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ANALYSIS OF THE GENERATION OF ANTIBODY DIVERSITY AND IDIOTYPE SPECIFIC IMMUNOREGULATION IN THE MURINE ANTI-ALPHA 1->3 DEXTRAN RESPONSE

by

ROBERT CHARLES STOHRER

A DISSERTATION

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ABSTRACT OF DISSERTATION
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Title Analysis of the Generation of Antibody Diversity and Idiotype Specific Immunoregulation in the Murine Anti-Alpha 1->3 Dextran Response

The network theory suggests that the immunoglobulin repertoire may be regulated through the unique idiotypic determinants expressed by each individual antibody. The response of BALB/c mice to alpha 1->3 dextran (DEX) is characterized by dominant expression of the lambda light chain, the expression of 2 individual idiotypes (IDI) present on the J558 and M104E myeloma proteins respectively and the expression of a cross-reactive idiotype (IdX) common to both myeloma proteins. Three monoclonal anti-idiotype antibodies (MAIDs) specific for each of the 2 IdIs or the IdX were administered (i) to adult BALB/c mice (ii) directly to neonatal mice or (iii) to neonatal mice by maternal routes. In each case the MAIDs suppressed only the idiotype bearing portion of the anti-DEX response to which they were directed.

The splenic focus assay was used to examine B cell clonal precursors for the production of DEX specific antibodies in the following aspects: (i) the effect of carrier primed T

cells on isotype and idiotype expression, (ii) the somatic generation of antibody diversity using a large panel of MAIDs as probes for detecting V region variants and (iii) the ontogeny of the idiotope repertoire of the anti-DEX response. The T cell independent (TI) and the T cell dependent (TD) B cell precursor responses to DEX appear to differ very little with respect to idiotope expression. The isotypes expressed by precursors responding to the TI form of DEX (dextran B1355S) were predominantly IgM, while the TD form of DEX (DEX linked to hemocyanin) resulted in a large increase in IgA and multiple isotype secreting foci. Idiotope differences between different isotypes were noted in about half of the multiple isotype secreting foci, indicating that somatic variation of V genes is occurring within clones at a high rate. B cell precursors from neonatal mice expressed a different but highly diverse idiotype pattern when compared to adult precursor idiotope patterns.

The results presented in this dissertation suggest that expression of the idiotope repertoire can be specifically regulated through idiotype interactions and the anti-DEX repertoire acquired during neonatal development is partially the result of somatic mutation events.

Abstract Approved by: Committee Chairman John F. Kearney

Program Director Richard V Campana

Date 12/5/88 Dean of Graduate School Dennis A Annwell

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INTRODUCTION

Review of the literature.

The vertebrate immune system possesses an exquisitely specific array of mechanisms for discriminating between self and non-self. Perhaps the most important of these mechanisms is the ability to synthesize an almost infinite variety of antibodies specific for almost any foreign antigen the organism may encounter. Immunologists have speculated over the question of how an organism's genes could possibly code for such an immense variety of antibodies and how precise expression of these antibodies could be regulated. The repertoire of immunoglobulins has been estimated to be well above a million and probably higher (1,2). Sequence analysis of myeloma proteins showed that the amino-terminal region of antibodies is diverse while the carboxy-terminal region is made up of several conserved sequences (3,4). The amino-terminal region was termed the variable (V) region and the carboxy-terminal region was termed the constant (C) region. This was somewhat of a paradox, since it was difficult to determine the genetic mechanisms that could explain the variability of only one part of a polypeptide. This seeming paradox was explained by a theory first proposed by Dreyer and

Bennett (5). They theorized that each immunoglobulin chain was coded for by two separate genes, one gene coding for the constant region (C) and one gene coding for the variable region (V). The C gene family could be relatively small while the V gene family would have to be exceedingly large to accommodate all antigens.

If one accepts the figure of 1 to 100 million different molecules as representative of the antibody repertoire, then even with separate V and C genes 1 to 100 million different V genes would still be required. As early as 1959 somatic mutation was postulated to play a role in the generation of antibody diversity. Somatic mutation, if it were to occur, was proposed to reduce the number of V genes necessary to explain the predicted antibody repertoire (6,7). The first evidence that somatic mutation may occur within immunoglobulin genes and be expressed at the protein level was obtained by comparing the amino acid sequences of lambda-1 light chains from 19 different myeloma proteins (8,9). Twelve of the 19 sequences were identical, while the other 7 were all different by only 1 to 3 amino acid residues. The 7 variant sequences were interpreted as being the results of somatic point mutations. It was later shown that mice possess only 1 gene coding for the lambda-1 light chain V and C regions which further reinforced the idea that somatic mutation was responsible for the variants (10,11). Amino acid sequences of heavy chain variable

regions also strongly support the concept of somatic mutation as a diversifier. Sequence analysis of BALB/c anti-DEX proteins has shown a tremendous amount of diversity. Of the 21 myeloma and hybridoma protein sequences obtained, no two are alike, which indicates that somatic mutation is probably at work or a large number of germline genes are used in the anti-DEX response (12,13 and B. Clevinger, personal communication).

The combination of a vast number of V_H and V_L genes coupled with the ability to somatically mutate and heavy and light chain combinaring anti-DEX response, and both the J558 and M104 idiotypes (except in a few isolated cases). Mice heterozygous for the a allotype responded the same as mice homozygous for the a allotype mice indicating the dominance of the lambda bearing portion of thenes coding for V regions associated with each light chain and heavy chain locus. In the germline state the lambda and the kappa chain variable region genes consist of a V segment and a joining (J) segment separated by intervening sequences of DNA which are spliced out when the V and J segments are brought together during differentiation (10,11,14). The heavy chain V region is encoded by three separate genes, the V (variable), the diversity (D), and the J (joining), each separated by an intervening sequence which is spliced out prior to transcription (15,16). In addition to this combinatorial diversity it has been shown

that DNA splicing events can be somewhat imprecise. The junctional regions between each of the V region gene segments have been shown to be "hot spots" of variability (17). It appears that part of the codon (at the junction such as position 96 for kappa chains) is supplied by one gene segment and the remainder supplied by the other gene segment (18,19). It has been shown that position 96 in kappa chains can be deleted entirely or an insertion can occur (18). Finally, the random association of the two (light chains) or three (heavy chain) different gene segments greatly increases the amount of antibody diversity which can be expressed by an individual immune system.

Recent sequence studies have strengthened the idea that somatic mutation plays an important role in the generation of antibody diversity. The best example of the role somatic mutation may play is illustrated by recent analysis of hybridomas binding phosphorylcholine (PC). It has been shown that the majority of PC binding proteins are encoded by only 1 V_H gene and express the T15 idiotype (20). Nineteen T15 positive myeloma and hybridoma proteins were sequenced and 10 were identical to that expected from expression of a germline V_H gene. Since the other 9 proteins all differed from the germline sequence and each other by substitution of 1 to 8 amino acid residues these variant molecules probably resulted from somatic point mutations (20,21). Somatic mutations were also shown to

have occurred in the V regions of the kappa chains from the MOPC167 and MOPC511 myeloma proteins (22). Studies to date show that antibody diversity is generated by 4 basic mechanisms: first, the combinatorial diversity created by the various combinations of V, D (H chain only), and J gene segments; second, junctional diversity created by the imprecise joining of the V region gene segments; third, somatic point mutations appear to occur quite frequently in all of the V region gene segments; fourth, the combination of different H and L chains.

The repertoire of antibody specificities was known to be very large even before confirmation by amino acid sequence data. It was realized that because of their diversity, immunoglobulins could themselves be antigenic. During studies of immunoglobulin allotypes Kunkel et al. (23) and Oudin and Michel (24) discovered that antibodies possessed other unique antigenic determinants that associated with certain antigen specificities. These variable region associated determinants were shown to differ between antibodies with similar specificities but from individual animals. These unique antigenic determinants on immunoglobulin molecules were termed idiotypes by Oudin. Following the discovery of immunoglobulin idiotypes, Niels Jerne formulated his network theory of immunoregulation (25). Jerne reasoned that since each antibody possessed a set of unique variable

region associated antigenic determinant(s), these antibodies (Ab-1) could be recognized and regulated by a complementary set of anti-idiotype antibodies (Ab-2). These anti-idiotype antibodies (which in turn may bind their own distict antigen) would themselves be regulated by another set of anti-(anti-idiotype) antibodies (Ab-3) some of which could be the same as Ab-1. These interacting sets of antibodies would link B cells into an extensive network of interacting V regions. Jerne also referred to the immune system as a "web of V-domains" (26). A number of investigators have since shown that passive administration of a small amount of anti-idiotype antisera will specifically suppresses the antibody response that is associated with that idiotype (27,28,29). Administration of anti-idiotype to adult mice induced a temporary state of suppression while administration of anti-idiotype to neonatal mice led to a chronic state of idiotype suppression (30,31). In addition to inducing a state of suppression, administration of appropriate amounts of anti-idiotype antibodies has also been shown to specifically enhance idiotype positive portions of antibody responses, including silent clones which are not expressed in normal immune responses (32,33).

Most of the initial studies on idiotype regulation were done using heterologous antisera which recognize a large number of different antibodies. The development of

monoclonal antibody technology has permitted a more detailed analysis of idiotype regulation than was possible in previous studies. A monoclonal anti-idiotype antibody (MAID) will recognize a smaller group of idiotype-bearing antibodies than conventional antisera. A MAID recognizing TEPC-15 was used to specifically suppress the T15 portion of the anti-PC response (34). Instead of the usually dominant T15 idiotype bearing anti-PC antibodies, variant molecules were detected which were negative for the MAID used for the suppression but were still T15 positive using other anti-idiotype antibodies recognizing T15. The cross-reactive idiotype (CRI) present on most anti-ARS antibodies was also suppressed in A/J mice using CRI specific MAIDS (35). MAIDs specific for (4-hydroxy-3-nitrophenyl)acetyl (NP) binding antibodies were used at high doses to specifically suppress the appropriate idiotype, and at lower doses to specifically enhance the idiotype that the MAID was directed against (36). Idiotype specific suppression can also be accomplished by natural passive administration, such as through maternal routes. The F1 progeny from BALB/c males crossed with SJL females which were immunized with J558 prior to mating were suppressed for the J558 idiotype (37). Mice were suppressed with rabbit anti-idiotype antisera specific for the cross-reactive p-azophenylarsonate idiotype then hyperimmunized with antigen. Sera from these

mice were transferred into pregnant mice which resulted in suppression of the offspring for the cross-reactive idiotype (38). A monoclonal anti-(anti-idiotype) antibody originally derived from mice undergoing a normal immune response to PC was used to specifically suppress the T15 idiotype by mechanisms not fully understood (39). The majority of these studies indicated that the specificities of the anti-idiotype antibodies administered precisely reflect the idiotypes which are affected. The studies summarized in this thesis attempt to explain some of the mechanisms involved in the generation and regulation of the immunoglobulin repertoire.

Scope of thesis

The scope of this thesis was to examine the diversity of the anti-DEX antibody response and to study aspects of regulation of this response by the use of MAIDs as markers for B cell clones. The anti-alpha 1->3 dextran (DEX) system was chosen for all of the major studies described in this thesis because of the apparent restricted nature ofthe response and because the antibodies produced in the BALB/c response to DEX are predominantly lambda light chain positive. Amino acid sequence data have allowed correlation of idiotope expression to primary structure. Virtually all monoclonal anti-idiotype antibodies produced

are kappa light chain positive reflecting the normal kappa lambda ratio of mouse immunoglobulins. All of the MAIDs produced against J558 and M104 are distinct and appear to recognize many different idiotopes expressed by anti-DEX proteins, allowing a detailed analysis of idiotope expression.

Manuscript I (J. Immunol. 131:1375, 1983) deals with: (i) the diversity of MAIDs produced against the J558 myeloma protein and (ii) the use of three monoclonal anti-idiotype antibodies to specifically suppress portions of the BALB/c anti-DEX response. Both neonatal and adult suppression was examined and compared. The idiotype expressed by normal BALB/c mice was determined using monoclonal antibodies for the first time, and the in vivo effects of administration of monoclonal anti-idiotype antibodies were determined.

Manuscript II (in press in Idiotypy; H. Kohler, J. Urbain and P. Cazenave, editors; Academic Press) is an extension of the neonatal idiotype suppression studies of manuscript I. The work in the DEX system in manuscript II is my own while the work on the PC system was done by B. Pollok. The ability of monoclonal anti-idiotype antibodies to suppress neonatal mice via the maternal route was the focus of this portion of the thesis.

In manuscript III (in press J. Exp. Med.) the splenic focus assay was used to examine the repertoire of BALB/c B

cell precursors responding to DEX *in vitro*. Isotype expression on the precursor level was compared to that expressed on hybridoma proteins and in serum antibody. A large panel of monoclonal anti-idiotype antibodies was used to dissect the idiotope expression of DEX stimulated B cell precursors. Comparisons of isotype and idiotype expression were made between precursors responding to T-independent and T-dependent forms of DEX. The role that somatic mutation may play in increasing anti-DEX antibody diversity was also examined using the panel of monoclonal anti-idiotype antibodies as probes.

Manuscript IV (submitted to J. Immunol.) is a study of the ontogeny of the anti-DEX response analyzed at the B cell precursor level. The frequency of anti-DEX precursors was determined at various stages of neonatal development. Isotype and idiotype expression by B cell precursor progeny was compared at various times during the ontogeny of the anti-DEX response.

These studies have been undertaken to answer questions pertaining to: (i) defining the idiotope repertoire expressed by anti-DEX antibodies, (ii) generation of idiotope diversity through somatic mutation events, (iii) the regulatory effects of MAIDs *in vivo* and (iv) the ontogeny of the anti-DEX response.

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ANALYSIS OF THE ANTI-α1->3 DEXTRAN RESPONSE WITH MONOCLONAL ANTI-IDIOTYPE ANTIBODIES[1]

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[2]Abbreviations used in this paper: DEX, α1->3 dextran; MAID, monoclonal anti-idiotype antibody; IdI, invididual idiotype; IdX, cross-reactive idiotype; TI, T cell independent; ELISA, enzyme linked immunosorbant assay; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing; PC, phosphorylcholine; NP, nitrophenyl.

ABSTRACT

The antibody response to α1->3 dextran $(DEX)^2$ in BALB/c mice consists of a family of closely related yet highly heterogeneous molecules. Although these antibodies have been previously characterized both idiotypically and structurally, detailed analysis of responding clones has not been possible using conventional anti-idiotype antibodies. Monoclonal syngeneic and allogeneic anti-idiotype antibodies (MAIDs) specific for anti-DEX antibodies were used in this study to dissect the serum antibody response to DEX in BALB/c mice. The MAIDs constructed showed considerable heterogeneity by isoelectric focusing and by their binding characteristics to a series of DEX specific myeloma and hybridoma proteins. The predominant heavy chain isotype of these MAIDS was γ_1. These antibodies were used to identify individual idiotypic structures (IdI) on J558, or M104E as well as cross-reactive determinants common to both (IdX). Although both IdX and IdI MAIDS were obtained, IdI specific antibodies were obtained more frequently. BALB/c mice immunized with DEX produced antibodies expressing both IdIs but in highly variable amounts. A large percentage of, but not all, DEX specific antibody, could be accounted for by IdX bearing antibodies. Suppression of adult and neonatal mice by IdI specific MAIDs was effective with precise elimination of only those clones expressing IdI determinants leaving the total λ bearing anti-DEX response intact. Suppression of adults and neonates by an IdX specific MAID resulted in a temporary and partial suppression of the total λ bearing anti-DEX response, along with total suppression of the IdX portion of the response. Unlike other systems these monoclonal antibodies produce only

suppression and under a variety of conditions enhancement of anti-DEX responses has not been observed.

INTRODUCTION

The immune response of BALB/c mice to α1->3 dextran (DEX), a thymus independent (TI) antigen, has been shown to consist almost entirely of antibodies bearing the λ light chain (1). Earlier studies on the diversity of the anti-DEX response in BALB/c mice revealed a restricted serum antibody response with respect to isoelectric focusing patterns (2). Furthermore, analysis with heterologous anti-idiotypic sera demonstrated that the majority of serum anti-DEX antibodies in BALB/c mice possessed idiotypic determinants cross-reactive with the BALB/c myeloma proteins J558 or M104E (1,3). More recently, however, amino acid sequence analysis of (DEX binding) hybridoma proteins has revealed that this family of antibodies, while showing many sequence similarities, is also highly diverse (4,5). The sequence analyses have shown that the greatest amount of heterogeneity within the group of anti-DEX proteins exists in the D region which is in turn largely responsible for individual idiotype identity (IdI) (5). Changes in the D region appear to radically affect expression of the IdI while not changing the ability to bind DEX to any great extent. Correlation of the cross reactive idiotype (IdX) to primary structure has also been made. It appears that V_H residue 55, a glycosylated asparagine, may be necessary for IdX expression (5). Although the response to DEX in BALB/c mice has been shown to be heterogeneous it is well characterized structurally and provides an ideal system for studies on regulation of idiotype expression.

In this study a panel of monoclonal antibodies was prepared against individual J558 and M104E idiotypes and against the IdX

present on most BALB/c anti-DEX antibodies. These monoclonal anti-idiotype antibodies were then used as probes to determine the quantities and proportions of the IdI and IdX-positive λ-bearing serum antibodies to DEX in immune BALB/c mice. Monoclonal anti-IdI and anti-IdX antibodies were able to suppress their respective idiotypes in vivo in neonatal and adult BALB/c mice. The total λ bearing anti-DEX response remained unchanged after suppression with IdI specific antibodies with deletion of only the appropriate IdI bearing antibodies. Administration of anti-IdX transiently and incompletely suppressed the λ bearing anti-DEX response in BALB/c mice.

MATERIALS AND METHODS

Mice. Adult A/J, SJL/J, BALB/c, BALB/cxSJL/J, BALB/cxA/J, were purchased from The Jackson Laboratory. Neonatal BALB/c mice and nude BALB/c mice were obtained from the Tumor Institute Immunocompromised Animal Facility, University of Alabama in Birmingham.

Antigens. Dextran B1355S, average M.W 4 x 10^7 daltons (35% α1->3 linkages) derived from Leuconostoc mesenteroides was a gift from Dr. Slodki, DEX-BSA was a gift from R. Ward (6). Nigerotriose prepared by Dr. Johnston (7) was a gift from W. Schuler.

Myeloma Proteins. Hybridoma HDEX9 was provided by Dr. Brian Clevinger (5). Mouse myelomas J558 (α,λ), M104E (μ,λ) and hybridoma HDEX9 (μ,λ) were grown as ascites tumors in Pristane primed BALB/c mice and the proteins were purified by absorption to α1->3 dextran coupled to Sepharose (8), followed by elution with 4M guanidine, pH 7.8. Purity was assessed in our laboratory by testing each purified

myeloma protein against a panel of alkaline phosphatase labeled goat anti-mouse isotype in a solid phase direct binding ELISA (9).

Monoclonal anti-idiotype antibodies. Monoclonal anti-J558 or anti-M104E antibodies (MAIDS) were prepared by immunizing A/J, SJL/J or BALB/c mice as described previously (9,10). Antibodies reacting with J558 or M104E were then screened against a panel of purified myeloma proteins of each mouse isotype. The antibodies which appeared to react with idiotypic determinants on J558 or M104E were subjected to further analysis in a solid phase inhibition assay described previously (10). The results of eight independent fusions are described in this paper and the characteristics of anti-idiotype antibodies selected for further studies are described in Table 1.

