24 genes. Forty percent of these mAbs express a V_K1 gene. This frequency suggests antigen-independent selection affecting the available antibody repertoire.

These mAbs displayed several patterns of binding to polysaccharide-containing antigens. One mAb, and the BALB/c galactan-binding myeloma protein XRPC44, (both expressing V_K4) bind to galactan and rye levan.

Idiotopes associated with V_HX24$^+$ antibodies, particularly that defined by IDA10, were expressed. Novel V_H-V_K combinations encoded these idiotopes. In mAbs derived from IDA10-treated mice, the IDA10 idiotope appeared at higher frequency, suggesting that mitogens may substitute for antigen in cooperation with anti-Id in clonal expansion. Nucleotide sequences show that the IDA10-defined idiotope can be germline-encoded. Thus, IDA10 may influence clonal selection from the virgin B cell repertoire.

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INTRODUCTION

In the beginning . . .

The phenomena we attempt to describe in the young Western science of _immunology_ have attracted the attention of deep thinkers for thousands of years. Survivors of plagues long ago discovered that their ordeal had left them somehow protected from serious debility or death in subsequent visitations of plague.

The Latin word _immunitas_ denotes an exemption from military service or taxation. In the first century A.D., this word was used to describe the exemption from disease acquired through experience (Silverstein and Bialasiewicz, 1980). The connotations of the Latin word survive in the usage of its English derivative, _immunity_. The generation of immunity by deliberate administration of a substance to an organism is _immunization_.

With the advance of technology and increasing focus on the material, late 19th century _immunologists_ observed that the sera of animals inoculated with infectious agents contained substances able to specifically neutralize or

destroy these agents. Thus, a tangible basis for the phenomenon of acquired immunity was discovered. In 1901, Landsteiner proposed the term antikörper (antibody) to designate these entities in immune sera. An antigen, then, is a substance which induces the appearance of antibodies reacting with it. Tiselius and Kabat (1939) electrophoresed immune sera and found that antibody activity was contained predominantly in the gamma globulin fraction. Heremans (1959) first proposed the name immunoglobulin (frequently abbreviated "Ig") for serum globulins with antibody activity.

What is an idiotype?

Long before molecular details of antibody structure became known, researchers observed that animals could produce antibodies against the Ig of other species. Many experiments utilizing anti-Ig antisera defined two types of reactivity which were ascribed to two types of antigenic determinants[1] present on antibody molecules: isotypes (see Natvig and Kunkel, 1973) and allotypes (Oudin, 1956, 1960).

Antibody molecules are comprised of two identical heavy chain polypeptides and two identical light chains (see Cohen and Porter, 1964). Isotypes or isotypic determinants distinguish several classes of heavy and light

[1]The phrase "antigenic determinant" describes the particular portion of the antigen molecule that interacts specifically with antibodies to which it binds.

chains within a species, and between species. Many vertebrate species produce five classes of immunoglobulin designated IgM, IgG, IgA, IgE, and IgD. In mice (and humans) the IgG class is further subdivided into subclasses. In mice these are named IgG1, IgG2a, IgG2b, and IgG3. Light chain isotypes also exist and are designated kappa and lambda. Allotypes or <u>allotypic determinants</u> distinguish Ig chains of one class between groups of individuals or inbred strains within a species.

Two groups of investigators simultaneously discovered a third type of antibody antigenic determinant. In 1963 Kunkel et al., and Oudin and Michel described the <u>individual antigenic specificity</u> of isolated human and rabbit antibodies, respectively[2]. These new antigenic determinants appeared to be restricted to only the individual antibody used to generate a particular anti-serum. Oudin and Michel later (1969) coined the term <u>idiotypy</u> to describe this idiosyncratic specificity. The noun idiotype, then, designates the individual determinant(s) of the antibody which elicit(s) specific <u>anti-idiotype</u> antibodies.

---

[2]Individual specificities in the gamma globulin fractions of the sera of patients with multiple myeloma had been described prior to this (Slater et al., 1955). However, Kunkel and Oudin were the first to demonstrate idiotypy in highly purified antibody preparations. Although suspected in the late 1950's, it was not until the late 1960's that most immunologists accepted myeloma proteins as <u>bona fide</u> immunoglobulins.

Henceforth, "idiotype" will frequently be abbreviated "Id."

In 1959, Porter hydrolyzed antibodies with papain and found that they were uniformly cleaved into two types of fragments called Fab (antibody binding) and Fc (crystallizeable). The Fab fragment contains the portion of the antibody reacting with antigen (the combining site), while the Fc has many of the effector functions associated with antibodies (e.g., complement fixation, interaction with specific receptors). Two Fab fragments and one Fc fragment are produced from one immunoglobulin molecule digested with papain. Fc fragments consist of approximately 50% of both heavy chains, while each Fab fragment contains one entire light chain and the remaining portion of the heavy chain.

Grey et al. (1965) prepared Fab and Fc fragments of human myeloma proteins and analyzed their reaction with anti-idiotype antisera. They made two important observations. First, in every case, the Fab fragment reacted with anti-Id antisera while the Fc fragment did not. Second, isolated antibody heavy and light chains reacted differently with different antisera. In some cases, anti-Id antisera could react with isolated heavy chains, in others with isolated light chains. Some antisera did not react at all with isolated heavy or light chains, they bound only to the intact immunizing antibody. Thus, idiotypes were seen to be formed by structures related, if not identical, to those which determined antigen binding, and we had our

first glimpse of the way in which idiotypic determinants relate to the overall structure of the antibody molecule.

Many investigators who analyzed amino acid sequences of antibody heavy and light chains discovered an unusual structural pattern. The C-terminal regions could be correlated with isotypic specificities. That is, the C-terminal portion of the IgM antibodies of a species are nearly identical to one another. IgM's from different species are mostly similar, but some species-specific differences are discernible. This pattern extends to the other immunoglobulin classes (of both heavy and light chains). On the other hand, the N-terminal regions of immunoglobulin chains displayed a remarkable heterogeneity. Quattrocchi and colleagues (1969) analyzed more than 100 human light chains, no two amino terminal sequences were identical. Thus, the C-terminal portion of an antibody heavy or light chain was called the constant region (C region), the N-terminal portion the variable region (V region).

As mentioned above, anti-Id antisera bind to antibody determinants located on Fab fragments. Early amino acid sequence analyses demonstrated that the Fab contained the N-terminal, or variable, portions of the heavy and light chains. It seemed reasonable to suppose that idiotypic specificities would be determined by V regions since antibodies with similar C regions had different idiotypes.

This supposition was strengthened when Oudin and Cazenave (1971) found that rabbit antibodies with different C regions (IgG and IgM) could have similar idiotypes. In 1972, Inbar et al. derived an antibody fragment consisting of both heavy and light chain variable domains, the Fv fragment. In 1973, Wells et al. demonstrated that one such Fv fragment carried an idiotype previously characterized on intact antibody molecules.

Categories of idiotypes and anti-idiotypes

Different anti-idiotypic reagents may have different patterns of reactivity when they are assayed for binding to various antibodies. Thus, an anti-Id may bind only to the Id used to generate it; it may bind to various antibodies with the same antigen specificity; or it may bind to antibodies with different specificities. Kunkel et al. (1973) designated idiotypes found on only one antibody as individual idiotypes (IdI). Idiotypes found on more than one antibody are cross-specific or cross-reactive idiotypes (IdX).

A uniform system of nomenclature for the participants in various Id-anti-Id interactions does not yet exist. However, two abbreviations have become widespread in the literature. The term Ab_1 denotes the antibody used to generate an anti-Id reagent, the Ab_2.

Since idiotypes are markers of antibody variable (V) regions, they are determined by the same structures which determine the antigen specificity of an antibody. In a protocol of immunization with an Ab_1, any structure exposed on the surface of the antibody V region may (theoretically) determine an idiotypic specificity. The antibody combining site is such a structure, and we may distinguish two types of Ab_2 based on their interaction, or lack thereof, with an Ab_1 combining site. Some bind to structures not related to the combining site, while others interact with part or all of the amino acid residues contributing to it. Considering the latter situation, can the Ab_1 distinguish between occupancy of its combining site by antigen or by Ab_2? Hence, the anti-Id which binds to the combining site may be considered the internal (within the immune system) representation of a particular antigenic structure, or its internal image[3]. Jerne et al. (1982) proposed the widely adopted abbreviations $Ab_{2\alpha}$ to designate Ab_2s binding outside the combining site, while $Ab_{2\beta}$ corresponds to the internal image.

---

[3]This phenomenon was first described by Sege and Peterson (1978) who characterized Ab_2s raised against antibodies specific for retinol-binding protein (RBP). These Ab_2s bound to cells possessing RBP receptors. The phrase "internal image" was first used by Jerne et al. (1982).

A brief molecular digression

The amino acid sequences of immunoglobulins were quite paradoxical in the context of the developing molecular biology of the 1960's and 70's. How could the constant region of an antibody, each class presumably originating from one gene, be associated with diverse variable regions, presumably the products of many genes? Dreyer and Bennet (1965) first proposed that the mRNA encoding an antibody chain is transcribed from two genes which are not contiguous on the chromosome. Wu and Kabat (1970), and Kabat (1980), extended this concept, proposing that the Ig amino acid sequences were better explained by a larger number of smaller gene segments.

These hypotheses have been shown to be essentially correct. Thus, "mature" immunoglobulin genes are generated by the somatic recombination of multiple germline gene segments (see Tonegawa, 1983). Mature heavy chain variable region (V_H) genes are assembled from germline V_H genes, D (diversity) genes and heavy chain joining region (J_H) genes. Each heavy chain isotype (C region) is encoded by a distinct gene. Mature light chain variable region (V_L) genes are comprised of germline V_L genes and J_L genes. Kappa and lambda light chain isotypes are encoded in different loci. We shall limit further discussions to kappa light chains, the relevant gene segments shall be abbreviated V_K and J_K.

The Network Theory

Until the late 1960's, investigators generated anti-idiotype reagents primarily across species or strain lines. In the late 1960's and early 1970's, researchers began to produce anti-idiotypic antibodies within the same strain (e.g., Sirisinha and Eisen, 1971). Thus, it was proven that an individual could produce anti-idiotypic antibodies against self Ig.

In 1974, Niels Jerne roiled the waters of immunological theory which remain somewhat turbid to this day. Dr. Jerne's daring proposal suggested that the auto-recognition of self antibody idiotypes by self antibodies or regulatory lymphocytes (T cells) actually controlled the state of activation or suppression of antibody-producing cells (B cells). This Network Theory viewed the "resting" immune system as a dynamic equilibrium of Id-anti-Id[4] interactions. An immune response reflected the disruption of these interactions and the establishment of a new dynamic steady state. Network theory stands in contrast to Clonal Selection Theory[5] in which lymphocytes are born and

---

[4]Dr. Jerne increased our immunological vocabulary with the introduction of three new words: an epitope is an antigenic determinant; a paratope is an antibody combining site; an idiotope is an antigenic determinant of an antibody which defines an idiotypic specificity. An idiotype, then, is the collection of idiotopes which may be distinguished in a particular antibody variable region.

[5]Mechanistic details of the requirements for activation or suppression of antibody-producing cells have been developed in the Associative Recognition Theory of

die passively awaiting positive or negative interactions determined principally by antigen, leading to an immune response (Burnet, 1959).

Connotations of dynamism or passivity aside, our knowledge of several important facts is insufficient for definitive refutal of one, or synthesis of both, of the above paradigms. We have yet to understand completely the spectrum of combining sites, or repertoire of antibody or T cell receptor specificities generated in an individual, and the processes which influence it (Alt, et al., 1987, Rajewsky, et al., 1987, Schroeder, et al., 1987). Furthermore, what is the potential for antibodies to interact with more than one structure (Ghosh and Campbell, 1986, Richards and Konigsberg, 1973, Richards, et al., 1975)? How does this question of antibody specificity relate to the potential for Id-anti-Id interactions that can arise in a defined repertoire? What are the details of lymphocyte modulation by Id or anti-Id at the level of interacting cells? These questions are equally important for models of immune regulation which accord primacy to antigen or idiotype. The data presented herein may cast some illumination on the answers to these questions.

Bretscher and Cohn (1970), an extension of the concepts of Clonal Selection Theory.

Levan and the ABPC48 idiotype system

As evidenced above, much knowledge of antibody structure has come from study of myeloma proteins. Prior to the advent of monoclonal antibody technology (see Köhler 1986) myeloma proteins were the only source of large amounts of homogeneous antibody. Particularly useful was the technique of inducing antibody-secreting lymphoid neoplasms by intraperitoneal injection into mice of viruses, mineral oil, or volatile hydrocarbons (Potter, 1967).

The majority of these myelomas, or plasmacytomas, secreted Igs of the IgA class. Of those whose antigen specificity could be determined, the majority bound to polysaccharide antigens of bacterial or plant origin (Potter, 1977). These characteristics may reflect the origin of these neoplasms in the lymphoid tissue of the intestines. The murine myeloma proteins have defined numerous idiotypic systems. Generation of monoclonal antibodies has allowed rapid extension of research concerning antigen-binding, idiotypy and antibody structure-function relationships into many species, vertebrate and invertebrate (extensively reviewed by Bona, 1987).

Naturally-occurring polyfructose molecules (levans) may be classified into three types according to the particulars of their chemical composition. Inulin, (INU) isolated from the tuberous roots of plants of the genus Dahlia (Haworth and Learner, 1928) consists of linear

polymers of ß(2-1)-linked fructose with a terminal glucose joined as in sucrose. Rye levan (RL) purified from perennial rye grass (Lolium perenne) is a linear polymer of ß(2-6)-linked fructose, also with a terminal glucose (Moreno et al., 1976, Tomasic, et al., 1978). Bacterial levan (BL), produced by a variety of bacteria (e.g., A. levanicum, Feingold and Gehatia, 1957), consists of branched polymers of ß(2-6)-linked fructose with ß(2-1) linkages at the branch points.

More than 100 myelomas have been induced in the BALB/c mouse strain (Potter, 1977). Of these, the largest fraction (13) have binding to one or more of the levan antigens just described. Two of these levan-binding myeloma proteins (LBMP), ABPC48 (A48) and UPC10 (U10), are specific for ß(2-6) linked polyfructose. They bind to rye levan and bacterial levan, but not to inulin (Vrana et al., 1976). The remaining 11 LBMP bind inulin and bacterial levan (Lieberman et al., 1975).

Since the ß(2-1) linkages in BL are relatively hidden in the branch points of this polysaccharide, some investigators considered it unlikely that INU-binding myeloma proteins had access to these determinants, and that their binding to BL represented a "dual specificity" (Vrana et al., 1976). Streefkerk et al. (1979) performed a detailed immunochemical analysis of some of these myeloma proteins and proposed an explanation for this apparent dual specif-

icity. ß(2-1)-linked fructose is restricted to a bulky secondary structure, while ß(2-6) levan can assume a relaxed, linear chain structure. A combining site capable of binding INU must be relatively wide to accommodate the molecule, while a strictly ß(2-6) levan-specific antibody may have a narrow combining site. Because of its relative aperture, the anti-INU combining site may accommodate the ß(2-6)-linked molecule.

Lieberman et al. (1975) extensively analyzed the idiotypy of these LBMP. In addition to an IdI associated with each myeloma protein, these authors identified 10 distinct IdX systems with variable expression in this group of antibodies. A48 and U10 were idiotypically distinct from the INU-binding proteins. U10 expressed none of the IdX, while A48 had only two of the ten. Each of the INU-binding proteins expressed at least five IdX.

The immune response to bacterial levan

Bona et al. (1978a) studied the kinetics and idiotypic characteristics of the BALB/c anti-BL immune response. These authors found that many anti-BL antibodies also bound to INU, and expressed the IdX of INU-binding myeloma proteins, designated the E109IdX. (E109 is an INU-binding myeloma protein.) Anti-E109IdX (anti-idiotypic) antibodies

were also produced during the anti-BL response[6]. Although the response to BL is T-independent[7], nu/nu BALB/c (nude, or athymic) mice develop an anti-BL response which differs from normal BALB/c in that expression of the E109IdX is much reduced. These latter two observations suggested the possibility of regulation of this response involving both E109IdX-specific T cells and antibodies.

Bona et al. (1979b) extended these observations and found that injection of anti-E109IdX antibodies prior to BL immunization suppressed the expression of the IdX in the anti-BL response. Suppression could also be observed in the small fraction of $E109IdX^+$ anti-BL antibodies in nude mice. Furthermore, spleen cells treated with anti-E109IdX antibodies when cotransferred with untreated spleen cells into irradiated recipients, did not suppress E109IdX production. These data suggested that the suppression was T-independent, and was due to a direct effect on IdX^+ B cell precursors.

---

[6]The occurrence of Ab_2 in the course of normal and pathologic immune responses has been documented by several investigators. A few additional examples are in the murine responses to trinitrophenyl (Schrater et al., 1979), and to phosphorylcholine (Kelsoe and Cerny, 1979); the rabbit response to human serum albumin (Jackson and Mestecky, 1979); and in myasthenia gravis patients (Dwyer et al., 1983).

[7]Many polysaccharide antigens are called thymus-independent type 2, or simply type 2 antigens, because a humoral immune response to these antigens does not require specific antigen recognition by a T cell (see Coutinho and Moller, 1973).

Bona et al. (1979c) also found that E109IdX$^+$ anti-BL antibodies could only be elicited in mice after 2-3 weeks of age. Before this time, mice produced IdX$^-$ antibodies which bound ß(2-6) fructosan, as did nude mice immunized with BL.

Lieberman et al. (1979) then found that nude mice treated with anti-E109IdX and BL-immunized, produced increasing amounts of A48Id$^+$ anti-BL antibodies. The transfer of enriched T cells from normal BALB/c mice into anti-E109IdX-treated mice ablated the increase in A48Id upon BL immunization. This suggested that A48Id-specific suppressor T cells might explain the low levels of the A48Id in the normal anti-BL response.

Hiernaux et al. (1981) injected one day old BALB/c mice with syngeneic anti-A48Id antibodies and immunized them with BL at age one month or older. These authors observed an increase in the percentage of A48Id$^+$ antibody-producing cells that varied directly with the dose of anti-A48Id administered neonatally. Ten ng anti-A48Id was sufficient to increase the fraction of A48Id$^+$ cells from 6% to 46% of the anti-BL response.

The regulatory idiotope hypothesis

According to the nomenclature described above, we may label A48 as Ab_1 and anti-A48Id antibodies as Ab_2. What will be the outcome of immunizing mice with Ab_2, thereby

producing "Ab_3," and continuing this process to generate "Ab_4[8]?" Bona et al. (1981) performed these experiments using A48 as Ab_1[9]. It was observed that both Ab_2 and Ab_4 bound to both Ab_1 and Ab_3. Ab_1 and Ab_3 did not interact, as did not Ab_2 and Ab_4. Ab_1, of course, bound BL, while Ab_3, for the most part, did not.

Results similar to these were obtained by Urbain et al. (1977) in an entirely different system. These authors immunized rabbits with Micrococcus lysodeikticus to produce Ab_1, and used this to generate Ab_2 and Ab_3. As above, Ab_2 bound to Ab_1 and Ab_3, and Ab_3 did not bind to the bacteria. Rabbits used to make Ab_3 (i.e., immunized with Ab_2) subsequently produced anti-bacterial antibodies which also bound to Ab_2.

According to "classical" notions of idiotypic immunization, we expect the paratopes of Ab_n to bind to the idiotopes of Ab_{n-1}. This interpretation cannot explain the interaction of Ab_4 and Ab_1. The simplest explanation of these results requires that Ab_1 and Ab_3 be idiotypically

---

[8]Note that the new labels Ab_3 and Ab_4 are not intended to define new theoretical entities in the idiotype network, they simply serve to distinguish the different sets of anti-Id antibodies. Each Ab_n is an anti-Id against Ab_{n-1}.

