IDIOTYPE CONNECTANCE IN THE IMMUNE SYSTEM

II. A Heavy Chain Variable Region Idiotope that Dominates the Antibody Response to the *p*-Azobenzenearsonate Group Is a Minor Idiotope in the Response to Trinitrophenyl Group

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Although idiotypic dominance was first observed over a decade ago in the antiarsonate (ABA)[1] system (1), its mechanism remains enigmatic. It was initially postulated that B cell clones expressing a dominant idiotype were preferentially expanded because they had higher affinities for antigen than their less successful competitors (2). Subsequent studies (2) showed, however, that anti-ABA antibodies expressing a dominant idiotype had median affinities for ABA, arguing persuasively against this explanation. Recent work suggests that multiple mechanisms may be responsible for idiotypic dominance.

Idiotypic dominance may be established at different levels in B cell ontogeny. In the T15 system, dominance has been demonstrated as early as the pre–B cell stage (3). In other responses, including that of A strain mice to ABA, dominance is established only after the expression of surface immunoglobulin (4). In this system, CRI^{a+} (cross-reactive idiotope) B cells accounted for ~3% of the total number of B cells responding to ABA in nonimmune mice. Upon immunization, this frequency increased to 20–80% (4), suggesting that the CRI^{a+} B cells were preferentially expanded. Since this observation cannot be directly accounted for by differences in affinity (2), idiotype-specific selection is implicated.

Idiotype-specific regulation could establish idiotypic dominance by either positive or negative selection of particular B cell clones. Dominance by positive selection could result from idiotype-specific helper T cells preferentially expanding clones that express a particular idiotype. Such helper cells have been reported in the CRI^{a} (5) and other systems (6, 7). Alternatively, dominance could result

This was supported by grants AI-17090 and AI-05664. Current address of P. Hornbeck is National Institute of Allergy and Infectious Diseases, Laboratory of Immunology, Bethesda, MD 20205. Current address of G. Lewis is University of Maryland, Department of Microbiology, Baltimore, MD 21201. He is the recipient of American Cancer Society Faculty Research Award FRA-264.

[1] *Abbreviations used in this paper:* ABA, *p*-azobenzenearsonate; ABP, *p*-azobenzenephosphonate; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; CGG, chicken gamma-globulin; CRI, cross-reactive idiotope; D segment, diversity segment; DANS, dansyl; ELISA, enzyme-linked immunosorbent assay; FL, fluorescein; J_H, joining segment; KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody; MHC, major histocompatibility complex; 4-MUP, 4-methyl-umbellifferyl phosphate; NPP, *p*-nitrophenly phosphate; OVA, ovalbumin; PC, phosphoryl choline; RGG, rabbit gammaglobulin; TNP, trinitrophenyl; V_H region, heavy chain variable region; V_L, light chain variable region.

from negative selection by preferentially suppressing cells that lack the dominant idiotype. In this case, idiotypes become dominant because they do not stimulate endogenous antiidiotypic suppressor activity. Such mechanisms are consistent with reports (8–10) that T and B cell antiidiotypic suppression play a role in limiting clonal expansion.

The idiotope CRI^{AD8}, a member of the CRI^{a} family, dominates the response of A strain mice to ABA (11). If this dominance is due to idiotype-specific regulation, then responses to other antigens that use CRI^{AD8} might also be dominated by this idiotope. Testing this hypothesis requires identification of antigens, other than ABA, which elicit CRI^{AD8}. We recently reported (11) that CRI^{AD8} is expressed on naturally occurring antibodies not specific for ABA. It was found on 0.1–0.5% of the naturally occurring antibodies in every A strain mouse studied, showing that this idiotope is frequently used in response to antigens other than ABA. Unfortunately, it was not possible to define the specificities of the naturally occurring idiotope, making it impossible to test the above hypothesis. Nevertheless, the recurrence of CRI^{AD8} on antibodies not specific for ABA suggested that this idiotope might be found on antibodies in diverse anti-hapten responses.

This suggestion was borne out in the studies described below, where we look at the dominance of CRI^{AD8} in antibody responses elicited by several haptenic groups and a single protein. Immunization with trinitrophenyl (TNP), and chicken gammaglobulin (CGG), but not with fluorescein (FL), azobenzenephosphonate (ABP), and dansyl (DANS), elicited significant levels of CRI^{AD8}, but in neither of these responses was it dominant. Attempts to increase CRI^{AD8} in anti-TNP responses by immunizing with double conjugates of TNP and ABA on the same carrier, failed. Analysis of hybridoma proteins and DNA showed the CRI^{AD8} correlates with the heavy chain variable (V_H)[2] and diversity (D) segments used by the prototypic CRI^{a} antibodies, but does not correlate with heavy chain joining (J_H) or V_L (light chain variable) segments (12, 13, 40, 54–56). Together, these observations suggest that V_H regions which contain CRI^{AD8} are necessary, but not sufficient for dominance; an additional specificity may be required. Subsequent studies showed that immunization with ABA calls up ABP-cross-reactive antibodies expressing CRI^{AD8}, but that immunization with ABP fails to elicit CRI^{AD8}, even on ABA-cross-reactive antibodies. These results are consistent with ABA itself being the additional specificity.

Materials and Methods

Animals. Female A/J mice, 6–8 wk old, were purchased from The Jackson Laboratory, Bar Harbor, ME and used after acclimatization to our animal colony.

Proteins and Antigens. Bovine serum albumin (BSA) was purchased from Sigma Chemical Co., St. Louis, MO. CGG was prepared from chicken serum (Gibco Laboratories, Santa Clara, CA) by successive ammonium sulfate precipitations. Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem-Behring Corp., San Diego, CA (No. 374805). Rabbit gammaglobulin (RGG) was purchased from Miles Laboratories, Elkhart, IN (No. 824551). Chicken ovalbumin (OVA) was purchased from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, NY. (No. 7819239).

[2] "V_H region" refers to the entire heavy chain variable region and includes the V_H, D, and J_H segments. "V_H segment" refers only to the region encoded by the V_H gene and includes positions 1–94 (13). The amino acid numbering system is that described by Kabat et al. (57).