Purification of anti-idiotype antibodies. Hybridoma lines secreting the monoclonal anti-idiotype antibodies were cultured in RPMI 1640 with 20% fetal calf serum and then purified by affinity chromatography. Antibodies which were of the IgG_{2a} class were purified by elution from Protein-A sepharose columns. Antibodies of IgG_1 or IgM classes were purified by absorption to and elution from purified goat anti-mouse immunoglobulin coupled sepharose (11). All antibodies were dialyzed against borate buffered saline and stored in the same buffer with 0.1% sodium-azide at 4°C.

Two dimensional gel electrophoresis. Two dimensional gel electrophoresis was carried out by a modification of the procedure described by Perlmutter et al. (12). Antibody heavy and light chains were separated by SDS-polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli (13) and then whole IgG molecules or the electrophoretically separated light chains were subjected to

isoelectric focusing (IEF) by methods described in detail elsewhere (14).

Five μg samples of purified antibody were loaded into each sample well and approximately 10 μg of purified fluorescamine-labeled (Fluram, Hoffman LaRoche, Nutley, NJ) reduced and alkylated M104E were included in two wells on each gel as a marker to locate the L chain band by fluorescence. A strip of the SDS-PAGE gel containing the L chains localized by the fluorescent λ chains was excised and transferred to equilibration buffer (7 M urea, 1% NP-40, 1% pH 3.5-10 ampholytes) for 45-60 min.

The equilibrated SDS-PAGE gel strip containing the light chains was laid across the top of and in contact with the IEF gel containing an ampholine mixture of 90% pH 3.5-10 and 10% pH 4-6 ampholines. The light chains were then focused at 200 V for 30-60 min, then at 4.5 mA constant current and a maximum of 2000 V for 14-16 hours at 4° C.

Upon completion of focusing, the light chains were precipitated in the gel using 5% trichloroacetic acid and 5% sulfosalicylic acid. The gels were then washed 2X in 40% ethanol and stained with Coomassie Blue. Photographs were made with Tri-X film (Kodak, Rochester, NY).

ELISA assays for serum antibody measurements. Polystyrene microtitration plates (Flow Laboratories, Hamden, CT) were coated with purified antiidiotype antibodies EB3-7, SJL18-1, CD3-2 or DEX-BSA at a concentration of 2 μg/ml in borate-buffered saline (BS) pH 8.5 for 4 hrs at 37° C. The wells were then blocked with 1% BSA-BS for 30 mins at room temperature. Two-fold serial dilutions of individual serum samples diluted 1:40 in 1% BSA-BS were added in duplicate and incubated at 4° C overnight. Purified myeloma or hybridoma derived anti-

DEX antibodies HDEX9 or M104E were used as standards at 80 μg/ml and gave the same results (5). The microtitration plates were then washed and alkaline phosphatase labeled goat anti-λ was added and incubated overnight at 4° C. The plates were then washed and substrate, p-nitrophenyl phosphate disodium (Sigma Chemical Co.) in diethanolamine buffer pH 9.8 was added at a concentration of 1 mg/ml. The enzyme reaction was stopped after 15-30 minutes by the addition of 3N NaOH. After allowing mixing of NaOH and substrate, OD_{405} was measured for each well using an automated ELISA reader (Flow Laboratories) and anti-DEX serum antibody and idiotype concentrations were calculated from standard curves constructed from antibody standards included on each plate.

RESULTS

Isoelectric focusing spectra of IgG monoclonal antibodies. In the course of this study 12 anti-IdI, 7 anti-IdX, 2 anti-V_H DEX and 1 anti-λ hybridomas were cloned and selected for further analysis (Table 1). All but three of the hybrids were IgG_1 and of the 12 anti-IdI hybrids, 11 were specific for IdI of J558 and only one was produced for IdI of M104E (Table 1). Our monoclonal IgG antibodies to both the IdX and IdI determinants were either of IgG_1 or IgG_{2a} isotypes (Fig. 1). When these purified IgG antibodies were subjected to isoelectric focusing (IEF), they exhibited a high degree of diversity when analyzed as intact molecules (Fig. 1) or as isolated light chains (Fig. 2). Not one IEF pattern repeat was observed within six independent fusions from different mice. These results are similar to those published by Schuler et al. (15) which showed considerable

TABLE I

MONOCLONAL ANTI-IDIOTYPE ANTIBODIES, IMMUNOGLOBULIN CLASSES AND SPECIFICITIES

Monoclonal Antibody	Strain	Isotype	Reactivity
EB3-16	A/J	γ_1,κ	IdI (J558)
AB3-7	A/J	γ_1,κ	IdI
BD5-3	A/J	γ_1,κ	IdI
RD3-1	A/J	γ_1,κ	IdI
UB6-1	A/J	γ_1,λ	IdI
JB2-1	A/J	γ_1,κ	IdI
B6-10	BALB/c	γ_1,κ	IdI
D8-3	BALB/c	μ,κ	IdI
EB3-7-2	A/J	γ_1,κ	IdI
TD6-1	A/J	γ_1,κ	IdI
PC4-1	A/J	γ_1,κ	IdX
CD3-2	A/J	γ_1,λ	IdX
HC4-1	A/J	γ_{2a},κ	IdX
LA6-9	A/J	γ_{2a},λ	IdX
LA4-8	A/J	γ_{2a},κ	IdI
AB2-7	A/J	γ_{2a},κ	IdX
FB1-8	A/J	γ_{2a},κ	IdX
WA2-1	A/J	γ_{2a},κ	V_H DEX
HB5-2	A/J	γ_{2a},κ	V_H DEX
UD6-1	A/J	γ_{2a},κ	anti-λ
N-20	BALB/c	μ,κ	IdX
SJL18-1	SJL	μ,κ	IdI (M104)

Figure 1. Isoelectric focusing spectra of affinity purified monoclonal anti-idiotype antibodies. The numbers at the top of the figure represent clone numbers. Antibodies were arranged on the gel grouped as IdI J558 or IdX in γ_1 or γ_{2a} heavy chain isotype groups. The numbers 1-6 indicate that a given hybridoma is a fusion product from one of six individual mice.

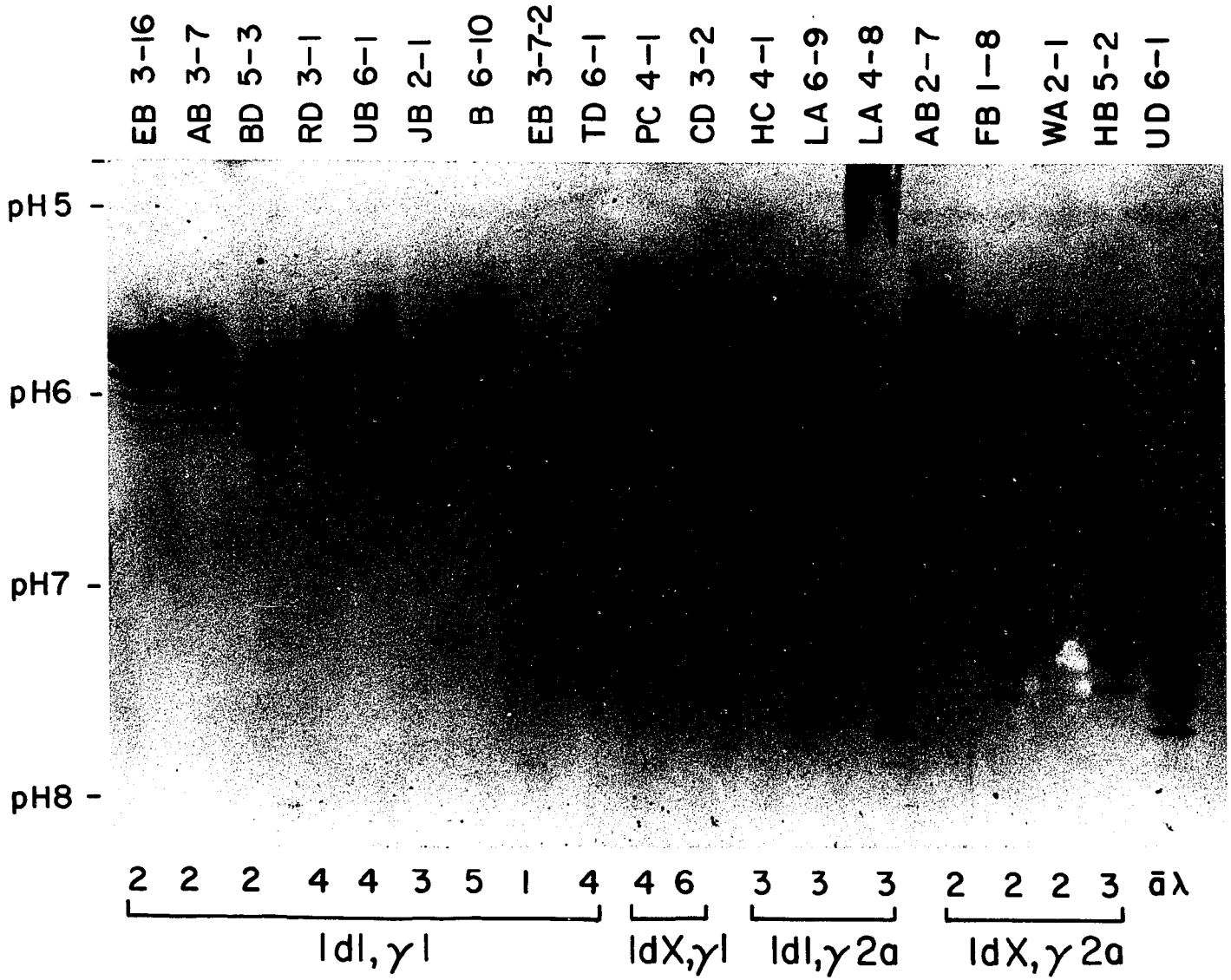
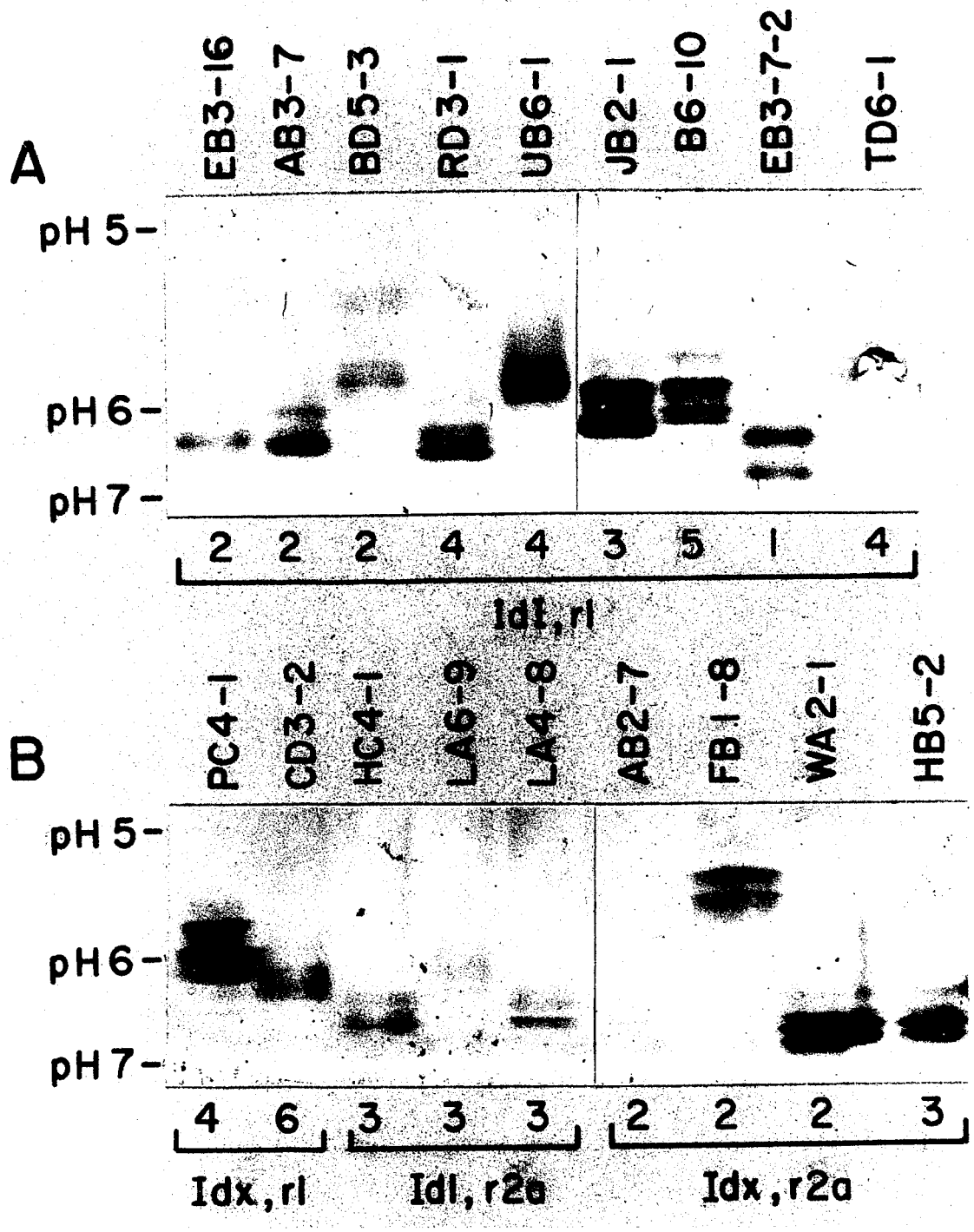

EB 3-16
AB 3-7
BD 5-3
RD 3-1
UB 6-1
JB 2-1
B 6-10
EB 3-7-2
TD 6-1
PC 4-1
CD 3-2
HC 4-1
LA 6-9
LA 4-8
AB 2-7
FB 1-8
WA 2-1
HB 5-2
UD 6-1
pH5
pH6
pH7
pH8
2 2 2 4 4 3 5 1 4
4 6
3 3 3
2 2 2 3
ā λ
Idl, γ1
IdX, γ1
Idl, γ2a
IdX, γ2a

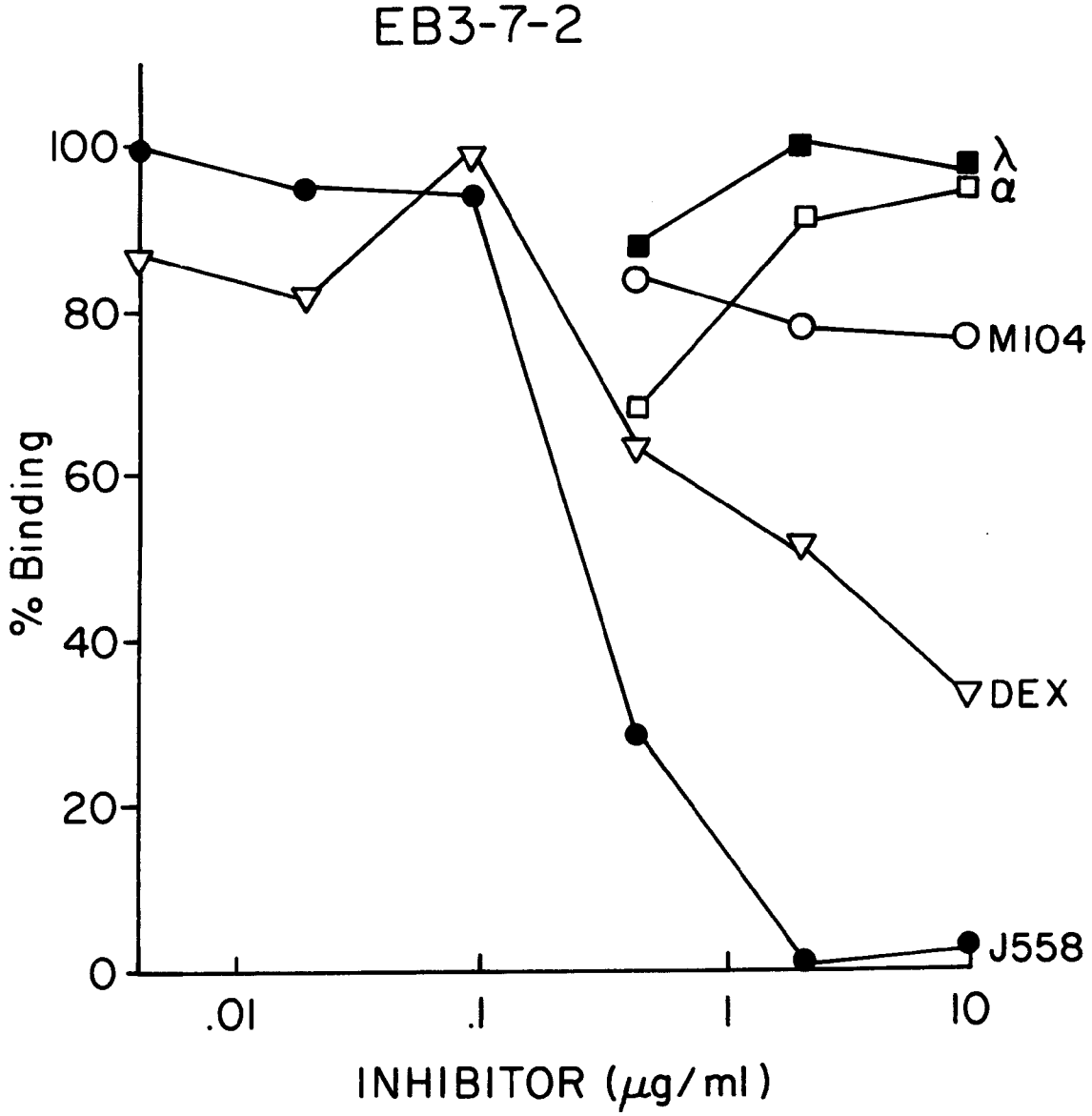
Figure 2. Isoelectric focusing spectra of light chains from the same antibodies described in Fig. 1 isolated by SDS-PAGE and transferred to an IEF gel. In panel A, all γ_1 IdI specific light chains are analyzed. In panel B, IdX γ_1 and IdX γ_{2a} associated light chains were analyzed. The numbers describe individual mice from which the hybridomas were isolated.

