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Analysis of the Immune Response at the Molecular Level

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INTRODUCTION

AN ANIMAL typically responds to challenge by a foreign antigen by producing a large number (>50) of different antibody molecules. Each molecule is believed to be the product of a single B cell clone. All these clonal products show specificity for the antigen but have different amino acid sequences, i.e., they are products of different structural genes. To the first approximation different inbred strains of mice respond with different collections of clonal products and for the most part individual members of a single strain also show diverse response. How this result is related to the genetic information in the animal is unclear. In order to address this problem we have begun an analysis of one particular immune response.

When the strain A mouse is immunized with azophenylarsonate coupled to a carrier, an immune response (anti-Ars) is observed. Approximately half of the antibody molecules present in the serum of an Ars-immune animal behave as conventional antibodies do (i.e., different amino acid sequences and different members of strain A each produce antibody molecules that differ in sequence). The other half of the antibody in the serum is apparently derived from a single B cell clone; they have the same or related amino acid sequences and share immunological determinants, i.e., they share the same idiotype. Thus, every member of strain A when immunized with Ars-carrier produces at least some anti-Ars antibody with the same cross-reactive idiotype (Ars-CRI).

The ability to respond to Ars-carrier with the same idiotype is a heritable trait and behaves as a single mendelian character in crosses with animals that make excellent anti-Ars antibodies but do not make the CRI. The interpretation of this phenomenon is that the structural gene for the heavy chain idiotype is in the germline and is expressed unaltered in the adult.

We have examined this system by producing many hybridomas expressing anti-Ars antibody from strain A mice that bear the CRI and ones that do not. The first and most unexpected result is that the amino acid sequences of anti-Ars antibodies bearing the CRI are similar but are significantly different from each other (fig. 1).

The two simplest interpretations of these data are that the heritable trait (i.e. the production of idiotype) is due to the inheritance of a single structural gene for the anti-Ars H chain and that this gene is expressed in the adult. In addition, during development of the immune system, somatic mutations accumulate in that gene and are expressed in the adult as well. The second interpretation is that the trait is due to the inheritance of a gene family whose members share an antigenic determinant (the idiotype). The hybridoma sequences observed are thus products of unaltered genes present in the germ line of strain A mice. Strains of animals not expressing the idiotype when immunized to Ars-carrier would then be expected not to have the structural information in either of the forms listed above. The possibility that all mouse strains have the same structural information and the id^+ strains are phenotypically positive only by virtue of control genes has not been excluded.

During the analysis of hybridoma-derived proteins that were not members of the major idiotype family, it was observed that several randomly selected proteins were in fact related to each other serologically and constituted another idiotype family present in A/J mice (fig. 2). This idiotype family (referred to as the 36-60 idiotype family) constitutes only about 5% of the total anti-Ars antibody whereas the major CRI constitutes about 50% of the anti-Ars antibody in A/J mice. The 36-60 idiotype also shows a different strain distribution than the CRI in that the latter is present in BALB/c mice and the CRI is not. Accordingly, all studies described below will use 36-60 as an additional element in all experiments. We can address questions as to why the 36-60 idiotype is present in only 20% of the CRI concentration and furthermore what enables it to be expressed in Balb/c and A/J but not in B10 mice.

Analysis of Genes Encoding the CRI

A nucleic acid probe has been prepared from the heavy chain variable region expressed in an idiotype-expressing hybridoma (id probe). The probe has been used to analyze the genetic constitution of A/J and other mouse strains either expressing the CRI idiotype or not. An unexpected result is that there are approximately 20 genes present in all mouse strains that cross-hybridize to the id probe (fig. 3). This is true regardless of whether or not they express the idiotype (compare lane 6 to 9). We do not yet know whether all of these genes produce protein products that constitute the idiotype family of antibodies. This question is currently under investigation. Restriction enzyme analysis of the genes expressed in the CRI-expressing hybridoma collection should shed light on the question. Analysis of genes in the cross-

