IDIOTYPE CONNECTANCE IN THE IMMUNE SYSTEM

I. Expression of a Cross-Reactive Idiotype on Induced Anti-*p*-azophenylarsonate Antibodies and on Endogenous Antibodies Not Specific for Arsonate*

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Lymphocyte clones that are individually specific for structurally dissimilar epitopes can be functionally connected through shared idiotypy. Although idiotype sharing between antibodies binding to different epitopes was first reported in 1971 by Oudin and Cazenave (1), followed by repeated confirmations at the immunochemical level (2–12), the biological consequences of such idiotype sharing have remained relatively uncharted. By incorporating unspecific parallel sets into the network hypothesis, Jerne (13) predicted the importance of shared idiotypy between lymphocyte clones specific for non–cross-reactive epitopes as a means of joining otherwise unconnected immune responses at the regulatory level.

Experimentally, the biological relevance of unspecific parallel sets is suggested by studies in the anti–hen-egg-white lysozyme (HEL)[1] system (2, 3) where antibodies directed against different epitopes on the lysozyme molecule share idiotype. Production of these antibodies is favored by an antiidiotypic helper T cell acting in concert with a classic lysozyme-specific helper T cell. Idiotypes such as those in the anti-HEL system that are coexpressed by different lymphocyte clones and that are targets for regulatory signals have also been called "regulatory idiotypes" by Bona et al. (14). Furthermore, it has been postulated that regulatory idiotypes are germ line encoded and that idiotype dominance during induced antihapten responses is due to the connection of these responses with ongoing responses to environmental or endogenous stimuli by virtue of shared regulatory idiotypes (8, 15). Idiotype sharing between induced antihapten responses and naturally occurring responses has been documented for the T15 (16), MOPC-460 (8), and A strain cross-reactive idiotope (CRI)[a] (7) idiotype systems. Only in the T15 system do the induced and naturally occurring antibodies bind the same epitope.

In this report, we describe our first experiments aimed toward understanding the

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[1] *Abbreviations used in this paper:* ABA, *p*-azobenzenearsonate; BBS, borate-buffered saline; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; CRI, cross-reactive idiotope; ELISA, enzyme-linked immunosorbent assay; H chain, heavy chain; HEL, hen egg-white lysozyme; HSA, human serum albumin; IFA, incomplete Freund's adjuvant; KLH, keyhole limpet hemocyanin; L chain, light chain; Nase, anti-staphylococcal nuclease system; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RID, radial immunodiffusion; SDS, sodium dodecyl sulfate; TBBD, BBS containing 1% BSA, 0.02% Tween-20, and 1 mg/ml dextran sulfate.

role and extent of idiotype coupling between immune responses to non–cross-reactive antigens. Our observations suggest a linkage between idiotype dominance in an induced anti-p-azobenzenearsonate (ABA) antibody response and the prior occurrence of an idiotypically related response that is not specific for ABA. These experiments were based on a monoclonal antiidiotype antibody AD8 detecting a new CRI family CRI^{AD8} that can be divided into two subpopulations. One subpopulation contains antibodies that are found naturally in the sera of unprimed strain A or related mice, and which do not bind ABA. This subpopulation is designated as the $ABA^- CRI^{AD8+}$ set and can be selectively expanded by immunization with a single low dose of AD8. The second subpopulation of the CRI^{AD8} family, found in strain A mice after immunization with ABA-conjugated proteins, binds ABA, and is fully cross-reactive in the CRI^a assay. This subpopulation is designated as the $ABA^+ CRI^{AD8+}$ set and can be selectively stimulated by immunizations with ABA-keyhole limpet hemocyanin (KLH). Regulatory interactions between these two idiotypic subpopulations are explored.

Materials and Methods

Animals.   Strain C.AL-20 mice were generously supplied by Dr. Claudia Henry, University of California, Berkeley, CA, or bred in our animal colony. Nude mice on a Swiss background were bred and raised in the University of California, San Francisco, Animal Care Facility. All other mouse strains were purchased from The Jackson Laboratory, Bar Harbor, ME. Generally, female mice were purchased at 6 to 8 wk old and were used after acclimatization to our animal colony. Male Lewis rats weighing 300–350 g were purchased from Simonsen Laboratories, Gilroy, CA. Female NZW rabbits were purchased from various local breeders.