[9]In these experiments antibodies were coupled to KLH (keyhole limpet hemocyanin, a very immunogenic carrier molecule), and immunization was performed with Freund's adjuvant. In subsequent experiments which describe activation of the A48Id in association with BL immunization, Id or anti-Id was in the native state, and injected without any adjuvants.

similar. This could arise in the following way: Ab_1 stimulates production of "classical" Ab_2 whose paratopes recognize Ab_1 idiotopes. In contrast to this, immunization by Ab_2 has the converse outcome. It is the paratopes of Ab_2 which stimulate expansion of clones with complementary idiotopes, that is, Ab_3 has idiotopes similar to those of Ab_1. Thus, immunization with Ab_3 generates antibodies similar to Ab_2. The failure of the majority of Ab_3 antibodies to bind BL is explained by the sharing of idiotopes by antibodies with different antigen specificity, a phenomenon that has been observed time and again (see, for example, Oudin and Cazenave, 1971).

This interpretation, then, requires an asymmetry in the recognition (or the generation) in the immune system of V region determinants. The "Ab_1 type" of antibody (which includes the Ab_3 above) bears idiotopes which can generate complementary paratopes. The "Ab_2 type" of antibody (which includes the Ab_4 above) does not bear such idiotopes, and stimulates the expansion of clones (Ab_1 type) which bear complementary idiotopes. The idiotopes which are capable of generating complementary paratopes were termed _regulatory idiotopes_. These concepts were formalized and presented as the _regulatory idiotope hypothesis_ by Paul and Bona (1982).

Some investigators have offered an alternative interpretation of the data which led to the regulatory idiotope

hypothesis (Sacks et al., 1983). If we consider the population of polyclonal anti-A48Id antibodies, it seems reasonable to suppose that the set of combining sites exhibited by the anti-Ids is smaller than the set of idiotopes exhibited. This is because all of these Ab_2 combining sites are restricted to specificity for A48 idiotopes, while the idiotopes which Ab_2s express have no such restriction, other than that they can be generated by the V genes creating their various anti-Id specificities. Since it has been observed in a number of systems (for example, the anti-BL immune response described above) that one antibody specificity may be associated with more than one idiotype, the above supposition seems tenable.

Immunization with anti-Id antibodies, then, is immunization with a restricted set of paratopes, and a less restricted set of idiotopes which we might _a priori_ assume to be equally immunogenic. Thus, the idiotypic similarity of Ab_1 and Ab_3 occurs because the higher concentration of similar Ab_2 paratopes versus the many dissimilar Ab_2 idiotopes results in more frequent activation of Ab_2 paratope-complementary clones, i. e., those bearing Ab_1-like molecules.

The resolution of these two alternative explanations might be achieved by immunizing with a monoclonal Ab_2, and by immunochemical and structural analysis of the resulting antibodies to determine, in this instance, whether most

elicited antibodies are Ab_1-like, or if they are "true" Ab_3 molecules (anti-Ab_2Id). Such an analysis has been performed using two monoclonal anti-A48Id antibodies (Legrain and Buttin, 1985; Legrain et al., 1985a). These data will be discussed below.

Further investigations concerning the A48Id

Rubinstein et al. (1982) discovered that not only can $A48Id^+$ clones be activated by injection of anti-Id before BL immunization, but they may also be activated by pre-treatment with A48 antibody itself. This pre-treatment leads to the appearance of A48Id-specific helper T cells since $Ly1^+$ T cells from treated mice transferred the ability to produce an $A48Id^+$ anti-BL response. Rubinstein and Bona (1983a) also demonstrated, through the use of various genetically disparate mouse strains, that this activation was not restricted to particular haplotypes of major histocompatibility, Ig heavy chain V region, or C region loci.

Rubinstein et al. (1983b) further showed that activation of the A48Id induced by neonatal treatment with anti-Id required an antigen stimulus, i.e., anti-Id treatment alone could not induce anti β(2-6) levan or $A48Id^+$ antibodies. B cells from anti-A48Id-treated mice were able to transfer the $A48Id^+$ anti-BL response to irradiated recipients. Thus, the mechanisms of Id-mediated versus anti-Id-

mediated Id expansion were seen to differ. The former is dependent on the activity of Id-specific T_h cells, the latter on a direct effect of anti-Id on B cells.

These authors also studied the effects of monoclonal anti-A48Id antibodies administered subsequently to polyclonal anti-A48Id antibodies. They found that one monoclonal anti-Id, 17-38, was able to substitute for antigen in the activation of BL-binding antibodies after treatment of mice with anti-A48. Interestingly, while the antigen-stimulated response after anti-Id-treatment is Id^+, the response stimulated by 17-38 after anti-Id treatment is Id^-. Thus, we have every indication that 17-38 carries the internal image of a BL determinant, but the immune system must, at some level, be able to distinguish quite well the difference between a non-Ig determinant versus the same determinant on an Ig molecule.

Rubinstein et al. (1984) were also able to demonstrate that placental transfer of maternal Id could alter the anti-BL response of progeny. The U10 myeloma protein is of the IgG2a class and is transferred across the placenta. Pregnant females were injected weekly with U10 during pregnancy. Newborn mice were not treated after birth. When the progeny were immunized with BL at age one month, 93% of the anti-BL response was $U10Id^+$ (versus 5% $A48Id^+$ in the progeny of females injected with a different IgG2a).

Studies with monoclonal anti-A48Id antibodies

Legrain et al. (1981) generated 17 monoclonal anti-A48Id antibodies called the IDA series. These were classified into six different groups based on their ability (or lack thereof) to mutually inhibit one another's binding to A48. BL was able to inhibit, to some degree, the binding of all IDA antibodies to A48. Apparently, A48 bears at least six distinct idiotopes capable of eliciting anti-idiotope antibodies. Three of these were found to be shared by U10. The fact that BL can affect IDA-A48 interaction in all cases shows that antigen binding has a marked effect on idiotope expression since it seems unlikely that six immunochemically distinct idiotypic determinants could be distinguished in one combining site.

Recall that BL is a secreted product of several enteric bacterial species, and for this reason is an "environmental" antigen. That is, animals may develop specific antibodies without experimental immunization. Legrain and Buttin (1983) examined the occurrence of IDA idiotopes on anti-levan antibodies in untreated and in BL-immunized mice. They found, in unimmunized mice, a substantial amount of anti-levan (about 100 μg/ml A48 equivalent) and of one idiotope defined by the monoclonal anti-A48Id IDA10 (about 10-30 μg/ml A48 equivalent). Some of the $IDA10^{+}$ antibodies were BL-binding, but not all. Furthermore, titers of IDA10-reactive antibodies did not

increase with age, while titers of anti-BL did. BL-immunization increased both anti-BL and IDA10$^+$ antibody titers, but the IDA10$^+$ component of the anti-BL response did not rise above 10% of the total. These data are consistent with previous studies using polyclonal anti-A48Ids in which the A48Id$^+$ anti-BL antibodies constituted 0-10% of the total anti-BL response.

Legrain et al. (1983) then assessed the effects of immunization with monoclonal anti-Ids on expression of A48 idiotopes. They used three anti-Ids, IDA10, IDA16, and IDA17, each of which had been shown to bind to a different determinant of A48. After injection of one or two or all three IDAs (coupled to bacterial lipopolysaccharide, or KLH), sera were assayed for the level of expression of each idiotope, and for the occurrence of more than one idiotope on the same antibodies. They found that each IDA induced a predominantly IDA-specific response; that expression of the IDA idiotopes did not occur on the same molecules; and that the A48Id (IDA10$^+$, IDA16$^+$, and IDA17$^+$) was extremely rare in these responses. Clearly, some important differences are seen between the consequences of treatment with polyclonal anti-Id versus monoclonal anti-Id. Furthermore, these studies pointed out the difficulty in distinguishing serologically the "directional" character of a particular Id-anti-Id interaction, that is, who's got the paratope and who's got the idiotope?

Legrain and colleagues suggested that molecular studies in conjunction with immunochemical analyses would be extremely valuable for more precise interpretation of the observed interactions of Id and anti-Id. These investigators have performed such analyses which will be described later.

Goldberg et al. (1983) prepared hybridomas from three groups of BALB/c mice treated in order to activate A48Id$^+$ clones. One group was injected at birth with A48 and at age one month with BL. A second group was injected with polyclonal anti-A48Id at birth and immunized at one month with BL. The third group consisted of adult mice immunized with a polyclonal anti-A48Id-KLH conjugate in Freund's adjuvant, and one month later with BL. Hybridomas were screened and selected for interaction with polyclonal anti-A48Id antibodies. These authors found that most antibodies derived from the neonatally-treated mice bound levan, while a substantial fraction of those from adult mice did not. This showed: a) the importance of antigen for expansion of clones in the former groups, and the sufficiency of vigorous anti-idiotypic immunization of adult mice for activation of A48Id$^+$ clones independent of antigen specificity; and b) that the A48Id could be found on antibodies which do not bind levan.

The latter point was further substantiated by the screening of 198 murine and 80 human myeloma proteins for

expression of the A48Id. The phosphorylcholine-binding murine myeloma protein MOPC167 (Potter,. 1977) was observed to inhibit the binding of A48 to anti-A48Id antibodies. Using polyclonal antibodies against idiotopes of A48, U10, and one representative mAb from each group, these authors also demonstrated heterogeneity of these idiotopes. Anti-Ids against different mAbs bound differently to mAbs all selected initially for binding to the same anti-A48Id antibody preparation. In addition, BL did not equally affect the binding of various anti-Ids to the mAbs.

Another brief molecular digression

Immunoglobulin V genes have been grouped into families based on nucleotide or amino acid sequence homology. In general, members of the same V_H or V_K gene familv have >80% homology with one another, while members o different families have <75% homology. Several systems of nomenclature for the various V_H gene families are in use currently (Brodeur and Riblet, 1984; Dildrop, 1984; Kabat et al., 1987; Potter, 1977). Two systems of V_K gene family nomenclature are also in use (Kabat et al., 1987; Potter et al., 1982).

In discussing V gene usage by the various myeloma proteins and monoclonal antibodies we will encounter, we will use for V_H genes the system of nomenclature established originally by Brodeur and Riblet (1984, Table I).

The V_K nomenclature established by Potter (1982) is quite simple. Twenty-eight V_K families have been distinguished, they are designated simply V_K1, V_K2, etc.

Table I. V_H gene families of the mouse.

Name	Reference
V_H36-30	Brodeur and Riblet, 1984
V_H7183	"
V_HJ558	"
V_HJ606	"
V_HQ52	"
V_HS107	"
V_HX24	"
V_H3609	Brodeur et al., 1984
V_HGam3.8	Winter et al., 1985
V_HSm7	Shlomchik et al., 1987
V_HRF24	Kroemer et al., 1987
V_H11	Reininger et al., 1988

Antigen specificities, idiotypes, and V_K genes associated with the V_HX24 gene family

Auffray and colleagues (1981) sequenced the expressed V_H genes of A48, U10, and MOPC173 (M173), another BALB/c myeloma protein of unknown antigen specificity. These sequences have been assigned to the V_HX24 gene family. Amino acid sequences of the light chains of these myeloma proteins showed that they all belonged to the V_K10 gene family (Hood et al., 1973; Hood et al., 1976; Potter et al., 1976).

Some other important V_K correlations have been made for antigen specificities associated with the V_HX24 gene family. Another extensively studied system are the galactan (GAL) binding myeloma proteins. Galactans are

another environmental antigen for the mouse, being present in the common rodent laboratory foods and bedding materials. This antigen specificity and associated idiotypes are encoded by the pairing of V_HX24 with V_K4. Examples include the BALB/c myeloma proteins XRPC24 (X24) and XRPC44 (X44) (Pawlita et al., 1981; Pawlita et al, 1982; Potter et al., 1979; Rudikoff et al., 1973, Rudikoff et al., 1984; Smith-Gill et al., 1986). The myeloma protein S117 which binds to group A streptococcal carbohydrate (GAC) also uses V_HX24 and V_K4 (Capra et al, 1976). Specificity and idiotypy of antibodies which bind fucosyllactosamine (a polysaccharide with varied distribution in the body) is determined by V_K24B in association with V_HX24 (Kimura et al., 1988). Finally, a monoclonal antibody which binds PR8 influenza virus hemagglutinin has been shown to use V_HX24 (Bona, 1987).

For their analysis of V_H-V_K association and structure in the A48-IDA system, Legrain et al. (1985) prepared hybridomas from mice immunized with IDA10- or IDA23-LPS conjugates, and selected them for binding to the immunizing anti-Id. Of 18 hybridomas selected, 10 were analyzed for V_H gene usage. Seven were found to produce mRNA hybridizing with a V_H441 gene probe. One of these also bound BL.

Nucleotide sequence analysis of the 3′ half of six of these antibody V_H regions showed that all were encoded by a

V_HX24 family gene[10]. Four of these six antibodies express the V_H441 gene, the particular gene expressed in the other two could not be determined. The D regions were quite heterogeneous, no obvious correlation being found with either anti-Id or anti-BL activity. These data strongly suggested that a) immunization with this Ab_2 elicited Ab_1-like (V_HX24^+) clones, and b) the basis of Id expression (in this particular case) may be V_H framework and/or V_L determined.

These suppositions were strengthened when Legrain and Buttin (1985) reported an additional V_H and seven V_K sequences from this group of mAbs. All were found to express V_K10 in association with V_HX24. Clearly, a majority (70%) of $A48Id^+$ (IDA10-binding) antibodies express this V_H-V_K combination. What other associations may create this idiotope? Furthermore, what particular antibody regions are important for Id expression? The latter question cannot be answered easily by comparison of sequen-

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[10]The V_HX24 gene family contains two 98% homologous germline genes (Hartman and Rudikoff, 1984). This degree of homology is too high to distinguish by hybridization techniques, nucleotide sequence analysis must be employed. Following the nomenclature of the first investigators to clone a germline gene from this family (Ollo, et al., 1981) and the family designation established by Brodeur and Riblet (1984), the gene expressed by A48, UPC10, and several galactan-binding myeloma proteins is V_H441. The gene expressed by the galactan-binding myeloma protein XRPC24 is V_HX24. These genes are designated VhGal 39.1 and VhGal 55.1, respectively, by Hartman and Rudikoff (1984). When not specifically stated, the abbreviation V_HX24 refers to the <u>family</u>.

ces deriving from the same V_H and V_K gene families since homology significant for Id expression is lost in the high background of germline homology.

Synopsis of experiments related below

The investigations discussed herein further probe the mechanisms of activation of A48Id$^+$ clones and the structural requirements for A48Id expression. Analysis of the hybridomas prepared by Goldberg et al. (1983) has been extended with detailed immunochemical and nucleotide sequence studies.

Antigen specificities, idiotypes, and V_K genes associated with the V_HX24 gene family in the virgin B cell population have also been examined. Hybridomas have been generated from untreated mice, from mice treated at birth with A48, and from mice injected with IDA10 at birth. IDA10 was chosen rather than polyclonal anti-A48 since it had been shown to occur with some frequency in non-BL-immune mice (Legrain and Buttin, 1983; see above), was found in high frequency among our A48Id$^+$ hybridomas, and would simplify interpretation of data attempting to correlate V gene structure with Id expression.

Spleen cells were taken at age one month and cultured in vitro with either one of two polyclonal B cell mitogens, bacterial lipopolysaccharide (LPS), or Nocardia water-soluble mitogen (NWSM). After fusion, hybridomas were

selected for expression of the V_HX24 gene family by dot blotting of cytoplasmic lysates and hybridization with a germline gene probe. After cloning by limiting dilution, expression of V_HX24 was confirmed by detection of an RNA of appropriate size in Northern blots.

The monoclonal antibodies secreted by these hybridomas have been analyzed for antigen binding, idiotype expression and V_K gene usage. In addition, nucleotide sequences were determined to further explore the structure-function relationships of these monoclonal antibodies.

MATERIALS AND METHODS

Animals

BALB/c mice were obtained from The Jackson Laboratories (Bar Harbor, ME).

Myeloma proteins and monoclonal antibodies

The BALB/c myeloma proteins ABPC48, UPC10, XRPC24, XRPC44, MOPC173, MOPC460, McPC870, and TEPC601 are from the collection of Dr. M. Potter (National Institutes of Health, Bethesda, MD). BALB/c myeloma proteins J606 and J539 are from the collection of Dr. M. Cohn (The Salk Institute, La Jolla, CA).

PY102, PY206, XY102 (Moran et al., 1984) are BALB/c mAbs specific for various influenza virus hemagglutinins, (provided by Dr. J. Schulman, Mount Sinai School of Medicine, New York, NY). The AIDA series hybridomas were provided by Dr. P. Legrain (Institut Pasteur, Paris, France). All other monoclonal antibodies were produced in our laboratory.

Series 1, 2, 3, and AIDA mAbs were purified from ascites by chromatography with Sephacryl G-300 (Pharmacia) in 10 mM Tris-HCl pH 8, 0.9% NaCl, and 0.3% NaN_3. Series N, A, and 10 mAbs were purified from culture supernatants by affinity chromatography with a monoclonal rat anti-mouse kappa chain antibody (187.1, from Dr. M. Scharf, Albert Einstein College of Medicine, New York, NY) coupled to Sepharose 4B (Pharmacia).

Preparation of hybridomas

The preparation of hybridomas from series 1, 2, and 3 have been described in detail (Goldberg et al., 1984).

Hybridomas of series N, A, and 10 were prepared as follows: BALB/c mice were injected at birth with either 10 µg A48 or 10 ng IDA10. At age one month spleen cells were removed and cultured in vitro according to previously published techniques (Bona et al., 1978b) for two days with either 25 µg/ml lipopolysaccharide (LPS) from E. coli (Gibco) or 30 µg/ml Nocardia water soluble mitogen (NWSM) from N. opaca (gift of R. Barot, Université Paris Sud, Orsay, France). Hybridomas were prepared by polyethylene glycol fusion with Sp2/0 cells (Shulman et al., 1978) using standard techniques (see Hudson and Hay, 1980).

Hybridomas from A48 or IDA10-treated mice were screened for expression of the V_HX24 gene family by preparing cytoplasmic lysates according to White and

Bancroft (1982), blotting and hybridization were performed by the methods of Thomas (1980). Hybridomas from untreated mice were screened by the method of Manser and Gefter (1984). Positive hybrids were cloned by limiting dilution and rescreened. Positive clones were grown, RNA prepared and hybridization with V_HX24 DNA verified in high-stringency Northern blots (see below).

Monoclonal antibody isotypes were determined using tritiated mouse isotype-specific rabbit antisera prepared in our laboratory. Kappa-bearing monoclonal antibodies were purified as described above.

Anti-idiotypic reagents

The syngeneic anti-A48Id hybridomas IDA3, IDA10, IDA16, IDA17, and IDA23 (Legrain et al., 1981) were provided by Dr. P. Legrain. Monoclonal anti-A48Id antibodies were purified from culture supernatants by affinity chromatography with A48 coupled to Sepharose 4B. HyX24-14 (Pawlita et al., 1981) is an A/J monoclonal anti-idiotype antibody specific for idiotopes expressed on BALB/c galactan-binding myeloma proteins and monoclonal antibodies and was provided by Dr. M. Potter. The (BALB/c X A/J)F1 monoclonal anti-UPC10Id antibody 10-1 (Bona et al., 1985) was prepared in our laboratory. The syngeneic polyclonal anti-A48Id antiserum, and the A/He polyclonal anti-XRPC24Id

antiserum were prepared according to previously published methods (Bona et al., 1981).

Anti-V_K10 antibodies

Purified U10 light chains were prepared according to Jaton et al. (1979). Light chains were coupled to KLH with glutaraldehyde according to standard techniques (see Hudson and Hay, 1980). Rabbits were immunized as described in Bona et al. (1979a). Rabbit antibodies were affinity purified over a column of U10 coupled to Sepharose 4B, followed by removal of antibodies adhering to columns containing other V_K groups. Specificity of the antibodies was confirmed by RIA with a panel of antibodies with known V_K usage.