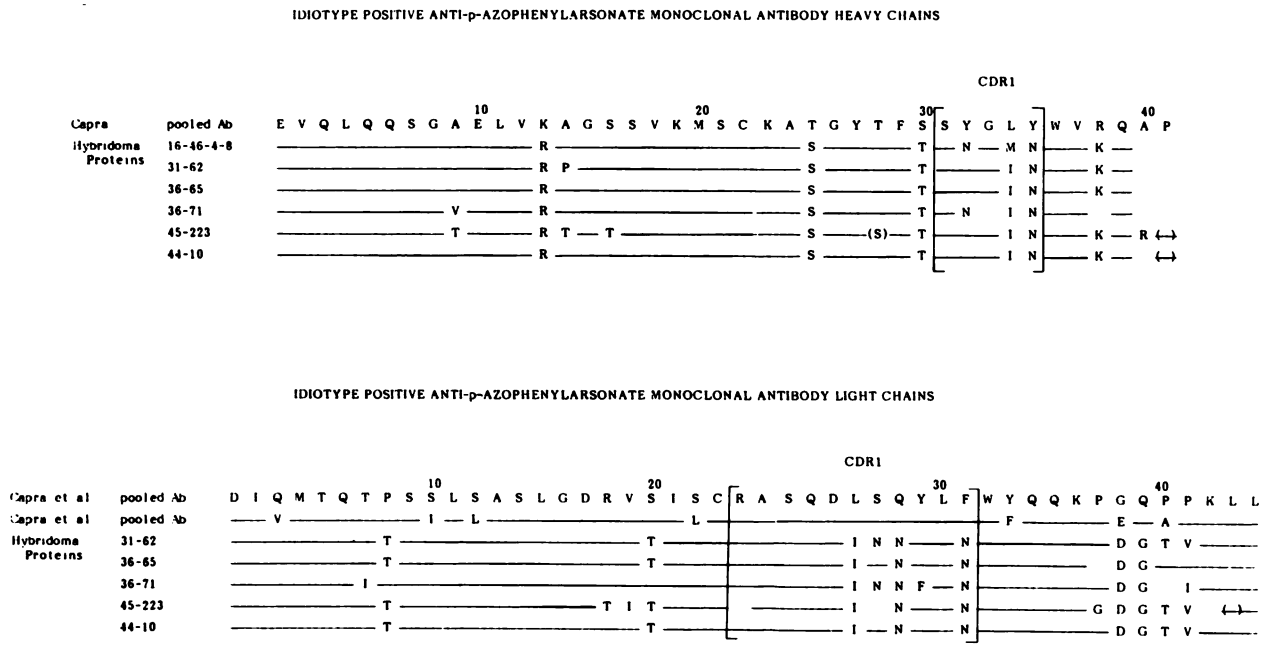

FIG. 1. *Top:* Amino acid sequences of murine A/J anti-Ars Id^{CR+} monoclonal antibody heavy chains compared to the sequence reported for pooled serum anti-Ars CRI^{+} heavy chains (3). Amino acids are indicated in the one letter code (IUPAC-IUB Commission on Biochemical Nomenclature, 1968). A line indicates identity with the topmost sequence. Unidentified residues are indicated by blanks. Numbering of residues and designation of complementarity-determining regions is according to Kabat et al. (19). All residues were identified by at least two independent methods or in two separate sequencer experiments, or both. Residues in parentheses are based on a single identification. We reported previously the sequences of 16-46-4-8, 31-62, 36-65, and 36-71 (17). *Bottom:* Amino acid sequences of murine A/J anti-Ars Id^{CRI+} monoclonal antibody light chains. These are compared to the two dominant light chain sequences for pooled serum CRI^{+} anti-Ars antibodies reported by Capra et al. (4). See the legend to figure 1, Top, for further details. (Abbreviations used are: Ars, azophenylarsonate; Id, idiotype; CRI, cross-reactive idiotype.)