Proteins and Antigens.   KLH and human serum albumin (HSA) were purchased from Calbiochem-Behring Corp., San Diego, CA. Bovine serum albumin (BSA) was purchased from Sigma Chemical Co., St. Louis, MO. *Brucella abortus* tube antigen, strain 1119-3, was obtained from the U. S. Department of Agriculture Animal and Plant Health Inspection Service, Ames, IA. Azobenzenearsonate-conjugated proteins were prepared according to Tabachnik and Sobotka (17).

Haptens.   ABA-conjugated amino acids were generously provided by Dr. D. E. Nitecki and Dr. J. W. Goodman.

Antibodies.   A/J anti-arsonate (ABA^+) antibodies, hereafter referred to as serum idiotype, were purified from hyperimmune anti-ABA-KLH ascites fluid (18) by affinity chromatography on columns of ABA-BSA-Sepharose or ABA-Tyraminyl-Affigel 10 (Bio-Rad Laboratories, Richmond, CA). ABA^+ antibody was eluted from the affinity matrix with either 2.5 M NaSCN in borate-buffered saline (BBS) or with 0.1 M p-arsarnilic acid. "Normal" A/J immunoglobulin (Ig) was purified from complete Freund's adjuvant (CFA)-induced ascites fluid by passage through ABA-BSA-Sepharose to remove any possible anti-ABA activity followed by Na_2SO_4 precipitation and exclusion chromatography through Biogel A 1.5 M (Bio-Rad Laboratories). Normal rat IgG and chicken gamma globulin were purified from serum (Gibco Laboratories, Santa Clara, CA) by Na_2SO_4 precipitation and chromatography on Biogel A 1.5 M. Mouse Ig subclasses were separated by differential pH elution from protein A-Sepharose (P-3391; Sigma Chemical Co.) according to Oi and Herzenberg (19). Rat Ig were purified using ion-exchange chromatography (20).

Antisera.   Rabbits were immunized by multiple intramuscular inoculations containing a total of 1 mg of protein emulsified in CFA (Difco Laboratories, Detroit, MI). The rabbits were boosted at multiple subcutaneous sites twice at 2-wk intervals with 1 mg of protein per boost emulsified in incomplete Freund's adjuvant (IFA) (Difco Laboratories). Antisera were collected beginning 1 wk after the second boosting. The rabbits were subsequently boosted once a month and bled at various times thereafter.

Rabbit anti-CRI^a sera were rendered idiotype specific by multiple passages through columns of normal A/J IgG conjugated to Sepharose. Depletion was monitored in an enzyme-linked

immunosorbent assay (ELISA), in which the amount of rabbit antibody in the column effluents reactive with CRI-coated polystyrene tubes (2054; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) was measured with protein A (P-9267) conjugated to alkaline phosphatase (type VII-S; Sigma Chemical Co.). Rabbit antiidiotype antibodies to be coupled to alkaline phosphatase were further purified from idiotype-specific antisera by affinity chromatography on, and chaotropic elution from, a serum idiotype-Sepharose column.

Two sets of antisera specific for mouse isotypes were used. One was generously supplied by Dr. Anne Good, University of California, Berkeley, and the other was purchased from Miles Laboratories, Inc., Research Products Div., Elkhart, IN. Other antisera include goat anti-mouse κ light chain (21-1079; Nordic, London, United Kingdom), rabbit anti-mouse λ light chain (64-367; Miles Laboratories), goat anti-rat IgG (12511; N.L. Cappel Laboratories Inc., Cochranville, PA), and goat anti-rat isotypes (Pel-Freeze Biologicals, Rogers, AR).

Mice were immunized variously as described in the text. CFA-induced and anti-ABA-KLH hyperimmune ascites were prepared as described by Tung et al. (18).