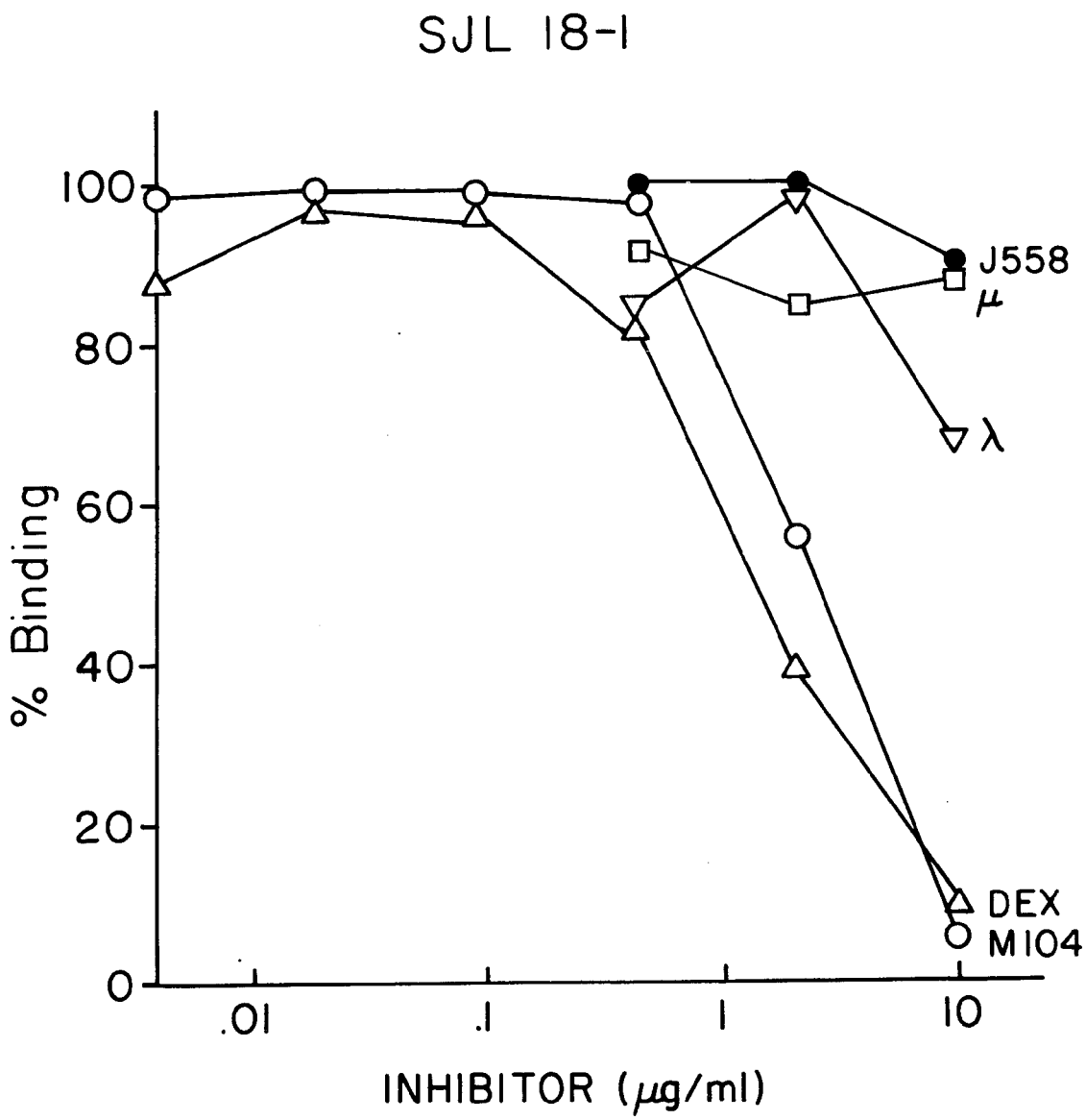

A
EB3-16
AB3-7
BD5-3
RD3-1
UB6-1
JB2-1
B6-10
EB3-7-2
TD6-1
pH 5-
pH 6-
pH 7-
2 2 2 4 4 3 5 1 4
Idl,rl
B
PC4-1
CD3-2
HC4-1
LA6-9
LA4-8
AB2-7
FB1-8
WA2-1
HB5-2
pH 5-
pH 6-
pH 7-
4 6 3 3 3 2 2 2 3
Idx,rl
Idl,r2a
Idx,r2a

heterogeneity in IEF patterns of syngeneic anti-idiotype antisera raised against hapten-modified J558.

Specificity of monoclonal anti-idiotype antibodies. The specificities of three selected monoclonal anti-idiotype antibodies were then determined using a solid phase inhibition ELISA. Hybridoma and myeloma proteins, hapten or DEX were used to inhibit binding of the alkaline phosphatase conjugated idiotype bearing protein to the plate bound purified anti-idiotype antibody. Figure 3a shows the idiotype specificity of each of these monoclonal antibodies for J558. DEX and J558 inhibited binding of AP-J558 to the plate bound EB3-7 as expected. Isolated J558 α and λ chains, M104E, and several unrelated mouse myeloma proteins all failed to inhibit binding of AP-J558 to the plate bound EB3-7. The monoclonal anti-M104E, SJL18-1 (μ,κ) was highly specific for M104E as shown in Figure 3b. Only M104E and DEX inhibit AP-M104E binding to plate bound SJL18-1. J558 and other unrelated myeloma and hybridoma antibodies all failed to inhibit AP-M104E binding. Isolated M104E μ chain and λ chain do not inhibit. Figure 3c shows that J558, M104E and DEX inhibit binding of AP-M104E to plate bound anti-IdX, CD3-2. The α1->3 linked trisaccharide, nigerotriose, inhibits binding of the appropriate myeloma to EB3-7, SJL18-1, and CD3-2 (Fig. 4) indicating that both the IdI and the IdX idiotopes are associated with the antigen binding regions of J558 and M104E in agreement with previous results using heterologous anti-idiotypic antisera (16). In summary EB3-7 and SJL18-1 recognize IdI determinants associated with J558 and M104E, respectively, while CD3-2 recognizes a determinant common to both. In the case of all three anti-

Figure 3. Solid phase inhibition ELISA assays showing specificity of monoclonal anti-idiotype antibodies. (a) Binding of EB3-7 to J558 is inhibited by the homologous protein J558 and α1->3 dextran but not by M104 or free α and λ chains. (b) binding of SJL18-1 to M104 is inhibited by M104 and DEX but not J558. Binding is not inhibited by free μ and λ chains. (c) Binding of the IdX specific antibody CD3-2 to J558 is inhibited by M104, J558, DEX but not by free M104 μ chains, J558 α chains or free λ chains.


EB3-7-2
100
80
60
40
20
0
% Binding
.01
.1
1
10
INHIBITOR (μg/ml)
λ
α
M104
DEX
J558


SJL 18-1
100
80
60
40
20
0
% Binding
.01
.1
1
10
INHIBITOR (μg/ml)
J558
μ
λ
DEX
M104

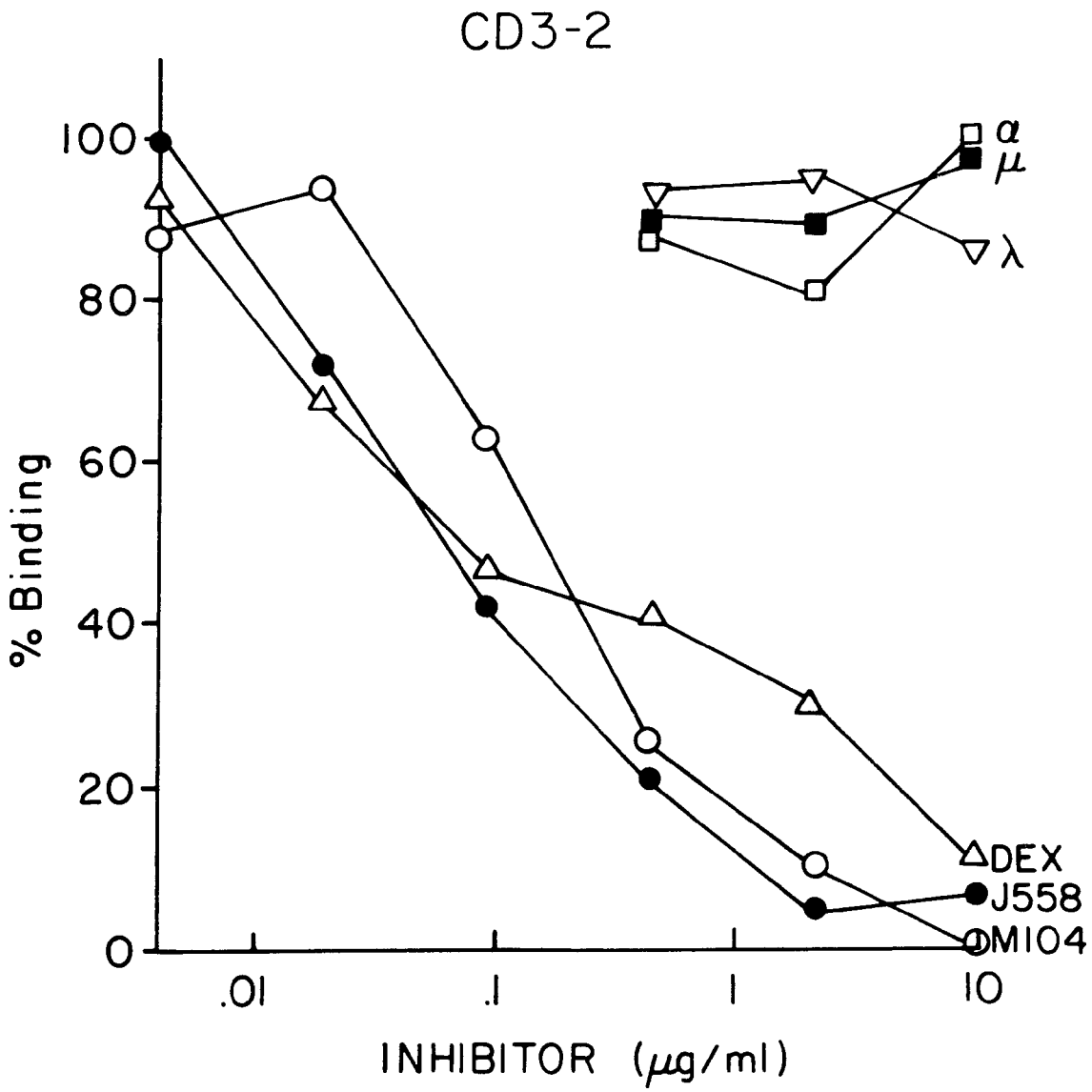
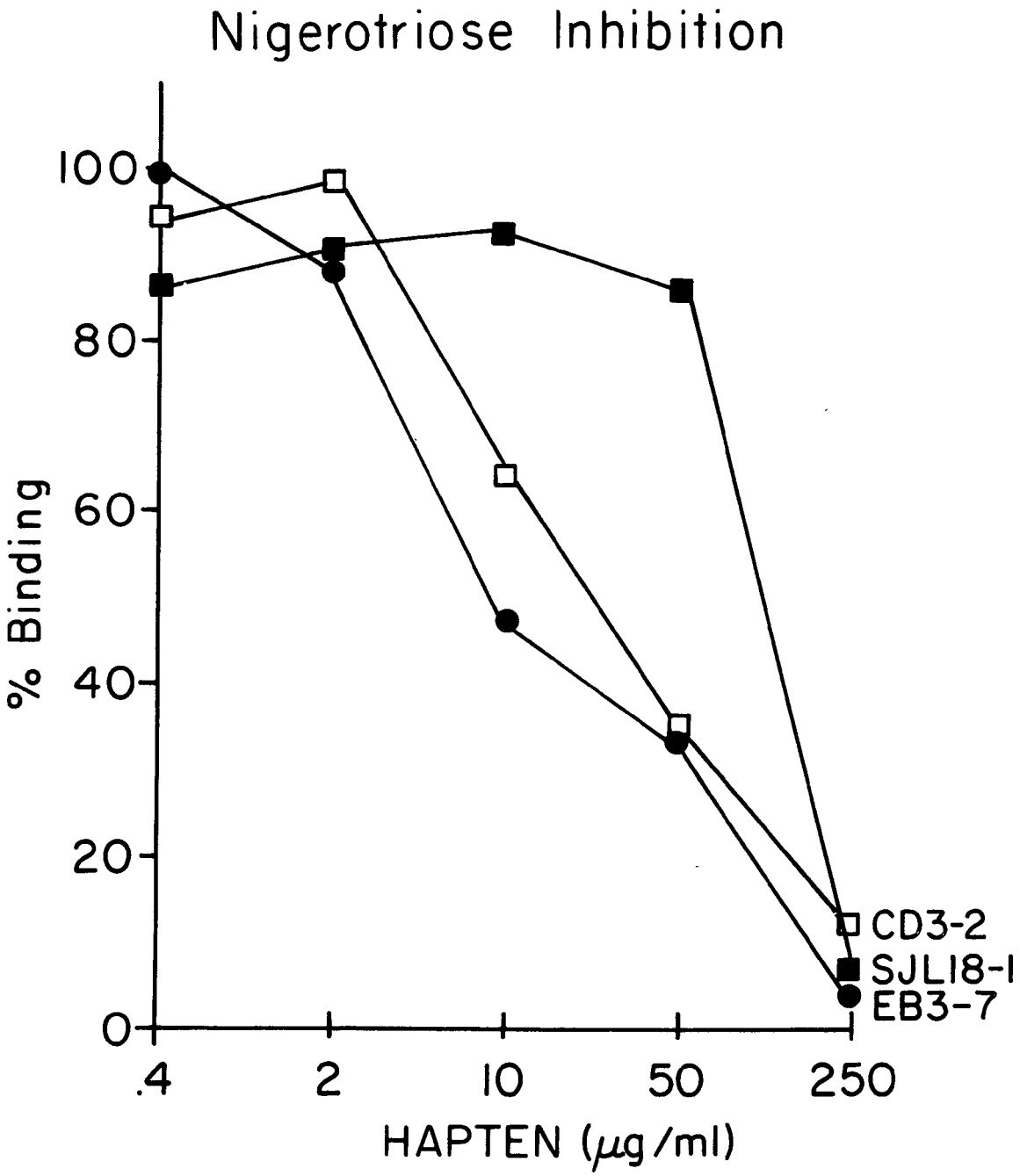

CD3-2
100
80
60
40
20
0
% Binding
.01
.1
1
10
INHIBITOR (μg/ml)
α
μ
λ
DEX
J558
M104

Figure 4. Inhibition curves showing that the α1->3 linked glucose trisaccharide nigerotriose inhibits the binding of EB3-7, SJL18-1 and CD3-2 to plate bound J558.


Nigerotriose Inhibition
100
80
60
40
20
0
% Binding
.4
2
10
50
250
HAPTEN (μg/ml)
CD3-2
SJL18-1
EB3-7

idiotype antibodies both light and heavy chain of the anti-DEX myelomas are required for expression of the idiotope specificity.

Suppression of the J558 IdI, the M104E IdI and the cross-reactive idiotype in adult BALB/c mice. Adult BALB/c mice were suppressed with affinity purified MAIDs from tissue culture supernatants and ascites or in some cases with diluted ascites fluid containing the appropriate MAID. No differences in effectiveness or specificity of Id suppression were observed between the different preparations. One hundred μg of either EB3-7, SJL18-1, CD3-2, or an irrelevant MAID, GB4-10 (anti-TEPC-15, ref. 10), were injected I.P. for 7 consecutive days. On day 8 the mice were challenged I.P. with 100 μg DEX and bled on day 15. The groups of mice were also rechallenged at later intervals and subsequent bleedings were taken 7 days after each boost.

The ability of EB3-7 to specifically suppress the J558 IdI when administered to adult mice is apparent in Table II. The J558 IdI was undetectable in mice suppressed with EB3-7, while mice given an unrelated anti-idiotype antibody produced normal amounts of J558 IdI. The M104E IdI levels of the EB3-7 suppressed mice did not differ significantly from the M104E IdI levels of the control GB4-10 treated mice. The total amounts of λ bearing anti-DEX antibody did not differ significantly between suppressed and normal mice. All mice suppressed with EB3-7 remained completely suppressed for the J558 IdI for 6 weeks after which some individuals began to escape from complete suppression but EB3-7 levels were still 10-100-fold lower than control mice at 9 weeks (data not shown). Considerable variation in λ bearing anti-DEX antibody and the IdI levels was observed among individual mice from the same source and mice from the same litter.

TABLE II

IDIOTYPE LEVELS IN ADULT MICE SUPPRESSED WITH MONOCLONAL ANTI-IDIOTYPE ANTIBODIES

Anti-Id Treatment[a]	Serum Antibody			
	λ-Anti-DEX	EB3-7	SJL18-1	CD3-2[c]
GB4-10 (anti-T15)	478.5[b] (1.1)	137.4 (1.3)	82.0 (1.7)	High
EB3-7 (anti-J558)	130.0 (1.5)	<0.5	81.8 (1.5)	High
SJL18-1 (anti-M104)	658.9 (1.4)	320.1 (1.5)	<0.5	High
CD3-2 (anti-IdX)	20.1 (1.2)	2.3 (1.3)	1.2 (1.2)	Absent

[a]Mice were given seven 100 μg doses of MAIDs on days 1-7, challenged on day 8 with 100 μg dextran B1355S and then bled on day 15.

[b]All values are given in μg/ml and are geometric means of sera from at least 8 animals; numbers in parentheses are the standard errors.

[c]Accurate quantitation of the IdX levels was not possible using CD3-2; only relative levels are given.

SJL18-1 was also effective in specifically suppressing the M104E IdI in adults. Table II shows that the M104E IdI was undetectable after SJL18-1 administration. J558 IdI, IdX, and the total λ bearing anti-DEX antibody levels did not differ significantly from the GB4-10 treated control mice. Total suppression lasted about 8 or 9 weeks after which time low levels (about 1-10 μg/ml) of the M104E IdI appeared in the sera of immunized mice.

CD3-2, which recognizes the cross-reactive idiotype on anti-DEX antibodies, was then used to suppress adult BALB/c mice (Table II). After DEX challenge CD3-2 treated mice showed no detectable CD3-2 positive antibody and there was also a dramatic reduction in total λ bearing anti-DEX antibody, as well as J558 IdI and M104E IdI in comparison to antibodies produced by the control group. Suppression of IdI bearing antibody and of the λ bearing antibody was incomplete. At 18 weeks, all CD3-2 treated mice still responded to DEX challenges with lower λ bearing anti-DEX antibody levels, and lower IdI bearing antibody levels than controls, IdX levels were detectable, but were well below normal (data not shown).

Suppression of the J558 IdI, the M104E IdI, and the IdX in neonatal mice. BALB/c neonates were injected with 50 μg of EB3-7, SJL18-1, or CD3-2 every second day for 14 days. At 5 to 12 weeks mice were challenged with 100 μg DEX and bled 1 week later. Table III shows that EB3-7 induced a chronic suppression of only the J558 IdI. The M104E IdI, the IdX, and total λ bearing anti-DEX levels were similar to those of control litters or control littermates. Suppression lasted for over a year in the case of individual mice followed for that length of time. Similarly, administration of SJL18-1 to neonates

TABLE III

IDIOTYPE LEVELS IN MICE NEONATALLY SUPPRESSED WITH MONOCLONAL ANTI-IDIOTYPE ANTIBODY

Anti-Id Treatment[a]	Serum Antibody			
	λ-Anti-DEX	EB3-7	SJL18-1	CD3-2[c]
Saline	383.9[b] (1.2)	226.8 (1.3)	90.8 (1.5)	High
EB3-7 (anti-J558)	241.7 (1.2)	<0.5	122.7 (1.3)	High
SJL18-1 (anti-M104)	344.8 (1.2)	54.0 (1.6)	<0.5	High
CD3-2 (anti-IdX)	75.6 (1.5)	0.8 (1.3)	<0.5	Absent

[a]Neonatal mice were given 50 µg of a monoclonal anti-idiotype antibody every second day for the first 14 days of life and were immunized with 100 µg dextran B1355S at age 8 weeks and bled 7 days later.