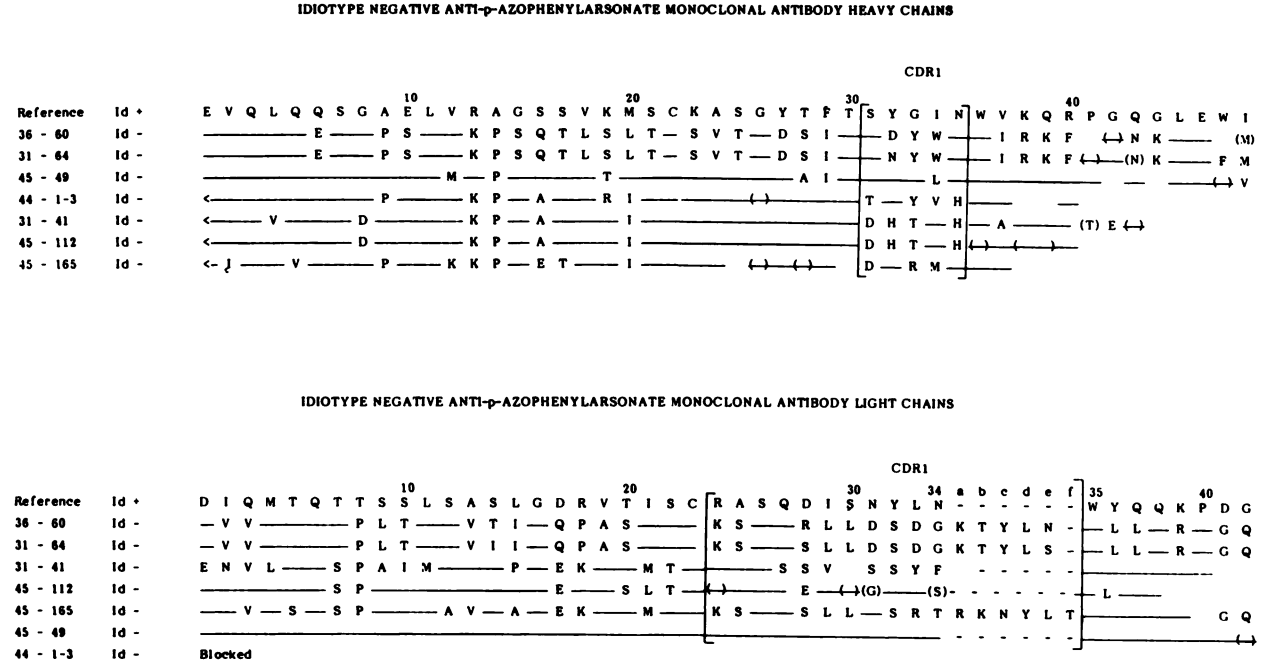

FIG. 2. *Top:* Amino acid sequences of murine A/J anti-Ars Id^{CR-} monoclonal antibody heavy chains. They are compared to a reference hybridoma protein Id^{CR+} sequence derived from the data in figure 1. The < indicates an amino-terminal pyrolidone carboxylic acid residue; the sequences of these chains were obtained after digestion with pyroglutamyl aminopeptidase (18). *Bottom:* Amino acid sequences of murine A/J anti-Ars Id^{CR-} monoclonal antibody light chains. They are compared to a reference Id^{CR+} light chain sequence derived from the data in figure 1. Insertions in CDR 1 are arbitrarily designated by letters carboxyterminal to positions 34 ending at the invariant tryptophan at position 35. (Abbreviations used are: Ars, azophenylarsonate; Id, idiotype; CRI, cross-reactive idiotype.)

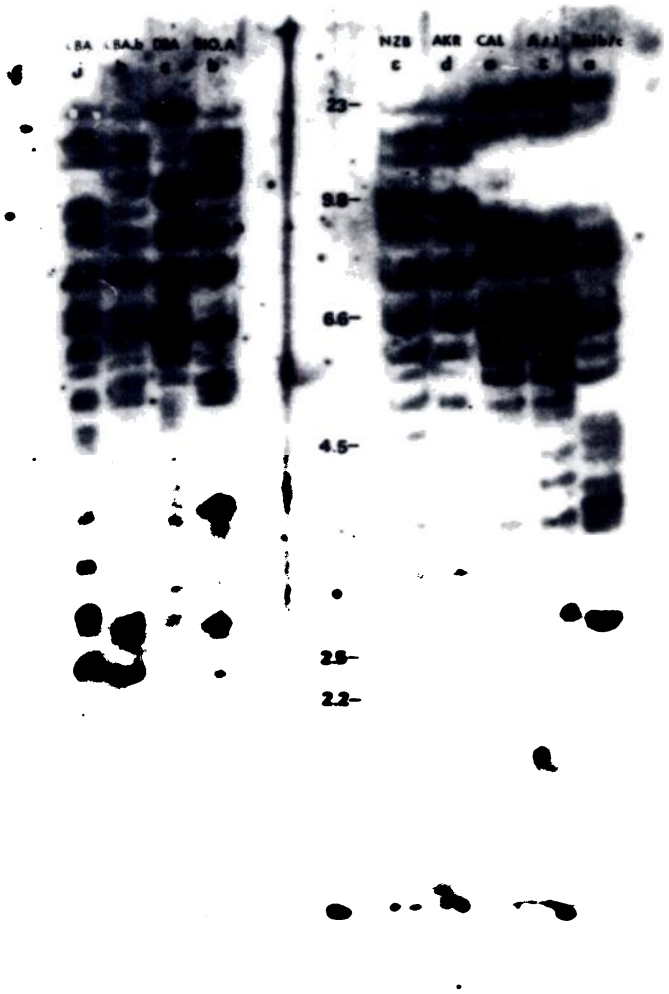

FIG. 3. *Southern blot analysis of embryonic DNAs of various mouse strains.* DNA was digested with Eco RI and hybridized to a ^{32}P probe containing the V_H gene of the anti-Ars hybridoma 36-65 on CRI^+ cell line. The lanes are numbered from left to right (1–9). The mouse strain and the V_H allotype is given above. (Abbreviations used are: RI, Igh recombinant strains; Ars, azophenylarsonate; CRI, cross-reactive idiotype.)

hybridizing family will also tell us what the relationship is between the germ-line encoded proteins and the expressed proteins, i.e., what is the relevancy of somatic mutations of germ-line sequences to expressed proteins. Until we know the answers to these questions, we cannot address the question as to why BALB/c does not express the CRI.