ABA-conjugated proteins were prepared from *p*-aminobenzenearsonic acid (No. 1369; Eastman Kodak Co., Rochester, NY) according to Tabachnik and Sobotka (14). ABP proteins were conjugated identically to the ABA proteins, except that *p*-aminobenzenephosphonic acid, synthesized by Dr. D. E. Nitecki, was used instead of *p*-aminobenzenearsonic acid. Proteins were reacted with dansyl chloride (No. D-2625; Sigma Chemical Co.) according to standard procedures (15). FL-modified proteins were prepared with fluorescein isothiocyanate (No. F-7250; Sigma Chemical Co.) according to Wofsy et al. (16). Proteins were coupled with TNP using trinitrobenzene-sulfonic acid (P-5878; Sigma Chemical Co.) as described by Good et al. (17). The bisubstituted carrier TNP-ABA-KLH was prepared by conjugating TNP-KLH with ABA as described above.

Haptens. TNP-lysine was from Sigma Chemical Co. (D-0380), and ABA-tyrosine was from Biosearch, San Rafael, CA.

Antisera. Mice were primed with 0.1 mg antigen in 0.15 ml complete Freund's adjuvant (CFA) and boosted 21 d later with 0.1 mg antigen in 0.15 ml incomplete Freund adjuvant. We collected antisera by tail-bleeding mice 7 d after immunization. Hyperimmune ascites against TNP-ABA-KLH and TNP-KLH were prepared as described (18).

Preparation of Immunoadsorbents. Biogel A-1.5 m (Bio-Rad Laboratories, Richmond, CA) or Sepharose 4B (Pharmacia, Inc., Uppsala, Sweden) were activated with CNBr (19) and coupled with AD8, ABA-BSA, ABP-BSA, DANS-BSA, FL-BSA, TNP-BSA, or TNP-OVA. >95% of the input protein was coupled to the activated matrix. The final coupling densities were usually around 5 mg protein/ml gel. ABA–tyraminyl–Affigel-10 was prepared from Affigel-10 (Bio-Rad Laboratories).

Antibody Purification. Specific antibodies were purified from sera, ascites, or hybridoma supernatants on affinity columns containing the immunizing protein or hapten conjugated to a heterologous carrier. After washing, the specific proteins were usually eluted with 2.5 M NaSCN and dialyzed against borate-buffered saline or phosphate-buffered saline. In some cases, the bound antibodies were eluted with step gradients of NaSCN beginning with 0.16 M and increasing the concentration by twofold increments until all the bound protein was eluted.

Preparation of AD8 F(ab')$_2$ Fragments. F(ab')$_2$ fragments of AD8 were isolated from a *Staphylococcus aureus* V8 proteinase (No. 39-900; Miles Laboratories) digest by stepwise elution from DE-52 (Whatman Ltd., Kent, England) according to the procedures of Rousseaux et al. (20). The products were characterized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and by inhibition of AD8-biotin–binding activity in an enzyme-linked immunosorbent assay (ELISA).

Idiotope and Antibody Assays. Idiotope, isotype, and specific antibody titers were determined with ELISA (11). In our standard inhibition idiotope assay, we coated microtiter plates with a solution of the CRI^{AD8+} protein R16.7 at 2 μg/ml. Binding of AD8, AD8-biotin, or 5Ci-biotin (200–300 ng/ml) to the R16.7 plate was inhibited by serial dilutions of the test protein or sera. We measured the amount of antiidiotope bound with goat anti-rat Ig–alkaline phosphatase or avidin–alkaline phosphatase. Idiotope titers are expressed either as protein concentration or serum dilution that inhibits 50% maximal binding. Bound alkaline phosphatase was usually revealed with the chromogenic substrate *p*-nitrophenyl phosphate (NPP) (No. N-3254; Sigma Chemical Co.). In some assays, we increased the sensitivity of the system by using the fluorogenic substrate 4-methylumbelliferyl phosphate (4-MUP) (No. M-8883; Sigma Chemical Co.), reducing the concentration of antiidiotope as indicated and reading the fluorescence on a microtiter plate fluorometer (No. 100-100; Allergenetics, Mountain View, CA).

Isotypes were determined by incubating antibody dilutions on plates coated with antigens (10 μg/ml) or AD8 F(ab')$_2$ (3 μg/ml) for 4–18 h. The isotypes of bound antibodies were determined with either rabbit anti-κ or anti-λ1 alkaline phosphatase–monoclonal antibody (mAb) conjugates.

Antigen specificities were assayed by incubating antibody dilutions on plates coated with antigens or haptenated proteins at 10 μg/ml, including ABA-BSA, ABP-BSA, TNP-BSA, and CGG (11). Bound antibody was measured with rabbit anti-mouse Ig–alkaline

phosphatase. In some cases, the antibody dilutions were set to allow measurement of the concentrations of antibody at half saturation.

Hybridomas and Hybridoma Proteins. We developed two protocols for enhancing hybridoma production, both of which yielded 5–10-fold more hybrids than did conventional recipes. The first procedure relied on a method, described by Haas (21), for enriching TNP-specific B cells from A/J mice primed with TNP-conjugated AD8. Donor spleen cells were incubated at 4°C on plates coated with TNP-gelatin. After removal of nonadherent cells, adherent cells were released by melting the gelatin at 37°C. After washing, we treated the cells with collagenase, cultured them for 6 d with lipopolysaccharide and rat thymocyte feeders (22), and fused the cells with SP2/0 cells, as described (23). We used ELISA to screen hybridomas for coexpression of CRI^{AD8}- and TNP-binding activity. One stable, anti-TNP, CRI^{AD8+} hybridoma, TL1-N5, was isolated using the first procedure, and is characterized in Results. In the second procedure, we injected mice with 50 μg TNP-CGG in 0.05 ml CFA once in each hind footpad. 10 d later, popliteal lymph node cells were harvested, fused with SP2/0 cells (23), and seeded at 6×10^4 precursor lymph node cells/well in 96-well microtiter plates. Peritoneal cells were added as feeders ($\sim 4 \times 10^5$ cells/well). We isolated six stable, anti-TNP, CRI^{AD8+} hybridomas, identified by the prefix TCP, using the second procedure. These are characterized in Results.

In addition to the new hybridomas of the TL1 and TCP series described in this paper, we have relied on many previously described hybridoma proteins. The rat antiidiotope mAb AD8 and 5Ci were prepared as described (11, 24). 5H5, 2E2, and 1F6 are anti-antiidiotope members of the CRI^{AD8} family: 5H5 and 2E2 were derived from A/J mice immunized with AD8 (11), and 1F6 from mice immunized with 5Ci (24). 5Ci and 1F6 were from L. Wysocki (24). AB2-24.1, AB2-37.2, and AB2-143.2 are arsonate-specific members of the CRI^{AD8} family and were derived from A/J mice immunized with ABA-brucella (11). The sequenced ABA^+ proteins 93G7, 123E6, 124E1, and 91A3 were from J. D. Capra (13, 25). The ABA^+ hydridomas 36-65, 31-62, 16-46-6, and 45-223 were from A. Marshak-Rothstein (26). The ABA^+ protein R16.7 was from E. Lamoyi and A. Nisonoff (27), while the protein L22.18.2, an antimurine λ1 reagent, was from R. Epstein (28).