Antigens

The AZO antigen (AZO) is p-aminophenyl-ß-N-acetyl-D-glucosaminide linked through an azo functional group to chicken ovalbumin. This antigen and group A streptococcal carbohydrate (GAC) were provided by Dr. M. McCarty (The Rockefeller University, New York, NY). Chicken ovalbumin (OVA) is from Sigma. ß(1-6)-poly-D-galactose coupled to BSA (GAL), gum ghatti, and arabino-galactan were provided by Dr. M. Potter (National Institutes of Health, Bethesda, MD). Bacterial levan (BL) from A. levanicum (ATCC 1552) containing both ß(2-6) and ß(2-1)

fructose linkages was prepared according to Lieberman et al. (1976). A conjugate of inulin containing only ß(2-1) fructose linkages coupled to BSA (INU) was prepared according to Chien et al. (1979). Perennial rye grass (Lolium perenne) levan containing only ß(2-6) fructose linkages (RL, Moreno et al.,1976) was provided by Dr. C. Moreno (Hammersmith Hospital, London, England). Chicken egg-grown A/PR/8/34 (PR8) and A/Aichi/2/68 (R) (X31) influenza viruses were provided by Dr. J. Schulman. A/WSN/1/33 (WSN) influenza virus grown in Madin-Darby canine kidney (MDCK) cells was provided by Dr. P. Palese (Mount Sinai School of Medicine, New York, NY). These viruses were purified by sucrose density gradient ultracentrifugation.

Radioimmunoassays

Polyvinylchloride 96 well plates (Falcon) were coated with antigen or antibody diluted in PBS for 2 hr. at 37°C or overnight at 4°C. After washing with PBS plates were blocked with 1% BSA in PBS for 30 min. at RT. In subsequent incubations, for series 1, 2, 3, and AIDA mAbs carried out overnight at 4°C, monoclonal antibodies or antisera, either labelled or unlabelled, with or without inhibitors were diluted in 1% BSA in PBS. For RIAs with series N, A, and 10 mAbs, antibodies were diluted in 1% BSA in PBS with 0.05% Tween 20 (Sigma) in order to reduce

background binding. After extensive washing, plates were cut apart and individual wells were counted in a gamma radiation counter (LKB 1272 CliniGamma). See figure and table legends for details about particular experiments. Monoclonal antibodies were labelled with ^{125}I by the method of Greenwood et al. (1968). The specific activity of the labelled antibodies averaged 9 X 10^6 CPM/μg.

ELISA

Plates were coated (100 μl volume) 2 hr. at 37°C, washed with PBS, then incubated with alkaline phosphatase-labelled antibodies, with or without inhibitors, overnight at 4°C. After washing with PBS-0.05% Tween 20, para-nitrophenylphosphate substrate (Sigma) 0.4 mg/ml in diethanolamine buffer was added. Reaction proceeded for 30 min.- 1 hr. at 37°C and was then stopped by addition of 25 μl 3N NaOH. A_{405} was read on an ELISA Microreader (Dynatech Laboratories, Inc.). See figure and table legends for further details about particular experiments.

Antibodies were coupled with alkaline phosphatase with glutaraldehyde according to standard techniques (see Hudson and Hay, 1980).

RNA blotting

Hybridoma RNA was prepared by the guanidinium isothiocyanate procedure essentially as described by Chirgwin et

al. (1979). Total RNA (5 μg) was electrophoresed in a 1.2% agarose-formaldehyde gel and transferred to nitrocellulose and hybridized with ^{32}P (Amersham) nick-translated probes and washed as described in Maniatis et al (1982). For dot blots, three dilutions (5, 1.5, and 0.5 μg total) of hybridoma RNA were spotted on nitrocellulose using a 96-well dot blot manifold (Bethesda Research Laboratories) and hybridized and washed identically to Northern blots. Labelling of DNA probes with ^{32}P by nick translation was performed using a kit manufactured by Amersham. Probe concentrations used for hybridization were 1-2 X 10^6 CPM/ml. Specific activity of probes was 1-3 X 10^8 CPM/μg. The final wash step was 0.1X SSC, 0.1% SDS at 65-68°C for 15-20 min.

V gene probes

The 500 bp V_H441 and V_HX24 gene probes (designated VhGal 39.1 and VhGal 55.1, respectively, by Hartman and Rudikoff, 1984) were provided by Drs. A. Hartman and S. Rudikoff (National Institutes of Health, Bethesda, MD). The V_K gene family probes used (V_K1, 2, 4, 8, 9, 10, 11, 19, 21, 22, 24) have been described in detail elsewhere (Painter et al., 1988). Probes were prepared according to techniques in Maniatis et al. (1982).

Nucleotide sequencing

Two techniques were employed. For antibodies 10L16-5, 10L126-7 and 10N17-4 mRNA purified by chromatography with oligo-dT cellulose (Aviv and Leder, 1972) was annealed with immunoglobulin-specific primers (prepared by OCS Laboratories) labelled with ^{32}P-dATP and polynucleotide kinase (Bethesda Research Laboratories); cDNA synthesis with reverse transcriptase (Molecular Genetics Resources) and isolation of full-length cDNA was performed according to Shlomchik et al. (1986). Maxam and Gilbert (1977) sequencing of 10-100,000 CPM labelled cDNA and 1 μg salmon sperm carrier DNA was performed using a kit manufactured by Sigma. Modifications to the protocols supplied were: G reaction was incubated 1 min. at RT; G+A reaction was incubated 20 min. at 37°C; C and C+T reactions were incubated 3-4 min. at 15°C. An additional A>C reaction was performed: DNA in 100 μl 1.2 N NaOH/1 mM EDTA was heated at 90°C for 10 min, the reaction was neutralized by addition of 150 μl 1 N acetic acid and 5 μl yeast tRNA (1 mg/ml). Sequencing reactions were electrophoresed on 40 or 80 cm 6-8% polyacrylamide, 7M urea gels with or without buffer gradients.

For hybridomas 10L15-15, NL104-1, NL112-9 and NL126-7, cDNA was synthesized using a kit manufactured by Amersham. Tailing of cDNA 3′ ends with dCTP, annealing with dG-tailed pUC9 (Pharmacia) and transfection of JM101 bacteria were

according to Sikder et al. (1985). Screening and selection of transformants were according to techniques in Maniatis at al. (1982). Plasmid preparation was by the method of M. R. Green (personal communication). Alkaline denaturation of plasmids for sequencing was according to Chen and Seeburg, (1985).

Dideoxy sequencing (Sanger et al., 1977) of double-stranded plasmid DNA with modified T7 polymerase (Sequenase^{TM}) was performed using a kit manufactured by US Biochemical Corp. Electrophoresis of sequencing reactions was as described above.

Searches of nucleotide sequence databases and sequence comparisons and analyses were assisted by computer software described by Devereux et al. (1984).

RESULTS

Analysis of A48Id$^+$ monoclonal antibodies

The immunochemical analysis of A48Id$^+$ monoclonal antibodies carried out by Goldberg et al. (1983) using polyclonal anti-A48Id antibodies, was extended by analysis with several monoclonal Ab_2s[11]. Table II lists the names and origins of the hybridomas prepared by Goldberg et al. (1983). We also included in our immunochemical analysis four hybridomas (of the AIDA series) prepared by Legrain and colleagues from mice immunized with monoclonal anti-A48Id antibody-KLH conjugates. These monoclonal anti-Ids (IDA series, Legrain et al., 1982) are described in the Introduction above. The AIDA series mAbs are described in Legrain and Buttin (1985), and Legrain et al. (1985).

Antigen binding of A48Id$^+$ monoclonal antibodies

These mAbs were initially tested for binding to three fructosans, inulin [INU, ß(2-1) linkages], rye grass levan

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[11]The results presented in this section have been published in Victor-Kobrin et al. (1985).

Table II. Designations and Origins of Series 1, 2, 3, and AIDA Monoclonal Antibodies

Designation	Isotype	Treatment of BALB/c donor mice
1-5-1	IgM[a]	10 μg A48 at birth, challenge at 1 month with 20 μg BL
2-1-3	IgG3	10 ng polyclonal anti-A48Id antibodies at birth, challenge at 1 month with 20 μg BL
2-1-10	"	
2-8-2	IgM	
2-9-17	"	
2-11-1	"	
2-11-3	"	
2-12-10	"	
2-12-19	"	
2-28-9	IgG3	
3-9-9	IgG1	Immunization with polyclonal anti-A48Id/KLH followed 1 month later by challenge with 20 μg BL
3-14-9	IgM	
3-27-6	IgG1	
3-76-4	IgM	
3-76-42	"	
3-101-14	IgG1	
AIDA10/16[b]	IgG1	Immunization with IDA10-KLH
AIDA10/21	"	
AIDA23/2	IgG1	Immunization with IDA23-KLH
AIDA23/3	"	

[a]All antibodies use kappa light chains.
[b]AIDA series antibodies were prepared by Legrain and colleagues (Legrain and Buttin, 1985; Legrain et al., 1985).

[RL, ß(2-6)] and bacterial levan [BL, ß(2-1) and ß(2-6), Table III]. As previously observed, A48 and U10 bind RL and BL but not INU, demonstrating their specificity for ß(2-6) linked fructosan. J606, an INU-binding myeloma protein, reacted with both INU and BL as do many other INU-binding myeloma proteins[12].

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[12]For a discussion of this see section entitled "Levan and the ABPC48 idiotype system" in the Introduction.

The mAb 1-5-1 binds to BL. Of the 9 mAbs of series 2, all bound to BL, some displaying cross-reactivity with either RL or INU, or both, indicating a combining site structure somewhat different from that of A48 and U10. Of the 10 mAbs from series 3 and AIDA, only five reacted with

Table III. Binding of A48Id$^+$ MAbs to Polyfructose Antigens

MAb	Antigens RL	BL	INU
ABPC48	5,659±275	12,819±945	-[a]
UPC10	1,353±95	19,233±723	-
J606	-	4,265±833	5,875±419
MOPC173	-	-	-
MOPC460	-	-	-
1-5-1	819±272	12,576±1,867	-
2-1-3	4,013±328	12,570±903	8,374±1,024
2-1-10	802±222	8,503±1,516	-
2-8-2	1,057±161	16,232±1,418	-
2-9-17	-	13,434±2,915	2,228±645
2-11-1	1,592±206	13,286±805	-
2-11-3	-	12,354±697	-
2-12-10	-	11,230±1,650	-
2-12-19	2,528±408	15,028±1,095	1,453±527
2-28-9	666±294	5,232±1,421	-
3-9-9	-	-	-
3-14-9	5,623±947	1,413±263	3,583±733
3-27-6	1,693±293	-	-
3-76-4	550±69	-	-
3-76-42	1,544±352	-	1,171±271
AIDA10/16	-	2,888±291	-
AIDA10/21	-	-	-
AIDA23/2	-	-	-
AIDA23/3	-	-	851±180

Plates were coated with 50 μl of RL (20 μg/ml), BL (20 μg/ml) or INU (3 μg/ml), then incubated with 50 μl of mAbs at 10 μg/ml. Binding of mAbs was detected with 50,000 CPM in 50 μl of ^{125}I-labelled monoclonal rat anti-mouse kappa chain antibody. Data are mean±SEM of triplicate determinations after subtraction of background binding of labelled antibody to BSA (214 CPM).
[a]A dash indicates <500 CPM.

one or more fructosan antigens. The binding to BL of the three mAbs reacting with all three fructosans (2-1-3, 2-12-19, and 3-14-9) could be inhibited by BL, but only very weakly or not at all by RL or INU (not shown). This suggests that these antibodies are specific for a determinant of BL and cross-react weakly with RL and INU.

Since it had been demonstrated that both levan and galactan-binding myeloma protein V_H regions were encoded by genes of the same (V_HX24) family (Auffray et al., 1981, Hartman and Rudikoff, 1984), mAbs which did not bind to levan antigens were tested for binding to galactan antigens (Table IV). Three antigens were used: a ß(1-6)-D-galactan-BSA conjugate (GAL), and two naturally occurring galactans, gum ghatti (GG), and arabinogalactan (ARA). The mAb AIDA23/2 bound very well to all three galactan-containing antigens. MOPC173 also bound galactan weakly.

Table IV. Binding to Galactans of Monoclonal Antibodies not Reacting with Fructosans

	Antigens		
Mab	ARA	GAL	GG
ABPC48	299±199	357±105	191±7
XRPC24	872±13	5,272±161	3,537±51
MOPC173	694±61	981±242	919±268
3-9-9	273±45	183±21	189±15
3-101-14	249±42	301±63	215±30
AIDA10/21	730±352	575±64	465±107
AIDA23/2	1,709±324	1,832±300	1,376±54
AIDA23/3	296±112	709±110	483±96

Plates were coated with 50 µl of ARA (20 µg/ml), or GAL (5 µg/ml, or GG (20 µg/ml), remainder as in Table III legend. Data are mean±SEM of triplicate determinations.

Expression of A48-U10 idiotopes by the monoclonal antibodies

Immunochemical analysis of the mAbs selected for binding to a polyclonal anti-A48Id reagent continued with a study of their binding to several monoclonal anti-Ids (Table V). Included in these studies were four Ab_2s of the

Table V. Binding of $A48Id^+$ MAbs to Monoclonal Ab_2s IDA10 and 10-1

	Anti-Ids	
Mab	IDA10	10-1
ABPC48	5,183±289	456±20
UPC10	1,492±141	2,213±278
MOPC173	519±233	681±212
MOPC460	184±17	51±13
1-5-1	991±301	215±184
2-1-3	679±228	319±128
2-8-2	804±276	404±15
2-9-17	356±257	246±43
2-11-1	728±313	454±93
2-12-10	1,140±136	532±288
2-28-9	795±181	932±222
3-9-9	3,078±111	349±144
3-14-9	3,745±152	554±175
3-27-6	6,030±369	640±186
3-76-4	1,548±128	217±86
3-101-14	2,093±41	204±92
AIDA10/16	1,616±457	531±216
AIDA10/21	3,197±674	667±148
AIDA23/2	9,619±107	747±258
AIDA23/3	9,578±553	678±185

For IDA10 binding plates were coated with 50 μl mAbs at 5 μg/ml, plates were incubated with 50 μl IDA10 or 10-1 at 20 μg/ml, then 50,000 CPM in 50 μl of ^{125}I-labelled A48 or U10. Data are mean±SEM CPM of triplicate determinations after subtraction of background binding to BSA (432 and 106 CPM for IDA10 and 10-1, respectively). MOPC460 is an IgA-kappa BALB/c myeloma protein with specificity for dinitrophenyl.

IDA series (IDA3, 10, 17, and 23), and a semi-syngeneic [(BALB/c X A/J)F_1] monoclonal anti-U10Id antibody, 10-1.

The majority of the antibodies tested bound, to some degree, to IDA10. In general, the binding to IDA10 of series 1 and 2 mAbs was much lower than the binding of series 3 and AIDA mAbs. Many antibodies also bound to the anti-U10Id, 10-1. However, binding to 10-1 did not appear to correlate with the method of generating the hybridomas. Not surprisingly, the mAbs AIDA23/2 and AIDA23/3 bound very strongly to IDA23 in addition to IDA10 (not shown). None of the other mAbs reacted with IDA23, and no mAbs (other than A48) reacted with IDA3, or IDA17 (not shown).

The binding of IDA10 to A48 can be inhibited by BL (Legrain et al., 1982). In Figure 1 we see that binding of IDA10 to U10 is also inhibitable by BL. The anti-Id 10-1 interacts differently with A48 and U10. Binding to U10 is inhibited well by BL, while binding to A48 is not.

Since it was demonstrated that at least one A48Id^+ mAb had anti-galactan activity, several galactan-binding myeloma proteins (GALBMP) were assayed for expression of A48-U10 idiotopes. Table VI shows the results of an ELISA inhibition experiment where GALBMP were used to inhibit the binding of alkaline phosphatase-labelled anti-Id reagents to A48 and U10. The GALBMP XRPC44 at 1 μg/ml was able to

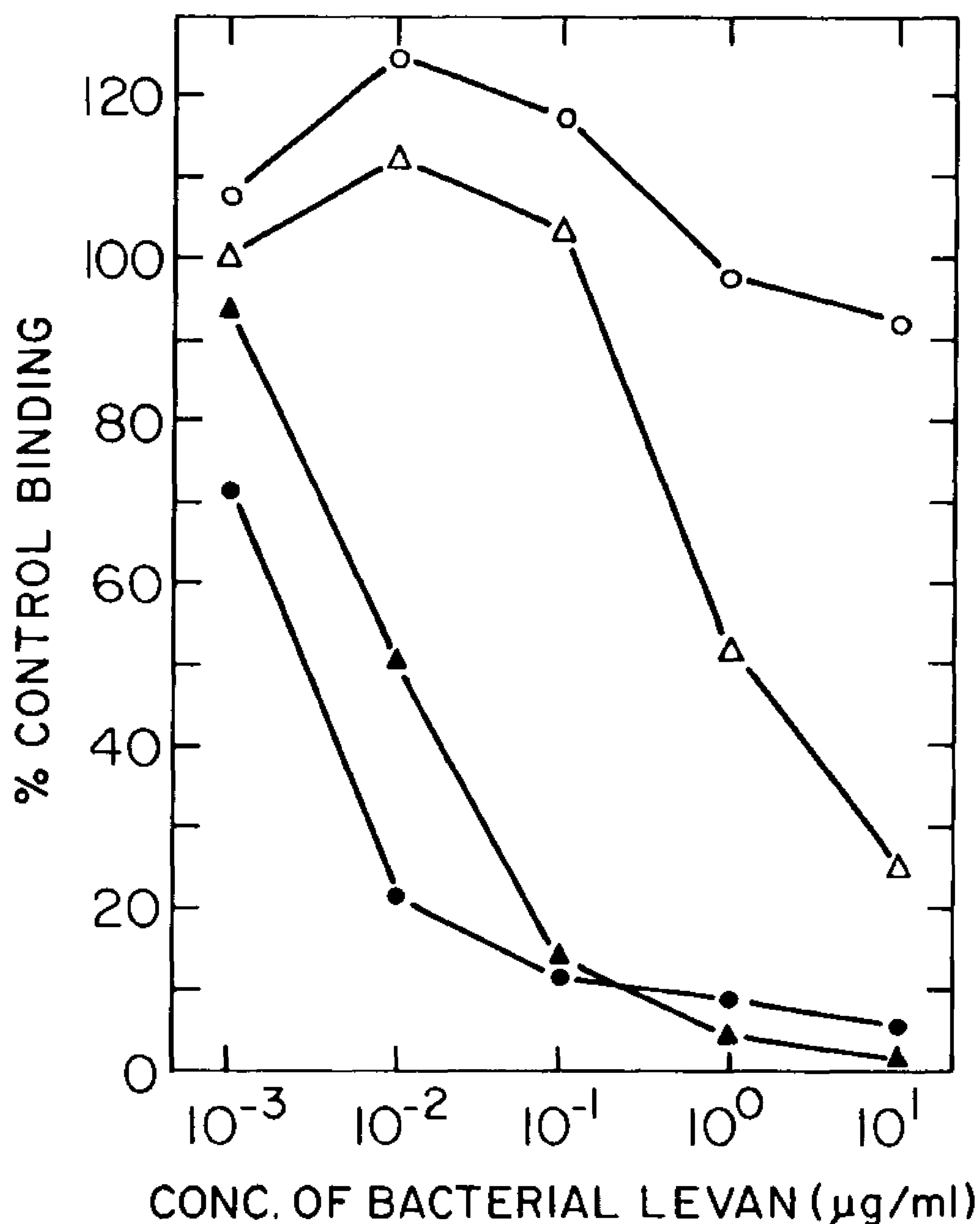

Figure 1. Inhibition by BL of the binding of IDA10 and 10-1 to A48 and U10. Plates were coated with 50 µl of A48 or U10 at 10 µg/ml. 50,000 CPM of ^{125}I-labelled IDA10 or 10-1 was mixed with varying concentrations of BL in 50 µl. Data points are means of triplicate determinations. Values for 100% CPM are: 10-1 with A48 (○), 789; 10-1 with U10 (△), 8,522; IDA10 with A48 (●), 8,797; and IDA10 with U10 (▲), 17,711.