Detailed analysis of the BALB/c genome (compare lanes X → Y) revealed that only about six to eight out of 20 cross-reacting genes are common to A/J and BALB/c. Thus, it is possible that not all 20 genes are used to express the CRI and it is therefore the nonidentical genes between BALB/c and A/J that are relevant for CRI expression.

Sequencing of the $idCRI^+$ and $idCRI^-$ hybridoma-derived V_H genes is being done to address their relatedness. All of these studies should provide us with a clear understanding of the nature of antibody diversity.

Control of Idiotype Expression

The idiotype is expressed as a family of related but distinct antibody sequences. Several reagents have been prepared that allow for a detailed analysis of the idiotype response. Conventional anti-idiotypic antibody recognizes all members of the idiotype family equally well. Determinants associated with the amino acid differences between these molecules (private determinants) can be recognized by conventional or hybridoma derived antibodies, i.e., antiprivate antibodies.

With these reagents, we asked whether the sequence variability associated with the immune response was reproducible from animal to animal, a result not consistent with a *rare* mutational event, giving rise to antibody diversity. We were able to show that to a first approximation, every animal that expresses the idiotype (the whole family) expresses all of the individual members of

the idiotype family. This result is consistent with all of the family being present in the germ line or that mutation events leading from the germ-line sequence to all of the somatically derived sequences are highly ordered and reproducible. The analysis of the DNA constitution of idiotype-producing strains (see above) will shed light on this question.

Another major question regarding the expression of idiotype is its capability of being regulated. Injection of animals with anti-idiotypic antibody before antigenic challenge leads to the synthesis of antiantigen antibody but not to the synthesis of idiotype. The work of others has shown that this is due to active suppression of the idiotype by suppressor T cells. Since we now know that idiotype is a family of unique molecules, is it the case that there is a family of individual T cells that is needed to regulate idiotype? To address this, animals were first given injections of an antiprivate antibody, then injections with antigen. If each molecular species is regulated independently then only the molecules bearing the private determinants recognized by the antiprivate antiserum should disappear from the immune sera. This result has been obtained. This raises the question as to how injected anti-id gives rise to selective suppression of just those determinants the id recognizes. This is especially interesting if one assumes that T cells contain idiotype encoded by V_H genes also found on B cells.

The analysis of hybridoma proteins derived from fusions between immune B lymphocytes and myeloma cell lines has contributed greatly to the understanding of the structural diversity associated with the A/J anti-Ars antibody response and the major cross-reactive idiotype (Id^{CR}) characteristic of this system (12). Various Id^{CR+} monoclonal antibodies identified on the basis of their extensive serological cross-reactivity in assay systems designed to detect the major idiotype have been examined. Amino acid sequence studies have shown that the Id^{CR+} molecules constitute a family of structurally related but nonidentical proteins (5, 6, 13–15, 17). As predicted by the occasional differences found in both the heavy and light chains, it was possible to prepare antisera that recognized "private" determinants associated with individual Id^{CR+} hybridoma proteins. The subsequent detection of these private determinants in Id^{CR+} immune sera confirmed that the Id^{CR+} hybridoma proteins were, in fact, representative of the normal in vivo Id^{CR+} antibody response (15, 16). Hybridoma proteins have also proved useful in the analysis of the Id^{CR-} portion of the anti-Ars response. In this case, amino acid sequence studies of randomly chosen Id^{CR-} monoclonal antibodies revealed "minor" cross-reactive idiotype families that were structurally unrelated to the major idiotype family (14). Again, antisera directed against representative hybridoma proteins indicated that the minor idiotype families also recur consistently among individual A/J mice (7, 8, 16).

Reagents specific for the major, private, and minor determinants have been applied to the study of various network interactions. One particularly intriguing aspect of the Ars antibody response is the sequence of cellular events that can result in idiotype suppression (9). Under the appropriate conditions, both Id^{CR+} antibody molecules and antisera directed against Id^{CR} have been shown to trigger Id^{CR} specific suppressor cell activity (11–13). The purpose of the present report is to examine the specificity and amplification capacity of suppressor cell networks induced by the various types of antisera.