[b]All values are given in µg/ml and are geometric means of sera from at least 8 animals; numbers in parenthesis are the standard errors.

[c]Accurate quantitation of the IdX levels was not possible using CD3-2; only relative levels are given.

(Table III) resulted in a chronic suppression of the M104E IdI, leaving the J558 IdI, the IdX, and the total λ bearing anti-DEX responses intact. Complete suppression of the M104E IdI lasted for at least 4 months for all individuals. SJL18-1, which is an IgM antibody, thus appeared to suppress as efficiently and as permanently as EB3-7 (γ_1,^{K}), indicating that the suppressive properties of these MAIDS when used at these concentrations may not associate with any particular isotype.

Neonates which had received CD3-2 (Table III) and were subsequently challenged at 6 weeks of age showed a marked depression in levels of total λ bearing anti-DEX antibody. The serum levels of J558 IdI, M104E IdI, and IdX in most individual CD3-2 suppressed mice were below detectable limits, 0.5 μg/ml. When these same suppressed mice were rechallenged at 12 weeks of age the total λ bearing anti-DEX antibody had recovered to about 150 μg/ml, low levels of the J558 IdI were detectable but were still far below those of control mice while the IdX remained completely absent (data not shown).

DISCUSSION

A large panel of syngeneic and allogeneic monoclonal anti-idiotype antibodies with specificities for the α1->3 dextran binding myeloma proteins J558 and M104E was constructed by immunizing appropriate strains of mice with J558 or M104E myeloma proteins in their native conformation, unmodified by maneuvers such as the coupling of a carrier protein or haptenation. Isoelectric focusing analysis of intact IgG molecules or electrophoretically separated light chains of MAIDS showed that this group of antibodies with IdI and IdX activities

for DEX binding antibodies was extremely heterogeneous. When either whole IgG immunoglobulins or isolated light chains from these MAIDs were subjected to IEF no repeat patterns were observed from either individual or different mice. Because of difficulties associated with isoelectric focusing of IgM molecules these were not analyzed by this method. Within the fusions described the majority of monoclonal allo-antibodies were anti-idiotype; allotype specific monoclonals were next most frequently obtained. One anti-λ as well as two anti-V_H antibodies which appeared to react with isolated J558 and M104E heavy chains were also obtained.

We used solid phase inhibition assays to confirm the idiotype specificity of 3 of these antibodies and to show that the idiotype-anti-idiotype reaction of these antibodies is inhibited by dextran and the hapten nigerotriose. The anti-J558 IdI, EB3-7, has been shown to bind a larger group of anti-DEX hybridoma proteins than a corresponding heterologous anti-IdI and in this respect appears to characterize a subgroup within the panel of anti-α1->3 DEX binding antibodies (16). The specificities of the anti-M104E IdI, SJL18-1 and the anti-IdX, CD3-2, appeared to be similar to those of conventional anti-idiotypic sera (unpublished results).

When the sera of DEX immunized BALB/c mice were analyzed for expression of these monoclonally defined idiotopes it became apparent that a great amount of variability occurred in the amounts of total λ bearing anti-DEX antibodies and the ratios of IdI expressing antibody between different mice. Similar findings were reported using heterologous anti-idiotype antibodies (17,18), although our results showed considerably higher values for idiotype bearing anti-DEX antibodies.

The kinetics of the response is always uniform, with peak antibody titers being reached on day six or seven (data not shown).

Heterogeneous anti-idiotypic sera have been used previously to suppress specific idiotypes in adult (19,20) and neonatal mice (21,22) in several antigen systems. Monoclonal anti-idiotype antibodies have also been used to suppress antibody responses in the PC and NP sytsems (10, 23) or to enhance expression of specific idiotype in the NP system (23). No enhancement of any of the DEX idiotopes was noted after treatment with monoclonal anti-idiotype antibodies over a wide range of doses (.1 μg/to 1000 g) in the DEX system in several experiments (unpublished results). This failure may be due to the dominance of expression of this group of λ antibodies to DEX in BALB/c mice or alternately to the thymus independent nature of anti-DEX responses. Similar lack of enhancement in the phosphorylcholine response in BALB/c mice has been observed with MAIDs specific for TEPC-15. We have also shown that monoclonal anti-idiotype antibodies of γ_1, and μ heavy chain isotypes, purified from ascites, tissue culture supernatant (or used in the raw ascites form) and directed against J558 and M104E IdI idiotopes specifically suppressed only those idiotopes to which they are directed, while having no effect on the IdX or total λ bearing anti-DEX antibody. When CD3-2 was administered to adults a partial and short term suppression of the entire λ bearing anti-DEX response was observed accompanied by partial suppression of the J558 and M104E IdIs and total suppression of the IdX. Recovery was slow in adults, with λ bearing anti-DEX antibodies returning to near normal levels within 18 weeks, while the M104E and J558 IdIs remained below control levels and the IdX levels remained well below controls at 18

weeks. However, a similar pattern of suppression following CD3-2 treatment was observed in adult mice suppressed as neonates as long as 6-12 weeks or longer after birth. The fact that an anti-IdX can eliminate most of the J558 IdI and M104E IdI indicates that the IdIs are usually associated with the IdX on in vivo serum antibodies, a fact which is in agreement with data obtained from idiotypic analyses of DEX binding hybridomas (4,5,16). The idiotypes expressed by adult mice suppressed as neonates are virtually identical to the idiotypes expressed by mice suppressed as adults. Although the idiotype specificifies of adult and neonatally administered MAIDs are identical, neonatally administered MAIDs led to a more permanent state of suppression. The ability of monoclonal anti-idiotype antibodies (purified from tissue culture supernatants on anti-isotype columns) to specifically suppress idiotypes rules out the possibility that suppression could be mediated by other exogenously produced molecules such as T-cell factors, Ab-3, or Ab-4, which may be present in conventionally produced anti-idiotypic sera. Furthermore, these studies show that injection of monoclonal anti-idiotype antibodies results in specific suppression of only antibodies bearing the idiotope to which the MAIDs are directed, leaving the remainder of the anti-DEX response unaffected. This observation suggests that the mechanisms involved in neonatal and adult suppression are highly specific and except for the action of IdX specific MAIDs do not affect other non-related clones.

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Selective alteration of the humoral response to alpha 1->3 dextran and phosphorylcholine by early administration of monoclonal anti-idiotype antibody

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In Idiotypy; H. Kohler, J. Urbain and P-A. Cazenave, editors; Academic Press, New York (in press).

I. Introduction

The capacity of anti-idiotype (anti-Id) immunoglobulin to chronically ablate the corresponding B cell response in mice exposed during the early stages of life has been documented by a host of investigators in several antigen/idiotype systems. In most of these studies, the anti-Id reagents used to manipulate a particular immune response were heterologous preparations (of xenogeneic or allogeneic origin) which probably contain antibodies recognizing a variety of idiotypic determinants, such as (i) V_L- and V_H-associated idiotopes (possibly reactive with T cells; Eichmann, 1978), (ii) framework-localized idiotypic determinants, and (iii) binding site-associated idiotopes (requiring paired V_H-V_L chains). The immunoglobulin subclasses of the anti-Id antibodies in most heterologous anti-Id preparations are also often not characterized; since certain isotypes appear to exhibit distinct immunoregulatory activities (Eichmann, 1974; Kelsoe et al., 1981), it is obviously important that the different functional properties of immunoglobulin isotypes be considered.

The above issues can be circumvented by the use of monoclonal anti-idiotype antibodies (MAIDs) to induce idiotype-specific unresponsiveness <u>in vivo</u>. We have used panels of hybridomas secreting well-defined MAIDs reactive

with the dominant T15 idiotype present in the anti-phosphorylcholine (PC) response and to major idiotypes found within the anti-alpha 1->3 dextran (DEX) response to establish an idiotype-specific suppressed state in neonatal mice (directly and maternally). The pattern and mechanism of B cell unresponsiveness resulting from both direct and maternal administration of these MAIDs are discussed.

II. Description of Methodology

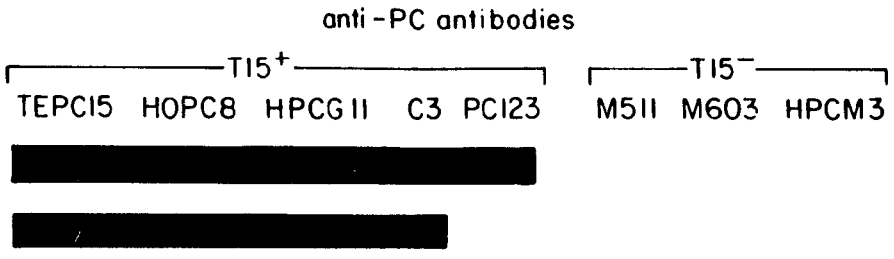
A. Generation and specificities of MAIDs

Monoclonal allogeneic (A/J,SJL) and syngeneic (BALB/c) anti-idiotype antibodies were constructed by fusing the non-secreting BALB/c plasmacytoma line P3x63Ag8.653 (Kearney et al., 1979) with lymph node cells from mice immunized with the myeloma proteins HOPC 8 (for anti-T15 Id), J558 (for anti-J558 Id), or MOPC 104E (for anti-M104 Id). The specificity and isotype of each MAID used in these studies is given in Table I. The BALB/c anti-(anti-T15 Id) antibody MM-60, which is specific for the anti-T15 MAID GB4-10, was derived from a fusion of S. Pneumoniae strain R36A-immunized spleen cells and Ag8.653.

Table I

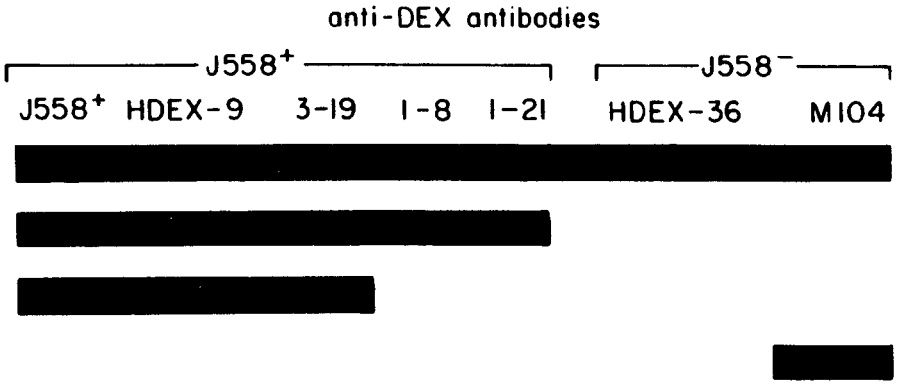
SPECIFICITY OF MONOCLONAL ANTI-IDIOTYPE ANTIBODIES

A. Phosphorylcholine system



MAID	Isotype	% Id positive of anti-PC IgM: Hybridomas	Serum antibody
ABI-2	$\gamma_1\kappa$	85	86-100
GB4-10	$\gamma_1\kappa$	80	83-100

B. α1-3 dextran system



		% Id positive of anti-DEX Ig	
CD3-2	$\gamma_1\lambda$	81	VARIABLE*
EB3-7	$\gamma_1\kappa$	57	5-95
EB3-16	$\gamma_1\kappa$	5	0-90
SJL18-1	$\mu\kappa$	35	1-85

B. Experimental procedures

1. Treatment with MAID *in vivo*

To induce T15 idiotype-specific unresponsiveness directly in neonatal BALB/c mice, 2 ug of MAID was given i.p. 24-48 hrs after birth. In the DEX system, Id-specific unresponsiveness was achieved by injecting 50 ug MAID on alternate days from day 1 to day 15. Exposure of neonates to MAID via the mother was accomplished either (i) *in utero*, where 100 ug of MAID was delivered i.p. 2 days prior to birth of the litter, or (ii) post-partum, in which case the mother was given 100 ug of MAID i.p. 24 hrs after giving birth. In all three cases, the MAID-treated mice were reared for at least 6 wks prior to challenge with antigen. Control and experimental mice (minimum of 5 per group) were always age-matched.

2. Assay for immunologic unresponsiveness

MAID-exposed mice were challenged i.p. at 6-8 wks with the appropriate T-independent type 2 antigen. In the PC/T15 system, 2 x 10^8 heat-killed *S. Pneumoniae* R36A (R36A vaccine) were given, and in the DEX system, 100 ug of dextran B1355S was injected. Mice were bled from the retroorbital plexus on Days 5 and 7 after challenge,

respectively. Antigen-binding and idiotype-bearing immunoglobulin levels present in the serum samples from suppressed and control mice were quantitated using a solid phase enzyme-linked immunosorbent assay (ELISA; Kearney et al., 1981).

3. Adoptive transfer

Cell transfer experiments were carried out using the procedures described by Owen et al. (1977). All x-irradiated (200 rads) recipients received a total of $2x10^7$ donor cells and were then challenged 3 days after transfer with a single dose of R36A vaccine and their serum antibody assayed 5 days afterward (as described). T lymphocytes were depleted using complement in conjunction with a monoclonal AKR anti-Thy 1.2 antibody (a gift from Dr. G. Hammerling). The resulting cell suspension contained <2% $Thy1^+$ cells (detected by immunofluorescence). B lymphocytes were depleted by two successive panning cycles on plastic petri dishes coated with purified goat anti-mouse Ig, resulting in <3% B cells remaining (Mage et al., 1977).

4. Splenic focus assay

Spleen cells from normal or EB3-7-suppressed BALB/c mice were transferred at limiting dilutions (approximately $3x10^7$ cells) to normal, irradiated (1400 rads) recipient BALB/c mice (65 and 6 recipients, respectively). Eighteen to 24 hours later, recipient spleens were diced into 1 mm cubes and cultured in complete Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum and 2 ng/ml dextran B1355S in sterile 96 well microtiter plates (Ward and Kohler, 1981). Culture supernatants were collected every four days and assayed for DEX-binding antibody, isotype expression, and idiotype expression by ELISA.

III. Id-specific B cell unresponsiveness after MAID exposure

A. Direct treatment of neonates

The panel of MAIDs used in this study of BALB/c anti-DEX responses is specific for individual idiotopes on either J558 or M104E myeloma proteins (i.e.,IdI), or for idiotopes present on both proteins (i.e.,IdX) as shown by Clevinger et al. (1980). Table II shows the effects on the adult anti-DEX response after administration of these MAIDs

TABLE II

DEX- and PC-specific Antibody Levels after Direct Neonatal Exposure to MAIDs

(a) DEX

	lambda-positive Ig (ug/ml + s.e.)[a]			
	anti-DEX	EB3-7+	EB3-16+	SJL18-1+
CD3-2	75.6 (1.5)	0.8 (1.3)	3.6 (2.6)	<0.5
EB3-7	166.4 (1.5)	<0.5	<0.5	138.1 (1.6)
EB3-16	218.9 (1.4)	31.8 (1.4)	<0.5	9.8 (1.9)
SJL18-1	344.8 (1.2)	54.0 (1.6)	26.5 (2.6)	<0.5
Saline	663.1 (1.2)	379.6 (1.1)	5.8 (1.7)	50.8 (1.4)

(b) PC

	serum IgM (ug/ml + s.e.)[b]		
	anti-PC	AB1-2+	GB4-10+
AB1-2	28.3 (3.7)	<0.1	<0.1
GB4-10	31.4 (3.7)	2.4 (1.0)	<0.1
MM-60	18.5 (3.8)	5.1 (1.4)	4.8 (1.4)
Saline	22.5 (2.7)	22.0 (3.1)	21.3 (2.6)

[a]Geometric mean (all tables).

[b]Arithmetic mean (all tables).

during the first 14 days of life. Treatment of neonatal mice with EB3-7 antibody resulted in complete and chronic suppression of the J558 IdI portion of the anti-DEX response. The M104E IdI and the IdX portions of the response were unaffected by EB3-7 treatment, as were the total lambda-bearing anti-DEX levels. There was no detectable EB3-7-positive antibody observed after DEX challenge for periods of up to one year after treatment. Analysis of DEX-binding hybridomas shows that EB3-7 defines a large subset of J558 IdI-positive hybridoma antibodies (Clevinger et al., 1981), while the idiotopes defined by EB3-16, BD5-3, and AB3-7 are expressed on smaller subsets of J558 Id^+ anti-DEX antibodies and are always associated with the EB3-7 idiotope (R.S. and J.K., unpublished). Neonatal exposure to EB3-7 completely abolishes expression of these idiotopes in the anti-DEX response (20-50% of control mice express high levels of the EB3-16, BD5-3, and AB3-7 idiotopes). When EB3-16 is injected into neonates, expression of the EB3-16^+ portion of the anti-DEX response is ablated along with the expression of the idiotopes defined by BD5-3 and AB3-7. The EB3-7 and SJL18-1 idiotope levels also appear to be lowered, although the levels are still within control ranges. The total lambda-bearing anti-DEX antibody was similar to controls.

An opposing pattern of unresponsiveness is obtained when neonatal mice are suppressed with SJL18-1 antibody,

which recognizes a M104E IdI determinant. When SJL18-1-treated mice are challenged at 6-8 weeks of age, the M104E IdI levels (defined by SJL18-1) are undetectable. The J558 IdI, IdX and total lambda-bearing anti-DEX antibody all are unaffected and comparable to levels found in control mice.

The results obtained by neonatal suppression with IdI-specific MAIDs contrast with the effects produced by CD3-2 antibody, which recognizes a cross-reactive idiotope expressed on J558, MOPC 104E, and on most lambda-bearing anti-DEX antibodies. Neonatal treatment with CD3-2 results in a slight depression of the lambda-bearing anti-DEX antibody level, accompanied by a complete failure in most mice to produce anti-DEX antibodies expressing the EB3-7, BD5-3, AB3-7, and SJL18-1 idiotopes. The EB3-16-defined idiotope is expressed at high levels in approximately 29% of suppressed individuals, and in these cases accounts for nearly all of the anti-DEX antibody. It is apparent that the EB3-16 idiotope can be expressed on CD3-2-negative immunoglobulins and that clones expressing this idiotopic profile are expanded in CD3-2-suppressed mice.

When certain idiotopes on anti-DEX antibodies are associated, they are concomitantly suppressed by a MAID directed against the inclusive idiotope, while the expression of non-associated idiotopes is unaffected. EB3-7 and SJL18-1 exhibit distinct specificities for

anti-DEX myeloma and hybridoma proteins of the J558 and M104E idiotype families, respectively. Since these MAIDs affect only those portions of the anti-DEX response for which they are specific, it may be inferred that whatever cellular mechanisms are involved in maintaining unresponsiveness into adult life, they exhibit the exquisite specificities characteristic of this panel of MAIDs.

In the PC system, treatment of BALB/c neonates with the monoclonal anti-idiotype antibody AB1-2 consistently produced complete and chronic suppression of the T15 component of the anti-PC response, while the total response was only slightly diminished (Table II). The difference in anti-PC antibody levels between the two groups after primary challenge is lost as the mice age or after they receive a secondary challenge of antigen. Pre-immune and immune serum from these mice also do not contain significant amounts of classically-defined T15-positive antibody (<1% of the total amount of anti-PC serum antibody), which was determined by using a goat anti-T15 Id antibody preparation in the solid phase ELISA (data not shown). Since the idiotope specificity of AB1-2 appears to encompass all immunoglobulins belonging to the T15 group of anti-PC antibodies (Kearney et al., 1981), the pattern of unresponsiveness following neonatal treatment with AB1-2

was expected to parallel that found using heterologous anti-"pan" T15 reagents (Augustin and Cosenza, 1976).