Table VI. Inhibition by GALBMP of Binding of Alkaline-Phosphatase-labelled Anti-A48Id and Anti-U10Id Antibodies to

A48 and U10

Inhibitor		Anti-Id/Id	
(1 μg/ml)	Anti-A48/A48	Anti-U10/U10	IDA10/A48
None	0.84±0.03	1.26±0.02	1.38±0.03
ABPC48	0.10±0.01	1.14±0.04	0.03±0.01
UPC10	0.80±0.02	0.10±0.01	0.91±0.04
XRPC24	1.08±0.01	1.28±0.03	1.38±0.03
XRPC44	0.96±0.02	0.63±0.03	1.31±0.03
TEPC601	1.01±0.03	1.23±0.03	1.29±0.03
J539	1.02±0.06	1.02±0.01	1.32±0.02

Plates were coated with 100 μl Id at 10 μg/ml, then incubated with 100 μl alkaline phosphatase labelled anti-Id with or without 1 μg/ml inhibitor, followed by substrate. Data are mean±SEM of triplicate determinations.

inhibit 50% of the binding of syngeneic polyclonal anti-U10Id antibodies to U10.

V gene usage of A48Id$^+$ monoclonal antibodies

V_H gene usage by these hybridomas was determined first by dot-blotting of RNA and hybridization with a V_H441 germline gene DNA probe. Subsequently, hybridization to RNA of an appropriate size was demonstrated in Northern blots. The mAb 1-5-1, and five of the nine series 2 hybridomas contained a hybridizing message. In addition, four of six series 3 mRNAs hybridized with the V_H441 gene probe (these data not shown). The two series 3 mRNAs not hybridizing with V_H441 also did not hybridize with a C_μ probe indicating loss of antibody secretory activity during passage in culture. The four AIDA series hybridomas

express a V_HX24 family gene as also reported by Legrain et al. (1985) who confirmed this by nucleotide sequence analysis.

Selected V_H441^+ hybridoma DNAs were also analyzed by Southern blotting. It was demonstrated that in each case rearrangement of the germline configuration of a gene of the V_HX24 gene family had occurred (also not shown).

Since both V_H and V_L may influence antibody specificity and idiotypy, it was important to investigate V_K gene usage by this group of mAbs. The first approach undertaken was use of a polyclonal rabbit antiserum obtained by immunization with U10 light chains, and rendered specific for the V_K10 family by adsorption on appropriate affinity columns.

Table VII shows the binding of alkaline phosphatase-labelled A48Id$^+$ mAbs to plates coated with anti-kappa or anti-V_K10 antibodies. The rabbit anti-V_K10 antibodies bind well to U10 and MOPC173, and somewhat less to A48. They do not bind to the mAb XY101, specific for influenza virus hemagglutinin, which uses a V_K21 light chain. The mAbs 1-5-1, 3-27-6, and AIDAs 10/16, 10/21, and 10/22 had binding to anti-V_K10 at least 2X over binding to BSA.

Table VII. Binding of Alkaline Phosphatase-labelled A48Id^{+} Monoclonal Antibodies to Rabbit Anti-V_K10 Antibodies

Labelled mAbs	Plates coated with: BSA	Anti-kappa	Anti-V_K10
ABPC48	-	0.84±0.02	0.29±0.01
UPC10	0.18±0.02	1.34±0.03	0.91±0.05
MOPC173	-	1.44±0.01	0.96±0.04
XY102	-	1.06±0.03	-
1-5-1	0.19	0.62±0.03	1.47±0.06
2-11-1	0.17±0.02	1.47±0.01	0.20±0.02
3-9-9	0.19±0.05	1.59±0.01	0.25±0.03
3-27-6	0.13±0.01	0.94±0.16	0.42±0.07
3-101-14	0.22±0.06	1.58±0.01	0.34±0.05
AIDA10/16	-	0.49±0.01	0.20±0.01
AIDA10/21	-	0.83±0.02	0.28±0.01
AIDA23/2	0.17±0.03	1.57±0.01	0.34±0.05
AIDA23/3	-	0.96±0.01	-

Plates were coated with 100 µl the indicated antibodies at 10 µg/ml, then incubated with the indicated labelled mAbs followed by substrate. Data are mean±SEM A_{405} values for triplicate determinations. A dash indicates mean $A_{405}<0.10$.

In subsequent work by Barak (1988), Northern blotting analysis of series 1, 2, and 3 hybridomas using a V_K10 specific DNA probe showed that all of the mAbs in Table VII do, in fact, use V_K10. Legrain and Buttin (1985) sequenced the V_K genes expressed by the AIDA series mAbs and determined that they also use V_K10.

Summary of the characteristics of A48Id^{+} monoclonal antibodies

Table VIII summarizes the analysis of the series 1, 2, 3, and AIDA mAbs described above. Antibodies derived from

Table VIII. Characteristics of A48Id^{+} Monoclonal Antibodies

Treatment	Name	Binding to: BL	IDA10	V_HX24	V_K10
	A48	++	++	+	+
10 μg A48 @ birth, BL 1 mo.	1-5-1	++	+	+	+
10 ng pc anti-A48Id @ birth, BL 1 mo.	2-1-3	++	+/-	+	+
	2-1-10	++		+	+
	2-8-2	++	+/-	-	
	2-11-1	++	+/-	+	+
	2-11-3	++		-	-
	2-12-10	++	+	+	+
	2-12-19	++		-	-
	2-9-17	++	-	-	
	2-28-9	++	+/-	+	+
Adult, pc anti-A48Id-KLH, BL 1 mo. later	3-76-4	-	+		
	3-76-42	-[a]			
	3-14-9	+[a]	++	+	+
	3-27-6	-[a]	++	+	+
	3-9-9	-	++	+	+
	3-101-14	-[a]	++	+	+
Adult, IDA10-KLH	AIDA10/16	+	++	+	+
	AIDA10/21	-	++	+	+
Adult, IDA23-KLH	AIDA23/2	-[b]	++	+	+
	AIDA23/3	-	++	+	+

Expression of V_K10 was determined by Barak (1988).
[a]These antibodies bind to RL or INU.
[b]This antibody binds galactan.

neonates are seen to be predominantly levan-binding, and the majority interact weakly with IDA10. MAbs derived from adult mice have generally weaker antigen binding with different specificity from those derived from neonates, and bind strongly to IDA10. Six of ten hybridomas from mice treated as neonates express V_HX24 and V_K10, as do eight of eight (for which Ig mRNA production was demonstrated) of the hybridomas derived from adults.

Thus, the characteristics of the mAbs derived from neonatally treated mice suggested that antigen played more of a role in expansion of these clones, since levan-binding was much more prevalent than in mAbs from adult mice. Interestingly, even though all mAbs were selected for A48Id expression, series 1 and 2 mAbs bound IDA10 weakly, while series 3 mAbs bound very well. In addition, a V_HX24-V_K10 association was quite strong (80-100%) in series 3 and AIDA, and accounted for 60% of series 1 and 2.

Rationale of subsequent experiments

These observations led us to question a) if the effects of neonatal Id or anti-Id and antigen on clonal expansion could be dissociated; b) the association of V_HX24 with various V_K genes in the absence of idiotype or antigen stimulation; and c) the structural determinants of A48Id expression.

Toward these ends, hybridomas were prepared from BALB/c mice which were untreated, or injected at birth with 10 μg A48 or 10 ng IDA10. At age one month, spleen cells were stimulated in vitro with polyclonal B cell mitogens (LPS and NWSM) prior to fusion. Hybridomas were selected for expression of genes of the V_HX24 family by hybridization of dot-blotted cytoplasmic lysates with specific radiolabelled DNA probes. After cloning, the mAbs secreted by these hybridomas were analyzed for antigen binding and

idiotype expression. Furthermore, nucleotide sequence analysis was undertaken in order to correlate specific V region structures with antigen or idiotypic specificities.

Frequency of clones expressing V_HX24

Table IX summarizes the frequency of clones expressing genes of the V_HX24 gene family in the different groups of hybridomas. A statistical analysis (Chi-square test) gave $p>0.25$ for all comparisons between any two treatment groups (disregarding different mitogens), between mitogens in one

Table IX. Frequency of Expression of the V_HX24 Gene Family Among Hybridomas Derived from LPS- or NWSM-stimulated Spleen Cells.

Treatment	Mitogen	Name	#Tested	#VHX24+	Frequency
None	LPS	NL	912	23	2.5%
	NWSM	NN	36	1	2.8%
A48, 10 µg	LPS	AL	99	1	1.0%
at birth	NWSM	AN	95	1	1.1%
IDA10,	LPS	10L	247	4	1.6%
10 ng	NWSM	10N	75	2	2.7%
at birth					
Totals			1464	32	2.2%

group, or between mitogens regardless of group. Thus, we calculate an overall frequency of expression of the V_HX24 gene family to be 32 in 1464, or 2.2% of LPS- and NWSM-responsive B cells in the BALB/c mouse.

Table X shows the isotypes and numerical designations of the antibodies in each of the groups introduced in Table IX. The two antibodies having lambda light chains (NL102-10 and NL114-1) were not analyzed further.

Figure 2 shows the results of high stringency Northern blotting of hybridoma total cellular RNA with a V_H441 germline gene probe. In each case we detected a hybridizing message of a size appropriate for the isotype.

Table X. Isotypes and Designations of VHX24+ Monoclonal Antibodies.

Isotype	Number	Antibodies
IgM, kappa	27	NL(101-10, 103-3, 104-1, 105-10, 106-2, 108-8, 109-2, 110-6, 112-9, 113-6, 115-1, 117-3, 119-3, 121-6, 122-5, 123-8, 124-1, 125-8, 126-4, 127-2, 128-1) NN101-8 AL42-7 AN18-5 10L(15-15, 107-1, 126-7)
IgG1, kappa	2	10L16-5 10N17-4
IgG3, kappa	1	10N109-1
IgM, lambda	2	NL(102-10, 114-1)

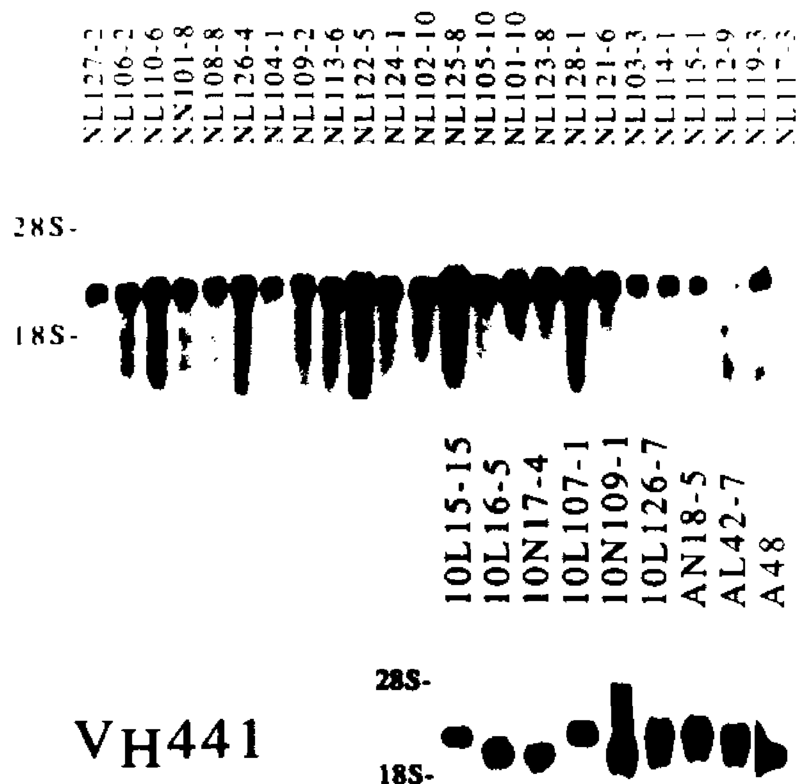

Figure 2. Northern blotting of hybridoma total cellular RNA with a V_H441 germline gene DNA probe. The positions of the 28S and 18S ribosomal RNA bands are indicated. Different sizes of hybridizing RNAs correlate with isotype (see Table X). Exposures range from 4 to 24 hr.

Antigen binding characteristics of the monoclonal antibodies.

We began our immunochemical analysis of these monoclonal antibodies (mAbs) by studying their binding in a solid-phase RIA to a panel of antigens previously shown to be recognized by myeloma proteins or mAbs deriving their V_H from the V_HX24 gene family. We tested binding to two antigens containing ß-N-acetyl-D-glucosamine, a hapten-carrier (chicken ovalbumin) conjugate (AZO), and group A streptococcal carbohydrate (GAC). Chicken ovalbumin (OVA) was included as a negative control for AZO. Also studied were the three polyfructosans described above, inulin (INU), rye levan (RL), and bacterial levan (BL). A ß(1-6)-poly-Dgalactose-BSA conjugate (GAL) and PR8 influenza virus (PR8) complete the list of antigens (see Materials and Methods for a more detailed description of these antigens).

All mAbs were tested at three dilutions for binding to antigen-coated plates. Figure 3 shows the increased binding with increasing amounts of antibody for four representative mAbs. Data for all mAbs at the highest concentration (10 µg/ml) are summarized in Table XI. A48 binds to RL and BL, but not INU, demonstrating its specificity for ß(2-6) linked fructosans (data for latter two antigens not shown). The mAb PY102 specific for PR8 influenza virus hemagglutinin binds well to PR8, but not

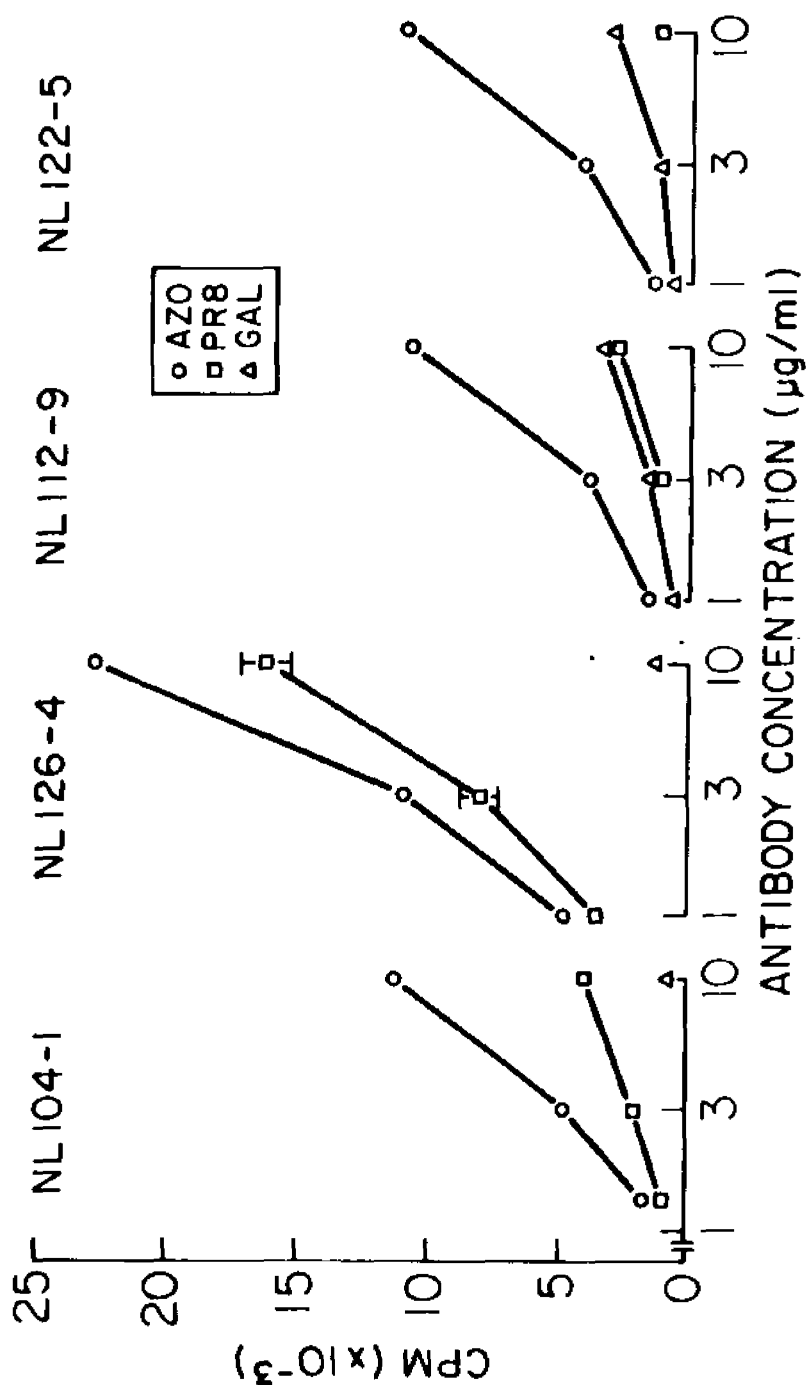

Figure 3. Increased binding to antigen with increasing concentrations of antibody. For experimental details see Table V legend. Data points represent mean±SEM of triplicate determinations.

Table XI. Antigen Binding Activities of V_HX24^+ Monoclonal Antibodies.

Antibody	Antigens AZO	PR8	GAL	RL
ABPC48	-[a]	-	-	11,014±567[b]
XRPC44	-	-	14,805±219	8,257±312
PY102	-	24,646±653	-	-
NL103-3	3,072±119	-	-	-
NL109-2	9,206±111	-	-	-
NL115-1	11,827±154	-	-	-
NL119-3	12,276±111	-	-	-
NL121-6	3,126±58	-	-	-
NL123-8	7,803±133	-	-	-
NL127-2	3,345±35	-	-	-
NL104-1	11,208±52	4,127±120	-	-
NL105-10	12,296±395	3,591±273	-	-
NL124-1	3,056±131	3,963±91	-	-
NL125-8	9,977±151	3,371±95	-	-
NL126-4	22,682±184	16,271±521	-	-
NL112-9	10,809±180	-	3,331±83	-
NL113-6	14,425±132	-	4,251±104	-
NL122-5	11,330±46	-	3,057±114	-
NL110-6	12,503±54	8,545±99	5,126±86	-
NL128-1	13,185±128	5,644±59	4,212±107	-
NL101-10	-	-	-	-
NL106-2	-	-	-	-
NL108-8	-	-	-	-
NL117-3	-	-	-	-
NN101-8	-	-	-	-
AL42-7	-	8,428±37	-	-
AN18-5	11,182±172	-	12,806±239	-
10L126-7	4,774±256	5,042±483	-	-
10N109-1	-	-	11,537±107	5,300±293
10L15-15	-	-	-	-
10L16-5	-	-	-	-
10L107-1	-	-	-	-
10N17-4	-	-	-	-

Plates were coated with antigen (50 μl/well) at 10 μg/ml, mAbs were added at 10 μg/ml, binding was detected with ^{125}I-labelled monoclonal rat anti-mouse kappa chain antibody at 50,000 CPM/50 μl. Binding to BL, INU, GAC, and OVA was also tested in this experiment. Binding to BSA was mean±SD of 269±190, n=30. The criteria for inclusion in the table were CPM > 2700 (10X above the mean background binding to BSA) or 5X background for a given mAb, whichever is higher.

[a]A dash indicates the CPM were below the criteria for inclusion in the table.

[b]Results are expressed as mean±SEM for triplicate determinations.

to any other antigen tested. The galactan-binding myeloma protein X44 displayed a surprising cross-reactivity, binding both to GAL and RL, but not to other antigens. This pattern was also displayed by the mAb 10N109-1, and by X24 (latter not shown).

Several patterns of antigen binding may be distinguished among the V_HX24^+ mAbs, binding to AZO alone, PR8 alone, or binding to two or more antigens. The cross-reactivities observed are between AZO and PR8, AZO and GAL, between all three, and between GAL and RL, as with X44. None of the mAbs bound to GAC or OVA. However, these AZO-binding mAbs may be specific for N-acetylglucosamine. The structure of this hapten has been shown to differ in AZO and GAC (McCarty, 1958). Five of 22 (23%) of the kappa-bearing mAbs from untreated mice failed to bind to any of the antigens tested. Among mAbs from IDA10-treated mice this number is four of six (67%). The antigen crossreactivities observed in the direct binding assay with some of these mAbs were further examined in inhibition experiments. Table XII summarizes the resultsof the first set of experiments where soluble antigen inhibited the binding to antigen-coated plates; mAb-binding was detected with radiolabelled anti-mouse kappa antibody. The binding of NL115-1 to AZO is inhibited by AZO, but not by GAC or OVA. The reaction of AL42-7 with PR8 is inhibited by PR8. Of

Table XII. Inhibition of the binding of V_HX24^+ monoclonal antibodies to antigen-coated plates by soluble antigen.