Hybridomas were grown either in culture or as ascites (11). We isolated hybridoma proteins of defined specificities on the appropriate immunoadsorbent matrices, and desorbed them with NaSCN. For instance, 36-65 and AB2-24.1 were isolated on ABA-BSA columns, while TL1-N5 and TCP-2A2 were isolated on TNP-BSA columns. Antibodies with unknown specificities, like 5H5 and 2E2, were grown in serum-free medium and isolated on protein A–Sepharose (11). We grew AD8 and 5Ci as ascites in nude mice and isolated them on DE-52, as described (11).

Results

CRI^{AD8+} Antibodies Are Differentially Elicited by Various Epitopes. In a previous report (11), we described CRI^{AD8} among naturally occurring antibodies, but were unable to determine their specificity. The possibilities, then, were that CRI^{AD8} is used by antibodies of many different specificities, as reported for certain V_H markers in rabbits (29) and mice (30, 31); or, that it is found only on anti-ABA antibodies and naturally occurring antibodies of a single specificity. To clarify this point, affinity-purified antibodies from several antihapten responses were examined for CRI^{AD8} content. Six groups of mice were immunized with CGG or CGG conjugated with one of five different haptens: ABA, TNP, DANS, FL, or ABP. Among these ABA and ABP are of particular interest because they have similar structures and are known to elicit cross-reactive antibodies in rabbits (32). Antibodies to each hapten and to CGG were affinity purified and analyzed for CRI^{AD8} content.

As shown in Fig. 1, the levels of CRI^{AD8} found in antibodies of different

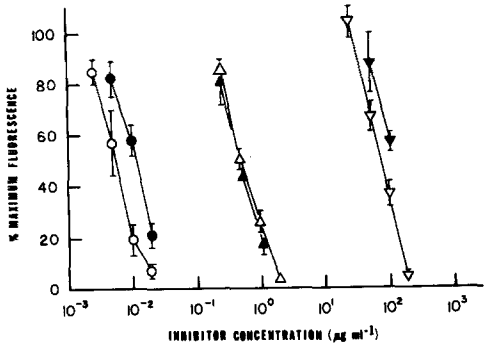

FIGURE 1. CRI^{AD8} content of representative affinity-purified antibodies of different specificities. CRI^{AD8} was measured with an ELISA, in which the binding of AD8 (50 ng/ml) to R16.7-coated plates was inhibited by serial twofold antibody dilutions. Bound AD8 was assayed with anti-rat Ig–alkaline phosphatase conjugates followed by the fluorogenic substrate 4-MUP. Percent maximum fluorescence is plotted on the abscissa, versus inhibitor concentration on the ordinate. Inhibitions, done in triplicate, were averaged; error bars span ±1 SD. Inhibitors were: 36-65 (○), anti-ABA (●), anti-CGG (▲), anti-TNP (△), anti-ABP (▽), and anti-DANS (▼).

TABLE I

CRI^{AD8} Levels in Antibodies of Various Specificities

Antibody*	CRI^{AD8} titer‡ at 50% inhibition	Percent antibody CRI^{AD8+}
	μg/ml	
36–65	0.0054	100
Anti-ABA	0.012	47
Anti-CGG	0.38	1.4
Anti-TNP	0.51	1.1
normal IgG	1.2	0.43
Anti-FL	74	0.007
Anti-DANS	76	0.007
Anti-ABP	81	0.007

* Specific antibodies were affinity purified from pooled antisera to ABA-CGG, CGG, TNP-CGG, FL-CGG, DANS-CGG, and ABP-CGG. Hybridoma protein 36-65 (26) was affinity purified from ascites fluid. Normal A/J IgG was isolated from CFA-induced ascites as described (11).

‡ CRI^{AD8} titers are expressed as the antibody concentration which inhibits 50% of the binding of AD8 to R16.7-coated plates in an enhanced ELISA. AD8 concentration was reduced from 200 ng/ml to 50 ng/ml and the amount of AD8 bound was measured with a goat anti-rat Ig–alkaline phosphatase conjugate followed by the fluorogenic substrate 4-MUP.

specificities varied over five orders of magnitude. Included for comparison is the standard hybridoma protein 36-65, which is strongly CRI^{AD8+} and binds ABA. Significantly, the slopes of inhibition for all the antibody populations shown in Fig. 1 are equivalent, suggesting that the CRI^{AD8} moieties in each antibody population bind to AD8 with similar affinities. This reinforces our earlier conclusion (11) that CRI^{AD8}, though intimately associated with the antigen-binding structure, is not identical to it. This equivalence of slopes allowed an

estimate of the percentage of each antibody population containing CRI^{AD8}, which we have summarized in Table I. The antibodies can be divided into three groups. The group expressing the highest proportion of CRI^{AD8} includes hp 36-65, all of which is assumed to express the idiotope, and anti-ABA serum antibodies, 47% of which contain CRI^{AD8}. The group expressing intermediate idiotope levels (Table I, rows *3–5*) includes normal A/J IgG as well as antibodies specific for CGG and TNP, which contain 0.4, 1.4, and 1.1% CRI^{AD8+} antibodies, respectively. Surprisingly, antibodies to ABP contained only traces of CRI^{AD8} and, along with antibodies to FL and DANS, comprised the group expressing the lowest levels of idiotope. Only ~0.007% of these antibodies express CRI^{AD8}, and possibly even this is due to nonspecific copurification of naturally occurring CRI^{AD8} along with specific antibodies; the vanishingly small amounts of CRI^{AD8} in these preparations precluded further characterization.

The ability of both CGG and TNP to elicit CRI^{AD8} was confirmed in a number of subsequent experiments. First, the possibility that CRI^{AD8} expression by anti-TNP antibodies is carrier dependent was ruled out by the experiment shown in Table II. We immunized mice with either TNP-RGG or RGG in CFA, bled them 7 d later, and analyzed the antisera for idiotope content. CRI^{AD8} levels in the antisera to the TNP-carrier conjugate were 12-fold higher than in the antisera to the carrier alone (groups *A* and *B*, Table II), indicating that the ability of TNP compounds to enlist CRI^{AD8+} antibodies is carrier independent. Second, two batches of CGG, independently prepared, consistently elicited CRI^{AD8+} antibodies (data not shown). Third, the possibility that CGG enlisted CRI^{AD8+} responses because it was contaminated with arsonate was directly ruled out by graphite furnace, atomic absorption spectrophotometry; no arsenic was detected in either batch of CGG (data not shown).