Results and Discussion

"Public" vs. "Private" Id^{CR} Determinants

A rabbit antiserum specific for the major ("public") cross-reactive idiotype (anti-Id^{CR}) was prepared by immunizing first with a pool of A/J anti-Ars serum antibody and then boosting with an Id^{CR+} hybridoma protein, 16-46 (17). A panel of nine hybridoma proteins recognized by this reagent were subsequently isolated and classified as Id^{CR+} on the basis of their ability to inhibit greater than 80% of the binding of [^{125}I]16-46 to rabbit anti-Id^{CR} (16; fig. 4A).

These nine hybridoma proteins could be divided into two distinct subsets on the basis of their reactivity with two additional reagents. A monoclonal BALB/c anti-

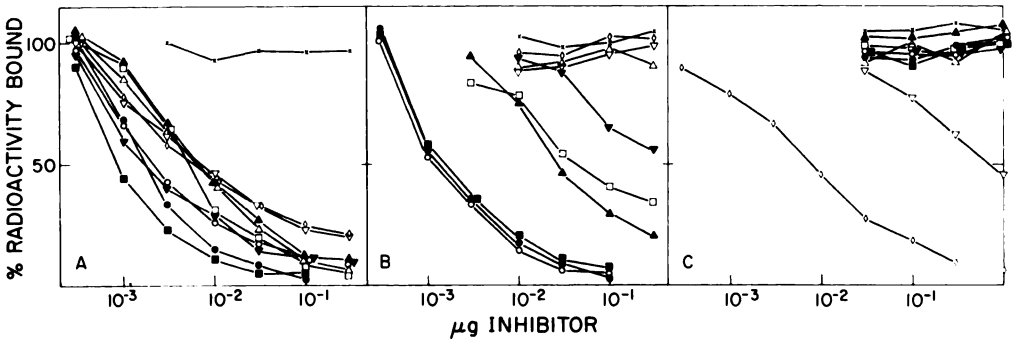

FIG. 4. Specificity of rabbit anti-Id^{CR}(A), BALB/c monoclonal anti-Id^{cr} (B), and rabbit anti-$Id^{44\text{-}10}$ (C). There were 3 ng of [^{125}I]36-65 (A and B) or [^{125}I]44-10 (C) added to polyvinyl microtiter dishes precoated with the appropriate antisera along with dilutions of the following unlabelled Ars-binding hybridoma proteins: 16-46-4-8 (□, IgG2a); 31-62; (○, IgG2a); 36-65 (●, IgGl); 36-71 (△, IgGl); 44-10 (◇, IgGl); 45-223 (▲, IgGl; 45-248 (■, IgM); 45-273 (▼, IgG3); 45-208 (▽, IgGl); and 36-60 (×, IgG2a). (Abbreviations used are: Id, idiotype; CR, cr, cross-reactive; Ars, azophenylarsonate.)

idiotype (MB-anti-Id^{cr}), prepared by fusing the spleen of a BALB/c mouse immunized with the Id^{CR+} hybridoma protein 36-65 (16), reacted with most of the Id^{CR+} proteins, but failed to bind the Id^{CR+} hybridoma proteins 44-10 and 45-208 (fig. 4B). In contrast, 44-10 and 45-208 were recognized by a heterologous antiserum (anti-Id$^{44-10}$) obtained from a rabbit immunized only with the hybridoma protein 44-10 (3; fig. 4C). Anti-Id$^{44-10}$ failed to recognize any of the hybridoma proteins detected by MB-anti-Id^{cr}. The determinants associated with MB-Id^{cr} and Id$^{44-10}$ were found only in A/J serum samples that contained Id^{CR}. As shown in table 1, both MB-anti-Id^{cr} and anti-Id$^{44-10}$ failed to react significantly with: 1) Ars-KLH immune A/J sera absorbed with Ars-BGG; 2) Ars-KLH immune sera from A/J mice neonatally suppressed with rabbit anti-Id^{CR}; 3) Ars-KLH immune BALB/c or B10.A sera; or 4) nonimmune A/J sera. Furthermore, the determinants detected by these two reagents were present in almost every individual A/J Ars-immune serum tested (6). Thus, these two reagents recognized nonoverlapping sets of Id^{CR+} hybridoma proteins as well as certain serum antibodies that displayed the same overall characteristics as the entire Id^{CR+} response.