Antibody/ antigen	Inhibitors AZO	PR8	GAL	RL	GAC	OVA	BL
NL115-1/AZO	3.5[a]		>10		>10	>10	
NL126-4/AZO	1.0	>10			>10	>10	
/PR8	>10	2.2			>10	>10	
NL122-5/AZO	>10		>10	>10	>10	>10	
/GAL	2.4	>10(31)[b]					>10
NL128-1/AZO	2.5	>10	>10	>10		>10	
/PR8	>10(41)	0.6	>10	>10			>10
/GAL	0.2	>10(38)	>10(45)	>10(37)			>10(33)
AL42-7/PR8		<0.1				DEX[c]	
10N109-1/GAL			<0.3	7.5		>10	
/RL			<0.1	<0.1		>10	>10

Plates were coated with 100 µl antigen at 10 µg/ml, five dilutions of antigen from 0.1 to 10 µg/ml were mixed with antibody at 10 µg/ml. Antibody binding was detected with ^{125}I-labelled rat anti-mouse kappa antibody at 100,000 CPM in 100 µl. The mean±SEM of 100% binding for each antibody/antigen combination is: NL115-1/AZO, 19,375±397; NL126-4/AZO, 27,309±932; NL126-4/PR8, 17,493±151; NL122-5/AZO, 21,068±1,056; NL122-5/GAL, 13,971±440; NL128-1/AZO, 21,846±794; NL128-1/PR8, 12,713±227; NL128-1/-GAL, 12,168±581; AL42-7/PR8, 13,911±186; 10N109-1/GAL, 21,002±267; 10N109-1/RL, 8,213±223. A space in the table indicates inhibition not tested in that combination.
[a]Data are concentration (µg/ml) giving 50% inhibition.
[b]In parentheses is shown the percent inhibition at 10 µg/ml soluble antigen if it is >30%.
[c]DEX is dextran B1355S used as a negative control where indicated.

the mAbs binding more than one antigen, 10N109-1 binding to GAL- or RL-coated plates is inhibited by both GALand RL, but not by a different polysaccharide, dextran B1355S.

The binding of NL126-4 to AZO is inhibited by AZO, but not by PR8. Conversely, binding to PR8 can be inhibited by PR8 but not by AZO. A possible explanation is that NL126-4 actually has higher affinity for the epitope

contained in PR8, but since PR8 is a complex mixture of antigens, the epitope density per unit mass is lower than in AZO. Thus, the low epitope density PR8 cannot inhibit binding to AZO-coated plates, while the lower affinity interaction with AZO cannot inhibit binding to PR8-coated plates. Avidity effects of binding of IgM antibody to antigen immobilized on a surface may also contribute to the observed inhibition pattern. As with NL115-1, the binding of NL126-4 to AZO is not inhibited by GAC or OVA.

The binding of NL122-5 to AZO was not inhibited by AZO, GAL, GAC or OVA, while binding to GAL was inhibited by AZO, also weakly (31% at 10 μg/ml) by GAL, and not by BL. These results may be explained by either a higher affinity or epitope density for the AZO antigen versus GAL, coupled with avidity effects enhancing the binding to the antigen coated on the plate. In a more sensitive experiment, both antigens inhibited binding to either (see below).

The mAb NL128-1 bound to three antigens, AZO, PR8, and GAL in the direct binding assay. Binding to AZO was inhibited by AZO, but not PR8, GAL, or OVA; binding to PR8 was inhibited weakly by AZO, strongly by PR8, and not by GAL, RL or BL. Binding to GAL was inhibited by AZO, and weakly by PR8 and GAL, but it was also weakly inhibited by RL and BL to which NL128-1 bound poorly in the experiment of Table XI. Apparently the interaction of this mAb with GAL is extremely weak compared to that with AZO and PR8.

In order to further establish the cross-reactivity among these antigens we studied the inhibition of binding to antigen-coated plates of the mAbs themselves radio-labelled. Figure 4a-g shows the results of these experiments. In Figure 4a, the binding of AL42-7 to a PR8-coated plate is inhibited strongly by PR8 in solution.

Figure 4b and 4c show inhibition of binding of NL126-4 to AZO and PR8, respectively. The results parallel those in Table VI. Binding to AZO is inhibited well by AZO, only partially by PR8, and not at all by GAC. Binding to PR8 is inhibited well by PR8, and not by AZO or GAC. To reiterate, we suggest that this pattern of antigen reactivity reflects higher apparent affinity of NL126-4 for PR8 which has a lower epitope density than AZO, coupled with enhanced binding to the antigen coated on the plate due to an avidity effect. In Figure 4d and 4e we see the inhibition of binding of NL122-5 to plates coated with AZO and GAL, respectively. In both cases binding is inhibited well by AZO, somewhat less by GAL, and not by GAC. We attempted to approach the issue of avidity by studying the competition between labelled and unlabelled antigens for binding to mAb-coated plates. However, iodination of AZO, GAL, and PR8 almost completely abolished mAb interaction with these antigens (not shown).

Finally, in Figure 4f and 4g is shown inhibition of the binding of X44 and 10N109-1 to GAL and RL. This

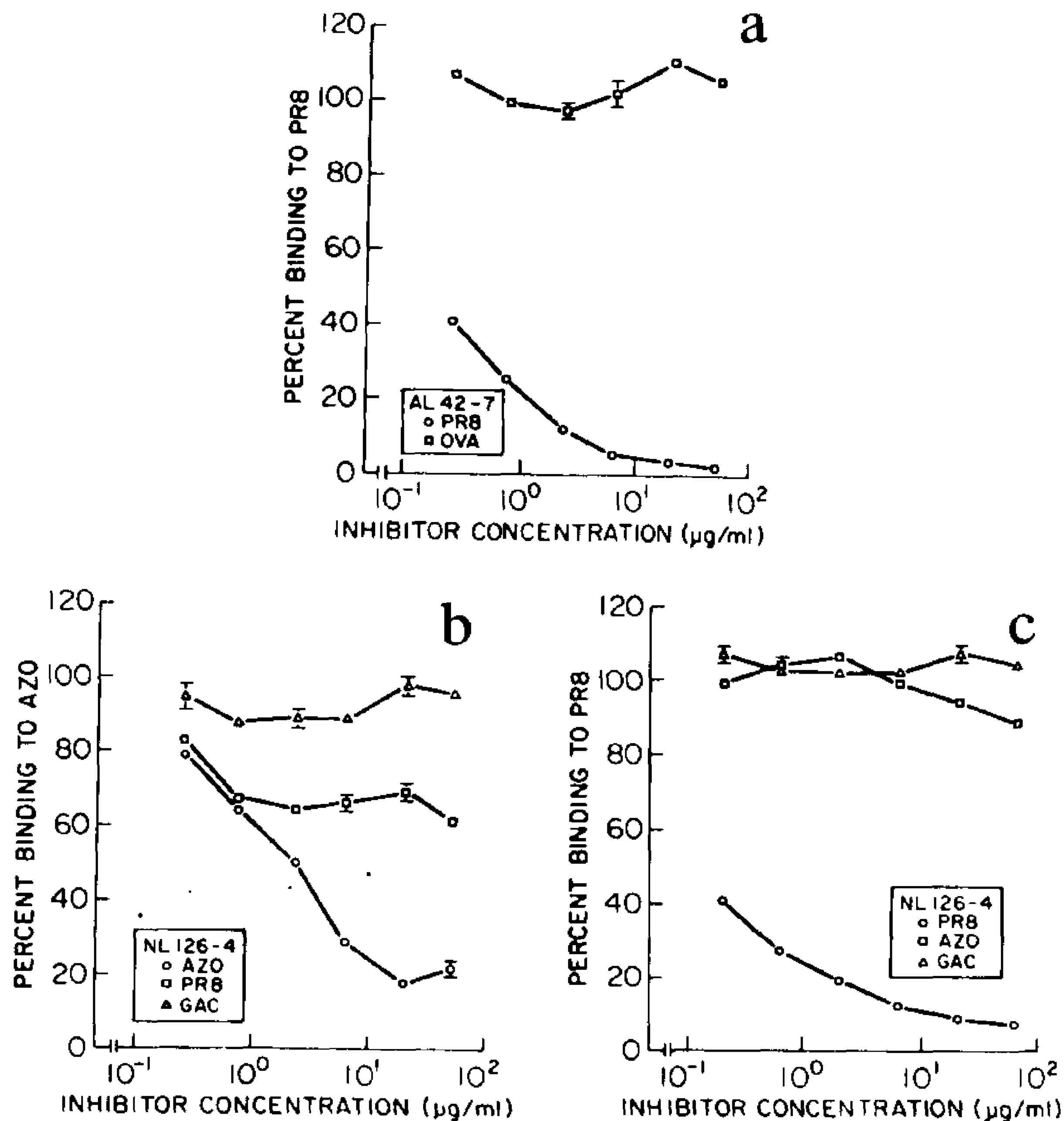

Figure 4. Inhibition of binding of radiolabelled mAbs to antigen-coated plates. Plates were coated with 100 μl of the indicated antigen at 5 μg/ml. Inhibitors were mixed with ^{125}I-labelled mAbs at 500,000 CPM in 100 μl. (For X44 inhibition only: GAL and RL were coated at 2 μg/ml and 20/μg/ml, respectively; labelled X44 was at 10,000 CPM in 100 μl.) Data points represent mean±SEM of triplicate determinations. The CPM representing 100% binding for each

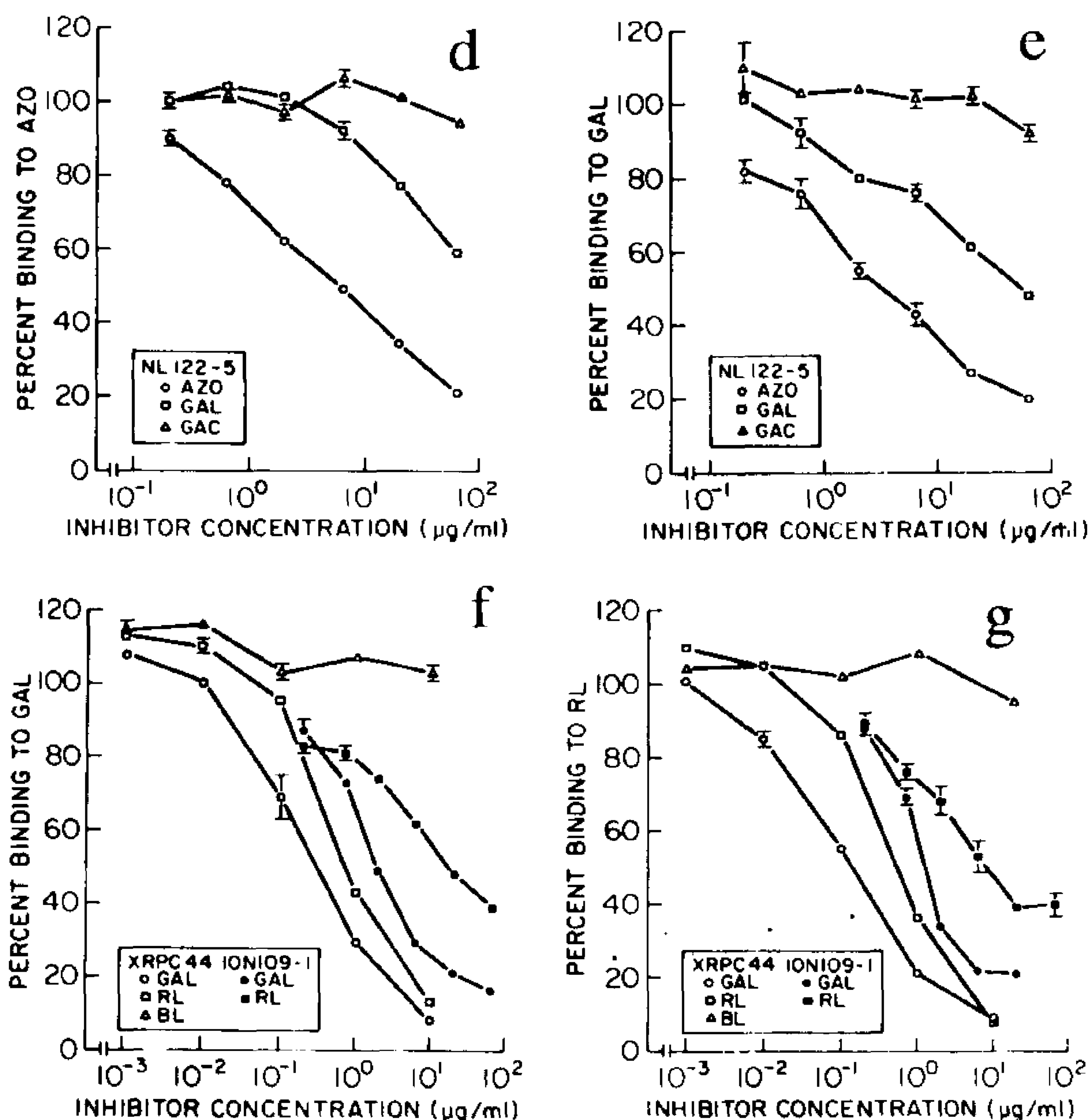

Figure 4, continued.
antibody/antigen combination are: a, AL42-7/PR8, 34,795±175; b, NL126-4/AZO, 7,946±166; c, NL126-4/PR8, 12,760±97; d, NL122-5/AZO, 3,535±27; e, NL122-5/GAL, 1,809±24; f, X44/GAL and 10N109-1/GAL, 3,156±30 and 6,923±-69, respectively; g, X44/RL and 10N109-1/RL, 1,670±13 and 2,483±20, respectively.

cross-reactivity was the most surprising since the antigen binding properties of X44 have been extensively studied (Jolley et al., 1973; Manjula and Glaudemans, 1976). Both GAL and RL inhibited the binding of X44 and 10N109-1 to GAL and RL. BL did not inhibit in these experiments even though it contains ß(2-6)-linked fructose, the major component of rye levan. The rye levan molecule has a terminal glucose moiety linked as in sucrose, but this is probably not responsible for X44 binding since the reaction of X44 and RL cannot be inhibited by sucrose (not shown). X44 has been considered to be highly specific for ß(1-6) poly-D-galactose (Manjula and Glaudemans, 1976). Molecules containing this type of polysaccharide have never been described in rye levan, despite extensive analysis (Moreno et al., 1976; Tomasic et al., 1978). Nevertheless, we considered it possible that a (galactan-containing?) polysaccharide molecule present as a minor contaminant of RL might be responsible for the apparent cross-reactivity of GAL and RL.

If the rye levan molecule itself reacts with X44, then it might be able to form an antigen bridge between X44 orA48 coated on a plate and labelled A48 or X44 in solution. The RL molecule is quite small, having an average chain length of seven fructose molecules (with a terminal glucose, Moreno et al., 1976). Assuming an average of 6 Å per fructose (Streefkerk et al., 1979), this gives a total

length of 40-50 Å for the RL molecule. It has been assumed that "only one immunoglobulin combining site would be capable of interaction per molecule of grass levan" (Streefkerk et al., 1979).

Table XIII shows the results of attempting to bridge A48 and X44 on plates and in solution with GAL, RL, and BL. As expected, GAL bridges X44 with X44 quite well, as does BL with A48. RL was able to form a bridge between X44-coated wells and labelled A48 in solution, but did not work as well (yet still 4X above background) with A48-coated wells and labelled X44. Considering the small size of RL, it is not difficult to imagine that this could be due to differences in the manner in which the two myeloma proteins interact with RL. Binding of A48 to RL first is incompatible with subsequent binding by X44, but not A48. X44 binds RL in a manner which is permissive for subsequent binding by A48 or X44. The only (dubious) alternative explanation for these data is that binding of RL to the

Table XIII. Antigen-bridging of Plates Coated with X44 or A48 and Labelled X44 or A48 in Solution

Coating antibody	Antigen added: BSA	GAL	RL	BL	Labelled antibody
BSA	319±14	115±11	1,643±63	1,325±274	A48
BSA	290±5	2,756±339	624±48	203±7	X44
A48	5,018±805	5,197±431	26,463±323	34,756±546	A48
A48	641±37	5,575±691	2,428±105	506±5	X44
X44	2,078±69	1,316±32	21,514±835	3,696±484	A48
X44	18,516±1,520	87,585±903	79,543±29	12,003±419	X44

Plates were coated with 100 μl antibody at 10 μg/ml, antigen was at 20 μg/ml and ^{125}I-labelled antibody at 200,000 CPM in 100 μl. Data are mean±SEM of triplicate determinations.

myeloma protein on the plate induces a conformational change and the labelled antibody then binds in an anti-idiotypic fashion to a new epitope/idiotope.

In Table XIII one can see that labelled X44 binds to X44 on the plate in the absence of antigen. Since this type of interaction has been observed in other systems (Kang and Köhler, 1986) we investigated the effect of varying concentrations of X44, GAL, RL, and BL mixed with labelled X44 under the same conditions as in Table XIII. X44 inhibits this interaction 50% at 3 μg/ml while BL has no effect up to 60 μg/ml. With GAL and RL however, the bridging interaction predominates even when high concentrations of antigen are present in solution. From 0.2 to 60 μg/ml of GAL or RL the CPM of X44 bound are 2-10X higher than the CPM bound in the absence of antigen (data not shown). We suspect that X44 self-binding is weak compared to antigen binding, but this type of experiment will be more easily interpreted when the reactivity of the myeloma protein Fab is studied.

We have also examined some PR8-binding mAbs for binding to other influenza virus preparations (Table XIV). We have used PR8 (hen egg-grown A/PR/8/34, H1N1), X31 [hen egg-grown A/Aichi/2/68(R), H3N2], and WSN (MDCK cell-grown A/WSN/1/33, H1N1 heterovariant). All three viruses were purified by sucrose density gradient ultracentrifugation. PR8 and X31 are identical except for their hemagglutinin

and neuraminidase. WSN differs from the other viruses in all components and in its growth conditions, although many antigenic determinants will be shared among all three viruses. AL42-7 binds well to PR8, less to X31, and not to WSN. This mAb may be binding to a surface glycoprotein. NL124-1, NL126-4, and NL128-1 bind best to PR8, and somewhat less to X31. NL124-1 does not bind well above background to WSN, but NL126-4 and NL128-1 do. Thus, AL42-7 appears to bind differently to these viruses than the other three mAbs tested, suggesting that more than one specificity may be present in the mAbs that bind to PR8.

Among 30 mAbs selected only for expression of a gene of the V_HX24 gene family, 21 (70%) were found to react with a limited panel of primarily polysaccharide antigens previously shown to be recognized by myeloma proteins or mAbs expressing these genes. At least six different

Table XIV. Binding of PR8-reactive Monoclonal Antibodies to Three Different Influenza Virus Preparations

	Influenza viruses			
Antibody	PR8	X31	WSN	BSA
PY102	31,593±143	237±41	161±3	318±37
PY206	393±13	27,000±117	250±65	176±12
AL42-7	8,098±145	1,406±27	469±17	284±10
NL124-1	4,690±465	3,684±84	1,311±126	549±40
NL126-4	11,950±242	8,302±82	2,472±185	301±24
NL128-1	5,148±139	3,601±212	2,738±81	557±69

Plates were coated with 50 μl virus suspension at 10 μg/ml. Mabs were at 10 μg/ml. MAb binding was detected with ^{125}I-labelled rat anti-mouse kappa antibody at 50,000 CPM in 50 μl. See Materials and Methods for a description of the viruses. PY102 and PY206 are mAbs specific for hemagglutinins H1 and H3, respectively (Moran et al., 1984). Data are mean±SEM of triplicate determinations.

binding patterns were identified (see Table XI) not including the possibility of more than one influenza virus antigen, or the mAbs which did not react with any antigen tested. The high degree of cross-reactivity among these antigens was confirmed in inhibition experiments.