CRI^{AD8} Dominance of Antibodies to TNP Cannot Be Elicited by Immunization With TNP-ABA-KLH. As shown above, CRI^{AD8} is a dominant idiotope in the response to ABA but a minor idiotope in the response to TNP. We attempted to increase its expression in the anti-TNP response by immunizing mice with the doubly haptenated immunogen TNP-ABA-KLH. We postulated that TNP-ABA-KLH might increase the CRI^{AD8} representation in the anti-TNP component of the response, based on two sets of observations. First, antibodies to different determinants on the same protein antigen can share a common cross-reactive idiotype

TABLE II

Influence of Immunizing With TNP on Serum CRI^{AD8} Levels

Group	*n*	Immunogen*	CRI^{AD8} titer‡
A	6	RGG	34 (2.19)
B	6	TNP-RGG	417 (1.99)

* Mice were immunized with 0.1 mg immunogen in CFA and bled 7 d later.

‡ CRI^{AD8} titers are expressed as serum dilutions that inhibit 50% of the binding of 200 ng/ml AD8 to R16.7-coated plates in our standard ELISA. The amount of bound AD8 was quantitated with a goat anti-rat IG–alkaline phosphatase conjugate followed by the chromogenic substrate NPP. Values are expressed as the geometric means, with the geometrical deviations in parenthesis.

(33-37), suggesting that different epitopes on the same immunogen can selectively call up idiotypically related antibodies. Second, idiotype-specific T helper cells, which have been reported in the CRI[a] system (5), would, presumably, be enlisted in response to TNP-ABA-KLH. If helper cells specific for the CRI^{AD8} V_H region are responsible for the dominance of the anti-ABA response by CRI^{AD8}, then they might also provide help to CRI^{AD8+}, TNP^+ clones. To test this possibility, A/J mice were hyperimmunized with either TNP-ABA-KLH or TNP-KLH. We then isolated anti-TNP or anti-ABA antibodies from pooled ascites fluid by affinity chromatography on a TNP-OVA–Sepharose immunoadsorbent. The anti-TNP antibodies were then depleted of possible contaminating anti-ABA activity by passage through ABA-tyrosine–Affi-gel columns and analyzed for CRI^{AD8}. As shown in Table III, anti-ABA antibodies contain 24 times more CRI^{AD8} than anti-TNP antibodies (row *1* vs. rows *2* and *3*). Significantly, anti-TNP antibodies isolated from TNP-ABA-KLH–immune mice did not contain elevated levels of CRI^{AD8} relative to anti-TNP antibodies from TNP-KLH–immune mice (Table III, row *2* vs. *3*). This result suggests that if antiidiotypic helper cells are responsible for the dominance of CRI^{AD8} in the ABA response, they are incapable of helping clones expressing CRI^{AD8} on immunoglobulin specific for TNP. Regardless of the mechanism of idiotope dominance, it is clear that CRI^{AD8} alone is not sufficient to establish it.

Relationship of Phosphonate-binding Site and CRI^{AD8} Use. The possibility that the phosphonate and arsonate groups elicit cross-reactive antibodies with overlapping specificities but dissimilar idiotopes (Table I) provides a particularly appealing system to examine idiotope use in the antigen-driven selection of the expressed repertoire. For example, although 5–40% of the antibodies raised against ABP cross-reacted with ABA (data not shown), they expressed only traces of CRI^{AD8} (Table I). This result implies that clones responding to ABP, even those which bind ABA, effectively lack CRI^{AD8}. This might be explained by inferring that structural constraints prevent ABP-binding antibodies from expressing CRI^{AD8} or that regulatory constraints limit the expansion of ABP-binding clones which also express this idiotope. These possibilities were explored, first, by determining if ABP–cross-reactive antibodies isolated from anti-ABA

TABLE III

Effect of ABA on CRI^{AD8} Levels in Antibody Response to TNP

Antibodies*	Immunogen	CRI^{AD8} titer‡ at 50% inhibition
		μg/ml
Anti-ABA	TNP-ABA-KLH	0.6
Anti-TNP	TNP-ABA-KLH	14.3
Anti-TNP	TNP-KLH	12.2

* Anti-TNP antibodies were affinity purified on TNP-OVA-Sepharose and depleted of possible anti-ABA contamination by passage over ABA-tyr-Affigel.

‡ CRI^{AD8} levels are expressed as the antibody concentration which inhibits 50% of the binding of 200 ng/ml AD8 to R16.7 plates in the standard ELISA using the NPP substrate. Data from three separate assays were normalized.

serum express CRI^{AD8}, and second, by determining if CRI^{AD8+} anti-ABA mAb also bind ABP.

That CRI^{AD8+} antibodies can bind ABP was demonstrated in both serum and hybridoma antibodies. We separated serum anti-ABA antibodies (from anti-ABA-KLH ascites fluid) into ABP-cross-reactive and non-cross-reactive fractions using an ABP-BSA immunoadsorbent, and analyzed them for CRI^{AD8} (Table IV). ABP-cross-reactive antibodies raised against ABA contained 4% of the CRI^{AD8} found in antibodies specific for ABA alone (compare rows *1* and *2*, Table IV). In contrast, anti-ABP antibodies raised against ABP contained ≤0.015% of the CRI^{AD8} in anti-ABA antibodies (rows *8* and *2*, Table I). Thus, anti-ABP antibodies raised in response to ABA expressed CRI^{AD8} at least 300-fold more effectively than those raised in response to ABP. Since the slopes of inhibition of the ABP-cross-reactive antibodies were the same as for anti-ABA antibodies (data not shown), we suspect that the reduced level of idiotope observed for the ABP-reactive antibodies reflects a lower content of CRI^{AD8}, rather than an alteration of the idiotope structure. In either case, however, this result shows that serum antibodies which express CRI^{AD8} can also bind ABP. One objection to this conclusion is the possibility that some CRI^{AD8+} antibodies nonspecifically adsorbed to the ABP-BSA columns. To circumvent this criticism, mAb were examined for the expression of CRI^{AD8} and the ability to bind phosphonate.