Neonatal treatment with GB4-10 antibody, a distinct anti-T15 MAID, provides more insight as to the nature of T15 idiotype-specific unresponsiveness which can be induced in this system. Mice treated neonatally with this antibody usually produce a significant proportion of $T15^+$ antibody within the total serum anti-PC IgM pool, but produce undetectable levels of anti-PC immunoglobulin expressing the GB4-10 idiotope. Evidently, the small set of $T15^+$ B cells which lack the GB4-10 idiotope are not inactivated by neonatal treatment with GB4-10 and expand in response to PC antigen to produce anti-PC antibodies of the $AB1\text{-}2^+$ $GB4\text{-}10^-$ phenotype (this situation can also be generated by treating adult mice with GB4-10, followed by repeated immunization with PC antigen; Kearney et al., 1981).

The induction of idiotype suppression discussed above resulted from administration of monoclonal anti-idiotype antibodies. However, T15 Id-specific unresponsiveness can also be accomplished by treating neonates with MM-60, a monoclonal BALB/c anti-(anti-T15) antibody that recognizes an idiotope on the GB4-10 molecule (Pollok et al., 1982). An important difference between the suppression protocols required when using MM-60 antibody as compared with AB1-2 or GB4-10 antibody is that MM-60 must be administered to the neonate after day 4 of life in order to detect

subsequent MM-60-dependent immunoregulatory effects on the anti-PC responses of adult mice. This requirement is probably due to the need for MM-60 antibody to be introduced *in vivo* at a point during the ontogeny of the "GB4-10" anti-T15 B cell clone when it is functional since clearance of MM-60, an IgM molecule, will be much more rapid than for IgG (Waldmann and Strober, 1969). The suppression of $T15^+$ immunoglobulin production in adult mice given MM-60 antibody as neonates is neither as complete nor as permanent as that resulting from the neonatal treatment with anti-T15 MAIDs. Partial recovery of responsiveness among $T15^+$ B cells in these mice is apparent at 12 weeks since 2-32% of their anti-PC IgM express the T15 idiotype (defined by reactivity with AB1-2). This variance reflects differences in the way unresponsiveness occurs in MM-60-treated mice as compared with anti- T15-treated mice (B.P. and J.K., manuscript in preparation) and may simulate regulatory mechanisms which occur during normal anti-PC responses since suppression of $T15^+$ idiotype B cells in MM-60-treated neonates and adults is a reversible phenomenon.

The pattern of B cell unresponsiveness produced by administration of those MAIDs which recognize a subset of antibodies within a major idiotype family (e.g., GB4-10 and EB3-16) is largely idiotope-specific. Naturally, if

concomitant expression of idiotopes occurs (e.g., all GB4-10$^+$ antibodies are also AB1-2$^+$), an idiotope-specific pattern of unresponsiveness will not develop in mice treated with the MAID of the broadest specificity (in this case, AB1-2). Similar to our findings, studies by Takemori et al. (K. Rajewsky, personal communication) in the NP^b system and by Marshak-Rothstein et al. (1981) in the arsonate system have shown that MAIDs specific for such private idiotopes can suppress mice when administered in vivo; the independent segregation in expression of certain idiotopes among serum antibodies produced by such suppressed mice was also observed.

If active suppression of B cells occurs as a result of anti-idiotypic suppressor T cells in these systems, then this kind of suppression must necessarily be idiotope-specific to allow idiotope-negative variants to escape from their regulatory action. In the case of neonatal treatment with GB4-10, any proposed cellular mechanism which would directly regulate GB4-10$^+$ anti-PC B cell clones could not be directed towards those idiotopes shared by GB4-10$^+$ and GB4-10$^-$ antibodies of the T15 family; these include all V_H and V_L framework, and binding site-associated determinants since the GB4-10-defined determinant is destroyed by amino acid substitutions within the middle of the T15 heavy chain D region (Kearney et al., in press).

B. Indirect treatment of neonates via maternal routes

Idiotype suppression via maternal routes was first reported by Weiler et al. (1977) using the alpha 1->3 dextran system. This was accomplished by immunization of SJL female mice with J558 prior to mating with BALB/c males. The vast majority (111 out of 112) of the F_1 progeny were suppressed for anti-DEX antibody and the J558-associated idiotype up to 12 weeks of age. With increasing age after 12 weeks, a greater percentage of the suppressed progeny was capable of producing anti-DEX antibody. Mice which escaped suppression at 20 weeks of age were tested for expression of the J558 Id and about 40% of these mice lacked this idiotype. It was also shown that the suppressive agent was transmitted through the milk; all F_1 mice from J558-immunized SJL mothers were suppressed when nursed by a J558-immunized SJL mother, but when members of the same litter were nursed by a normal SJL mother only some of them were found to be suppressed. Maternal suppression has also been reported by Bona (1979) in the levan-inulin system and by Cosenza et al. (1977) in the PC system.

Administration of 100 ug EB3-7 antibody to BALB/c mothers 1-4 days before birth produces a pattern of B cell

unresponsiveness identical to that resulting from direct neonatal treatment with EB3-7 (Table III), even though a lower total effective dose of antibody per neonate is probably realized. Administration of 100 ug SJL18-1 antibody to pregnant females failed to specifically suppress the M104E IdI component of the anti-DEX response. The failure of SJL18-1 to effect maternally transmitted suppression in contrast to its efficacy when directly administered to newborn mice is attributable to SJL18-1 being an IgM antibody, thus being unable to cross the placental barrier (Waldmann and Strober, 1969).

Suppression of the J558 IdI can also be induced by administration of EB3-7 antibody to the mother after birth of the litter. As shown (Table III), the EB3-7 idiotope is not expressed in the anti-DEX response when these mice are challenged with antigen in adult life. This suppression is most likely due to transport of EB3-7 immunoglobulin into the milk, which after ingestion is absorbed into the blood and tissues of the suckling neonate. Evidence for this mechanism has been provided by independent investigators using heterologous anti-Id reagents (Weiler et al., 1977) and also appears to be involved in the maternal transmission of $T15^{+}$ B cell unresponsiveness using AB1-2 as outlined below.

TABLE III

DEX- and PC-specific Antibody Levels after Maternal Treatment with MAIDs

(a) DEX

	Route	lambda-positive Ig (ug/ml + s.e.)[a]		
		anti-DEX	EB3-7+	SJL18-1+
EB3-7	in utero	318.2 (1.6)	<0.5	48.3 (1.9)
EB3-7	post-partum	794.0 (1.3)	<0.5	157.3 (1.5)
Control	post-partum	478.5 (1.1)	137.4 (1.3)	82.0 (1.7)

(b) PC

		serum IgM (ug/ml + s.e.)[b]		
		anti-PC	AB1-2+	GB4-10+
AB1-2	in utero	26.9 (2.4)	<0.2	<0.2
AB1-2	post-partum	29.7 (3.7)	1.8 (1.3)	1.7 (1.1)
Saline	in utero	22.5 (3.3)	21.2 (3.0)	20.4 (3.1)

[a]Geometric mean.

[b]Arithmetic mean.

A previous study on maternal transmission of idiotype-specific suppression in the PC system (Cosenza et al., 1977) reported that injection of a heterologous anti-T15 idiotype antibody into the mother prior to birth did not alter the idiotypic profile of the anti-PC response among the progeny. In contrast, we found that injection of a monoclonal anti-T15 idiotype antibody (AB1-2) into the mother (i.p.) prior to birth permanently stifled the T15 component of the humoral anti-PC response in the progeny, in a pattern identical to that observed after direct neonatal treatment with AB1-2 (Table III). This discrepancy is resolved once it is recognized that all AB1-2 molecules which are injected (i) have the potential to cross the placenta (AB1-2 is an IgG_1 antibody), and (ii) are directed at an idiotope which will mediate the regulatory effects of the antibody - properties that many anti-T15 antibodies in a heterologous preparation may not possess.

Treatment of the mother with AB1-2 antibody after birth can also prevent the dominance of the T15 component within the anti-PC response of the adult progeny, although there is a substantial level of $T15^+$ B cell responsiveness present in such mice (Table III). To determine whether transfer of AB1-2 immunoglobulin from the mother to the neonate via the colostrum occurs, the stomach fluids and sera from day 3 neonates were separately pooled and assayed by ELISA for the presence of AB1-2 antibody. Five hundred

seventy-two ng/ml and 156 ng/ml of anti-T15 IgG_1 antibody (presumably AB1-2) were detected in stomach fluid and serum, respectively. These amounts of AB1-2 compare favorably with a dose of 250 ng of AB1-2 antibody, which if delivered intraperitoneally to neonatal mice, is sufficient to permanently inactivate $T15^+$ B cells (B.P. and J.K., unpublished).

IV. Mechanism of MAID-induced Id-specific B cell unresponsiveness

The striking similarity in the pattern of B cell unresponsiveness among mice treated as neonates with MAID, independent of the route of exposure and the antigen system used, suggests that a similar mechanism may be responsible for the maintenance of the idiotope-specific unresponsiveness observed in such adult mice. We examined this question in multiple ways: (i) B cells from EB3-7 neonatally suppressed mice were tested for functional activity in a splenic focus assay, (ii) in the PC system, spleen cells from AB1-2 neonatally suppressed mice were tested for their pattern of responsiveness to PC in an adoptive transfer system, and (iii) spleen cells from mice neonatally suppressed with GB4-10 antibody were examined for expression of the GB4-10-defined idiotope before and after stimulation with lipopolysaccharide (LPS).

A. Splenic focus of B lymphocytes from EB3-7-suppressed donors

The splenic focus assay provides a means of comparing the functional activities of precursor B lymphocytes in MAID-exposed and normal mice. Table IV shows the predominant expression of the EB3-7 idiotope among the antibodies produced by the progeny cells of anti-DEX precursor B lymphocytes in normal BALB/c mice. When B cells from mice neonatally treated with EB3-7 are examined by identical means, there is a clearcut lack of evidence for functional precursor B lymphocytes expressing the EB3-7 idiotope. Explanations for this observation include (i) physical deletion of idiotope-positive precursor B lymphocyte clones, (ii) co-transfer of DEX- and/or idiotype-specific suppressor T cells into the irradiated recipients, and (iii) permanent inactivation of idiotype-positive precursor B lymphocytes. Co-transfer of suppressor T lymphocytes is unlikely, since the dilution factor used would make the likelihood of a specific suppressor T cell ending up in each fragment containing a complementary B cell quite small.

TABLE IV

Idiotope Expression Among DEX-specific B cell Precursors

	% of anti-DEX precursors		
	EB3-7+	SJL18-1+	CD3-2+
Control	74	26	83
EB3-7-suppressed	0	50	63

B. Adoptive transfer of lymphocytes from AB1-2-suppressed donors

As shown in Table V, significant T15 idiotype unresponsiveness can be adoptively transferred to adult syngeneic recipients using spleen cells from AB1-2 neonatally suppressed mice. A slightly lower and more variable degree of responsiveness to PC antigen is also present in those recipients receiving cells from suppressed donors. B cells are the only detectable cell type responsible for causing the lack of $T15^+$ serum IgM in the humoral anti-PC response among recipients; no evidence could be shown for the existence of suppressor T cells when AB1-2 is used as the suppressing agent. This finding supports work of Kim and Hopkins (1978) and Weiler (1981), who showed that chronic idiotype unresponsiveness could be obtained by treating neonatal nude BALB/c mice with anti-idiotype antibody. Transferring a 1:1 mixture of suppressed and normal spleen cells (both PC-primed) leads to a partial recovery in the proportion of the recipient's anti-PC serum IgM expressing the T15 idiotype. Consequently, active suppression of $T15^+$ B cells by regulatory B or T lymphocytes is not evident in this system.

TABLE V

Adoptive Transfer of Lymphoid Cells From AB1-2 Neonatally-Treated BALB/c Mice

Source of spleen cells	Treatment	recipient serum IgM (μg/ml ± S.E.)	
		anti-PC	AB1-2$^+$
Normals	-	27.5 (2.1)	25.9 (2.4)
AB1-2 suppressed	-	15.8 (3.2)	1.2 (0.8)
"	anti-Thy 1 + C'	18.4 (2.7)	1.9 (1.1)
"	B cell-depleted	14.4 (1.9)	13.7 (1.6)
Normal + AB1-2-suppressed (1:1)	-	27.7 (1.6)	10.8 (1.7)

C. Analysis of T15 Id expression on B cells from anti-T15 MAID-treated mice

B cells from anti-T15 MAID neonatally suppressed BALB/c mice were examined for cell surface expression of the GB4-10 idiotope by indirect immunofluorescent staining using a GB4-10/biotin conjugate as the primary reagent, followed by an avidin-fluorescein conjugate as a detection reagent (Pohlit et al., 1979). While approximately $1.5/10^4$ splenic lymphocytes of normal BALB/c mice express this idiotope on their surface immunoglobulin under these conditions, no GB4-10-bearing cell has yet been detected in AB1-2 or GB4-10 neonatally-treated mice (resulting in a frequency of $<1/10^5$ spleen cells; Table VI). This finding confirms the observations made by Kohler et al. (1974) which stated that $T15^+$ B cells as detected by immunofluorescence were absent in anti-T15 neonatally suppressed BALB/c mice.

To determine whether $T15^+$ B cell clones were actually physically deleted or merely lacked detectable levels of membrane immunoglobulin, spleen cells from GB4-10-suppressed mice were cultured in the presence of LPS for 4 days prior to staining with GB4-10/biotin. After LPS stimulation, a significant proportion of spleen cells from suppressed mice expressed the GB4-10-defined idiotope, at approximately 25% of the frequency present among similarly

cultured normal BALB/c spleen cells (Table VI). In accordance with the findings of Fung and Kohler (1980a) in the splenic focus assay, it appears that idiotope-positive B cells are not physically deleted after neonatal exposure to anti-Id antibody, but remain nonfunctional in adult life due to an inability to express an antigen receptor in sufficient quantity or native form. Receptor modulation by residual anti-Id antibody cannot account for the lack of surface Ig expression since in vitro culture of spleen cells from anti-T15 neonatally-treated mice did not allow $T15^+$ B cells to escape from an inactive state (Strayer et al., 1975). Functional immunoglobulin gene structure is obviously conserved in these inactive B cells since interaction with LPS can lead to production of Id-positive immunoglobulin; a blockade at the transcriptional or translational levels of antibody synthesis is the most probable explanation for the absence of surface Ig expression.

These and similar independent studies using anti-T15 MAIDs strongly suggest that dominance of the PC response by T15-negative B cells is the basis for T15 clonal unresponsiveness in neonatally-treated mice. Studies supporting the existence of suppressor T cells after neonatal suppression regimes (DuClos and Kim, 1977) are not necessarily incompatible with these results, as

heterologous anti-Id preparations may contain antibodies which stimulate regulatory T cell sets. Taken together, the results from the cell transfer and immunofluorescent experiments presented here support the concept that establishment of clonal dominance by T15-negative B cells acts to create and/or maintain the functional unresponsiveness among T15-positive B cell clones in mice treated neonatally with anti-idiotype antibody. The permanent nature of the T15 unresponsive state in these mice has been informally suggested by several investigators (in Idiotypes: Antigens on the Inside, 1982) to exist because establishment of an active $T15^-$ anti-PC response somehow prevents subsequent maturation of T15-positive B cell precursors. This concept is also supported by the work of Fung and Kohler (1980a), who found that T15-positive B cells were present in BALB/c mice treated neonatally with anti-T15 antibody, but were in a functionally immature state. Also, Etlinger et al. (1982) have shown that altho dugh $T15^-$ B cells from anti-T15 neonatally treated mice have a lower avidity for PC than that of normal $T15^+$ B cells, the avidity of the former increases over time to become equal to that of $T15^+$ B cells, thereby cementing their selective advantage over any newly developed $T15^+$ B cells in the competition for antigen. Differences in the avidity for antigen of T15-negative and T15-positive B cells, as well as disparity in the number of PC-reactive B

cells transferred, may help to explain the observed decrease in the total PC response among mice receiving T15-negative B cells (from suppressed donors) in comparison with those receiving T15-positive B cells (from normal donors; Table V).

V. Summary

Treatment of neonatal BALB/c mice with monoclonal anti-idiotype antibodies (MAIDs) specific for idiotopes associated with anti-DEX or anti-PC immunoglobulins ablates the corresponding B cell response as effectively as heterologous anti-idiotype antibody preparations. Idiotype-specific B cell unresponsiveness can be accomplished in both antigen systems via maternal routes of MAID administration, as well as by direct treatment of neonates. Following neonatal treatment with MAIDs which distinguish among idiotope variants of a major idiotype family, the anti-DEX and anti-PC humoral responses reflect an idiotope-specific pattern of B cell inactivation. The immunologic defect in MAID-suppressed mice is apparently at the B cell level, since idiotope-positive B cells seemingly exist in an inactive and immature state, and because suppressor T cell activity is not detectable among lymphocytes from such mice. These findings demonstrate that a mechanism based on the dominance of

idiotope-negative B cells over non-functional idiotope-positive B cells sufficiently accounts for the maintenance of idiotype-specific humoral unresponsiveness in mice given anti-idiotype antibody early in life.

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FINE IDIOTYPE ANALYSIS OF B CELL PRECURSORS IN THE T-DEPENDENT AND T-INDEPENDENT RESPONSES TO α1->3 DEXTRAN IN BALB/c MICE[1]

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Running Head: Idiotopes of Anti-α1-3 Dextran B Cell Precursors

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Footnotes

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Abbreviations used in this paper: DEX, dextran B1355S; IdX, cross-reactive idiotype; IdI, individual idiotype; TI, thymus independent; TD, thymus dependent; MAID, monoclonal anti-idiotype antibody; DNP, dinitrophenyl; Hy, Limulus hemocyanin; ELISA enzyme linked immunosorbant assay; BSA, bovine serum albumin.

Introduction

The humoral immune response of BALB/c mice to α1->3 dextran (DEX) has been well characterized with respect to isotype and idiotype expression. The serum antibody induced in BALB/c mice by DEX consists almost entirely of the IgM and IgG_3 heavy chain and lambda light chain isotypes (1,2). The majority of serum antibody which binds DEX possesses a cross-reactive idiotype (IdX) present on both the J558 and M104E myeloma proteins and individual idiotypes (IdI) present on either J558 or M104E (3-5). The serum antibody from DEX immune BALB/c mice had been previously shown to be homogeneous when examined with heterologous anti-idiotype antibodies and by isoelectric focusing (4, 6). However, more recent amino acid sequence analysis of anti-DEX hybridoma antibodies has shown that while the majority of these antibodies are closely related, there is a considerable amount of heterogeneity mainly involving differences in only a few V_H region amino acid residues (7-9). Several mechanisms have been proposed for the generation of this limited amount of sequence diversity including imprecise joining of V-D-J gene segments, use of a large number of different D region genes and somatic mutation of variable region genes (9-12). It has also been suggested that somatic mutation of V segments is associated with isotype switching during B cell differentiation. This proposal is based on the observed increase in V_H sequence heterogeneity of IgG versus IgM hybridomas that bind phosphorylcholine (13,14).