Among mAbs derived from normal mice, 77% (17/22) bound to an antigen, while only 33% (2/6) of the mAbs derived from IDA10-treated mice had an identifiable specificity. This difference may reflect an effect of anti-idiotype treatment.

Idiotype expression of the monoclonal antibodies

Eight anti-Id reagents against A48, U10, X24 or X44 were used to assess the idiotypes expressed in the mAbs. Polyclonal reagents included a BALB/c (syngeneic) anti-A48Id, and an A/He (allogeneic) anti-X24Id. Monoclonal anti-Ids included IDA3, IDA10, IDA16 and IDA17 anti-A48Ids (Legrain et al., 1981), 10-1, a semisyngeneic [(BALB/c X A/J)F1] anti-UPC10Id (Bona et al., 1985), and HyX24-14, an allogeneic (A/J) anti-Id which recognizes an idiotope shared by X24 and X44 (Pawlita et al., 1981).

MAbs were initially tested for Id expression by examining their ability to compete at a high concentration for the binding of labelled Id to anti-Id-coated plates (not shown). Antibodies which inhibited this binding were further tested in dose-effect inhibition experiments. At

least one mAb was found to inhibit these Id-anti-Id interactions for three of the reagents studied, IDA10, IDA16, and HyX24-14 (Table XV).

The mAb NL125-8 inhibited the binding of labelled X44 to HyX24-14, and 10L107-1 inhibited the interaction of IDA16 and A48. Three mAbs (NL112-9, 10L16-5, and 10N17-4) inhibited binding of the mAb 3-14-9 to IDA10. The mAb 3-14-9 has previously been shown to react with IDA10, and expresses V_HX24 and V_K10 genes (Victor-Kobrin et al., 1985;

Table XV. Idiotype Expression of V_HX24^+ Monoclonal Antibodies

		Anti-Id	
	IDA10	IDA16	HyX24-14
Ligand:	3-14-9	A48	X44
Inhibitors:			
A48	0.3[a]	0.6	-[b]
UPC10	1.8	-	-
X44	-	-	0.8
3-14-9	0.2		
MOPC870		-	-
MOPC173	-		
NL112-9	12	-	-
NL125-8	-	-	7.9
10L16-5	45	-	-
10L107-1	-	2.4	-
10N17-4	1.0	-	-

For IDA16 and HyX24-14 plates were coated with 100 μl anti-Id at 3 μg/ml, varying amounts of mAbs were mixed with the indicated labelled ligand at 100,000 CPM in 100 μl. Mean±SEM 100% binding for IDA16 and HyX24-14 are 3,075±235 and 9,058±124, respectively. Data for IDA10 is shown graphically in Figure 5, see Figure 5 legend for details. See text for a description of the mAb and myeloma protein controls. A space in the table indicates a combination not tested.

[a]Data are the concentration in μg/ml of inhibitor giving 50% inhibition.

[b]A dash indicates <50% inhibition by up to 50 μg/ml of inhibitor (IDA10) or 30 μg (IDA16 and HyX24-14).

Zaghouani et al., 1988). The negative controls used in these experiments are the BALB/c myeloma proteins MOPC173 (M173) which uses V_HX24 and V_K10 genes (Auffray et al., 1981; Hood et al., 1973), and McPC870, a myeloma protein which binds S. tel aviv LPS and uses V_K24 (Hood et al., 1973; Potter, 1977, its V_H gene sequence is not known.

Expression of the idiotope defined by IDA10 is important since one of the central questions in this investigation concerns the frequency of this idiotope in mAbs derivedfrom mice not immunized with levan and not selected for A48Id expression. Figure 5 shows the dose-effect of inhibition of the IDA10-3-14-9 interaction. Two mAbs from hybridomas derived from IDA10-treated mice (33%) and one from untreated mice (5%) clearly express this idiotope, giving >50% inhibition in this assay. Two other mAbs, 10L126-7 and 10L15-15 give weak inhibition, 30% and 25%, respectively at 50 μg/ml, but still above the level of M173 (5% at 50 μg/ml) which uses genes from the same V_H and V_K families as A48. A statistical analysis by the Fisher exact test (see Zar, 1984) for the contingency table including one mAb from untreated mice and only two mAbs from the IDA10 group gives $0.1>p>0.05$ for this difference in frequency ofId expression arising at random. Inclusion of one additional mAb from the IDA10 group (bringing that frequency to 50%) gives $0.02>p>0.01$.

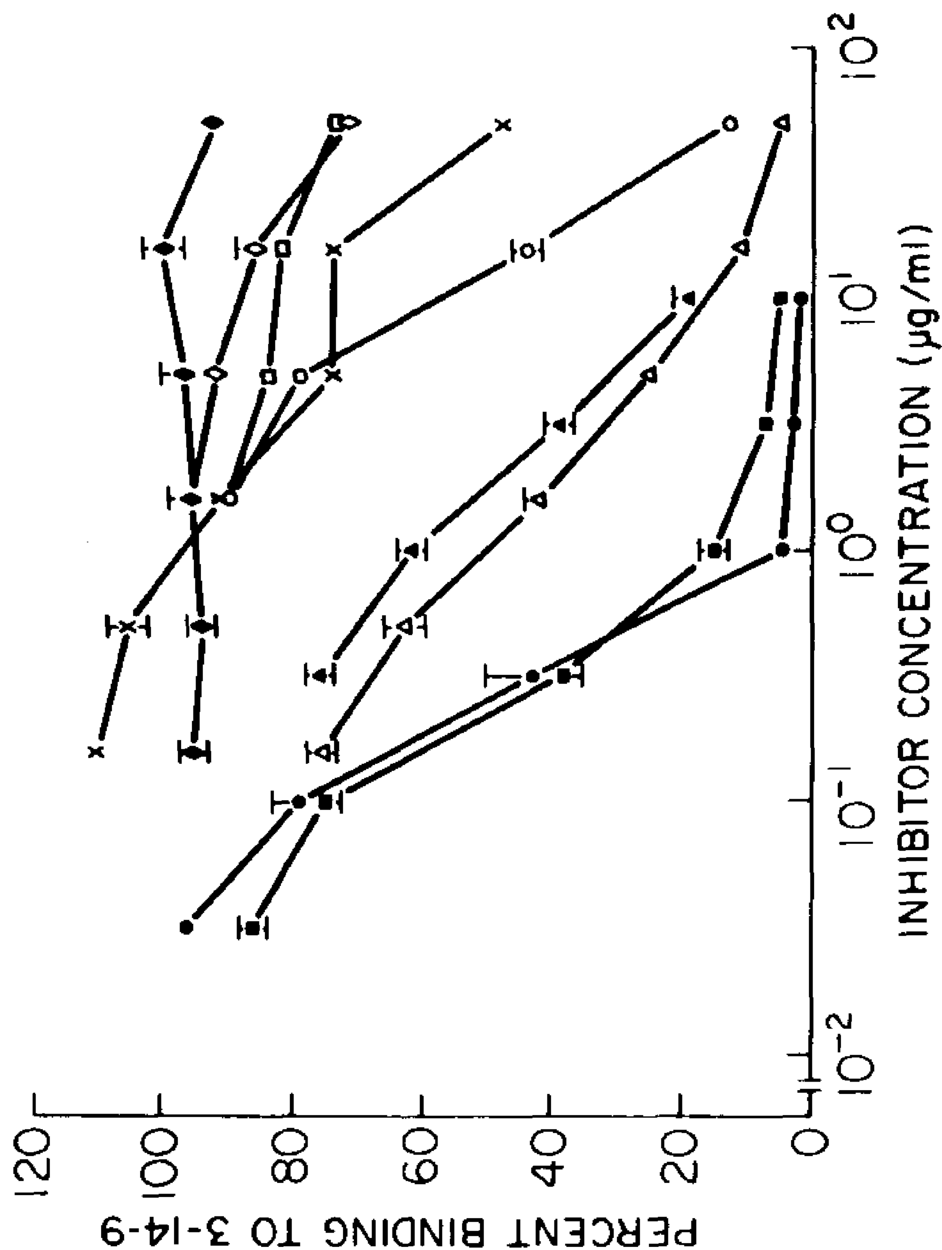

Figure 5. Inhibition of binding of labelled 3-14-9 to IDA10 by $V_H X24^+$ monoclonal antibodies. Plates were coated with 100 µl IDA10 at 10 µg/ml, varying concentrations of mAbs were mixed with ^{125}I-labelled 3-14-9 at 25,000 CPM in 100 µl. Shown are inhibition by: A48 (●); 3-14-9 (■); 10N17-4 (△); UPC10 (▲); NL112-9 (○); 10L16-5 (✕); 10L126-7 (◇); 10L15-15 (□); and MOPC173 (◆). Mean±SEM of CPM of 100% binding is 5,736±106. Data points represent mean±SEM of triplicate determinations.

Of eight idiotypic systems associated with antibodies expressing a $V_H X24$ gene, three arise in our panel of mAbs. Two of these, IDA16, and HyX24-14 appearing once, while the idiotope defined by IDA10 occurs on (at least) three mAbs. This idiotope does not appear in high frequency among mAbs from untreated mice, but appears at higher frequency in mAbs from IDA10-treated mice. This may reflect an expansion of IDA10Id$^+$ cells by neonatal administration of IDA10.

V_K gene usage among the monoclonal antibodies

As mentioned in the introduction, the pairing of particular V_K gene families is important in determining the antigen specificity and idiotypes of antibodies expressing a $V_H X24$ gene. The majority of levan-binding A48Id$^+$ antibodies use $V_K 10$ (Barak, 1988; Legrain and Buttin, 1985), while specificity and idiotypes of anti-galactan antibodies are associated with $V_K 4$ (Rudikoff, 1984).

Total cellular hybridoma RNA was first tested at three dilutions in high stringency dot blots with 11 probes representative of different V_K gene families ($V_K 1$, 2, 4, 8, 9, 10, 11, 19, 21, 22, and 24). RNAs which hybridized well at all dilutions with only one of the probes used was analyzed further by Northern blotting to show hybridization with a mRNA of appropriate size (Figure 6).

A high number (12/30 or 40%) of these hybridomas express the $V_K 1$ gene family. Although hybridization

appears weak in some cases, this correlates with a weaker signal with the V_H441 probe (Figure 2) and probably reflects a lower quantity of specific message. Expression of V_HX24 and V_K1 for one of these weakly hybridizing RNAs, NL112-9, has been confirmed by nucleotide sequence analysis (see below). A V_K1 gene occurs in mAbs having most of the antigen-binding patterns detected, i.e., association with V_K1 does not appear to determine a particular pattern of reactivity among the antigens studied.

The mAb 10N109-1 which has antigen binding identical to X24 and X44 also uses the same V_K gene family, V_K4. The two mAbs from IDA10-treated mice which reacted well with IDA10 also use the same V_K10 gene family used by A48. The IDA10-binding mAb from normal mice, NL112-9, uses V_K1. Another mAb which interacts weakly with IDA10, 10L15-15, uses V_K1. Finally, an mAb with low binding to AZO and PR8, NL124-1, uses V_K9.

Nucleotide sequence analysis of selected monoclonal antibodies.

Figure 7 shows the V_H nucleotide sequences determined for six of the mAbs compared to sequences of A48, 3-14-9, and the two germline genes of the V_HX24 gene family. Of thesix new V_H sequences analyzed, four derive from the V_H441 gene, while one derives from the V_HX24 gene. The

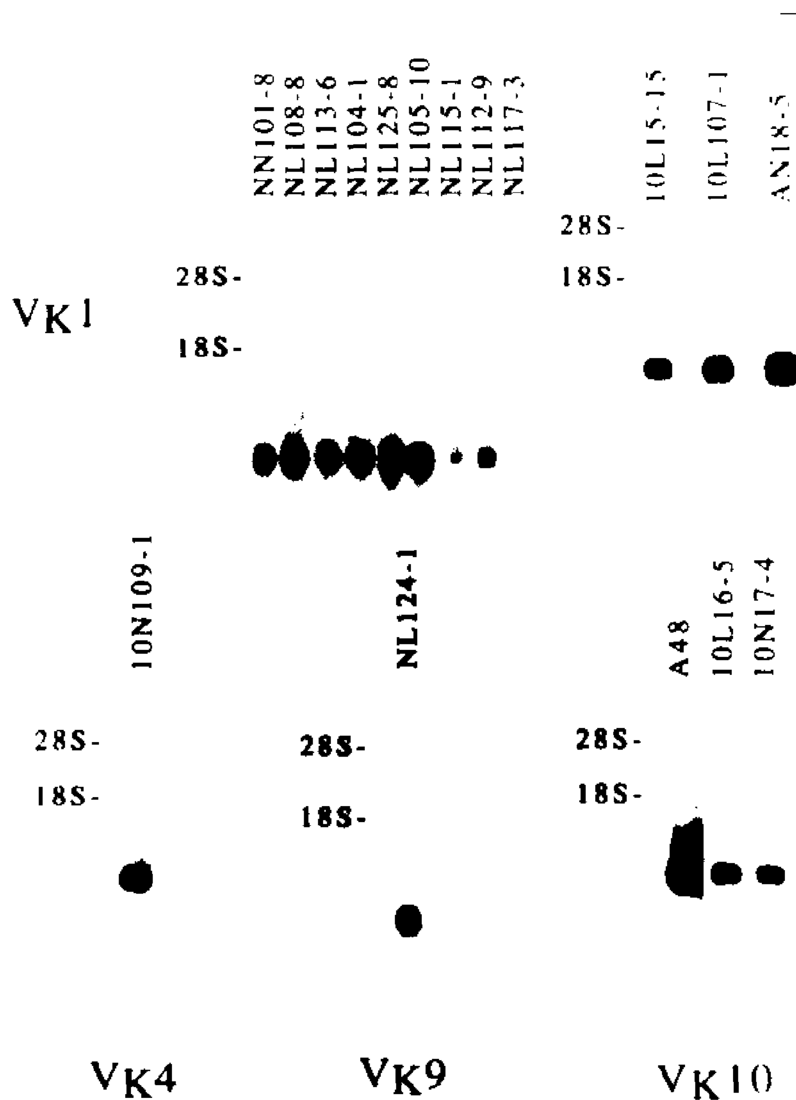

Figure 6. V_K genes identified by Northern blotting among $V_H X24^+$ monoclonal antibodies. Total cellular hybridoma RNA was Northern blotted and hybridized with ^{32}P-labelled DNA probes representing the indicated V_K gene family. Positions of the 28S and 18S ribosomal RNA bands are indicated.

sixth sequence does not extend far enough 5′ to distinguish the particular germline gene origin. Somatic mutation appears to be very infrequent in the V_H regions of the mAbs under study. In 1,263 sequenced bases, there occurs only one somatic mutation which leads to replacement of Ser with Ile in codon 35 of the V_H CDR1 of 10L16-5. As has been observed previously (Victor-Kobrin et al., 1987), usage of either germline gene is consistent with expression of the IDA10-defined idiotope.

CDR3 structure is quite heterogeneous among the mAbs. All three D region families (Kurosawa and Tonegawa, 1982) occur in the new sequences, as well as D regions without obvious homology to these families. Three antibodies have a juxtaposition of Arg-Gly in the D region, A48, 10L126-7, and NL104-1. Both NL104-1 and 10L126-7 bind to AZO and PR8, while A48 binds ß(2-6) fructosan. This difference is probably due to influence of V_K on antigen binding. Three antibodies also share Pro-(two or three amino acids)-Asn: 10N17-4, 10L126-7, and NL112-9. The antigen binding of10N17-4 is unknown, while 10L126-7 binds AZO and PR8, and NL112-9 binds AZO and GAL. These D amino acids may play a role in AZO binding, however, NL104-1 does not have this combination, and uses the same V_K gene family as NL112-9. In the six new sequences, four contain JH4; two contain JH1.

```
                                  5                                 10                                  15                                  20                                  25
          E   V   K   L   L   E   S   G   G   G   L   V   Q   P   G   G   S   L   K   L   S   C   A   A   S   G   F   D
Vh441     GAG GTG AAG CTT CTC GAG TCT GGA GGT GGC CTG GTG CAG CCT GGA GGA TCC CTG AAA CTC TCC TGT GCA GCC TCA GGA TTC GAT
10N17-4                                                        - --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
10L16-5                                                                                                   - --- --- --- --- ---
10L126-7  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                                                                      N
VhX24     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --T --- --- --- --- --- --- --- --- ---
3-14-9    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --T --- --- --- --- --- --- --- --- ---
10L15-15                                                       - --- --- --- --- --- --T --- --- --- --- --- --- --- --- ---

                  ****** CDR1 *******                                                                   ********** CDR2 ***********
              30                  35                      40                      45                      50              52a          55
          F   S   R   Y   W   M   S   W   V   R   Q   A   P   G   K   G   L   E   W   I   G   E   I   N   P   D   S   S
Vh441     TTT AGT AGA TAC TGG ATG AGT TGG GTC CGG CAG GCT CCA GGG AAA GGG CTA GAA TGG ATT GGA GAA ATT AAT CCA GAT AGC AGT
                                                                  R
ABPC48                --- --- --- --- --- --- --- --- --- --- --- -G- --- --- --- --- --- --- --- --- --- --- --- --- ---
10N17-4   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                  I
10L16-5   --- --- --- --- --- --- -T- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
10L126-7  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
NL104-1                                                                                                -- --- --- --- ---
                                      A                               Q                               G
VhX24     --- --- --- --- --- --- --- --- -CT --- --- --- --- --- --- --- -AG --- --- --- --- --- --- --- --- -GA --- ---
                                                      L
3-14-9    --- --- --- --- --- --- --- --- -CT --- --- --- -T- --- --- --- -AG --- --- --- --- --- --- --- --- -GA --- ---
10L15-15  --- --- --- --- --- --- --- --- -CT --- --- --- --- --X X-- --- -XG --- --- --- --- --- --- --- --- -GA --- ---

          ************** CDR2 ******************
                              60                  65                      70                      75                      80          82a
          T   I   N   Y   T   P   S   L   K   D   K   F   I   I   S   R   D   N   A   K   N   T   L   Y   L   Q   M   S
Vh441     ACG ATA AAC TAT ACG CCA TCT CTA AAG GAT AAA TTC ATC ATC TCC AGA GAC AAC GCC AAA AAT ACG CTG TAC CTG CAA ATG AGC
                                          R                                               G
ABPC48    --- --- --- --- --- --- --- --- -G- --- --- --- --T --- --- --- --- --- -G- --- --C --- --- --- --- --- --- ---
10N17-4   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
10L16-5   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
10L126-7  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
NL104-1   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
VhX24     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
3-14-9    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
10L15-15  --- --- --- --- --- -X- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --X
NL112-9                                                   - --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

          b   c           85                      90                            **************** CDR3 *****************
          K   V   R   S   S   D   T   A   L   Y   Y   C   A   R
Vh441     AAA GTG AGA TCT GAG GAC ACA GCC CTT TAT TAC TGT GCA AGA
                          D                           Y             A   N   W   D   R   G
ABPC48    --- --- --- --- --C --- --- --- --- --- --- -A- ---       GCT AAC TGG GAC AGG GGG                      DQ52,Jh3
                                                                                P   Y       N
10N17-4   --- --- --- --- --- --- --- --- --- --- --- --- --- ---               CCG TAT --T AAC                DSP2.(5,7,8),Jh4
                                                                        K   G   Y   F   Y   Y   G   S   S
10L16-5   --- --- --- --- --- --- --- --- --- --- --- --- --- ---     --G G-- T-T TTT TAC TAC GGT AGT AGC      DFL16.1,Jh1
                                                                                P           N
10L126-7  --- --- --- --- --- --- --- --- --- --- --- --- --- --              G CCG C-- --T AAC                DSP2.(5,7,8),Jh4
                                                                            L   G           Y   Y   F
NL104-1   --- --- --- --- --- --- --- --- --- --- --- --- --- ---         CT- -GA C-- --- TAC TAC TTT CC         DQ52,Jh1
VhX24     --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                                                        L   L   A   K
3-14-9    --- --- --- --- --- --- --- --- --- --- --- --- --- ---     CTA -T- -C- -A-                          D?,Jh4
                                                                        L   S   P   G   K   D
10L15-15  X-- --X --- --- --  --- --  --- --- --- --- --- --- ---     CT- -CT CCT G-T AA- GAT                  D?,Jh4
                                                                            P   L   I       N
NL112-9   --- --- --- --- --- --- --- --- --- --- --- --- --- ---         CCT CTA -TC --- AAT                  D?,Jh4
```

Figure 7.