Anti-ABA hybridoma proteins were compared for their expression of CRI^{AD8} and their abilities to bind ABP and ABA. Hapten-binding activity was measured with ELISA in which serial dilutions of antibodies were reacted with ABP-BSA- or ABA-BSA-coated plates. Antibody concentrations at 50% maximum binding were determined, when possible. As shown in Table V, there is no apparent correlation between CRI^{AD8} and the relative ability to bind ABP or ABA. In the group of molecules that express CRI^{AD8} best, 16-46-6 binds ABP twice as effectively as ABA, AB2-24.1 binds ABP nearly as strongly as ABA, while 36-65 and AB2-143.2 bind ABP at least 8- and 44-fold less effectively than ABA, respectively. Similarly, no trend is seen among CRI^{AD8-} antibodies: 31-62 binds phosphonate twice as effectively as arsonate, while 123E6, 124E1, and 45-223

TABLE IV

CRI^{AD8} Expression in the Anti-ABA Response: A Comparison of ABP-cross-reactive and Non-cross-reactive Antibodies

Antibodies*	Protocol	CRI^{AD8} titer‡ at 50% inhibition
		μg/ml
Anti-ABA	Depleted of anti-ABP	0.09
Anti-ABP	Desorbed with 0.16 M NaSCN	2.10

* Antibodies to ABA were isolated from anti-ABA-KLH ascites fluid on an ABA-BSA column. These antibodies were subsequently fractionated into ABP-cross-reactive and non-cross-reactive portions by affinity chromatography on an ABP-BSA immunabsorbent. Anti-ABP antibodies were desorbed from the column using 0.16 M NaSCN in borate-buffered saline.

‡ Idiotope titers are expressed as the antibody concentration which inhibits 50% of the maximum binding of AD8 to R16.7-coated plates in an enhanced ELISA (as described in Table I).

TABLE V

Comparison of Ability of CRI^{AD8+} and CRI^{AD8-}, Arsonate-specific mAb to Bind Phosphonate

mAb	Antibody concentration ($\times 10^{-6}$/ml) at 50% maximum binding to plates coated with:*		ABP/ABA ratio	CRI^{AD8}‡
	ABA	ABP		
16-46-6	4.8	2.3	2.1	++
AB2-24.1	2.2	2.8	0.78	++
36-65	1.9	>16	<0.12	++
AB2-143.2	5.2	>231	<0.02	++
AB2-37.2	5.6	7.5	0.75	+
R16.7	1.3	>8.1	<0.16	+
93G7	1.9	>6.2	<0.31	+
31-62	1.2	0.56	2.1	−
91A3	2.2	>10	<0.22	−
123E6	0.81	>6.2	<0.13	−
124E1	1.6	>125	<0.01	−
45-223	1.6	>625	<0.003	−

* Specific antibody titers were measured with ELISA in which dilutions of mAb were incubated on plates coated with either ABA-BSA or ABP-BSA. Bound antibody was assayed with rabbit anti-mouse–alkaline phosphatase conjugates. Antibody molarity at 50% maximal binding was determined in those cases where saturation binding was achieved.

‡ CRI^{AD8} expression, assessed in our standard inhibition ELISA, was scored relative to 36-65, which was assigned a value of 100%, as follows: 10–170% (++); 1–10% (+); and not detected (−).

all bind phosphonate less effectively than arsonate. These results show that CRI^{AD8+} antibodies can bind ABP, and that some even bind ABP more effectively than ABA. Furthermore, CRI^{AD8-} antibodies bind ABP no better than ABA. Thus, it is difficult to explain why immunization with ABP fails to elicit CRI^{AD8}. It might be argued that CRI^{AD8+} clonal precursors bind ABP relatively weakly, and that affinity selection in anti-ABP responses preferentially expands CRI^{AD8-} clones. However, since antigen is not limiting, this possibility appears unlikely. Apparently, the differential abilities of ABP and ABA to expand CRI^{AD8+} clones cannot be explained simply by the capacity of B cell antigen receptors to bind antigen. This result is consistent with the existence of an auxiliary mechanism that preferentially expands CRI^{AD8+} clones in the response to ABA but not ABP. In the Discussion, the possibility is raised that ABA itself is an auxiliary factor required to establish dominance.

Production of TNP^+, CRI^{AD8+} Hybridomas. While the above results strongly suggest the existence of TNP^+, CRI^{AD8+} antibodies, proof of their existence requires mAb with this phenotype. To this end, two protocols were developed that increased the frequency of anti-TNP hybrids. The first procedure, using LPS-stimulated, TNP-specific spleen cells, yielded one stable CRI^{AD8+} hybridoma out of 110 TNP^+ clones (TL1-N5, Table VI). The second procedure, using popliteal lymph node cells from mice immunized 10 d previously with TNP-CGG, yielded six stable CRI^{AD8+} hybridomas out of 237 TNP^+ wells (TCP proteins, Table VI).

As shown in Table VI, all heavy chain isotypes except IgD and IgA were found on TNP^+, CRI^{AD8+} hybridomas. Interestingly, two out of seven of these use λ1 light chains. In contrast, all other CRI^{AD8+} antibodies previously studied use κ

TABLE VI

Isotypes of Hybridoma Proteins Which Express CRI^{AD8} and Are Specific for TNP

Protein	Isotype	
	Heavy chain	Light chain
TL1-N5	IgM	λ1
TCP-2C3	IgG3	λ1
TCP-2A3	IgG1	κ
TCP-2B2	IgG2b	κ
TCP-1B3, -2A1, -2A2	IgG2a	κ

Isotypes were determined using ELISA in which hybridoma supernatants were incubated on plates coated with AD8-F(ab')$_2$. The isotypes of the bound antibodies were determined with antiisotype–alkaline phosphatase probes. Antigen specificity was determined by depletion of CRI^{AD8} on TNP-BSA immunoabsorbents.

TABLE VII

Comparison of Light Chain Isotypes Used by mAb Against Either TNP or CGG

Antigen	Total number of hybridomas	Percent λ1	Percent κ
CGG	168	3	97
TNP	110	23	77

Hybridomas to TNP and CGG were prepared from popliteal lymph nodes of mice immunized once with TNP-CGG and fused with SP2/0 cells. Light chain isotypes were determined with ELISA. Plates were coated with either TNP-BSA or CGG, reacted with hybridoma supernatants, and developed with anti-λ1 mAb or polyclonal anti-κ–alkaline phosphatase conjugates.

light chains (11). The use of λ1 light chains on antibodies specific for TNP might be explained by the ability of λ1 light chains to bind nitrophenyl groups (38, 39). To determine if λ1 is used preferentially by anti-TNP hybridoma proteins in this system, λ1 expression by anti-TNP antibodies was compared to λ1 expression among anti-CGG antibodies isolated from the same fusion. As seen in Table VII, 23% of the anti-TNP antibodies used λ1, while only 3% of the anti-CGG antibodies did so. Thus, in this system, the use of λ1 by two out of seven TNP$^+$, CRI^{AD8+} hybridoma proteins is proportional to its use by all anti-TNP hybridomas.