We have used a panel of monoclonal anti-idiotype antibodies (MAIDs) with distinct specificities to ask the following questions in

relation to the above problems: (i) do thymus independent (TI) and thymus dependent (TD) B cell precursors produce antibodies with differing idiotope profiles, (ii) what effects do T cells have on the generation of clones secreting multiple immunoglobulin isotypes and (iii) within a single clone secreting more than one isotype do the IgM and non-IgM molecules produced by differentiation of single clonal precursor express different idiotope profiles. By using this approach we planned to determine if T cells play a role in the generation of idiotype diversity and to examine the time frame within which V region diversity may result from genetic events associated with immunoglobulin isotype switching.

Materials and Methods

Animals. Adult BALB/c mice were purchased from The Jackson Laboratory, Bar Harbor, ME, and were used at ages 8 to 20 weeks.

Antigens. Dextran B1355S (DEX), 35% α1->3 linkages, derived from L. mesenteroides, was a gift from Dr. Slodki. Dextran-Limulus hemocyanin (DEX-Hy) and dextran-bovine serum albumin (DEX-BSA) were gifts from R. Ward (15). Dinitrophenyl-ficoll (DNP-ficoll) was a gift from J. Quintans. Limulus hemocyanin (Hy) was obtained from Sigma Chemical Co., St. Louis, MO.

Monoclonal Anti-idiotype Antibodies. The construction of monoclonal anti-idiotype antibodies has been described previously (16). Briefly, they were prepared by immunizing A/J, SJL/J or BALB/cJ mice with purified J558 or M104E proteins. The lymph nodes were then fused with the non-secreting myeloma Ag8.653 (16) and screened according to the protocol previously described (17). Antibodies reactive with J558

or M104E were then tested against a panel of purified myeloma proteins of each mouse isotype. The antibodies which appeared to react with idiotypic determinants on J558 or M104E were subjected to further analysis in a solid phase inhibition assay as described previously (17). Hybridoma lines secreting the monoclonal anti-idiotype antibodies were cultured in RPMI 1640 with 20% fetal calf serum (Grand Island Biological Co., Grand Island, NY) and then purified by affinity chromatography appropriate for the particular isotype. Antibodies which were of the IgG_{2a} class were purified by elution from protein-A sepharose 4B columns (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ). Antibodies of IgG_1 or IgM classes were purified by absorption to and elution from purified goat anti-mouse immunoglobulin coupled to Sepharose 4B (18). All antibodies were dialyzed against borate buffered saline and stored in the same buffer (containing 0.1% sodium azide) at 4°C.

Splenic Focus Assay. The splenic focus assay was performed with minor modifications to that previously described by Klinman (19). Spleens from normal 8- to 10-week-old BALB/c mice were teased apart, washed and passed over a glass wool column. This single spleen cell suspension was transferred intravenously into lethally irradiated (1400 rad) normal or Hy-primed recipients. Eighteen to 24 hours later the recipient spleens were removed and diced into 1 mm cubes. The spleen fragments were then cultured in complete Dulbecco's modified Eagle's medium (Grand Island Biological Co.) with 10% fetal calf serum and antigen, in 96 well tissue culture plates (Costar, Data Packaging, Cambridge, MA) at 37°C. The optimum concentration of each antigen was initially determined by titration in several independent assays.

Dextran B1355S was present in culture at 10 ng/ml while DEX-Hy was present in culture at 1 μg/ml both from day 0 to day 4. TNP-Ficoll was injected intravenously into the recipient mice 1 hour prior to dicing of the irradiated recipient spleens. Supernatants were harvested from each fragment culture every 3 to 4 days and assayed on days 15 or 19 by ELISA.

Assays for Anti-DEX Activity in Supernatants. Polystyrene (Costar) or polyvinyl microtitration plates (Dynatech Corp., Alexandria, VA) were coated with 3 μg/ml DEX-BSA or with 10 μg/ml of each MAID for at least 18 hours at 4°C. Because of the small volume of supernatants (10 μl) to be tested, it was necessary to centrifuge the 96 well microtitration plates after each addition to ensure symmetrical coating of the plastic wells. The plates were then blocked with 1% BSA in borate saline for 30 minutes at room temperature. Ten μl of supernatant was added to each well and incubated at 4°C for at least 18 hours. The appropriate alkaline phosphatase conjugated goat or monoclonal rat (anti-α) anti-mouse isotype antibodies, prepared as previously described (16), were then added to each well and incubated for 3-4 hours at 37°C. The wells were then washed and the (substrate) p-nitrophenyl phosphate, disodium (Sigma Chemical Co.) was added in diethanolamine buffer pH 9.8. The optical density (405 nm) of each well was measured using an automated ELISA reader (Flow Laboratories, Rockville, MD). The limit of detection of anti-DEX antibody in this assay was about 10 ng/ml.

Results

Specificity of Monoclonal Anti-idiotype Antibodies. A summary of the specificities of syngeneic (BALB/cJ) and allogeneic (A/J and SJL/J) monoclonal anti-idiotype antibodies (MAIDs) produced against J558 and M104E which were used in these experiments is shown in Table I. Each of the MAIDs has been shown previously to be hapten (nigerotriose) and DEX inhibitable (5, and unpublished results). The DEX cross-reactive idiotope (IdX) is defined by CD3-2 (γ_1,λ) which recognizes determinants on both J558 and M104, and the majority of DEX binding hybridomas produced in this laboratory and in the laboratory of Dr. B. Clevinger. SJL18-1 (μ,κ) was produced against M104E and discriminates between M104E and J558 in ELISA inhibition assays. It also does not recognize any other myelomas or hybridomas contained within the J558 family (5). The remaining MAIDs EB3-7, B6-10, EB3-16, TD6-4, LA4-8, RD3-2, and JB2-2 were all prepared by immunization with J558, and do not bind M104E or hybridomas possessing the SJL18-1 idiotope. Each of these MAIDs was found to be distinct by their isoelectric focusing patterns and binding specificities (4,20, and unpublished results). Each MAID defines an idiotope present on J558 and recognizes subgroups within the EB3-7 positive family of anti-DEX hybridomas. Some of these idiotopes are also expressed by anti-DEX antibodies outside of the EB3-7 IdI family. A further point to be noted in Table I is that the idiotopes defined by this panel of MAIDs do not preferentially associate with any particular immunoglobulin isotype.

TABLE I

Specificity of monoclonal anti-idiotype antibodies to hybridoma or myeloma proteins[a]

MAID	J558 α,λ	3-19 γ_1,λ	8-21 γ_{2b},λ	1-8 α,λ	M104 μ,λ	B14-1 γ_3,λ
CD3-2	+	+	+	+	+	+
EB3-7	+	+	+	+	-	-
LA4-8	+	+	+	+	-	-
TD6-4	+	+	+	-	-	-
EB3-16	+	+	+	-	-	-
RD3-2	+	+	-	-	-	-
JB2-2	+	+	-	-	-	-
B6-10	+	-	-	-	-	-
SJL18-1	-	-	-	-	+	+

[a]The specificities of monoclonal anti-idiotype antibodies were determined in a solid phase ELISA as previously described (5).

Frequency of Antigen Responsive B-cell Precursors. Since the clonality of the splenic focus assay is critical to the interpretation of data in this study the number of donor cells injected into recipients was adjusted to give a plating efficiency of about 10% in all assays. At this plating efficiency only 0.53% of the wells would be expected by chance (Poisson distribution) to contain two or more DEX positive B cell precursors. As a further test for clonality in this assay, B cell precursor frequencies for two independent antigens, DEX and DNP, were analyzed within the same splenic fragments. The frequencies of DEX-positive and DNP-positive precursors in splenic focus assays stimulated simultaneously with both dextran B1355S and DNP-Ficoll are described in Table II. It can be seen that the frequency of DNP-positive foci is about 30% higher than the frequency of DEX-positive precursors. The observed frequency of foci secreting both anti-DNP and anti-DEX antibody was 1.52% of the total fragments, which is only slightly higher than the frequency of 1.16% expected from a chance association of independent DNP and DEX specific precursors. These results indicate that the homing of DEX-specific and DNP-specific B cell precursors to the spleen occurs randomly and that there do not appear to be certain sites within the spleen that are more conducive to individual precursor homing and maturation.

The frequencies of precursors responding to various TI and TD antigens (including DEX) have been previously described (15,21). Assuming that B cells comprise approximately 40% of the adult spleen cell population and assuming a 4% homing efficiency (15,22) the frequency of precursors responding to the TI antigen B1355S is about 1 in

TABLE II

Frequency of precursors secreting anti-DEX and anti-DNP antibody[a]

Assay Number	Foci Examined	DEX^+ only	DNP^+ only	DEX^+ DNP^+	Expected Doubles
1	288	32[b]	21	2	2.3[c,d]
2	192	30	27	6	4.2
3	192	18	46	4	4.3
4	288	22	35	4	2.7
5	288	15	26	3	1.4
Totals	1248	117	155	19	14.5

[a]Splenic focus cultures were simultaneously stimulated with dextran B1355S and DNP-Ficoll and fragments secreting both anti-DEX and anti-DNP antibody were scored.

[b]The number of fragments in each culture secreting antibody binding the appropriate antigen.

[c]The expected number of foci secreting both anti-DEX and anti-DNP antibody.

[d]The differences between the values for expected and obtained values are all non-significant and P values are not shown.

1.28×10^5 and the frequency of precursors responding to the TD antigen DEX-Hy is about 1 in 7.04×10^4.

Isotype Expression by T-independent and T-dependent B Cell Precursors. The *in vivo* serum antibody response of BALB/c mice to TI-2 antigens such as dextran B1355S has been reported to consist of antibody primarily of the IgM and IgG_3 isotypes (2). The response of carrier primed mice to a TD antigen such as DEX-Hy has been shown to result in production of a larger proportion of antibody of the other IgG subclasses (23). Previous studies on isotype expression by clonal progeny of B cell precursors responding to TD antigens *in vitro* showed a high percentage of foci secreting more than one isotype while the response to TI antigens consists of fewer multiple isotype secretors (21,24,25). Table III compares the isotype distribution of anti-DEX antibodies secreted by the progeny of B cell precursors stimulated by B1355S or by DEX-Hy. The most frequently expressed isotype in response to B1355S was IgM, which was secreted by 90% of all DEX positive foci. IgA was the next most frequently expressed (13.5%) followed by IgG_3 and IgG_{2b} at 2.7% and 1.8%, respectively. No IgG_1 or IgG_{2a} producing clones were detected in the TI assays. Two-thirds of the IgA positive foci in the TI assay also expressed IgM suggesting that most of the IgA positive foci may have originated from IgM precursors. B cell precursors stimulated by the TD antigen DEX-Hy showed a marked decrease in the number of foci secreting IgM anti-DEX antibody; only 42.9% were IgM positive, while the frequency of IgA anti-DEX precursors increased 5-fold to about 81.8%. There was also an increase in the number of foci secreting IgG_{2b} and IgG_1, at

TABLE III

Analysis of the isotypes expressed by splenic foci stimulated by dextran B1355S or DEX-Hy

Isotypes Expressed	Antigen[a]	
	Dextran B1355S	DEX-Hy
IgM	89.7 (79.8)[b]	42.9 (9.1)
IgG_1	0	3.8 (1.3)
IgG_{2a}	0	0
IgG_{2b}	1.8 (1.3)	16.9 (2.6)
IgG_3	2.7 (2.2)	0
IgA	13.5 (4.5)	81.8 (46.8)
2 isotypes	9.9	32.5
3 isotypes	0	5.2
4 isotypes	0	1.3

[a]The TI-2 antigen dextran B1355S or the TD antigen DEX-Hy were used to stimulate precursors in the splenic focus assay.

[b]The percentage of DEX positive foci expressing the appropriate isotype alone or in association with any other isotype. The number in parenthesis indicates the percentage of foci expressing only the indicated isotype.

16.9% and 3.8%, respectively. The frequency of IgG_3 positive anti-DEX foci showed little change when TI and TD assays were compared.

In addition to increased expression of most of the non-IgM isotypes in the TD assays, the frequency of foci secreting more than one isotype also increased, even though the overall frequencies of DEX-positive precursors were similar for both the TI and TD assays. The frequency of foci secreting two isotypes in the TI assay was about 9.9% of the total DEX positive foci, and none secreted more than two isotypes. The frequency of foci secreting two isotypes in the TD assay was 32.5% of total DEX positive foci, and 6.5% of the foci secreted more than two isotypes. In both the TI and TD assays the most frequently associated isotypes were IgM and IgA (data not shown). When the observed frequency of clones secreting multiple isotypes was compared to the predicted frequency of clones that would contain multiple isotypes resulting from two independent precursors it is apparent that most of the multiple isotype secreting foci are progeny of one precursor and the result of class switching events (Table IV).

Idiotype Expression of Antibody Secreted by B Cell Precursors Responding to Dextran B1355S and DEX-Hy. Although monoclonal anti-idiotype antibodies exhibit exquisite specificity they cannot be used to define clonal markers on B cells since it has been shown previously in our laboratory and others that most individual MAIDs bind a number of hybridoma and myeloma proteins which possess small but distinct differences in amino acid sequences (20, and J. Kearney, unpublished results). However, by using a large panel of distinct MAIDs, an idiotope profile can be obtained for different clonally derived DEX binding antibodies from splenic foci. We have used our panel of MAIDs

TABLE IV

Frequency of multiple isotype secreting foci responding to the TD antigen DEX-Hy

Isotypes	Observed	Predicted
IgM-IgA	29.4[a]	3.11[b]
IgM-IgG_{2b}	2.2	0.65
IgG_{2b}-IgA	3.7	1.23
IgM-IgG_{2b}-IgA	2.9	0.047
IgM-IgG_1-IgG_{2b}-IgA	1.5	0.0001

[a]The percentage of DEX-positive splenic foci secreting multiple isotypes in the TD assays.

[b]The predicted percentage of DEX-positive splenic foci secreting multiple isotypes.

specific for DEX binding proteins (Table I) to analyze, in fine detail, the expression of idiotopes on antibodies secreted by splenic foci stimulated with DEX and DEX-Hy. Table V illustrates independent expression of the various MAID defined idiotopes on DEX binding foci derived antibodies. It is apparent that when the idiotopes expressed in the TI assays are compared to those expressed in the TD assays no significant differences are apparent in the selection of idiotopes expressed in each assay. These results indicate that generation of and/or selection of diverse idiotopes in the DEX system is independent of the presence of carrier primed T cells.

The majority (84%) of TI and TD foci secreted antibody expressing the CD3-2 idiotope (IdX). About 73% of the foci expressed the EB3-7 idiotope (J558 IdI). Next most frequently expressed is the LA4-8 idiotope at 57%. The LA4-8 idiotope is also associated with a portion of both the J558 and M104E IdI families. The SJL18-1 idiotope (M104E IdI) is found on about 32% of antibody secreted by foci. Although the EB3-7 and SJL18-1 idiotopes defined two separate non-overlapping groups of myeloma and hybridoma proteins, 8.5% of DEX positive foci expressed both idiotopes. This is due in part to the random association of two different IdI-bearing precursors, and the other possibility is that the two idiotope families can on rare occasions overlap. The majority of DEX positive antibodies expressed either the EB3-7 or SJL18-1 idiotopes, along with the cross-reactive CD3-2 idiotope in agreement with previous analysis using heterologous anti-idiotype antibodies that J558 and M104E-like antibodies constitute most of the λ anti-DEX response in BALB/c mice (3-5). The minor idiotopes EB3-16 and B6-10 (expressed by J558) are detected on only about 10% and 3% of

TABLE V

Analysis of the idiotopes expressed by splenic foci stimulated by dextran B1355S or DEX-Hy[a]

Idiotope	Dextran B1355S	DEX-HY
EB3-7	74.1[b]	71.9
B6-10	2.3	4.7
EB3-16	6.8	14.0
TD6-4	17.3	29.0
LA4-8	59.0	55.1
RD3-2	4.2	12.7
JB2-2	4.8	5.1
SJL18-1	26.5	38.0
CD3-2	83.6	84.4

[a]The TI-2 antigen dextran B1355S or the TD antigen DEX-Hy were used to stimulate precursors in the splenic focus assay.

[b]The percentage of DEX positive splenic foci expressing the MAID defined idiotope.

DEX positive foci. The EB3-16 idiotope is always associated with the EB3-7 idiotope, and the B6-10 idiotope is always associated with both EB3-16 and E3-7 idiotopes. EB3-16 and B6-10 defined smaller subsets within the J558 idiotype family. The TD6-4, RD3-2 and JB2-2 idiotopes are expressed on about 23%, 8% and 5% of antibodies, respectively, and are usually associated with the J558 idiotope defined by EB3-7.

When the 209 anti-DEX producing monofocal supernatants (from a total of 2349 splenic fragments) were assayed against the full panel of 9 MAIDs, 35 different idiotope profiles were obtained. Most foci-derived antibodies therefore expressed several idiotopes simultaneously, resulting in an idiotope profile which can be considered equivalent to idiotype. There was no preferential association of any idiotope profile with any particular isotype.

Intraclonal Idiotope Profiles of Monofocal Antibodies Differing in Isotypes. The diverse fine specificities of the MAIDs used in this study provided a method to detect minor differences in idiotopes expressed by distinct antibodies generated within individual splenic foci. Accordingly if somatic mutation occurred frequently during the antigen stimulated maturation of precursors to plasma cells then the panel of MAIDs used in this study would be expected to detect some of these changes if they occurred at critical residues and resulted in changes in idiotypes. The straightforward approach which we used was to examine foci secreting more than one isotype and determine if there were any differences in idiotope expression by the different isotypes detected within the same focus. A total of 45 double and triple isotype-secreting foci were examined for idiotope-isotype associations. Twenty-three of these exhibited identical idiotope patterns for all

isotypes expressed (several foci expressed only one or two idiotopes). In 22 foci containing multiple isotypes, distinct idiotope profile differences were detected on the IgM and non-IgM isotypes. Table VI lists all examples of multiple isotype secreting foci in which differences were detected. Most of these differences were probably not due to the presence of more than one DEX specific precursor in the original fragment because of the low plating efficiency used. The frequency of fragments which would statistically be expected to contain more than one DEX responsive precursor at a 10% plating efficiently is 0.53%. The frequency of foci coexpressing IgM and IgA by chance is about 3.1% of DEX positive foci which is 10-fold lower than the actual frequency of IgM-IgA doubles obtained (29.4%) suggesting that multiple isotype secreting foci are the result of isotype switching. The idiotope differences detected on the different isotypes originating from one precursor could possibly be due to a competitive inhibition of binding by one isotype over another. Such a possibility is very unlikely because of the high coating concentration used in the ELISA, 10 μg/ml. The low concentration of antibody in the monofocal supernatants (100-400 ng/ml) did not saturate the MAID coated wells. The possibility of competitive inhibition was also tested by mixing monofocal supernatants of different isotypes and known idiotope profiles. No inhibition of idiotope deletion was observed in all of these cases.