Two of the mAbs reacting with IDA10 were shown by Northern blotting to express the same V_K gene family (V_K10) as A48. In Figure 8, the V_K sequences of 10N17-4 and 10L16-5 are compared to a germline V_K10 sequence from the A/J mouse strain (Meek, et al., 1987), to a BALB/c consensus sequence derived from A48 and seven A48Id+ mAbs (Legrain and Buttin, 1985), and to the V_K sequences of A48 and 3-14-9 (Zaghouani et al., 1988). As with the V_H genes, very little somatic mutation is evident in these two V_K genes. Of the 23 nucleotide differences between 10L16-5 and the BALB/c consensus sequence, 19 are shared by a sequence reported by Legrain and Buttin (1985, designated 10/1). The 10/1 sequence is not long enough to determine if three additional changes in 10L16-5 are shared as well. Thus, the single silent C to T change in codon 86 may represent the only somatic mutation in the 483 V_K10 nucleotides sequenced. Both 10L16-5 and 10N17-4 have unknown antigen binding activity and are of the IgG1 isotype.

Figure 7 (preceding page). Comparison of V_H nucleotide sequences of selected monoclonal antibodies to sequences of germline genes of the V_HX24 family, and the V_H sequences of levan-binding, A48Id$^+$ antibodies. The germline sequences Vh441 and VhX24 are from Hartman and Rudikoff (1984) where they are designated V_HGal 39.1 and V_HGal 55.1, respectively. The ABPC48 sequence is from Auffray, et al. (1981), and the 3-14-9 sequence is from Victor-Kobrin et al. (1987). Codon numbering is according to Kabat et al. (1987).

```
                                                                                                       ****** CDR1 *******
                             5                   10                  15                  20                  25
             D   I   Q   M   T   Q   T   T   S   S   L   S   A   S   L   G   D   R   V   T   I   S   C   R   A   S   Q   D
VK-GL       GAT ATC CAG ATG ACA CAG ACT ACA TCC TCC CTG TCT GCC TCT CTG GGA GAC AGA GTC ACC ATC AGT TGC AGG GCA AGT CAG GAC
CONS.K10                                    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                                                                 I
ABPC48                                      --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- --- --- ---
3-14-9      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---  .  --- --- --- --- --- --- --- --- --- --- ---
10N17-4                                                       - --- --- --- -       --- --- --- --- --- --- --- ---
                                                                                                     S   V           G
10L16-5                                                         --- --- --- --- --- --- --- --- --T -T- --- --- -G-

            ******* CDR1 **********                                                            ********* CDR2 ***********
                 30                  35                  40                  45                  50                  55
             I   S   N   Y   L   N   W   Y   Q   Q   K   P   D   G   T   V   K   L   L   I   Y   Y   T   S   R   L   H   S
VK-GL       ATT AGC AAT TAT TTA AAC TGG TAT CAG CAG AAA CCA GAT GGA ACT GTT AAA CTC CTG ATC TAC TAC ACA TCA AGA TTA CAC TCA
CONS.K10    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                                                                                         I       Q
ABPC48      --- --T --- --- --- --- --- --- --- --- --  --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --G ---
                         L                                                                                           Y
3-14-9      --- --- --- -TA --- --- --- --- --- --- --- --- ---  .  --- --- --- --- --- --- --- --- --- ---     --- --- -AC
10N17-4     --- --- --- --- --- --- --- --- --- --- --- --- -    .- --- --- -   --- --- --- --- --- --- --- --- --- --- ---
                                                                                                 N           T       Y
10L16-5     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---      -- --T A-- --- --- -CT --- T-- ---

                         60                  65                  70                  75                  80
             G   V   P   S   R   F   S   G   S   G   S   G   T   D   Y   S   L   T   I   S   N   L   E   Q   E   D   I   A
VK-GL       GGA GTC CCA TCA AGG TTC AGT GGC AGT GGG TCT GGA ACA GAT TAT TCT CTC ACC ATT AGC AAC CTG GAG CAA GAA GAT ATT GCC
CONS.K10    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
ABPC48      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
3-14-9      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
10N17-4     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -   --- --- --- ---     --- --- --- --- --- --- ---
                                                                                                     P
10L16-5     --- --- --- --- --- --- --- --- --- --- --- --G --- --- --- --- --- --- --C - T     --- --A -CT --- --- --- ---

                                ********** CDR3 *************
            85                  90                  95
             T   Y   F   C   Q   Q   G   N   T   L   P
VK-GL       ACT TAC TTT TGC CAA CAG GGT AAT ACG CTT CCT C
                     L
CONS.K10    --- --- --A --- --- --- --- --- --- --- --- -
                                         H
ABPC48      --- --- --A --- --- --- --- C-- --- --- --- -          Jk1
3-14-9      --- --- --- --- --- --- --- --- --- --- --- -          Jk1
10N17-4     --- --- --- --- --- --- --- --- --- --- --- -          Jk1
                     Y               Y   S   K
10L16-5     --- --T -A- --T --G --- TA- -G- -A- --- --- -          Jk1
```

Figure 8.

The mAb NL104-1 binds AZO and PR8, while NL112-9 binds AZO and GAL. The antigen specificity of 10L15-15 is unknown. These mAbs use a V_K1 gene. Figure 9 shows the comparison of these V_K sequences to two BALB/c V_K1 germline genes (Corbet et al., 1987). These genes encode the VK-1A and VK-1C homology subgroups defined by Hum et al. (1984). We found the NL112-9 and 10L15-15 V_K genes to be encoded by K5.1 (VK-1A) and NL104-1 expresses K1A5 (VK-1C). Once again, somatic mutations are rare. The G in the last position occurs in many expressed V_K genes in the GAT system, and is ascribed to "junctional diversity" by Corbet et al. (1987). This leaves the silent A to C change in codon 66 of NL112-9, and the replacement and silent mutations in codons 41 and 73, respectively, of 10L15-15 relative to K5.1 as the only potential somatic mutations detected in these two genes.

The two germline V_K1 genes have 5 amino acid differences in their CDRs. The V_H gene of NL104-1 is germline-encoded from CDR2 onward, while NL112-9 is germline encoded in FR3 (the remainder of the sequences not being deter-

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Figure 8 (preceding page). Comparison of V_K nucleotide sequences of two A48Id$^+$ monoclonal antibodies to germline and expressed V_K10 genes. The A/J germline sequence VK-GL is from Meek et al. (1987), the BALB/c consensus V_K10 (CONS.K10) and ABPC48 sequences are from Legrain and Buttin (1985). The 3-14-9 sequence is from Zaghouani et al. (1988). Codon numbering is according to Kabat et al (1987).

Figure 9.

```
                                                                                                  ******* CDR1 ******
                           5                  10                  15                  20                  25          27a
           D   V   V   M   T   Q   T   P   L   S   L   P   V   S   L   G   D   Q   A   S   I   S   C   R   S   S   Q   S
K5.1      GAT GTT GTG ATG ACC CAA ACT CCA CTC TCC CTG CCT GTC AGT CTT GGA GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT CAG AGC
NL112-9                                                                       --- --- --- --- --- --- --- --- --- --- ---
10L15-15                                                              --- --- --- --- -X- --- --- --- --- --- --- --- ---
                   L
K1A5      --- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
NL104-1                                                                                                         - --- ---

          **************** CDR1 *********************                                                         *******
           b   c   d   e          30                  35                  40                  45                  50
           L   V   H   S   N   G   N   T   Y   L   H   W   Y   L   Q   K   P   G   Q   S   P   K   L   L   I   Y   K   V
K5.1      CTT GTA CAC AGT AAT GGA AAC ACC TAT TTA CAT TGG TAC CTG CAG AAG CCA GGC CAG TCT CCA AAG CTC CTG ATC TAC AAA GTT
NL112-9   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                                                                   S
10L15-15  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---   - A-- --- --- --- --- --- --- --- --- --- ---
           I                                       E
K1A5      A-- --- --T --- --- --- --- --- --- --- G-A --- --- --- --- --A --      - --     -- --- --- --- --- --- --- ---
NL104-1   A-- --- --T --- --- --- --- --- --- --- G-A --- --- --- --- --A -   --- --- --- --- --- --- --- --- --- --- ---

          ***** CDR2 ********
                       55                  60                  65                  70                  75
           S   N   R   F   S   G   V   P   D   R   F   S   G   S   G   S   G   T   D   F   T   L   K   I   S   R   V   E
K5.1      TCC AAC CGA TTT TCT GGG GTC CCA GAC AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC AGC AGA GTG GAG
NL112-9   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --C --- --- --- --- --- --- --- --- --- --- --- --- ---
10L15-15  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --T --- --- --- --- --- ---
K1A5      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
NL104-1   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --  --- --- --- --- --- --- --- --- ---

                                                    ****** CDR3 ******* *******
          80                  85                  90                  95
           A   E   D   L   G   V   Y   F   C   S   Q   S   T   H   V   P
K5.1      GCT GAG GAT CTG GGA GTT TAT TTC TGC TCT CAA AGT ACA CAT GTT CCT
NL112-9   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --G           Jk2
10L15-15  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---           Jk1
                                       Y       F       G   S
K1A5      --- --- --- --- --- --- --- -A- --- -T- --- G-- T-- --- --- ---
NL104-1   --- --- --- --- --- --- --- -A- --- -T- --- G-- T-- --- · · --G           Jk1
```

mined). Considering the overall very low rate of observed somatic mutation, we consider it unlikely that many mutations exist in the portions of V_H not sequenced. Thus, we believe that the more or less subtly different antigen binding of NL104-1 (AZO and PR8) and NL112-9 (AZO and GAL) may be determined by differences in the D region and in the germline encoded light chains.

The mAb NL126-4 also binds to AZO and PR8, but uses a different V_K gene than NL104-1. In dot blots, NL126-4 RNA hybridized weakly with a V_K8 probe, but a computer database search for sequences homologous to the NL126-4 V_K gene identified a V_K8 sequence from an aberrantly rearranged gene in the plasmacytoma PC3609 (Feddersen and Van Ness, 1985) as the murine gene with highest homology[13].

The PC3609 and NL126-4 sequences are compared in Figure 10. The overall homology is fairly high, with the exception of the last four codons. There are 36 base differences (including six ambiguities in the NL126-4 sequence) over 245 bases compared, or 85% homology. Homology at the amino acid level is 88% counting ambiguities in the first or second bases of a codon as amino acid changes

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Figure 9 (preceding page). Comparison of V_K nucleotide sequences of monoclonal antibodies NL104-1 and NL112-9 to two germline V_K1 sequences. The K5.1 and K1A5 sequences are from Corbet et al. (1987).

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[13]The gene identified with highest homology is a human V_KIV gene (Klobeck et al., 1985).

```
                                  5                       10
          D   I   V   M   S   Q   S   P   S   S   L   A   V   S
PC3609    GAC ATT GTG ATG TCA CAG TCT CCA TCC TCC CTA GCT GTG TCA

                                                  ****** CDR1 *******
          15                  20                  25          27a
          V   G   E   K   V   T   M   S   C   K   S   S   Q   S
PC3609    GTT GGA GAG AAG GTT ACT ATG AGC TGC AAG TCC AGT CAG AGC
NL126-4                       --  --- --- --T --- --- --- --A --T

          ****************** CDR1 *********************
          b   c   d   e   f           30                  35
          L   L   Y   S   S   N   Q   K   N   Y   L   A   W   Y
PC3609    CTT TTA TAT AGT AGC AAT CAA AAG AAC TAC TTG GCC TGG TAC
          V
NL126-4   G-- --- --C --- TCA --- --G --- --- --- --- --- --- ---

                                                              ***
                      40                  45                  50
          Q   Q   K   P   G   Q   S   P   K   L   L   I   Y   W
PC3609    CAG CAG AAA CCA GGG CAG TCT CCT AAA CTG CTG ATT TAC TGG
                      G
NL126-4   --- X-- --- GG- --- --- --- --- --- --- --- --C --- ---

          ***** CDR2 ************
                          55                  60
          A   S   T   R   E   S   G   V   P   D   R   F   T   G
PC3609    GCA TCC ACT AGG GAA TCT GGG GTC CCT GAT CGC TTC ACA GGC
NL126-4   -X- --- --- --- --- --- --T --- --- --- --X --- --- ---

          65                  70                  75
          S   G   S   G   T   D   F   T   L   T   I   S   S   V
PC3609    AGT GGA TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC AGT GTG
NL126-4   --- --- --- --- --- --- --T --- --T --X --- --- --- --A

                                                  ***** CDR3 ****
              80                  85                  90
          K   A   E   D   L   A   V   Y   Y   C   Q   Q   Y   Y
PC3609    AAG GCT GAA GAC CTG GCA GTT TAT TAC TGT CAG CAA TAT TAT
          Q                                                   L
NL126-4   C-A --- --- --- --- --- --- --- --- X-- X-T --- --C CTC

          ***********. . .
                  95
          S   Y   P
PC3609    AGC TAT CCT                  NL126-4   ATCAATA.CCTCTCCTC
              S   Y                    VH104,5'  -------C--C------
NL126-4   TC- -CG TAC   Jk2
```

Figure 10. Comparison of the V_K nucleotide sequence of monoclonal antibody NL126-4 to a V_K8 sequence. The PC3609 sequence is from Feddersen and Van Ness (1985). A portion of the 3' end of the NL126-4 V_K sequence is also compared to a fragment of the 5'flanking region of a V_H pseudogene (Cohen et al., 1982).

(93% not counting ambiguities). The high number of base substitutions as well as the demonstrable lack of a high degree of somatic mutation in the other genes sequenced argues for an origin of the NL126-4 V_K gene from a germline gene other than that represented by PC3609.

The final GTAC in the NL126-4 V_K sequence shown is most probably encoded by the JK2 gene. The 16 bases immediately preceding have high homology with a fragment of the 5' flanking region of a mouse V_H pseudogene (Figure 10). This portion of the NL126-4 V_K gene may have arisen as a consequence of gene conversion (Clarke et al., 1982), either during evolution or somatically, with a similar sequence within the V_K locus.

RNA from the hybridoma 10L126-7, which binds to AZO and PR8, hybridized weakly in dot blots with the V_K19 probe. Partial nucleotide sequencing has confirmed this assignment (not shown).

The immunochemical characteristics and V_K gene associations of monoclonal antibodies selected for expression of V_HX24 family genes are summarized in Table XVI.

Table XVI. Summary of Antigen Binding, Idiotype Expression and V_K Gene Usage by V_HX24^+ Monoclonal Antibodies

Antibody	Isotype	Antigen Binding	Idiotype Expression	VK gene family
NL115-1	IgM	AZO		1
NL104-1	"	AZO, PR8		1
NL105-10	"	AZO, PR8		1
NL125-8	"	AZO, PR8	HyX24-14	1
NL112-9	"	AZO, GAL	IDA10	1
NL113-6	"	AZO, GAL		1
NL108-8	"			1
NL117-3	"			1
NN101-8	"			1
NL126-4	"	AZO, PR8		8
NL124-1	"			9
AN18-5	"	AZO, GAL		1
10L15-15	"		(IDA10)[a]	1
10L107-1	"		IDA16	1
10N109-1	IgG3	GAL, RL		4
10L16-5	IgG1		IDA10	10
10N17-4	"		IDA10	10
10L126-7	IgM	AZO, PR8	(IDA10)	19

[a]Parentheses indicate weak interaction with IDA10.

DISCUSSION

Hybridomas selected for expression of the A48Id

Differences in the antigen binding characteristics of series 1 and 2 versus series 3 and AIDA monoclonal antibodies suggest different influences responsible for expansion of the clones immortalized as hybridomas. The predominance of levan binding of series 1 and 2 mAbs indicates that antigen primarily accounted for expansion of these clones. The same treatments of mice used in production of series 1 and 2 result in an anti-BL response that is approximately 50% $A48Id^{+}$ (Hiernaux et al., 1981, Rubinstein et al., 1982). From analysis of monoclonal antibodies, it appears that few $A48Id^{+}$ antibodies elicited by these treatments have specificity for antigens other than BL.

On the other hand, the vigorous anti-idiotypic immunization of mice yielding mAbs of series 3 and AIDA appears to have expanded Id^{+} clones independently of specificity for BL (BL was not used in production of AIDA mAbs). In fact, one $A48Id^{+}$ antibody binds galactan. The very strong

association of V_HX24 and V_K10 genes among these antibodies (all four series) is very suggestive of their "Ab_1-like" quality. This is the outcome predicted by the regulatory idiotope hypothesis. Ab_2-immunization expands Ab_1-like clones.

Although all mAbs of series 1, 2, and 3 were selected for binding to syngeneic polyclonal anti-A48Id antibodies, they display some heterogeneity when analyzed with monoclonal antibodies specific for A48 or U10 idiotopes. IDA10 recognizes a determinant present on most mAbs reacting with a polyclonal anti-A48Id reagent, although this determinant is more faithfully reproduced in antibodies elicited with vigorous anti-idiotypic immunization. The monoclonal anti-U10Id antibody 10-1 also reacts with many of these mAbs, but not in a manner correlating with the particular treatment used to expand $A48Id^+$ clones. Thus, the determinant defined by 10-1 is expressed independently of that defined by IDA10. Similarly, other A48 idiotopes defined by other IDA mAbs are also expressed independently in these monoclonal antibodies, confirming the analysis of anti-IDA antisera reported by Legrain et al. (1985b). The idiotope defined by IDA10 may be a marker of (a) germline gene(s) alone or in combination since it is the only A48 idiotope appearing in relatively high frequency among these mAbs.

If the immune network is to be a network indeed, then antibodies of one specificity should have diverse idio-

topes, and/or antibodies with the same idiotope should have diverse specificities. The former has been shown clearly for A48 by Legrain et al. (1981). The latter has also been demonstrated for anti-levan (Lieberman et al., 1975) and anti-galactan (Rudikoff et al., 1973) myeloma proteins. The proportion of idiotopes expressed on an antibody which might be classified as regulatory remains undetermined. It is interesting to note that immunization with both IDA10 and IDA23 elicited predominantly Ab_1-like antibodies (Legrain and Buttin, 1985; Legrain et al., 1985).

That antibodies bearing the same idiotope may have different antigen specificities has been observed in many systems (one of the earliest observations made by Oudin and Cazenave, 1971). The results above also demonstrate this fact. The mAbs of series 1 and 2 have antigen-binding similar, but not identical to A48. Series 3 and AIDA are even more different, one AIDA mAb binding galactan. Furthermore, the galactan-binding myeloma protein X44 shares some idiotypy with U10.

Does anti-Id directly induce clonal proliferation, or does anti-Id treatment favor clones for antigen-driven expansion? In the former instance we would suppose that Ab_2 treatment followed by an antigen non-specific (mitogen) stimulus would result in an increased frequency of the idiotype independently of the specificities with which it is (known to be) associated. Assuming this scenario, the

mitogen stimulus is actually gratuitous for idiotype expansion. In the second case, the same treatments might not result in idiotype expansion if a) specific antigen is really necessary, or b) the specificity(ies) associated with the idiotype is not represented in mitogen-sensitive cells. Idiotype expansion would occur if specific antigen stimulation is, in fact, unnecessary, i.e., mitogen is able to substitute for antigen. With respect to BL-specific antibodies, LPS or NWSM stimulate approximately 30,000/10^6 spleen cells to secrete BL-binding antibodies (C. Bona, unpublished data). We will return to the questions above after discussing the immunochemical characteristics of V_HX24^+ mAbs, and information regarding structure-function relations in this system.