Preparation of Ig Chains and Fragments. Fab fragments were prepared from IgG by digestion with papain (Sigma Chemical Co.) for 5 h at 37°C (21). Fab fragments were purified by gradient elution from DEAE cellulose by using a 2.5–100 mM phosphate gradient at pH 8.0. The fragments were sized by gel filtration on Sephadex G-100 and passed over a protein A Sepharose CL-4B column at pH 8.5 to remove residual Fc fragments and IgG. Analysis by gradient sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and immunoelectrophoresis revealed only Fab fragments. Heavy (H) and light (L) chains from normal A/J IgG and serum idiotype were prepared by reduction with 0.02 M dithiothreitol and alkylation with 0.12 M iodoacetimide in N_2 (22). The H and L chains were separated in 1 M acetic acid by chromatography on Sephadex G-100 at 4°C. Serum idiotype and nIgG controls were similarly processed, but without reduction and alkylation. The separated chains and the Ig were dialysed vs. phosphate-buffered saline (PBS), centrifuged at 15,000 rpm for 30 min, and the protein concentrations of the supernatants were determined from their optical densities. The molecular weights of the various species were determined on gradient SDS-PAGE. The H chains were mixed in all combinations with the L chains so that the final H/L optical density ratio was 2.2:1.0. H-L recombination was promoted by dialysing (*a*) 6 h vs. 0.5 M acetic acid at room temperature, (*b*) 24 h vs. 0.1 M acetic acid, pH 5.5, at 4°C, (*c*) 24 h vs. 0.02 M acetic acid, pH 5.5, at 4°C, and (*d*) 24 h vs. BBS.

Idiotype and Antibody Assays. An ELISA (Engvall and Perlman [23]) was adapted to 96-well polystyrene microtiter plates so that an optical reader (Flow Laboratories, Inc., Irvine, CA) could be used to measure the extent of reaction. Microtest plates (Cooke type R, M220-29; Cooke Engineering Co., Alexandria, VA or Dynatech Immulon II, Dynatech Laboratories, Inc., Alexandria, VA) were coated with 90 μl of the relevant ligand for 5 h at 37°C or overnight at ambient temperature. The ligands, which were dissolved in BBS with 0.04% NaN2, included 10 μg ml^{-1} ABA-HSA, or 1.5–2.5 μg ml^{-1} of (*a*) serum idiotype or Fab fragments of serum idiotype, (*b*) normal A/J IgG or its Fab fragments, or (*c*) hydridoma proteins R16.7 or 36-65. Before use, the wells were filled for a minimum of 30 min at room temperature with TBBD (BBS containing 1% BSA, 0.02% Tween-20 [P-137; Sigma Chemical Co.], and 1 mg/ml dextran sulfate). 95 μl of antibody solution or antibody-enzyme conjugate, which was premixed for 30 min with dilutions of various inhibitors when appropriate, was added in duplicate to the coated plates and allowed to react overnight at ambient temperature. Occasionally, this incubation was shortened to 5 h at 37°C. The wells were then rinsed by washing three times with H_2O, incubating for a minimum of 10 min with BBS-0.02% Tween-20, and washing again three times with H_2O. When an additional developing reagent was required, 100 μl of the appropriate alkaline phosphatase-conjugated anti-Ig was diluted in TBBD, added to the plates, and incubated for 5 h at 37°C. The wells were then rinsed as above, and 100 μl of the substrate *p*-nitrophenyl-phosphate (104-0; Sigma Chemical Co.) at 1 mg ml^{-1} in 0.05 M Na_2CO_3, pH 9.6 was added. The plates were usually incubated at 37°C until the positive control wells had an OD_{405} >0.5. The data were analyzed on a Hewlett-Packard model 85 computer (Hewlett-Packard Co., Palo Alto, CA) using programs generated in our laboratory. Anti-Ig antibodies, including affinity-purified rabbit anti-CRI, goat anti-rat Ig depleted of anti-mouse Ig activity,

5 Ci, and rabbit anti-mouse κ and λ L chains, were conjugated to alkaline phosphatase using the method described by Engvall and Perlmann (23).