To further test the association between the expression of Id^{CR} (determinants recognized by rabbit anti-Id^{CR}) and MB-Id^{cr} and Id$^{44-10}$ (determinants recognized by MB-anti-Id^{cr} and anti-Id$^{44-10}$), 15 Igh recombinant (RI) strains derived from matings between either A/He and BALB/c or BAB/14 and C.AL-9 and selected on the basis of intra-Igh-V region recombination events were examined (20). A/He and C.AL-9 Ars-immune sera are Id^{CR} positive; BALB/c and BAB/14 are not. In all cases, immune sera from strains that were Id^{CR} positive on the basis of reactivity with the rabbit anti-Id^{CR} antiserum also reacted with the monoclonal BALB/c anti-idiotype; those that were not recognized by the rabbit antiserum failed to react with the monoclonal reagent. Preliminary results obtained with the rabbit anti-Id$^{44-10}$ antiserum indicate a similar trend. All of the strains that failed to express Id^{CR} also lack Id$^{44-10}$ determinants, but as yet only seven out of 10 of the Id^{CR+} positive strains have been shown to express Id$^{44-10}$. The discrepancy may be due to the relatively low titers of anti-Ars antibody obtained from the three inconsistent strains. Alternatively, anti-Id$^{44-10}$ may recognize a determinant dependent on a combination of V and D region gene segments that were separated by recombination in the construction of the strains. The correlation between Id^{CR} expression and the expression of the "private" determinants (MB-Id^{cr} and Id$^{44-10}$) suggests that the entire Id^{CR} family is derived from a single germ-line V_H gene. Alternatively, if every antibody sequence were encoded in the germ line then one might expect that the phenotypes could be separated by recombination.

TABLE 1

*Id$^{44-10}$ and MB-Id^{cr} are present only in sera containing Id^{CR}**

Serum Sample	Anti-Ars Antibody	Id^{CR}	Id$^{44-10}$	MB-Id^{cr}
Immune A/J	2920	1920	76	1150
Immune A/J—adsorbed with Ars-BGG	<3.5	<2	6	<3
Immune A/J—suppressed with anti-Id^{CR}	3570	<2	0	<3
Immune BALB/c	1110	<2	0	N.T.
Immune B10.A	2380	<2	0	<3
Normal A/J	N.T.	3	1	<3

* The various pools of immune sera were obtained from mice immunized at least twice with Ars-KLH as described previously (17). Suppression of Id^{CR} was induced by neonatal injection of rabbit anti-Id^{CR}. Id^{CR}, MB-Id^{cr}, and anti-Ars antibody; titers are expressed in terms of gram equivalents of 36–65/ml as determined by solid phase radioimmunoassay (10, 16). Id$^{44-10}$ is expressed in terms of the percent inhibition of binding of [^{125}I]44-10 by rabbit anti-Id$^{44-10}$ by 1 μl of immune sera in the radioimmunoassay described in (15). Abbreviations used are: Ars, azophenylarsonate; Id, idiotype; CR, cr, cross-reactive; MB, monoclonal BALB/c; BGG, bovine gamma globulin; KLH, keyhole limpet hemocyanin; N.T., not tested.)

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These experiments strongly suggest that all of the members of the idiotype family of antibody (probably >300 members) are derived from a small number (possibly 1) of germ-line encoded genes. Thus somatic mutation, i.e. changing of the germ-line sequence by mutations, gives rise to serum antibodies. We do not yet know what fraction of serum antibodies are encoded in the germ line and what fraction are mutants.

A "Minor" Cross-Reactive Idiotype. Serological evidence for minor cross-reactive idiotypes associated with the A/J anti-Ars response emerged from the study of Ars-binding hybridoma proteins that failed to express the major cross-reactive idiotype (7, 16). The structural basis for one such minor idiotype family became apparent when the N-terminal amino acid sequences of eight Id^{CR-} A/J Ars-binding hybridoma protein heavy and light chains were determined (14). These eight monoclonal antibodies had been selected solely on the basis of their inability to react with rabbit anti-Id^{CR}, yet three of the eight proteins were remarkably similar; only a few minor differences were found in the framework and CDR 1 (complementarity determining region one) regions of both the heavy and light chains (16). All three hybridoma proteins reacted with a rabbit antiserum prepared against the single protein 36-60 (rabbit anti-Id$^{36-60}$). Comparison of the Id^{36-60+} sequences to the Id^{CR+} sequences showed that they differed by as many as 29 out of the first 48 N-terminal heavy chain residues and by 31 out of the first 47 light chain residues, and were probably encoded by separate germ-line genes (14, 16). Further evidence for the relatively high frequency of this minor idiotype is that Id^{CR-} protein sequences homologous to the Id^{36-60+} group have also been reported by others (5, 1). It was, therefore, not surprising that rabbit anti-Id$^{36-60}$ reacted with almost all the A/J anti-Ars immune sera we examined. Unexpectedly, rabbit anti-Id$^{36-60}$ also reacted with sera from B10.A (16). A similar interstrain cross-idiotype has been found by Brown et al. (2).