Hybridomas selected for expression of the V_HX24 gene family

The analysis of V_K gene usage, idiotypy and antigen specificity in this system has now been extended to include the virgin B-cell repertoire, or "steady state" by immortalizing in vitro polyclonally-activated spleen cells from one month old untreated mice, and from one month old mice injected at birth with A48 or IDA10.

Frequency of V_HX24

We screened 1,464 hybridomas and selected 32 (2.2%) on the basis of their expression of a gene of the V_HX24 gene

family. We detected no difference in the frequency of expression of this gene family in the three treatment groups. The overall frequency we observed agrees well with the value of 2.5% reported by Schulze and Kelsoe (1987).

Antigen binding characteristics of V_HX24^+ monoclonal antibodies

The antigen binding properties of these mAbs are markedly different from those observed in series 1, 2 ,3, and AIDA. Reactivity with ß(2-6) fructosan is rare, occurring with only one antibody which displayed an unexpected binding to GAL and RL. Even more surprising was the same double reactivity displayed by the myeloma proteins X24 and X44.

The ability to form a bridge between immobilized A48 or X44 and A48 or X44 in solution with RL indicates that these myeloma proteins are binding the same RL molecule, although their interaction with the antigen appears to be different. The ability of certain myeloma proteins to interact with both beta(2-1) and beta(2-6) linked fructosans has been explained by the ability of these polysaccharides to form homologous arrangements of chemical functional groups (Streefkerk et al., 1979). We suggest that the interaction of X24, X44 and 10N109-1 with GAL and RL has the same basis. The mAb 10N109-1 uses the same V_K family (V_K4) as X24 and X44 (and other GAL-binding myeloma proteins and mAbs, Rudikoff, 1984).

The predominant antigen binding activity detected among these mAbs is to AZO. Several mAbs also bind variably to PR8, to GAL, or to all three. Although AZO-binding antibodies do not bind GAC, they may be specific for N-acetylglucosamine (NAG). McCarty (1958) found that while antibodies elicited by GAC immunization bound AZO, the opposite was not true, even though AZO-binding was inhibited by NAG in all cases. Carrier (OVA) alone also fails to interact with these mAbs.

The binding to more than one antigen in solid-phase RIA was confirmed in most instances by reciprocal competitive inhibition. In some cases (e.g., NL126-4) binding to AZO could be inhibited by AZO, but only weakly by PR8. Binding to PR8 was inhibited by PR8 but not by AZO. We believe these effects to be due to the differing strengths of interaction between the mAb and the antigen, the epitope density of the antigen, and the effect of increased avidity of binding to antigen immobilized on the plate relative to antigen in solution (Rubin et al., 1980).

Although not measured directly, we believe the affinity of interaction of the majority of the mAbs with the antigens to be low because coating of plates with very low antigen concentrations (reducing avidity effects) decreased binding below levels suitable for accurate quantitation of inhibition. A study of the binding of labelled antigen to mAb-coated plates was not possible

because iodination of the antigens abolished their interaction with the mAbs.

V_H-V_K associations and the antibody repertoire

A surprisingly high number (12/30 or 40%) of the kappa-bearing mAbs use V_K1. This does not appear to correlate precisely with antigen binding or idiotype expression. What is the origin of this high frequency? Four possibilities, alone or in combination, may be considered: 40% of normal B cells use V_K1; 40% of LPS-responsive B cells use V_K1; 40% of B cells expressing V_HX24 also express VK1; 40% of the LPS-responsive B cells which express V_HX24 also express V_K1.

Direct measurements of V_K1 usage have shown expression of this family to be very frequent, though not 40%. Ten percent of hybridomas from LPS-stimulated spleen cells of NZB mice expressed V_K1 (Bailey et al., unpublished data), as did 28% of LPS-stimulated BALB/c spleen cells (Kaushik et al., unpublished data).

Zhu et al. (1984) have studied light chain expression by unstimulated or LPS-stimulated spleen cells indirectly. Hybridomas secreting antibody specific for trinitrophenyl or sheep red blood cells were selected for loss of production of H or L chains. Unstimulated or LPS-stimulated spleen cells were fused with these mutants; the resulting hybrids were screened for reconstitution of antigen bind-

ing. V_K genes were grouped on the basis of restriction analysis in Southern blots, and 2-dimensional gel electrophoresis. These authors observed that a) unstimulated or LPS-stimulated B cells reconstituted antigen binding with equal frequency; b) different V_K families could reconstitute the same specificity; and c) antigen binding was most often reconstituted by the same, or nearly identical, light chain as that whose expression was lost in the original hybridoma.

The situation in our experiments has some parallels with those of Zhu et al. In this case, we examined the antigen binding of different light chains "reconstituting" antibody activity with a given H chain. As did they, we observed that the same antigen binding pattern may be created by pairing of the same H with different L chains. The data of Zhu et al. argue against a role for LPS in the observed high frequency of V_K1 expression. Thus, it may be that V_K1 accounts for 40% of the antibody repertoire, or that there is preferential association of V_HX24 with V_K1 genes. The resolution of this question holds important implications for the generation of antibody diversity and establishment of the antibody repertoire.

Preferential association of V_H and V_K gene families would reduce the amount of antibody diversity ascribed to random V_H-V_K pairing. Dominance of one V_K family in the repertoire would also seem to reduce the diversity of the

repertoire. Such an antigen-independent selection operating in establishment of the repertoire has been postulated by Primi et al. (1987). These authors used antibodies specific for V_HT15 or V_K21D and E determinants to demonstrate strain (BALB/c)-specific non-random preferential association of these V_H and V_K genes in LPS-stimulated B cells.

The role of mitogen stimulation in these observations remains unclear. While LPS stimulation may be responsible for the high frequency of V_K1 expression in BALB/c spleen cells mentioned above, some preferential V_HX24-V_K1 pairing is indicated by the frequency of 40% we observed in this study. Perhaps this issue will only be resolved by an analysis of the frequencies of expression of V_H and V_K gene families both independently and in association, in unstimulated and mitogen-stimulated cells.

Idiotype expression of V_HX24+ monoclonal antibodies

Two mAbs from IDA10-treated mice use a V_K10 gene and react with IDA10. Both of these mAbs are of the IgG1 isotype which is very infrequent among LPS-stimulated B cells (Yuan and Vitetta, 1983). These cells may have received an Id-specific stimulus exclusively or in addition to a mitogen stimulus. An additional mAb from untreated mice binds IDA10 and express V_K1. Two other mAbs from

IDA10-treated mice bind weakly to IDA10 and use V_K1 and V_K19.

As is the case for IDA10, the idiotopes recognized by IDA16 and HyX24-14 can also be encoded by V genes other than those expressed in the antibodies against which they were developed.

Molecular basis of the idiotope defined by IDA10

Zaghouani et al. (1988) studied in Western blots the binding of IDA10 to antibodies in the native or reduced state. IDA10 bound to intact antibody, but not to isolated heavy or light chains. These results indicated that some contribution of both chains is necessary for expression of the idiotope defined by IDA10. Amino acid residues from both chains do not necessarily have to interact with IDA10, the function of one chain may be simply to mold its partner into a conformation appropriate for the Id-anti-Id reaction.

These authors also studied expression of this idiotope among 70 monoclonal antibodies derived from various mouse strains and having specificities for several antigens (self antigens, INU, dextran, phosphorylcholine, influenza virus hemagglutinin, nitrophenyl, trinitrophenyl). Five of these mAbs were found to inhibit the binding of IDA10 to 3-14-9.

The V gene usage of these mAbs was known through prior Northern blotting analyses using DNA probes specific for

various V gene families. Surprisingly, the antibodies binding IDA10 used gene families other than V_HX24 and V_K10. The V_H and V_K genes of these antibodies were sequenced and compared in an attempt to determine residues contributing to the idiotope. Instead of searching for homology throughout V_H and V_K regions, analysis was directed by the hydrophilicity profiles of the V regions. Amino acid sequences were processed according to the hydrophilicity algorithm of Hopp and Woods (1981). Amino acid residues having calculated hydrophilicity values greater than or equal to zero are considered likely to be surface-exposed in solution. Comparison of these restricted areas of the V regions revealed four surface-exposed segments having homology. One of these is in the V_H while three are in the V_L.

Kieber-Emmons and Köhler (1986) described a "surface variability analysis" in which the variability (calculated in the manner of Wu and Kabat, 1970) of the hydrophilicity value at a given position in a V_H or V_L was determined in a comparison of many sequences from different species. The resulting profile of calculated values for each residue in the sequences has large peaks in areas that have high variability in conjunction with hydrophilicity. These authors suggested that the portions of the V regions preserved on the surface through evolution, and having high variability, were likely to be regions which formed idio-

topes. Thus, they were designated "idiotope-defining regions."

The homologous surface-exposed regions of IDA10-reactive mAbs overlap with four of the idiotope defining regions (IDRs). Figure 11 extends the analysis of Zaghouani et al. to include previously published sequences of IDA10-binding mAbs, and those presented here. As mentioned in the Introduction, an analysis restricted to sequences derived from V_HX24 and V_K10 is not very instructive since "idiotope-specific" homology is lost in the high background of germline homology. Comparison of sequences from different V_H and V_K gene families highlights homology related to idiotope expression.

Although all of the Id^+ antibodies listed in Figure 11 have not been assayed for binding to IDA10 together in the same experiment, they may be roughly ordered based on the strength of this interaction using information from experiments in which several mAbs from this list have been used in different combinations. The antibodies from A48 through NL112-9 have "high" binding. MAbs from M56 through Y19-10 have "medium" binding, while mAbs from 10L15-15 through 2-28-9 are "low" binding. Also included in Figure 11 are the known sequences from antibodies found not to react with IDA10.

Clearly, the combination of V_HX24 and V_K10 is neither necessary nor sufficient to confer high binding to IDA10.

```
          [HEAVY CHAIN ]    [      LIGHT CHAIN        ]
                  IDR D     IDR A     IDR C         IDR E
                  ------    ------    -----         ---
         62      70        22       38        77
Id+:      !       ! *++     !*+++*   !** +     !  +**+
ABPC48    SLKDKFIISRDNAK    SCRASQ   QKPDGTV   NLEQEDI
AIDA10/1             ---               X-X--   ---P---
AIDA10/3  -----------X--              ------   -------
AIDA10/9  ------------X-    ------   -------   -------
AIDA10/14 -X----------X-                       XX-----
AIDA10/16 --------------              ------   -------
AIDA10/17 --------------                       X------
AIDA23/3  --R-E-------X-                       X--X---
3-14-9    --------------    ------    ------   -------
3-27-6    --------------    ------    ------   -------
Z26       TV-GR-T------R    T-KS--   ---GQSP   SVQA--L
10N17-4   --------------    ------   -------   -------
UPC10     --------------
NL112-9          -------    ---S--   ---GQSP   RV-A--L
M56       KF-G-ATLTS-KSS    --KS--   ---GQSP   SVQA--L
10L16-5   --------------    --SV--   -------   ---P---
PY102     ---GX-T------R    --KS--   K--GQPX   SVKA--L
Y19-10    RF-GRATLTV-KSS
10L15-15  --------------    ---S--   ---SQSP   RV-A--L
10L126-7  --------------
1-5-1     --------------    --G---   -------   -------
2-1-3     -------------A    --S---   -------   ---P---
2-1-10    --------------
2-28-9    --------------    --S---   -------   ---P---
-----------------------------------------------------------
Id-:
HPCG8     -V-GT--V---TSQ    --T--E
HPCG11    -V-GR--V---TSQ    --T--L
HPCG14    -V-GR-FV---TSQ    --T--E
HPCG15                      --LS--
J558      KFNGLATLTV-KSS
MOPC167   KF-GR--V---TSQ    ---S-K   -R-GQSP   RVKA--V
CP5       -I--R-T-F---D-    T-K---   ---WXSL   S--SD-T
J606      -V-GR-T----DS-    T-Q--A   ---GKAP   S--D--M
W3082     -V-GR-T----DS-    T----E   --QGKSP   S-QP--F
EPC109    -V-GR-T----DS-    T-Q--Q   ---GKAP   S--D--M
MOPC315   ---NRVS-T--TSE
129-48    TVTGR-T-------
MOPC460                     ---S--   ---GQSP   SR-A--L
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Figure 11. Comparison of surface-exposed segments of the heavy and light chains of monoclonal antibodies which do or do not react with IDA10. Sequences labelled Id$^+$ are ordered roughly in descending order of binding to IDA10. Numbering of variable regions and CDRs (complementary determining regions) are according to Kabat et al. (1987). IDRs

Some residues appear to correlate with Id expression. All IDA10-binding antibodies have Asn-Ala or Lys-Ser at positions 73-74, while only 2 of 11 Id- sequences have one of these combinations. In the light chains, 8 of 12 Id^+ sequences have Arg-Ala or Lys-Ser in positions 24-25, while 1 of 10 Id^- sequences has Arg-Ala in these locations. Gly or Gln is identified at position 42 in 16 of 17 Id^+ sequences, and only in two of six light chains from Id^- mAbs. Finally, 15 of 20 Id^+ light chains have Gln or Ala at position 80, and all have Ile or Leu at position 83. Two of six Id^- light chains have Ala at position 80, while only one of six has Leu at position 83. No Id^- antibody has the residues associated with Id expression in all four of the common surface-exposed areas.

Although some combinations of residues occur with higher frequency in Id^+ versus Id^- sequences, we cannot assume that these residues are all of those which contact

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Figure 11 continued.
(idiotope defining regions) are according to Kieber-Emmons and Köhler (1986). References for Id^+ sequences are: A48 V_H, Auffray et al., (1981); AIDA series V_H, Legrain et al. (1985a); A48 and AIDA series V_K, Legrain and Buttin, (1985); 1-5-1 and 3-14-9 V_H, Victor-Kobrin et al. (1987); 2-1-3, 2-1-10, and 2-28-9 sequences, Barak (1988); 3-14-9 V_K, Z26, M56, Y19-10, and PY102 sequences, Zaghouani et al. (1988). All other sequences are presented fully in Figures 7-10. All Id^- sequences are from Kabat et al. (1987) except for CP5 (Reininger et al., 1987) and 129-48 (Manheimer-Lory et al., 1986). Residues marked with asterisks (*) are conserved in the majority of murine V region sequences. Residues marked with pluses (+) may be important in expression of the idiotope recognized by IDA10.

the anti-Id, or that they are all themselves contacting residues. The amino acid residues we have focused on may affect idiotope expression by determining the spatial location of residues which may also occur in Id^- mAbs in positions inappropriate for Id-anti-Id interaction. A study of synthetic peptides corresponding to these surface-exposedregions, in combination with molecular techniques such as site-directed mutagenesis may permit further elucidation of the structural requirements for IDA10 binding.

An effect of anti-Id treatment?

The increased frequency of IDA10-binding mAbs derived from IDA10-treated mice did not reach a standard criterion of statistical significance (α=0.05) by the test we considered most appropriate (Fisher exact test). However, the probability we calculated was not much higher (approximately 0.08). Furthermore, we consider it suggestive of an effect of anti-Id treatment that both 10L16-5 and 10N17-4 are of the IgG1 subclass. IgG1-producing hybridomas were frequent among those selected for Id expression after immunization with monoclonal IDA10 or IDA23 (Legrain et al., 1985a).

Rubinstein et al. (1983) showed that the levels of A48Id+ antibodies are not increased by neonatal anti-Id antibody injection in the absence of an antigen stimulus.

This strongly suggests that in the experiments reported here, specific antigen or mitogen is capable of stimulating Id+ B cells which have interacted with anti-Id antibody in a manner rendering them preferentially inducible relative to antigen or mitogen-sensitive Id^- cells. That is, neonatal administration of very small amounts of anti-Id favors expansion of Id+ clones by a subsequent antigen or mitogen stimulus without directly causing expansion of Id+ cells. This process is distinct from more vigorous immunizations utilizing anti-Id antibodies emulsified in adjuvants and/or coupled to immunogenic carrier molecules where Id+ clones are activated without additional antigen stimulation (Bona et al., 1981).

Nucleotide sequence analysis of 12 V genes from these hybridomas indicates that the antigen binding patterns and idiotypes of these mAbs are predominantly germline encoded. Nevertheless, association of predominantly germline V_H and V_K genes will not always reproduce an idiotype, while association of V_H and V_K genes different from the "original" pair that defined the idiotype may also form the idiotype. It remains to be seen if anti-idiotypes in this system are also germline-encoded. If Id-anti-Id interactions are to be invoked in determining the expressed antibody repertoire (Jerne, 1975), it is important to determine the requirements for the appearance of particular Ids or anti-Ids, i.e., whether or not their expression is

dependent on somatic mutation (stimulation of clones by antigen or mitogen, or perhaps other Ids or anti-Ids).

Thus, the association of V_HX24 with V_K10, levan-binding and IDA10-binding in mAbs arising from anti-A48Id treatment and levan-immunization may be ascribed to two interdependent influences. The V_HX24-V_K10 association previously observed is not clearly ascribable to levan immunization or anti-Id treatment. No mAbs with antigen binding similar to A48 or U10 which use different V_H or V_K genes have been found. In addition, binding to polyclonal anti-A48Id appears to be more dependent (but not exclusively so) on this specific V_H-V_K association than binding to monoclonal anti-A48Ids. We detected no mAbs among hybridomas derived from mitogen stimulated spleen cells which bound to the polyclonal anti-A48Id, and Goldberg et al. (1983) found only one mouse myeloma protein (among 178 mouse and human myelomas tested) which bound to polyclonal anti-A48Id, MOPC167. This myeloma protein uses V_HS107 and V_K24 genes (Rudikoff and Potter, 1976; Potter et al., 1982).

And what of regulatory idiotopes?

To reiterate, the regulatory idiotope hypothesis predicts that the idiotopes expressed on immunoglobulin molecules do not function equally as stimulators or as targets of anti-Id lymphocytes and antibodies. From the

perspective of the antibody, this situation may simply be a result of the frequency with which particular idiotopes are expressed in a given immune response. Only those found in sufficiently high concentration may elicit anti-Id antibodies or T cells. From the point of view of the lymphocyte, preferential recognition of particular idiotopes may result from biases in the repertoire (more frequent paratopes complementary for a given idiotope) or from peculiarities (antigen presentation?) inherent in the response to autologous immunoglobulin.

These factors may operate in concert to determine which idiotopes behave in a regulatory fashion, that is, those whose expression can be controlled by small amounts of Id or anti-Id antibodies, or demonstrable populations of Id-specific regulatory cells.

Does IDA10 define a regulatory idiotope? We have seen that this idiotope occurs on antibodies with different specificities, and that small amounts of IDA10 influence expression of this idiotope. Do T cells play a role? We cannot say for certain, but recall that Rubinstein et al. (1983) did not observe T cells participating in expansion of the A48Id induced by polyclonal anti-A48Id antibodies and antigen. The nucleotide sequences reported here indicate that this idiotope can be encoded by germline genes. Injection of small amounts of IDA10 followed by a B cell mitogen activates Ab_1-like clones. This parallels the

results of Legrain and Buttin (1985) after hyperimmunization with IDA10. The body of data analyzing the expression of A48 idiotopes utilizing monoclonal antibodies supports the regulatory idiotope hypothesis. Attempts to define the role played by T cells, as well as similarly detailed analyses of other idiotype systems are necessary before definitive statements regarding the hypothesis may be made.

Regarding the A48Id system, it behooves us now to inquire about V gene usage in other selected groups of hybridomas: levan immunization selected for levan binding or IDA10 binding, and mitogen stimulation selected for the same characteristics. These experiments will complete the analysis, in this system, of the frequencies with which various specificities, idiotypes and V genes occur together under different circumstances. New techniques of in situ hybridization of unfused B cell colonies (Schulze and Kelsoe, 1987) and their adaptation for use with labelled immunoglobulin reagents will allow this analysis to performed rapidly with very large numbers of clones yielding more precise information.

The regulatory idiotope hypothesis, and the network theory itself, have not yet met their final challenge. What form the critical question will take is not clear, however, the molecular tools required for its construction are being rapidly set in order.

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