The efficiency of CRI^{AD8} expression by anti-TNP hybridoma proteins was explored in more detail by comparing its expression on three affinity-purified TNP$^+$, CRI^{AD8+} proteins with the canonical CRI^{AD8+}, anti-ABA protein 36-65. We included 91A3 as a negative control. ELISA inhibition curves (Fig. 2) show that all three anti-TNP proteins express CRI^{AD8} as effectively (TCP-2C3), or nearly as effectively (TL1-N5 and TCP-2A2), as 36-65. It is noteworthy that the expression of CRI^{AD8} can be similar in antibodies using either λ1 (TCP-2C3) or κ (36-65) chains (also see Table VIII). In summary, hybridoma production proves that CRI^{AD8} can be expressed on anti-TNP antibodies as well as on anti-ABA antibodies, and that its expression is largely determined by the heavy chain.

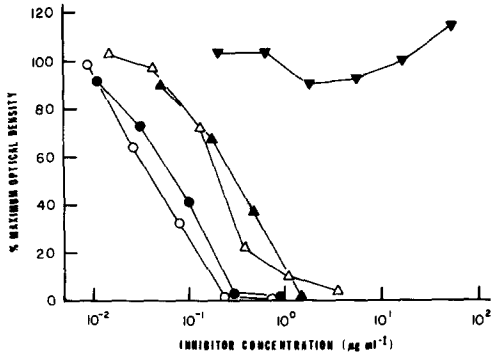

FIGURE 2. CRI^{AD8} expression by three anti-TNP hybridoma proteins compared with anti-ABA hybridoma 36-65. The anti-ABA hybridoma protein 91A3 was included as a negative control. Anti-TNP antibodies were affinity purified on TNP-BSA immunoabsorbents and chaotropically eluted. CRI^{AD8} was measured with an ELISA, in which the binding of AD8-biotin (200 ng/ml) to R16.7-coated plates was inhibited by dilutions of the five proteins. Bound AD8-biotin was assayed with avidin–alkaline phosphatase conjugates followed by the chromogenic substrate NPP. Inhibitors were: 36-65 (○), TCP-2C3 (●), TL1-N5 (△), TCP-2A2 (▲), and 91A3 (▼).

TABLE VIII

Light Chain, J_H, and V_H Usage Compared With CRI^{AD8} Expression

Hybridoma protein	Light chain*	J_H	V_H CRI^{a}‡	CRI^{AD8}§ (μg/ml at 50% inhibition
36-55	$V_{\kappa-10}$	2	+	0.07
2E2	$V_{\kappa-9}$	2	+	0.10
AB2-143.2	$V_{\kappa-?}$	3	+	0.04
5H5	$V_{\kappa-?}$	4	+	0.06
TCP-2C3	$V_{\lambda 1}$	?	?	0.13
TL1-N5	$V_{\lambda 1}$	?	?	0.30

* V_L subgroups and isotypes were determined either from the amino acid sequences or serologically. The sequence of 36-65 was reported previously (54, 55), and that of the 48 N-terminal residues of 2E2 has recently been determined by M. Margolies. All other isotypes were determined by ELISA.

‡ J_H and V_H CRI^a usage was determined by L. Wysocki (personal communication) as described (12). Briefly, DNA from various hybridomas was digested with EcoRI, electrophoresed in agarose, transferred to nitrocellulose paper, and hybridized with a labeled V_H CRI^a probe as described (12).

§ CRI^{AD8} content is expressed as the antibody concentration which inhibits 50% of the maximum binding of AD8 to R16.7-coated plates in an ELISA (as described in Table III).

CRI^{AD8} Correlates With the CRI^a V_H Region, Exclusive of J_H. We have suggested, here and elsewhere (11), that CRI^{AD8} depends upon V_H regions that contain the germline CRI^a V_H segment (12). Additionally, the expression of CRI^{AD8} may depend upon a particular D segment gene that is characteristic of CRI^{a+} V_H regions (13). Six arguments support mapping CRI^{AD8} to the V_H region: (*a*) The expression of CRI^{AD8} is linked to the A strain Igh locus (11), which contains both V_H CRI^a and the D segment alleles. (*b*) All CRI^{AD8+} hybridomas that have been

examined at the DNA level have undergone rearrangements of the V_H CRI^a gene (L. Wysocki, personal communication). For example, as summarized in Table VIII, restriction mapping and hybridization with a labeled V_H CRI^a probe showed that all four of the CRI^{AD8+} hybridomas examined have undergone genomic rearrangements of the V_H CRI^a gene. (*c*) CRI^{AD8} expression, like that of CRI^a (40, 41), is independent of a particular J_H segment. As summarized in Table VIII, proteins 36-65 and 2E2 use J_H2; AB2-143.2 uses J_H3; and 5H5 uses J_H4 (L. Wysocki, personal communication). The CRI^{AD8} titers of all four proteins are very similar, ranging from 0.04 μg/ml for AB2-143.2 to 0.10 μg/ml for 2E2. The independence of CRI^{AD8} and J_H might be explained by the largely internal location of J_H (42), which suggests that this region does not directly contribute to the idiotopic surface structure. (*d*) CRI^{AD8} is expressed on antibodies with different light chains. As shown in Table VIII, proteins that have $V_{\kappa}10$, $V_{\kappa}9$, or λ1 all effectively express CRI^{AD8}. (*e*) CRI^{AD8} is expressed by isolated heavy chains (11, 43). (*f*) CRI^{AD8} correlates with the sequence encoding 36-65, which expresses the germline-encoded CRI^a V_H sequence and a distinctive D segment (13), and is successively lost in proteins known to diverge from this sequence. To illustrate this, the number of amino acid substitutions, in both the V_H and D segments of nine idiotypically related hybridoma proteins, relative to 36-65 sequences, is compared with CRI^{AD8} expression (Table IX). CRI^{5Ci} expression is included as a control. Seven of the V_H regions shown in Table IX have been sequenced entirely (12, 13, 25, 41, 54) and another (2E2) has been sequenced through 51 of the first 52 amino-terminal residues (M. N. Margolies, unpublished observations). CRI^{AD8} correlates with the V_H and D sequences of protein 36-65, while the control idiotope CRI^{5Ci} shows no such correlation. For instance, protein 2E2, which is identical to 36-65 in the region that has been sequenced, expresses