The standard inhibition assay for CRI^{AD8} used idiotype-coated plates, 200 ng ml^{-1} AD8 incubated with serial dilutions of sera or hybridoma proteins to be assayed for idiotype, and goat anti-rat Ig alkaline phosphate conjugates. An alternate assay for CRI^{AD8} substituted AD8-biotin for AD8 and was developed with 1:300 dilutions of avidin-alkaline phosphatase (A-2100; Vector Laboratories, Inc. Burlingame, CA). These two assays, when compared with a battery of eight monoclonal proteins, produced identical patterns of reactivity. The antiidiotypic probes in the CRI^a and CRI^{AD8} assays were, respectively, rabbit antiserum idiotype-alkaline phosphatase and 5 Ci-alkaline phosphatase. The idiotype content was determined from the linear regression of the linear portion of the inhibition curves, and is reported as either the serum dilution or the protein concentration giving 50% of the maximum OD_{405} value.

Anti-arsonate antibody was measured by incubating duplicate dilutions of sera on ABA-HSA-coated plates followed by rabbit anti-mouse κ and λ conjugates. The specificity of this reaction was investigated by including a 3-mM concentration of the hapten 4-azo-his in the reaction mix, a concentration that completely inhibited specific interactions. Anti-ABA content was determined by interpolating the reactivity of the experimental dilutions onto the curve of a reference anti-ABA serum with a known anti-arsonate titer. The concentration of anti-arsonate antibodies in our standard antiserum was established using the quantitative precipitation assay and serial dilutions of ABA-BSA. Isotypes were determined by Ouchterlony analysis and relative concentrations of isotypes were assayed using radial immunodiffusion (RID).

Hybridomas. The characteristics of the hybridomas used in this study are outlined in Table I. Clones AD8 and BA6 were derived from male Lewis rats immunized intraperitioneally with 500 μg serum idiotype in 0.2 ml CFA, and boosted 10 d later with 500 μg serum idiotype in IFA. 3 wk later, the rats were boosted on four consecutive days with 300 μg serum idiotype in PBS. These boosts were given intraperitoneally, except for the second boost, which was administered intravenously. The spleen was removed on the day following the last boost, and the spleen cells were hybridized with the Y3-Ag 1.2.3 rat myeloma cell line (24) using the protocol of Oi and Herzenberg (19), except that the cells were initially plated at 5×10^5 total cells per well in Microtest II plates (Dynatech Laboratories, Inc.). Supernatants were characterized using ELISA. Rat hybridomas were expanded in vitro before intraperitoneal transfer into nude mice that had been primed with 0.5 ml of pristane on two occasions 7 d apart and at least 7 d before tumor transfer.

Mouse hybridomas were produced by fusing cells from immune A/J mice with the NS1 or SP2 cell lines using either the method of Oi and Herzenberg (19) or of de St. Groth and Scheidegger (25). Hybridomas were grown either as ascites in pristane-primed CAF_1 mice or in

TABLE I

Hybridoma Characteristics

Cell line*	Myeloma parent	×	Fusion partner	Immunogen	Antigen specificity	Idi-otype‡	Isotype
AD8	Y3		Lewis rat	Serum idiotype	CRI^{AD8}	−	IgG_{2b}
BA6	Y3		Lewis rat	Serum idiotype	Mouse IGFc	−	IgM
5Ci	NS1		CD rat	Serum idiotype	CRI^{5Ci}	−	IgG_{2a}
5H5	SP2		A/J mouse	AD8	?	+	IgG_{2a}
3D10	SP2		A/J mouse	5Ci	?	+	IgG_1
31C3	NS1		A/J mouse	ABA-KLH	ABA	−	IgG_1
36-65	SP2		A/J mouse	ABA-KLH	ABA	+	IgG_1
R-16.7	SP2		A/J mouse	ABA-KLH	ABA	+	IgG_1
AB2-37.2	SP2		A/J mouse	ABA-Brucella	ABA	+	IgG_1

* 5Ci and 3D10 were gifts from L. Wysocki; 36-65, from A. Marshak-Rothstein; and R-16.7, from E. Lamoyi.