A further way of strengthening the evidence for the clonal origin of multiple isotype secreting foci is to examine the expression of the infrequent idiotopes on each of the isotypes secreted in one fragment (Table V). The EB3-16 idiotope is ideal for this purpose since it is

TABLE VI

Intraclonal idiotope profiles of monofocal antibodies differing in isotypes[a]

		Idiotopes							
Focus	Isotypes	EB37	B6-10	EB3-16	TD6	LA4	RD3	SJL18[b]	CD-3
D484	IgM	-	-	-	-	-	-	-	-
	IgA	+	+	+	-	-	-	-	+
D4C5	IgG2b	+	-	+	+	+	+	-	+
	IgA	+	+	+	+	+	+	-	+
D6E12	IgM	+	-	+	+	+	+	-	+
	IgG_{2b}	+	-	+	-	-	-	-	+
	IgA	+	-	+	+	+	+	-	+
D6A8	IgG_{2b}	-	-	-	-	-	-	-	+
	IgA	-	-	-	-	-	-	+	+
D7A2	IgM	-	-	-	-	-	-	-	+
	IgG_{2b}	-	-	-	-	-	-	-	-
	IgA	+	-	-	-	+	-	-	+
D7F9	IgG_{2b}	+	-	+	-	+	-	-	+
	IgA	+	-	-	-	-	-	-	+
D7D7	IgM	+	-	+	+	+	-	-	+
	IgG_{2b}	+	-	-	-	-	-	-	+
	IgA	+	-	-	-	-	-	-	+
D7A12	IgM	+	-	-	-	-	-	-	+
	IgG_{2b}	-	-	-	-	-	-	-	-
D7E5	IgM	+	-	-	+	+	-	-	+
	IgA	+	-	+	+	+	-	-	+
D7B11	IgG_{2b}	-	-	-	-	-	-	-	+
	IgA	+	-	-	-	-	-	-	+
T10E11	IgM	+	+	+	-	-	-	-	+
	IgA	+	-	+	-	-	-	-	+
I11E11	IgM	-	-	-	-	-	-	-	+
	IgA	-	-	-	-	-	-	+	-
I12C1	IgM	+	+	-	-	-	-	-	+
	IgA	+	-	+	-	-	-	-	+
D73D7	IgM	-	-	-	-	-	-	?	+
	IgA	+	-	-	-	-	-	+	+
I16F8	IgM	-	-	-	-	-	-	?	+
	IgA	-	-	-	-	-	-	+	+
I4D10	IgM	+	-	-	-	-	-	-	+
I15C6	IgA	+	-	-	-	-	-	-	-
I17H5									
I18DF12									
D6C7	IgM	+	-	-	-	+	-	-	+
D6B7	IgA	+	-	-	-	-	-	-	+
D6H7									

[a]Monofocal supernatants were added to 96 well microtiter plates coated with all 9 MAIDs, and then developed with each AP-conjugated anti-mouse isotype corresponding to the isotypes expressed by each individual focus.

[b]? indicates that IgM reactivity with SJL18-1 could not be determined since SJL18-1 is of the IgM isotype; - indicates that the IgM containing focus in the SJL18-1 column contained no lambda bearing anti-DEX antibody bearing the SJL18-1 idiotope.

expressed on only about 6-14% of DEX positive foci. The frequencies (Poisson distribution) at which this idiotope would be expected to occur on DEX positive antibodies secreted by two independent precursors is 0.098% for focus D4C5, 0.023% for focus T10E11, and 0.0005% for EB3-16 to appear on all three different isotypes secreted by focus D6E12. The actual frequencies at which the EB3-16 idiotope appears on individual isotypes is therefore 10-5000 fold higher than that expected. These statistical analyses strongly support the origin of multiple isotype secreting foci from one precursor cell. However, within these same foci it can be seen that there are idiotope shifts on the different isotypes detected.

The most likely explanation for the idiotope differences between the different isotypes within many of these foci is that they result from somatic variations occurring after in vitro antigenic stimulation and may be related to isotype switching events.

Discussion

The restricted and well-defined nature of the idiotype response of BALB/c mice to α1->3 dextran provides a model in which to study the regulation and generation of antibody diversity (1-5). Amino acid analysis of hybridomas binding DEX (and other antigens) has shown that considerable heterogeneity exists between closely related families of antibodies binding the same antigen (8,9,14). However, these observed differences were usually limited to only a small number of amino acid residues. Somatic mutation has been exposed to account for this slight drift from the germline sequences which may be an important mechanism for the generation of antibody diversity (9,11-13).

By examining the sequences of a number of hybridomas with our panel of MAIDs, a correlation between idotype and certain amino acid residues can be made. For example, it has been shown that certain D region sequences were essential for reactivity with certain IdI specific MAIDs and a glycosylated asparagine is important for reactivity with the IdX specific MAID CD3-2 (20, and B. Clevinger, personal communication). The same data also suggests that some amino acid substitutions can also be silent and have little or no effect on id-anti-id recognition. Each of the nine MAIDs used in this study is distinct by (i) isoelectric focusing (5), (ii) by their reactivity with a large panel of anti-DEX hybridomas and (iii) by their reactivity with a preliminary set of monofocal supernatants (unpublished results). Since the substitution of as few as one residue can be detected by appropriate MAIDs in some cases, we reasoned that this large panel of MAIDs would increase the likelihood of detecting minor idiotope shifts on monofocal antibodies which would occur as the result of somatic mutation. The primary goals of this study were: (1) to determine the potential heterogeneity of B cell precursors capable of responding to DEX, (ii) to examine the heterogeneity associated with each isotype expressed in response to DEX, and (iii) to determine whether idiotope differences occur within foci which have undergone an isotype switch and which secrete more than one isotype.

The majority (Table III) of DEX positive splenic foci stimulated by the TI form of the antigen secrete antibody bearing only the IgM isotype. Only about 4.5% of the foci secreted antibody of any of the IgG isotypes and only 13.5% secrete IgA, most of which also contained IgM. In contrast, only 9.1% of DEX positive foci stimulated by the TD

form of the antigen expressed IgM alone. An increase in detectable IgG and IgA isotypes was also apparent. There was also a 4-fold increase in foci secreting 2 or more isotypes. The difference in isotypes of antibodies secreted by foci responding to TI or TD forms of α1->3 dextran implies a direct role for carrier primed T cells in regulating the expression of the various isotypes in the splenic focus assay. The serum of DEX-Hy immunized BALB/c mice (Hy primed 6 weeks earlier) contains little or no detectable IgA. The striking increase in IgA secreting foci after stimulation by the TD antigen DEX-Hy may then be the result of T cell mediated isotype switching or the result of enhanced IgA specific T helper cell activity in this in vitro system. As suggested by others the IgA bearing B cell precursors may, in vivo, migrate to mucosal sites and as a result low levels of IgA would be expected to be found in serum (26,27). The distribution of the various isotypes in the DEX TI assay is considerably different from the results published in other antigen systems such as the TNP system (28). In the TNP (TI assays) system most foci expressed multiple isotypes, and a much higher frequency of IgG secreting foci were detected. However, nude mice were used as recipients, a different antigen was used and a higher frequency of positive foci was obtained.

An idiotope profile can be prepared for antibodies in each monofocal supernatant based on the reactivity of each individual MAID. A total of 35 different idiotope profiles were expressed by a total of 223 DEX positive monofocal supernatants. This is probably a conservative indication of V region diversity within this group of DEX specific clones since not every amino acid sequence difference in individual proteins will be detected by this panel. Variations which are

silent and do not affect idiotopes will not be detected. There appeared to be very little difference between the idiotope profiles of IgM, IgG and IgA antibodies, indicating that there was no preferential association between an idiotope profile and any particular isotype. This is in contrast to data derived from amino acid sequence analysis of anti-PC hybridomas which showed a greater degree of heterogeneity among IgG hybridomas versus IgM hybridomas (13,14). Increased heterogeneity may exist in IgG compared to IgM antibodies in these foci but are not detectable by the panel of MAIDs used in this study. Despite this drawback it is quite evident that the IgM foci derived antibodies already express considerable V region diversity unrelated to class switching events. The idiotopes which were detected by the monofocal antibodies in this study correlated well with the frequencies of idiotopes expressed by anti-DEX hybridomas and detectable in DEX immune sera of BALB/c mice. We have previously reported that the B6-10 idiotope is not detectable in DEX immune sera of BALB/c mice (5). The reason for non-expression of the B6-10 idiotope in sera is probably due to the very low frequency of responsive B6-10 positive precursors observed in our splenic focus assays. The B6-10 idiotope may be the result of an infrequently expressed germline gene, or the result of somatic variation of a germline gene.

One of the major questions to be asked in this study was whether any differences in idiotope profiles exist between the multiple isotypes secreted by the progeny of one precursor in any given fragment. It has been suggested of anti-PC hybridomas which showed a greater degree of heterogeneity among IgG hybridomas versus IgM hybridomas (13,14). Increased heterogeneity may exist in IgG compared to IgM

antibodies in these foci but are not detectable by the panel of MAIDs used in this study. Despite this drawback it is quite evident that the IgM foci derived antibodies already express considerable V region diversity unrelated to class switching events. The idiotopes which were detected by the monofocal antibodies in this study correlated well with the frequencies of idiotopes expressed by anti-DEX hybridomas and detectable in DEX immune sera of BALB/c mice. We have previously reported that the B6-10 idiotope is not detectable in DEX immune sera of BALB/c mice (5). The reason for non-expression of the B6-10 idiotope in sera is probably due to the very low frequency of responsive B6-10 positive precursors observed in our splenic focus assays. The B6-10 idiotope may be the result of an infrequently expressed germline gene, or the result of somatic variation of a germline gene.

One of the major questions to be asked in this study was whether any differences in idiotope profiles exist between the multiple isotypes secreted by the progeny of one precursor in any given fragment. It has been suggested that somatic variation may accompany a class switch by the activation of a hypermutation mechanism, or somatic variation may occur throughout the life of a B cell with the result that older cells accumulate more mutations; these cells would also be more likely to have undergone a class switch (11-14). Splenic foci secreting more than one isotype provide a convenient means for examining the above hypothesis. Table VI clearly shows that in about 50% of the cases examined that the multiple isotype secreting foci express different idiotope profiles. This should be considered a conservative estimate since not all differences will be detected by the panel of

MAIDs used in this study. It must be concluded that in order for these idiotope differences to be detectable the idiotope shift must have occurred about the same time as the class switch. If not, then the antibodies examined would consist of heterogeneous populations of idiotypes associated with each isotype which would not be detectable in our assay. If the rate of somatic variation is very high with variation occurring well before or after class switching, then our assay may only be detecting somatic mutations which occurred by chance at about the same time as a class switch and are maintained thereafter during further clonal growth. A high degree of idiotype heterogeneity was also detected among the IgM precursors. This suggests that somatic variation may not always be associated with class switching. It is also evident that somatic variation involving B cell precursors which results in these distinct antibodies can occur within a very short time frame, of less than 15 days which is the time period during which the precursor B cells are cultured.

In conclusion, the splenic focus assay in conjunction with the use of MAIDs provides a powerful tool with which to probe possible mechanisms for the generation of antibody diversity. When comparing TI and TD assays it becomes apparent that carrier primed T cells are important in inducing expression of isotypes downstream from $C\mu$. The presence of carrier primed T cells does not appear to affect the expression of any particular idiotope or idiotope profile and the time frame in which the splenic focus assay is run (11-20 days) provides enough time for somatic mutation and subsequent idiotope changes to occur following antgenic stimulation.

What role does the generation of somatic variants play within the immune system? Are there redundant or degenerative antibodies? Isolation of clonally derived cell line variants by cell fusion procedures and structural analysis of immunoglobulins that they produce would provide more insight into the significance of and the mechanisms involved in generation of such variant immunoglobulins.

Summary

In this study BALB/c B cell precursors responsive to the T independent type 2 (TI-2) antigen, dextran B1355S (DEX) and the T dependent derivative, dextran-Limulus hemocyanin (DEX-Hy) were examined for isotype and idiotope expression using the splenic focus assay. The predominant isotype detected in the TI assay was IgM while IgA was the predominant isotype expressed in the TD assay. There was also a 4-fold increase in the number of foci secreting more than one isotype in the TD assay versus the TI assay without an overall change in anti-DEX precursor frequency suggesting that carrier primed T cells enhance the expression of non-IgM isotypes possibly by increasing the frequency of isotype switching by individual B cell precursors.

A panel of distinct monoclonal anti-idiotype antibodies (MAIDs) was then used to examine idiotope expression by antibodies secreted in splenic foci responding to DEX and DEX-Hy. This analysis revealed considerable diversity in the idiotope profiles expressed by all isotypes tested. There appeared to be no differences in idiotope diversity among the various isotypes. A similar diversity of idiotope profiles was obtained from both TI and TD splenic foci indicating that a comparable degree of diversity was associated with the antibodies

generated by TI and TD precursors. Idiotype analysis of IgM-IgA secreting foci with a panel of monoclonal anti-idiotope antibodies revealed slight idiotypic differences between the two isotypes secreted in the same focus in about half the cases. These results suggest that somatic variation occurs during the antigen driven maturation of B cell precursors, within the 15-day time frame of the splenic focus assay, and may be associated with isotype switching.

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ONTOGENY OF B CELL PRECURSORS RESPONDING TO ALPHA 1->3 DEXTRAN IN BALB/c MICE[1]

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Footnotes

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[2]Abbreviations used in this paper: LPS, lipopolysaccharide; TI, T cell independent; TNP, trinitrophenyl; PC, phosphorylcholine; Ars, arsonate; DNP, dinitrophenyl; IdX, cross-reactive idiotype; DEX, alpha 1->3 dextran; MAID, monoclonal anti-idiotype antibody; BSA, bovine serum albumin; Hy, hemocyanin.

ABSTRACT

The ontogeny of the BALB/c B cell repertoire with specificity for alpha 1->3 dextran (DEX) was examined using monoclonal anti-idiotype antibodies (MAIDs). Anti-DEX B cell precursors were absent from donor mice less than 5 days old. Precursors were first detected in mice from 5-11 days of age but at a very low frequency of <1 in 10^8. As the mice aged anti-DEX precursors increased in frequency gradually, but spleens from 30-day-old donors still contained 20 fold less anti-DEX precursor than adult mice. Seventy-five percent of splenic foci from 5-11 day old donors expressed IgA and 70% expressed both IgM and IgA. The frequency of DEX positive IgA and IgM-IgA secreting foci decreased as the age of the donors increased. The frequency of IgM secreting foci remained constant at about 90% of DEX positive foci regardless of donor age. The frequency of the MAID defined cross-reactive idiotope CD3-2 and the EB3-16 idiotope changed very little in frequency with age, while the EB3-7 and LA4-8 idiotopes increased in frequency as donor age increased. Conversely, the SJL18-1 idiotope which was predominant at days 5-11 decreased in frequency relative to DEX positive foci as the age of the donors was increased. The ratios of M104E-like and J558-like molecules from neonatal B cell precursors is

reversed from that expressed by adult B cell precursors and may reflect some type of regulatory mechanism.

INTRODUCTION

The ability of the immune system to respond to various antigens in vivo is an age dependent process. Fetal lambs are able to mount antibody responses to certain viruses, ferritin, and hemocyanin, while the responses to diphtheria toxin and Salmonella O antigen are delayed until about 40 days of age (1). One day old BALB/c mice can mount an antibody response after challenges by lipopolysaccharide (LPS) , galactan, and levan (2). However, mice immunized before day 14 with a T-independent type 2 (TI-2) antigen, trinitrophenyl-ficoll (TNP-ficoll) are unable to produce anti-TNP antibodies, while immunization with a T-independent type-1 (TI-1) antigen trinitrophenyl-lipopolysaccharide (TNP-LPS) does elicit an antibody response (2). Analyses of B cell precursors responsive to phosphorylcholine (PC) have demonstrated that PC responsiveness does not arise until after day 4 in BALB/c mice (3,4). Results obtained in the arsonate (Ars) antigen system also suggest that the anti-Ars response arises at about the same time as PC responsiveness at about day 4 (2). In contrast to these relatively early arising responses the response to alpha 1->6 dextran has been shown to develop after 1 month of age (5). The ability of BALB/c mice to respond to inulin is another example of late development of responsiveness. The earliest age at which

these mice can mount a plaque forming cell response or a serum antibody response appears to be at 14 days (2,6).

Antigen responsiveness and the dominant idiotypes are not always expressed simultaneously during ontogeny. When the anti-inulin response is first detected at about day 14 the usually dominant (in adult mice) E109 idiotype is absent (2,6). At about 28 days of age the E109 idiotype begins to appear and eventually becomes the dominant idiotype in adult mice. The expression of two idiotypes associated with dinitrophenyl (DNP) binding antibodies have been shown to change considerably between days 1 and 13 (7). The TF2-36 idiotype represents about 14% of anti-DNP hybridomas at days 1-4 but less than 2%, at 7-8 days after birth. At days 1-3 the TF2-76 idiotype represents less than 1% of anti-DNP hybridomas but by days 12-13 TF2-76 represents almost 7% of the anti-DNP hybridomas.

We have examined in detail the ontogeny of the anti-DEX response in BALB/c mice. The isotypic and idiotypic makeup of adult BALB/c B cell precursors stimulated by DEX has been well characterized (8,9). We have previously prepared a large panel of anti-idiotype antibodies (MAIDs) which enable us to examine idiotope expression by neonatal B cell precursors in fine detail (8). Precursors from mice less than 5 days of age are unable to respond to DEX. After 5 days low frequencies of DEX positive foci are detected. As the neonatal mice age the frequency of anti-DEX precursors

increases, while the isotypes expressed and the idiotope patterns change.

MATERIALS AND METHODS

Animals. Adult BALB/c mice were purchased from The Jackson Laboratory, Bar Harbor, ME, and were used as recipients at ages 8 to 20 weeks. Neonatal BALB/c mice used as donors were obtained from our own breeder colonies.

Antigens. Dextran B1355S (DEX), 35% alpha 1->3 linkages, derived from L. mesenteroides, was a gift from Dr. Slodki. Dextran-bovine serum albumin (DEX-BSA) was a gift from R. Ward (10).

Monoclonal Anti-idiotype Antibodies. The construction of monoclonal anti-idiotype antibodies has been described previously (11). Briefly, they were prepared by immunizing A/J, SJL/J or BALB/cJ mice with purified J558 or M104E proteins. The lymph nodes were then fused with the non-secreting myeloma P3x63 Ag8.653 and screened according to the protocol previously described (12). Antibodies reactive with J558 or M104E were then tested against a panel of purified myeloma proteins of each mouse isotype. The antibodies which appeared to react with idiotypic determinants on J558 or M104E were subjected to further analysis in a solid phase inhibition assay described previously (12). Hybridoma lines secreting the monoclonal

anti-idiotype antibodies were cultured in RPMI 1640 with 20% fetal calf serum (Grand Island Biological Co., Grand Island, NY) and then purified by affinity chromatography appropriate for the particular isotype. Antibodies which were of the IgG_{2a} class were purified by elution from protein-A sepharose 4B columns (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ). Antibodies of IgG_1 or IgM classes were purified by absorption to and elution from purified goat anti-mouse immunoglobulin coupled to sepharose 4B (13). All antibodies were dialyzed against borate buffered saline and stored in the same buffer (containing 0.1% sodium azide) at $4^{o}C$.