Independent Regulation and Minor Idiotype Expression. It has been reported previously that expression of

the major idiotype could be suppressed by injection of the appropriate heterologous antisera without affecting the overall anti-Ars antibody titer (6). It was of interest to establish whether the minor idiotype family, Id^{36-60}, could be similarly suppressed. In addition, the suppressive capacity of the BALB/c monoclonal anti-Id^{cr} was examined. As shown in table 2, neonatal injection of MB-anti-Id^{cr} before Ars-KLH immunization as adults resulted in a profound decrease in the level of Id^{CR} (the idiotype defined by the rabbit antiserum), but did not alter Id^{36-60} expression. Determinants recognized by MB-anti-Id^{cr} were totally absent in these suppressed sera (data not presented). Similarly, the rabbit anti-Id^{36-60} suppressed the expression of Id^{36-60} but not Id^{CR}. The independent regulation of the major and minor idiotype families further supported the finding that they were structurally distinct.

Extent of MB-Anti-Id^{cr}-Induced Suppression. Careful examination of the data in table 2 shows that the relative proportion of Id^{CR} in the group of mice suppressed with MB-anti-Id^{cr} is approximately 20-fold lower than the control group. The extent of this suppression was somewhat greater than expected considering that MB-anti-Id^{cr+} reacted strongly with only three out of nine of our Id^{CR+} hybridoma proteins (fig. 4B). These results suggest that if suppression were induced with a reagent that recognized private determinants present on a subset of Id^{CR+} molecules, then Id^{CR+} molecules that expressed the major cross-reacting determinants but lacked the original more restricted determinants would also be suppressed. In order to address this question more directly, suppressed and nonsuppressed sera were compared for their content of Id^{44-10}, since MB-anti-Id^{cr} and rabbit anti-Id^{44-10} recognized nonoverlapping subsets of Id^{CR+} hybridoma proteins (fig. 1). The data presented in table 3 demonstrates that the injection of MB-anti-Id^{cr} into either neonatal or adult mice before antigen challenge results in suppression of both MB-Id^{cr} and the majority of Id^{44-10}. This result would seem to be in keeping with expected "network"-related interactions.

TABLE 2

*Independent regulation of major and minor idiotype**

Antiserum Used to Induce Suppression	Anti-Ars Antibody	Id^{CR}	Id^{36-60}
None[a]	1860	520	81
MB-anti-Id^{cr}	600	10	85
None[b]	2630	1394	56
Rabbit anti-Id^{36-60}	1428	894	8

* A/J mice were suppressed by neonatal injection of either MB-anti-Id^{cr} or rabbit anti-Id^{36-60} and then immunized twice with Ars-KLH as adults. The results are summarized as the median values of 10^a or 4^b mice per group. Id^{CR} and anti-Ars antibody titers were determined in solid phase radioimmunoassays and are presented in terms of microgram equivalents of 36–65/ml. Id^{36-60} is expressed in terms of percent of inhibition of binding of [^{125}I]36-60 by rabbit anti-Id^{36-60} as described in table 1 (16). (Abbreviations used are: Ars, azophenylarsonate; Id, idiotype; CR, cr, cross-reactive; MB, monoclonal BALB/c; KLH, keyhole limpet hemocyanin.)

TABLE 3

*Suppression induced by BALB/c monoclonal anti-Id^{cr}**

Serum Sample	Suppression Regimen	MB-Id^{cr}	Id^{44-10}
D117-1	None	680	17
D117-2		450	30
D140-1	MB-anti-Id^{cr} ascites, 10 μl as neonates	<1	2
D140-2		<1	<1
D140-5		<1	<1
D140-6		<1	10
D112-1	Sp2 ascites, 10 μl as adults	1560	26
D112-2		925	38
D112-3		333	2
D112-4		300	10
D114-1	MB-anti-Id^{cr} ascites, 10 μl as adults	<1	2
D114-2		<1	<.5
D114-3		<1	5
D114-4		<1	<.5

* A/J mice were suppressed as neonates or adults by injection with MB-anti-Id^{cr} and subsequently immunized twice with Ars-KLH. MB-Id^{cr} titers are expressed as microgram equivalents of 36-65/ml. Id^{44-10} titers are expressed as microgram equivalents of 44-10/ml. (Abbreviations used are: Id, idiotype; cr, cross-reactive; MB, monoclonal BALB/c.)