‡ Hybridoma proteins that contain any of the CRI associated with the strain-A anti-arsonate response, including CRI^a, CRI^{AD8}, and CRI^{5Ci}. See Fig. 2 and Results.

vitro using standard conditions. Arsonate-specific antibodies were isolated using affinity chromatography on ABA-tyraminyl-Sepharose. 5H5 was grown in serum-free medium (50% F-12, 50% RPMI, 10 μg ml^{-1} transferrin, 10 μg ml^{-1} human insulin, 2 mM glutamine, and 5 $\times$ 10^{-5} M 2-mercaptoethanol), and was isolated on protein A-Sepharose columns. Proteins and hybridomas generously supplied by other investigators include 5 Ci and 3D10 (L. Wysocki, Harvard University, Cambridge, MA [7]), 36-65 (Ann Marshak-Rothstein, Massachusetts Institute of Technology, Cambridge, MA [26]), and R16.7 (E. Lamoyi, Brandeis University, Waltham, MA [27]).

Results

Summary of Preliminary Observations. A number of rat $\times$ rat hybridomas were produced that reacted with serum idiotype. Of this number, the antibody from hybridoma AD8 was found to be exceptionally reactive with monovalent Fab fragments of serum idiotype. In the studies that follow, AD8 and 5Ci refer to the monoclonal antiidiotypic hybridoma antibodies, CRI^{AD8} and CRI^{5Ci} refer to the antibodies containing the cognate idiotopes, and CRI^a collectively refers to idiotopes recognized by our affinity-purified rabbit antiidiotypic probe. In the initial screen, it was found that 10 μg/ml of serum idiotype or its Fab fragments completely inhibited the binding of AD8 to serum idiotype Fab-coated plates, whereas similar concentrations of Fab fragments from normal IgG failed to inhibit. Fortuitously, this concentration of normal Fab fragments was just below that which reveals endogenous CRI^{AD8}. For this reason, we initially failed to detect CRI^{AD8} in normal Ig. The presence of ~9 μg/ml CRI^{AD8} in normal sera and CFA-induced ascites will be documented below. Further preliminary studies showed that the interaction between AD8 and serum idiotype Fab-coated plates could not be inhibited by the CRI$^-$ ABA$^+$ hybridoma protein from clone 31C3. Additionally, it was shown that several different rabbit anti-CRI^a antisera would compete with AD8 for binding to serum idiotype. To determine if CRI^{AD8} is enriched in antibodies specific for ABA, A/J antisera to ABA-KLH and ABA-CGG were extensively depleted of anti-arsonate activity by passage through ABA-BSA-Sepharose columns. The CRI^{AD8} titers of these ABA-depleted sera were reduced ~200-fold, indicating that most of the CRI^{AD8} moieties in ABA-immune sera co-purify with the anti-arsonate activity. Immunochemical and electrophoretic analyses showed that AD8 was a rat IgG$_{2b}$ protein. Because these preliminary studies demonstrated that AD8 was indeed antiidiotypic and seemed to have a high affinity for its cognate idiotope, we selected this protein for further study.

Strain Distribution of CRI^{AD8} is Similar to that of CRI^a. V$_H$ loci that are linked to the Igh-C^e haplotype, or to the related Igh-C^o haplotype, encode for a cross-reactive idiotype (CRI^a, above) that is expressed on a substantial fraction of anti-arsonate antibodies (28). Additionally, most idiotopes of this major cross-reactive idiotype can be found on single hybridoma proteins (26, 27, 29).

In light of the above findings, expression of CRI^{AD8} was examined during anti-ABA-KLH responses in nine strains of mice. As shown in Table II, only mice possessing A-strain V$_H$ genes produced CRI^{AD8} during this response. In contrast, no CRI^{AD8} was found in mice lacking A-strain V$_H$ genes. Regardless of their ability to make CRI^{AD8}, all strains of mice produced similar amounts of anti-arsonate antibodies. These results show that the strain distribution of CRI^{AD8} closely parallels that of CRI^a (28).