Splenic Focus Assay. The splenic focus assay was performed with minor modifications to that previously described by Klinman (14). Spleens from normal 1-to 28-day-old BALB/c mice were teased apart, washed and passed over a small glass wool column. This single spleen cell suspension was transferred intravenously into lethally irradiated (1400 rad) adult BALB/c recipients. Eighteen to 24 hours later the recipient spleens were removed and diced into 1 mm cubes. The spleen fragments were then cultured in complete Dulbecco's modified Eagle's medium (Grand Island Biological Co.) with 10% fetal calf serum and antigen, in 96 well tissue culture plates (Costar, Data Packaging, Cambridge, MA). Dextran B1355S (10 ug) was injected intravenously into the recipient mice 1 hour prior

to dicing of the irradiated recipient spleens. Supernatants were harvested from each fragment culture every 3 to 4 days and assayed on days 15 or 19 by ELISA.

Assays for Anti-DEX Activity in Supernatants. Polystyrene (Costar) or polyvinyl microtitration plates (Dynatech Corp., Alexandria, VA) were coated with 3 ug/ml DEX-BSA or with 10 ug/ml of each MAID for at least 18 hours at 4°C. Because of the small volume of supernatants (10 ul) to be tested, it was necessary to centrifuge the 96 well microtitration plates after each addition to ensure symmetrical coating of the plastic wells. The plates were then blocked with 1% BSA in borate saline for 30 minutes at room temperature. Ten ul of supernatant was added to each well and incubated at 4°C for at least 18 hours. The appropriate alkaline phosphatase conjugated goat or monoclonal rat anti-mouse isotype antibodies, prepared as previously described (12), were then added to each well and incubated for 3-4 hours at 37°C. The wells were then washed and the (substrate) p-nitrophenyl phosphate, disodium (Sigma Chemical Co.) was added in diethanolamine buffer pH 9.8. The optical density (405 nm) of each well was measured using an automated ELISA reader (Flow laboratories, Rockville, MD). The limit of detection of anti-DEX antibody in this assay was about 10 ng/ml.

RESULTS

Frequency of lambda bearing anti-DEX B cell precursors in neonatal BALB/c mice. As previously reported and as shown in Table I the frequency of B cell precursors capable of responding to DEX in adult BALB/c mice (8 to 12 weeks old) is about 1 in 1.28×10^5 (9). Before day 5 no anti-DEX precursors could be detected in spleen as shown or from fetal livers (R. Stohrer, unpublished observations). Day 5-11 spleen cells yielded DEX positive foci at a frequency of 1 in 1.1×10^8. As the age of the donor mice increased the frequency of DEX precursor in spleens also increased. Although the proportion of B cells in the spleen is approximately 10 fold lower in neonatal mice than in adults, the absolute difference between adult and neonatal B cell frequencies is not sufficient to account for the 1000 fold difference in anti-DEX precursor frequencies seen between the youngest age group of neonates and adults.

Isotype expression by B cell precursors from neonatal mice. The response of adult mouse B cell precursors to T-independent type 2 (TI-2) antigens is usually predominantly IgM (8,15,16). IgM is secreted by 89.7%, of the foci after stimulation with DEX (Table 2); however, the IgG isotypes are expressed infrequently by DEX stimulated precursors from adult mice. IgG_{2b} and IgG_3 producing foci represent only 1.8%, and 2.7%, of the DEX positive splenic

TABLE I

Frequency of DEX positive precursors from donors of various ages

Age group[a]	Frequency[b]
0-4	-[c]
5-11	1 in 1.1×10^8
12-15	1 in 1.6×10^7
16-20	1 in 4.2×10^6
21-30	1 in 6.0×10^6
>30	1 in 2.5×10^6

[a] Age of donor mice in days.
[b] Frequency of DEX positive precursors as a function of spleen cells injected.
[c] No DEX positive foci were detected before 5 days of age.

TABLE II

Isotype expression by dextran reactive precursors from BALB/c mice of different ages

Isotypes Expressed	Age of Donors 5-11 days	12-15 days	16-20 days	21-30 days	>30 days
IgM	95.0[a]	81.1	89.1	85.7	89.7
IgG_{2b}	5.0	<6.3	4.3	3.6	1.8
IgG_3	<5.0	<6.3	4.3	<3.6	2.7
IgA	75.0	56.3	41.5	53.6	13.5
IgM-IgA	70.0	31.3	31.7	39.3	9.1

[a]Percentage of DEX positive splenic foci.

foci, respectively. IgA is expressed by 13.5% of the DEX positive precursors from adult mice and IgM-IgA double isotype secreting foci account for 9.1% of the DEX positive foci. While the expression of IgM is constant for all of the age groups at about 90% of foci, there is a much higher frequency of IgA secreting precursors, especially in the youngest age group. The frequency of IgM-IgA double secreting foci is also dramatically higher in the youngest age group. The high frequency of IgA and IgM-IgA secreting precursors in the youngest age groups may be because the neonates probably first encounter antigens through mucosal surfaces which would trigger IgA production.

Variable idiotype expression by B cell precursors during neonatal development. A panel of 9 monoclonal anti-idiotype antibodies (MAIDs) was used previously to analyze the idiotypic repertoire of adult B cell precursors responding to DEX (8). The same panel of MAIDs was used to examine the ontogeny of the BALB/c antibody response to DEX at the B cell precursor level. The idiotopes defined by B6-10, TD6-4, RD3-2 and JB2-2 are usually expressed at such low frequencies (Table III) that they were not always detected in each age group, so that it was not possible to make statistically signifigant comparisons between each age group. The cross-reactive idiotype (IdX) defined by CD3-2 was consistently expressed by 90% of the DEX positive precursors in each age group. The EB3-16 idiotope (J558

TABLE III

Effect of donor age on the expression of idiotopes by splenic foci.

Idiotopes Expressed	Age of Donors 5-11 days	12-15 days	16-20 days	21-30 days	>30 days
EB3-7	10.0[a]	56.3	71.1	73.9	74.1
B6-10	<5.0	<6.3	6.6	<2.2	2.3
EB3-16	5.0	6.3	11.1	4.3	6.8
TD6-4	<5.0	<6.3	17.7	<2.2	17.3
LA4-8	30.0	43.8	51.1	78.2	59.0
RD3-2	<5.0	<6.3	6.6	<2.2	4.2
JB2-2	<5.0	<6.3	6.6	<2.2	4.8
SJL18-1	80.0	37.5	28.8	30.4	26.5
CD3-2	90.0	81.3	75.5	95.6	83.6

[a]Percentage of DEX positive foci expressing the idiotope.

family) was expressed by about 6% of DEX positive precursors from all age groups. However, the major idiotope within the "J558 family" EB3-7, was expressed by only 10% of the DEX positive precursors from the 5-11 day old age group. This frequency then increased until at days 12-15, 56.3% of the foci were EB3-7 positive and at days 16-20, 71.1% of precursors were EB3-7 positive (the approximate adult frequency). While the EB3-7 idiotope frequency is very low on days 5-11, the SJL18-1 idiotope (M104 family) is expressed by 80% of precursors. The SJL18-1 idiotope frequency rapidly decreases to 37.5% at days 12-15, and at days 16-20 and 21-30 the frequencies are equal to the adult frequency of about 27%. The LA4-8 idiotope expression also increases with the age of the donor mice from 30% to 60% of DEX positive foci, which is not to the same degree as EB3-7 or SJL18-1 positive foci change frequency.

DISCUSSION

Neonatal mice do not appear to possess the ability to respond to antigenic stimulation with the same antibody repertoire that adult mice normally express (3,18,19). During the development of the neonate there appears to be a sequential activation of V genes. It has been noted that certain antigenic specificities appear much earlier than

others (1-4). Although the sequence and time during ontogeny which antibody producing precursors with these antigen specificities appear is well documented, there have been few studies on the ontogeny of the fine idiotype repertoire expressed within a particular antigen system. MAID defined idiotope profiles of anti-DEX antibodies can be used as indicators of immunoglobulin diversity. The main purposes of this study were: (i) to examine and the idiotype repertoire of neonatal mice and compare it to that of adult mice, (ii) to determine if there is any chronological expression of any particular idiotopes over others and (iii) to examine and compare differences in isotype expression among precursors from neonatal and adult mice.

The low frequency of DEX responsive B cell precursors recovered from mice less than 21 days of age hindered the examination of expression of the less frequent idiotopes such as B6-10, TD6-4, RD3-2 and JB2-2. The frequency of precursors (expressed as a function of spleen cells) is less than 1 in 10^8 for the youngest age group, which required running a large number of splenic focus assays to get even a small number of DEX positive precursors. As the age of the mice increased so did the frequency of DEX positive precursors, but even at day 30 the frequency was still 20 fold less than adult anti-DEX precursor frequencies.

It has been reported that BALB/c mice are unable to mount an IgA plaque forming cell response to DEX before 4 weeks of age (17). It is apparent from this study that there is a high frequency of DEX positive B cell precursors secreting the IgA isotype from donors only 5 days of age. Virtually all of the IgA positive foci also secreted IgM. This is similar to the pattern of IgA expression by foci from adult donors where 67%, of IgA positive anti-DEX foci also secreted IgM. Very few IgG secreting foci were detected among neonatal or adult ant-DEX precursors. There are a number of reasons why IgA may be expressed at such a high frequency during the early days of DEX responsiveness. During the first few days of life neonatal mice are first exposed to many environmental antigens via mucosal routes. The high frequency of IgA may be because of the need for seeding of the mucosal surfaces with IgA positive B cells. Another alternative is that the high IgA frequency may be the result of some maternally derived factors present in the milk which could enhance IgA expression.

Along with sequential acquisition of antigen specificities, idiotypes have also been reported to be differentially expressed during neonatal development (2,7). The immunoglobulin repertoire of neonatal mice has been reported to be somewhat limited compared to adult mice (3,18,19). By utilizing our panel of 9 monoclonal anti-idiotype antibodies (MAIDs) we have examined the

idiotope repertoire of B cell precursors responding to DEX from neonatal mice and have shown that there appeared to be little difference in the heterogeneity of the idiotope profiles compared to those expressed by adult precursors. The only idiotope which appeared much less frequently among neonatal foci was the TD6-4 idiotope, which indicates that the neonatal repertoire may be slightly smaller than the adult anti-DEX repertoire. The CD3-2 idiotope (IdX) is expressed by about 90%, of DEX positive foci regardless of the age of the donors. This indicates that the IdX gene(s) is(are) expressed early, although there are IdX negative variants.

Whether the IdX negative antibodies are germline encoded or somatically derived remains to be seen. The EB3-16 idiotope is also expressed at a constant frequency of about 6% of DEX positive foci regardless of the age of the donor. The LA4-8 idiotope increases in frequency from 30% at days 5-11 to 60% for adults. The increase in EB3-7 idiotope frequency and the reciprocal decrease in the SJL18-1 frequency during neonatal development is very striking. Amino acid sequence studies have shown that the EB3-7 idiotope differs from the SJL18-1 idiotope by only the 2 D region amino acids. The light chains and V_H and J_H sequences are identical. Therefore, it appears that the SJL18-1 positive D region(s) is somehow selected for use before the EB3-7 positive D-region(s). One D segment may

be germline (SJL18-1) while the other may be somatically derived, which would be a time dependent process. The EB3-7 D region may be the result of imprecise joining of V-D and,or D-J, or the result of a point mutation (20,21,22). Both D regions may be formed in a similar manner but the formation of the SJL18-1 positive D region may initially be a more likely event. Somatic variation in V and J (and lambda) may also play a role in the expression of the SJL18-1 and EB3-7 idiotopes. As the mice age, somatic events would accumulate increasing the frequency of the EB3-7 variants. Of course there would need to be selective pressures, either via antigen or via idiotype regulation. An important point to be made is that some of the diversity is probably generated during the course of the splenic focus assay as previously reported (8,9). However, it is apparent that as soon as DEX responsiveness appears there is already a diverse response to DEX, even though some diversity may be generated <u>in vitro</u>. The point remains that neonatal B cell precursors have the potential to generate plasma cells secreting a fairly large repertoire of anti-DEX antibodies. The repertoire does change during neonatal development indicating some sort of regulation possibly by environmental antigens or anti-idiotype is occurring which results in prototype anti-DEX response.

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SUMMARY

The work presented in the four papers and the appendix addresses two separate but closely related problems. The first problem addressed is the specific effects of monoclonal anti-idiotype antibodies on the humoral response to alpha 1->3 dextran (DEX). The second problem is the potential variable region repertoire expressed by B cell precursors. The B cell precursor is the probable target of anti-idiotype either directly or indirectly through the network. Knowledge of the B cell precursor idiotope repertoire coupled with precise knowledge of the alterations of that idiotope compartment induced by monoclonal anti-idiotype antibodies will result, it is hoped, in a better understanding of the mechanisms underlying the regulation of the V region repertoire.

Administration of monoclonal anti-idiotype antibodies to neonatal mice induces a chronic state of idiotype suppression, while administration of monoclonal anti-idiotype antibodies to adults induces only a temporary state of suppression. Administration of the CD3-2 (anti-IdX) not only suppressed the IdX but the J558 IdI, M104 IdI and the total anti-DEX response. This is understandable since the IdX is found on the vast majority

of anti-DEX antibodies, and the two IdIs are almost always co-expressed with the IdX. Administration of the anti-IdIs suppressed only the IdIs they were specific for. In all cases the specificity of the suppression was identical to the serological specificity of the anti-idiotype. This indicates that the target of anti-idiotype may be the B cell compartment itself, or if T cells are involved, then the specificity of the T cells is identical to that of the anti-idiotype administered. The induction and maintenance of suppression has been shown in many studies (some conflicting) to involve both the B cell and T cell compartments. The evidence to date does suggest that in the alpha 1->3 dextran system T cells are not needed for anti-idiotype induced suppression. There could be two completely different suppression mechanisms at work for neonatal and adult suppressions; however, their specificities would appear to be identical. A major difference between adult mice and neonatal mice could be in the state of their B cells. Adults possess a heterogeneous population of B cells with respect to B cell maturity. Neonates possess a B cell population consisting of mostly immature B cells. The fact that neonates are susceptible to permanent alteration of their B cell response through direct and maternal administration of anti-idiotype suggests that early exposure to anti-idiotype (or possibly antigen) via maternal routes may play an important role in

determining the repertoire to be expressed by the neonates.

Whether mice are suppressed with MAIDs as adults or neonates, they are still capable of generating an antibody repertoire against DEX. Sequence analysis of anti-DEX hybridomas and myelomas suggests a diverse repertoire of anti-DEX antibodies available to the mouse. Although it appears that several V and J genes may be in use, the greatest heterogeneity occurs in the D region which is usually only 2 amino acid residues in length. It is interesting to note that most of the monoclonal anti-IdI antibodies generated and used in this study were most sensitive to changes in the D region sequences. In contrast the ability to bind DEX seems relatively insensitive to D region differences. An important role for the D region may be to provide idiotope markers for specific regulatory functions. Analysis of B cell precursors specific for DEX with a panel of MAIDs indicate that they possess an extensive antibody repertoire. It has been reported that IgG anti-PC antibodies are more diverse than their IgM counterparts. This situation does not seem to hold true in the DEX system where there is extensive heterogeneity also among IgM precursors. Carrier primed T cells appear to have little effect on idiotope expression or antibody diversity since DEX (TI-2) and DEX-hemocyanin (TD) stimulated precursors exhibit very similar idiotope profiles. The carrier primed T cells do have a very strong

effect upon isotype expression, increasing the frequency of multiple isotype and IgA secreting foci. Idiotope analysis of each isotype within multiple isotype secreting foci has revealed that idiotope differences between the different isotypes occur in about half the cases examined. These results indicate a rapid rate of somatic variation and that somatic variation can occur within the 18 day time frame of the splenic focus assay.

B cell precursors from neonatal BALB/c mice less than 5 days of age are unable to respond to DEX. After day 5 a low frequency of anti-DEX precursors can be detected. The frequency of DEX positive precursors increase as the mice age. Precursors from neonatal mice stimulated with DEX express different isotypes and different idiotope patterns. The younger the neonate, the greater the proportion of DEX positive foci that will secrete IgA (mostly as IgM-IgA doubles). There is also a shift towards expression of the SJL18-1 idiotope (M104E) in place of the usually dominant EB3-7 idiotope (J558). The differences in isotype expression between adult and neonatal precursors may reflect seeding and activation of anti-DEX precursors in the gut. The predominance of SJL18-1 positive precursors at the earlier neonatal ages may reflect an ordered expression of D region genes or the EB3-7 D region may take more time to be somatically derived from germline D segments. There also do not appear to be major differences

between the anti-DEX repertoire expressed by adult and neonatal B cell precursors. Whatever the mechanism is, the expression of all components of the BALB/c anti-DEX response is an ordered and tightly controlled series of events. This dissertation presents information which will aid in the understanding of the complex sets of interactions that form the immune network, and the generation of antibody diversity. The main points put forward are: (i) administration of monoclonal anti-idiotype antibodies suppress exactly according to their serological specificities, (ii) somatic variation of V regions is a frequent event following antigenic stimulation of precursors, and (iii) the adult idiotope repertoire is a result of ordered events occurring during neontal development

APPENDIX

Allotype restriction of the lambda bearing anti-alpha 1->3 dextran response in mice.

It has been previously reported that the lambda bearing anti-DEX response and the associated idiotypes are linked to the Igh-C^a locus (the BALB/c allotype). Using monoclonal anti-idiotype antibodies produced against J558 and M104E the question of what gene locus is most closely linked to the expression of the lambda bearing anti-DEX response and the IdIs is addressed. A number of different allotype mice along with various F_1 mice and a number of different recombinant strains were examined for lambda and idiotype expression in the sera after immunization with DEX. All mice possessing the Igh-V^a allotype were able to mount a strong lambda bearing anti-DEX response, and both the J558 and M104 idiotypes (except in a few isolated cases). Mice heterozygous for the a allotype responded the same as mice homozygous for the a allotype mice indicating the dominance of the lambda bearing portion of the anti-DEX response and the associated idiotypes. The congenic strain, BAB.14, possesses the constant region from C57/BL6 mice on a BALB/c background. The BAB.14 mice responded to DEX exactly as BALB/c mice would. In collaboration with R. Riblet, a large number of recombinant strains were tested for lambda anti-DEX and the DEX idiotypes. In all strains

tested the ability to respond to DEX with the BALB/c prototype response always segregated with the BALB/c variable region. Most but not all of the non-a allotype mice were unable to respond to DEX with any lambda bearing antibody, or the associated idiotopes. However, 20% of A/J mice and about 50% of C57/BL6 mice did respond with high levels of lambda bearing anti-DEX antibody. The A/J mice were totally negative for the J558 and M104E idiotypes, but a very small proportion of the C57/BL6 mice made what appeared to be small amounts of the J558 and M104E idiotypes. Whether they were using the same structural genes as a allotype mice or whether it is an unrelated gene family with weak cross-reactivity remains to be seen.

Allotype restriction of the anti-DEX response

Strain	Igh-C	Igh-V	percentage of mice expressing[a]		
			lambda	J558 IdI	M104 IdI
BALB/c	a	a	100.0	100.0	100.0
SJL/J	b	b	0	0	0
A/J	e	e	20.0	0	0
C57/BL6	b	b	48.6	5.4	2.7
BALB x SJL	a/b	a/b	100.0	100.0	80.0
BALB x A/J	a/e	a/e	100.0	100.0	100.0
BALB x C57	a/b	a/b	100.0	100.0	100.0
BAB.14	b	a	100.0	91.6	100.0

[a]A quantitative ELISA was carried out on each serum sample the minimum level of detection was about 0.5 ug/ml.

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