TABLE IX

CRI^{AD8} Is Most Strongly Expressed on the CRI^a Prototype Protein and Is Lost Upon Divergence From the Prototype

Hybridoma*	Amino acid substitutions‡		CRI^{AD8}§ titer	CRI^{5Ci}§ titer
	V_H	D		
36-65 (54)	0	0	1	1
2E2 (56)	0	?	1.3	>10,000
93G7 (13)	3	1	12	42
R16.7 (13)	4	1	11	6
1F6 (40)	9	2	71	3
123E6 (25)	7	1, and 1 insertion	>10,000	8
124E1 (13)	8	2	>10,000	1
91A3 (13)	10	2, and 1 deletion	>10,000	>10,000

* Sequences of V_H regions were determined as described in the references in parentheses. All V_H sequences are complete except 2E2, which has been sequenced through 51 of the first 52 N-terminal residues.

‡ Substitutions in the V_H segment (residues 1–94) are relative to 36-65, which is the unmutated product of the V_H CRI^a gene (12, 54). The D segments (residues 95–100), with substitutions relative to 36-65, also have an amino acid insertion (123E6) and a deletion (91A3).

§ Idiotope content is expressed as the relative amount of protein which inhibits 50% of the binding of either AD8-biotin or 5Ci-biotin to R16.7-coated plates in our standard ELISA. The units are arbitrary and adjusted so that the CRI^{AD8} titer of protein 36-65 is 1.0.

CRI^{AD8} with about the same efficiency as 36-65, but does not express CRI^{5Ci} Protein 1F6, which contains nine V_H and two D segment substitutions relative to 36-65, expresses CRI^{AD8} 70-fold less effectively than 36-65, but expresses CRI^{5Ci} with about the same efficiency as 36-65. In contrast, 124E1, which has eight V_H and two D segment amino acid differences compared with 36-65, has lost CRI^{AD8} but expresses CRI^{5Ci} as strongly as 36-65 does. Thus, CRI^{AD8} correlates with both the CRI^a V_H segment and the 36-65 D segment, and is independent of the light chain or J_H.

While the precise location of CRI^{AD8} is unknown, the ability of both hapten and antigen to inhibit the binding of AD8 to CRI^{AD8+} antibodies suggests that the idiotope resides in the hypervariable region of the molecule (56). This assumption, in concert with available Ig structural data, and the possibility that the majority of amino acid substitutions in functional proteins have predominantly local effects (44), allowed us to postulate that CRI^{AD8} is determined by CDR-2 or the D segment (unpublished observations). This provisional assignation awaits further structural data for verification. It will be particularly interesting to study the complete structures of the TNP^+ members of the CRI^{AD8} family, which should reveal the relative contributions of V_H and D regions to the idiotope, as well as increase our understanding of how the same V_H structures can be used to construct antibodies of different specificities.

Discussion

The main finding of this report, that CRI^{AD8} dominates some antihapten responses but not others, shows that the CRI^{AD8} V_H region is necessary but not sufficient for idiotype dominance, and suggests that an additional specificity is involved. This suggestion introduces a new constraint on the mechanism of idiotype dominance, with the caveat that these conclusions may be limited to the A strain anti-ABA response, because no single mechanism currently accounts for all observations made in all systems.

For example, in the T15 system, idiotype dominance can be traced back to the pre–B cell stage where most of the surface Ig^- precursors (which, in the splenic focus assay, differentiate in response to phosphorylcholine; PC) are $T15^+$ (3, 45). While these findings do not exclude a role for idiotype-specific helper T cells, it is not necessary to invoke them to explain idiotype dominance in this system. On the other hand, the situation is different in the CRI^a system, where only 3% of the nonimmune, arsonate-specific B cells responding in the splenic focus assay are CRI^{a+} (4). This frequency increases 7–30-fold after immunization with T-dependent conjugates, suggesting that the precursors bearing this idiotype are selectively expanded. This expansion might be explained by either idiotype-specific helper cells or preferential suppression of idiotype-negative precursors. In the latter, suppression could be mediated by antiidiotypic suppressor T or B cells.

Our data strongly suggest that CRI^{AD8} is expressed by CRI^a V_H regions, and thus provides a marker for analyzing CRI^a dominance in anti-ABA responses. This assumption is based on the observations that CRI^{AD8} has been found only on CRI^a V_H regions, and that the CRI^{AD8+} subset, like the rest of the CRI^a family, is selectively expanded after immunization with ABA. This prediction has not

been tested by precursor analysis; however, it is supported by two other observations. First, CRI^{AD8} is not as dominant in T-independent anti-ABA responses as it is in T-dependent responses (46), arguing that CRI^{AD8-} precursors are relatively frequent in nonimmune mice. By contrast, in the T15 system, both T-dependent and T-independent responses are dominated by T15 (3, 47). Second, idiotype suppression experiments argue that CRI^{AD8} is a smaller fraction of the ABA-specific precursor pool than T15 is of the PC-specific precursor pool. In CRI^{AD8}-suppressed mice, anti-ABA responses are normal even shortly after suppression (unpublished observations), suggesting that CRI^{AD8-} precursors readily compensate for the deleted clones. This result would be predicted if only 3% of the ABA-specific precursor pool was CRI^{AD8+}. On the other hand, in T15-suppressed mice, anti-PC responses are lost for several months after idiotype suppression (48, 49), suggesting that the majority of PC precursors are $T15^+$ (3, 45). Taken together, these two observations are most consistent with dominance in the CRI^{AD8} system being due to selective expansion of CRI^{AD8+} precursors after immunization with ABA.

If this expansion is mediated by an antiidiotypic helper cell, two questions arise. First, what additional specificity, expressed by CRI^{AD8+} anti-ABA antibodies, does the helper recognize? And, second, why is there more help for CRI^{AD8+} anti-ABA clones than for those lacking this idiotope?