TABLE 4

*Activity remaining after absorption with monoclonal anti-idiotype**

Serum Sample	Anti-Ars Antibody	MB-Id^{cr}	Id^{CR}	Id^{44-10}	Id^{36-60}
D112					
Pre-	3850	1280	2970	10.0	4.9
Post-	625	<2	210	0.8	6.7
D118					
Pre-	3940	1442	2500	28.6	8.8
Post-	1330	<2	105	.8	10.7
D119					
Pre-	4760	870	3800	33.3	13.3
Post-	770	<2	190	1.2	14.6
XD43 + Protein 44-10					
Pre-	3615	1052	1250	25.1	N.T.
Post-	1875	<2	32	17.8	

* Three pools of Ars-immune sera (D112, D118, D119) were selected on the basis of relatively high percentages of Id^{CR} and Id^{44-10}. Serum sample XD43 contained a low level of Id^{44-10} but was mixed with affinity purified 44-10 hybridoma protein. Aliquots of each serum sample were diluted in PBS containing 50% fetal calf serum, and a portion of each sample was passed through a monoclonal anti-Id^{CR}-coupled Sepharose column. Pre- and post-column samples were adjusted to equal protein concentrations and compared by radioimmunoassay (15-17). MB-Id^{CR}, Id^{CR}, and anti-Ars antibody titers are presented in terms of microgram equivalents 36-65 per ml; Id^{44-10} and Id^{36-60} in terms of microgram equivalents of 44-10 and 36-60 per ml respectively. (Abbreviations used are: MB, monoclonal BALB/c; Id, idiotype; cr, CR, cross-reactive; Ars, azophenylarsonate; PBS, phosphate-buffered saline.)

Two alternative explanations for the results presented in table 3 were immediately apparent: 1) the specificity of the suppressor effector cells induced with MB-anti-Id^{cr} was broader than the original specificity of the MB-anti-Id^{cr} antibody due to sequential anti-idiotype idiotype anti-idiotype T cell interactions (12) that resulted in the recognizition of cross-reactive determinants; or 2) the

subsets serologically defined on the basis of reactivity with our panel of hybridoma proteins did not reflect subsets normally occurring in immune sera. In other words, typical antibodies in immune sera express determinants recognized by both MB-anti-Id^{cr} and anti-$Id^{44\text{-}10}$, such that suppression with one reagent leads to the loss of the other determinant. To test the latter possibility, several pools of immune serum were absorbed with MB-anti-Id^{cr}-coupled Sepharose 4B. As shown in table 4, this procedure removed all antibodies reactive with MB-anti-Id^{cr} and reduced the amount of Id^{CR} by greater than 95%. In addition, the level of $Id^{44\text{-}10}$ was markedly reduced. These results could not be attributed to nonspecific effects, since the level of $Id^{36\text{-}60}$ remained unchanged. Furthermore, affinity purified 44-10 protein, even when mixed with immune serum, did not bind to the column, indicating that the retention of $Id^{44\text{-}10}$ molecules in the immune sera was not due to either a low affinity interaction between MB-anti-Id^{cr} and 44-10 protein or idiotype-anti-idiotype complexes that might be present in immune serum. Rather, immune sera must contain molecules that express both $Id^{44\text{-}10}$ and MB-Id^{cr} determinants.

In summary, the extensive suppression of Id^{CR} induced by the injection of MB-anti-Id^{cr} appears to reflect the actual specificity of MB-anti-Id^{cr} for antibodies found in Ars-immune sera. Presumably, MB-anti-Id^{cr} reacts with approximately 90% of the Id^{CR+} molecules found in immune sera. These results strongly suggest that the microheterogeneity of the idiotype response as seen in the hybridoma sequences is an underestimate of the total heterogeneity characteristic of the Id^{CR+} serum response since some molecules in serum are composites of the sequences studied so far. It is noteworthy that not all $Id^{44\text{-}10}$ associated-determinants present in immune serum can be removed by adsorption on an MB-anti-Id^{cr} column. This indicates that there are Id^{CR+} molecules in serum that are similar to 44-10 in that they fail to bind to MB-anti-Id^{cr}. Combined with the fact that small amounts of $Id^{44\text{-}10}$-like molecules are present in immune sera when suppression is induced with MB-anti-Id^{cr}, these results strongly suggest that specificity does not degenerate as the various elements of the regulatory network operate to suppress idiotype production.

These results also speak to the question as to how many distinct antibody sequences can be derived from one germ-line gene. Previous estimates based on hybridoma-derived sequences suggest that the number was about 50. This serum analysis suggests a number of about 300. The mutational mechanism is unknown. We are equally ignorant of factors that may control this process. It is clear, however, that since somatic mutation is responsible for generating a considerable portion of the normal immune response that interference with the process might lead to an individual being immune different.

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