Binding of AD8 to CRI^{AD8} is Hapten Inhibitable and H Chain Determined. The relation-

TABLE II

Idiotype and Anti-Arsonate Antibody Content in Antisera to ABA-KLH from Nine Strains of Mice[*]

Strain	Igh-C type	n	CRI^{AD8},[‡] 50% Dilution	Anti-ABA,[§] μg equivalent ml^{-1}
C.AL/20	o	7	519 (1.89)	97 (2.07)
CAF1	a/e	6	1,820 (1.48)	337 (2.10)
A/J	e	8	2,677 (2.39)	367 (2.92)
DBA/2	c	6	None	ND
BALB/C	a	6	None	480
CBA/J	j	6	None	740
C57Bl/6	b	8	None	220
NZB	n	8	None	590
C3H/He	j	6	None	440

[*] Mice were primed with 150 μg ABA-KLH CFA intraperitoneally, and boosted 3 wk later with 150 μg ABA-KLH/IFA i.p. Antisera were collected 11 d after boosting.

[‡] CRI^{AD8} levels determined using a standard ELISA, in which the binding of AD8 (200 ng ml^{-1}) to microtiter plates coated with 1.5 μg ml^{-1} of serum CRI was inhibited by duplicate serial dilutions of each antiserum, and the amount of bound AD8 was measured using goat anti-rat Ig enzyme conjugates. The level of idiotype is reported as the serum dilution that inhibits 50% of the maximum OD_{405} value. No CRI^{AD8} was observed in non-A strain mice. Results are expressed as geometrical means, with geometrical deviations in parentheses.

[§] Antibody equivalents of anti-arsonate Ig were determined with a standard ELISA in which microtiter plates were coated with 10 μg ABA-HSA ml^{-1}, incubated with 1:500 antisera dilutions, and developed using anti-mouse κ and λ L-chain enzyme conjugates. Antibody equivalents were calculated by comparison with the binding curve of a reference anti-ABA-KLH serum pool of known anti-ABA titer. Values followed by parentheses are geometrical means; geometrical deviations are in parentheses. All other values are from pooled antisera. ND, not determined.

ship of CRI^{AD8} to the antigen-binding site was investigated by using ABA haptens to compete the interaction between AD8 and idiotype-coated plates. As a specificity control, the Fc-specific hybridoma protein BA6 was similarly assayed. In a preliminary study, neither AD8 nor BA6 were inhibited by concentrations of phenyl-glu-tyr-ABA $<5 \times 10^{-3}$ M. Although these results suggested that the CRI^{AD8} idiotope is not within the arsonate-binding site, subsequent studies using hybridoma protein R16.7 instead of serum idiotype have shown that the CRI^{AD8} determinant is indeed hapten inhibitable (Table III). Microtiter plates were coated with serum idiotype or hybridoma proteins R16.7 or 36-65, and the binding of AD8 to these plates was inhibited by dilutions of various ABA haptens. The results using R16.7-coated plates show that binding was inhibited by all haptens tested. ABA-tyrosine inhibited most efficiently (3.9 $\times$ 10^{-6} M at 50% inhibition) whereas ABA-phenyl-propionic acid was the least efficient inhibitor (7.4 $\times$ 10^{-4} M at 50% inhibition). However, when the inhibition of binding of AD8 to 36-65 or serum idiotype was assayed, inhibition was observed only at very high hapten concentrations ($\sim 10^{-3}$ M). At least two factors might explain this inconsistency: (*a*) The affinity of AD8 for 36-65 may be higher than for R16.7. Results presented in Figure 2 B suggest that, indeed, the affinity of AD8 for 36-65 is $\sim$100-fold higher than for R16.7 (50% inhibition of AD8 binding is 0.11 μg ml^{-1} for 36-65 and 11 μg ml^{-1} for R16.7). (*b*) The affinity of R16.7 for ABA may be higher than that