There are at least five candidates for the additional specificity recognized by an antiidiotypic T helper cell: the light chain V and J segments; the heavy chain D and J segments; and ABA itself. Helper cells that recognize light chain idiotopes have been reported (50), and it is possible to explain the lack of recognition of the TNP- and CGG-specific members simply by postulating that the helpers only recognize the light chain V_κ regions used by the ABA-specific members. The anti-ABP response argues differently. Immunization with this hapten does not call up any significant CRI^{AD8+} antibody, even though immunization with the ABA group calls up ABP cross-reactive antibodies expressing CRI^{AD8}. Similarly, anti-ABA hybridoma proteins that express CRI^{AD8} also bind ABP. If the idiotype-specific T helpers are simply recognizing V_κ determinants, then immunization with ABP (like immunization with ABA) should call up significant levels of CRI^{AD8}, which in fact, it does not.

The hypothesis most consistent with the above data is that a complex between ABA and the CRI^{AD8+} V_H region is recognized by the helper cell. This would explain the lack of dominance in the anti-TNP and -CGG responses, as well as the absence of CRI^{AD8} in anti-ABP cross-reactive responses. In the latter, immunization with ABP would not call up CRI^{AD8+} antibodies because ABP would not cross-react with ABA at the T cell level (51). Additionally, the requirement for ABA in this regulatory circuit accounts for observations, in both the CRI^a (24) and CRI^{AD8} (11) systems, that CRI^+ ABA^- components are not expanded in antibody responses to ABA. This hypothesis is also consistent with the lack of carrier specificity in CRI^{AD8} dominance of T-dependent antibody responses (11). One potential objection to this hypothesis is that Woodland and Cantor (5) were able to isolate CRI^a-specific helpers on idiotype-coated plates, suggesting that the helpers are specific for idiotype alone. This objection can be countered in two ways. First, it is possible that the helpers have sufficient avidity for the idiotope itself to stick to idiotype-coated plates but still require ABA for activation.

Second, the idiotype used to coat the plates was purified on ABA columns, and it is possible that some ABA desorbed with the antibody. In fact, we have invariably found small amonts of haptenated proteins in affinity-purified antibody preparations when we have looked with sensitive techniques (unpublished results).

If a complex between ABA and a CRI^{AD8+} V_H region is recognized by T helper cells, why is this complex more immunogenic than ones made with CRI^{AD8-} V_H regions? That the former complex is more immunogenic is suggested by the dominance of CRI^{AD8} in anti-ABA responses. The simplest possibility is that idiotype dominance in this system is due to an *Ir* gene effect, where A/J mice are high responders to the ABA-CRI^{AD8} complex (52). In this instance, the idiotype-specific helper cell need only be a conventional major histocompatibility complex (MHC)-restricted helper cell. The ABA-idiotype complex could be the nominal antigen, with the helper restricted by MHC. Alternatively, ABA could be the nominal antigen, with the helper restricted by a complex of MHC and idiotype. In either case, the result would be preferential expansion of anti-ABA clones that express CRI^{AD8}.

The patterns of CRI^{AD8} expression are also consistent with a suppression model of idiotype dominance. In this case, the complex between CRI^{AD8} and ABA would elicit lower levels of suppression than either complexes between ABA and CRI^{AD8-} antibodies, or complexes between CRI^{AD8} and the other haptens. The difference in levels could be accounted for by an *Is* gene (52) where strain A suppressor cells respond poorly to CRI^{AD8} plus ABA.

There is evidence that antiidiotypic antibodies (53) and antiidiotypic suppressor T cells (9) can limit the expansion of clones expressing CRI^{a}. Under the usual conditions of a T-dependent anti-ABA response, these mechanisms may not be as effective for CRI^{a+} responses as they are for either CRI^{a-} responses or CRI^{a+} responses specific for other epitopes. A corollary of this hypothesis is that CRI^{a} dominance should be increased by inactivation of suppression. Such a role for antiidiotypic regulation in the CRI^{a} system has been confirmed by recent studies showing that tolerance of auto-antiidiotypic antibody responses (induced by immunization with CRI^{a}) led to the complete dominance of anti-ABA responses by this idiotype (53).

In summary, the patterns of idiotype dominance observed in the CRI^{a} system are consistent with a mechanism of idiotype-specific regulation. The possible mechanisms include: selective expansion of idiotype-positive precursors by idiotype-specific help, and selective inhibition of idiotype-negative precursors by selective suppression. The available data do not permit a choice between these two mechanisms; however, they do impose a new constraint on the specificity of regulation in the CRI^{a} system: antigen, in addition to idiotype, is an important factor in establishing idiotypic dominance. Understanding the mechanism awaits further experimentation, but knowing that the specificity may involve antigen should make this understanding more accessible.

Summary

We describe the recurrence of a cross-reactive idiotope (CRI^{AD8}) in antibody responses to different epitopes, and explore factors leading to its dominance in some responses, but not others. Serological and genomic DNA analyses showed

that CRI^{AD8} is a marker of the CRI^a heavy chain variable region (V_H) that encodes the majority of anti-*p*-azobenzenearsonate (anti-ABA) antibodies. The independence of CRI^{AD8} from any particular light chain or antigen specificity was underscored by the fact that we could isolate hybridomas that secrete antitrinitrophenyl (TNP) antibodies expressing CRI^{AD8}, with λ1 light chains. CRI^{AD8} is dominant in anti-ABA responses, recurrent but nondominant in anti-TNP and anti–chicken gammaglobulin responses, and is virtually absent in other antihapten responses, including that to *p*-azobenzenephosphonate (ABP), which contains an ABA–cross-reactive component (~5–40%). Dominance in the anti-TNP response could not be achieved by immunization with doubly haptenated TNP-ABA-KLH (keyhole limpet hemocyanin), where the anti-ABA response was dominated by CRI^{AD8}. These observations suggest that, while the CRI^{AD8} V_H region is necessary for idiotypic dominance, it is not sufficient. Apparently, an additional specificity is required. Since immunization with ABA calls up anti-ABP antibodies that express CRI^{AD8}, but not vice versa, it is possible that the additional specificity is ABA itself. This possibility imposes a new constraint on the specificity of the putative idiotype-specific regulation that may establish dominance in the CRI^a system.

We thank Dr. Joel Goodman for support, Drs. L. Wysocki, A. Marshak-Rothstein, E. Lamoyi, J. Capra, A. Nisonoff, D. Nitecki, and R. Epstein for reagents, Dr. C. Henry for reading the manuscript, and Ms. Inge M. Stoltenberg for technical assistance. We especially thank Drs. M. Margolies and L. Wysocki for analyzing our hybridoma lines and sharing unpublished results.

Received for publication 13 August 1984.

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