TABLE III

Hapten Inhibition of AD8 Binding to Different CRI Proteins *

Hapten	Plate coated with		
	R 16.7	36-65	Serum CRI
ABA-tyrosine	3.9×10^{-6}	8.4×10^{-4}	$>2.5 \times 10^{-3}$
4-azo-A-his	1.0×10^{-5}	$>1.3 \times 10^{-3}$	$>1.3 \times 10^{-3}$
benzyl-glu-tyr-ABA	2.2×10^{-5}	$>4 \times 10^{-3}$	$>4 \times 10^{-3}$
ABA-phenyl-proprionic acid	7.4×10^{-4}	$>2.5 \times 10^{-2}$	$>2.5 \times 10^{-2}$

* Plates were coated with 2 μg ml^{-1} of idiotypic protein in BBS, and were assayed
using ELISA as in Table II, with the following modification: AD8 reactivity was
measured using AD8-biotin, followed by avidin-alkaline phosphatase. The values
are molar concentrations of hapten that inhibit 50% of the idiotype-binding activity.

of 36-65. Recent experiments indicate that R16.7 has a 4.4-fold higher affinity for ABA-tyrosine than does 36-65 (K_a of R16.7 = 1.1 × 10^6 LM^{-1} [30]; K_a of 36-65 = 2.5 × 10^5 LM^{-1} [T. Rothstein, personal communication]).

Genetic studies have shown that, although CRI^a is coded for by an A strain V$_H$ locus, optimal CRI^a expression requires the appropriate L chain (31). Although data presented in Table I on the strain distribution of CRI^{AD8} are consistent with the involvement of the H chain variable region in the expression of CRI^{AD8}, they do not speak to the role of the L chain in CRI^{AD8} expression. The relative roles of both H and L chains in the formation of the idiotope CRI^{AD8} was investigated by separating H and L chains from both serum idiotype and normal A/J IgG. Each H chain preparation was recombined with one or the other L chains. These recombinant mixtures and the separated chains were assayed in the standard competition ELISA using AD8 and serum idiotype Fab-coated plates. Because our chain-separation protocol does not yield pure H chains, the role of the L chain in CRI^{AD8} expression must be inferred by comparing the relative effectiveness of the idiotype L chain vs. the normal L chain in restoring the idiotype to a CRI^{AD8} H chain preparation.

This chain-recombination experiment revealed that the relative amounts of protein necessary to restore equivalent idiotypic antigenicity are: idiotype H and idiotype L, 1.0; idiotype H and normal L, 2.4; and idiotype H alone, 3.4. The idiotype L chain alone or combined with the normal H chain failed to inhibit at concentrations up to 20 μg ml^{-1}. Endogenous CRI^{AD8} occurs at 1 part in 1,000 in normal Ig and was below the range needed to influence our chain-recombination experiments. This experiment suggests that, although the AD8 idiotope is a an H chain-associated structure, the correct L chain is required for optimal expression. These findings are similar to the genetic and sequence studies in the CRI^a system (31, 32).

CRI^{AD8} is Found in Normal Serum.   In a series of control experiments we found that high concentrations of normal A/J IgG inhibited in our CRI^{AD8} assays, suggesting that CRI^{AD8} might also be found in normal A/J serum. To test this hypothesis, sera from 11 nonimmunized A/J mice were analyzed individually for CRI^{AD8} and for anti-ABA activity. These values are compared with those of an ascites pool from A/J mice hyperimmune to ABA-KLH in Table IV. The average CRI^{AD8} content in normal serum was estimated to be 9.4 μg ml^{-1} (range, 2.7–16.3 μg ml^{-1}). By contrast, the hyperimmune ascites pool contained 2,400 μg ml^{-1} of the CRI^{AD8+} molecules. The CRI^{AD8} in normal serum is probably not associated with an ABA-binding Ig because

TABLE IV

Idiotype and Anti-Arsonate Content of Normal A/J Sera Compared with an Ascites Pool from A/J Mice Hyperimmune to ABA-KLH *

	n	Antibody equivalents, $\mu g\ ml^{-1}$	
		CRI^{AD8}‡	Anti-ABA§
Normal sera	11	9.4 (1.83)	0 ($P < 0.1370$)
Hyperimmune ascites	—	2,400	9,700

* Hyperimmune ascites prepared as in (18).

‡ Determinations for normal sera as in Table II. Antibody equivalents were estimated by comparison of the inhibition curves of each normal serum with the inhibition curve of the hyperimmune pool. CRI^{AD8} content of the hyperimmune pool was determined by measuring the amount of antibody that was affinity purified on an AD8-Sepharose column. Normal sera value is expressed as the geometrical mean, followed parenthetically by geometrical deviation.

§ Anti-ABA values of normal sera were determined using ELISA in which sera were diluted 1:20, either with or without 3 mM 4-azo-his, incubated 3 h at 21°C, added to the plates, and processed in the standard fashion (Table II). Control anti-ABA serum dilutions were completely inhibited by this procedure, but no specific binding of antibodies from normal sera to ABA was observed ($P < 0.1370$). The anti-ABA content of the hyperimmune pool was determined in a quantitative precipitin assay.

we failed to detect anti-ABA Ig in the serum with an assay sensitive to 10–100 ng Ig ml^{-1} and also because the CRI^{AD8} in CFA-induced ascites is not removed by passage through an ABA-affinity column. This result is similar to that of Wysocki and Sato (7) for the CRI^a system and to that of Dzierzak and Janeway (8) for the MOPC-460 system.

IgG Isotypes Associated with CRI^{AD8} on Arsonate-binding Serum Antibodies Differ from Those on Arsonate-nonbinding Serum Antibodies. Initial studies of isotype distribution of ABA^+ CRI^a serum antibodies isolated from ABA-KLH ascites indicated that most of the idiotype was found on the IgG_1 (33) subclass. Subsequent investigations using both hybridoma technology (26, 27) and conventional immunization schemes (34) have shown that CRI^a can be expressed on most subclasses. Because the isotype of a given antibody population may reveal clues about both the biological function of that population and the nature of its cognate antigen (i.e., TD, TI_1, TI_2 [35]), we determined the isotypes of ABA^- CRI^{AD8+} antibodies found in normal sera and compared them with the isotypic profile of anti-arsonate antibodies isolated from hyperimmune anti-ABA-KLH ascites.

The experiment shown in Table V was designed to reveal IgG subclass restrictions of CRI^{AD8} in anti-arsonate IgG and in normal IgG. ABA^+ IgG was affinity purified on ABA-tyramine-Sepharose-4b from ascites induced by ABA-KLH in CFA, whereas normal IgG was purified from ascites induced by CFA alone. This choice was dictated by the need for a CFA control to compare with ABA-KLH/CFA-induced ascites and by the need for relatively large volumes of normal A/J serum to do the experiment. Additional experiments have shown that CRI^{AD8} levels in normal sera and CFA-induced ascites fluid are indistinguishable. Nevertheless, we emphasize that we cannot presently rule out the possibility that CFA priming during ascites production alters the subclass distribution of CRI^{AD8}. Although no anti-ABA activity was found in either CFA ascites or normal sera, the CFA ascites was extensively absorbed on an

TABLE V

Isotype Associations of CRI^{AD8} in A/J Anti-Arsonate Antibodies Compared with Normal ABA^- A/J Antibodies

Elution* pH	IgG isotypes‡				Anti-ABA Ig§			Normal Ig‖		
	1	2a	2b	3	IgG	CRI^{AD8}**	CRI^{AD8}/IgG	IgG¶	CRI^{AD8}**	CRI^{AD8}/IgG
					%	%		%	%	
8.6	1.4	0.0	0.1	0.0	40	39	1.0	37	<8	<0.2
5.5	0.2	2.0	0.6	0.2	43	44	1.0	41	51	1.2
4.5	0.0	1.0	0.6	4.2	10	12	1.2	18	41	2.3
Total percent					93	95		96	100	
Percent of total IgG containing CRI^{AD8}						24			0.1	

* Elution pH from *Staphylococcus aureus* protein A-Sepharose columns.

‡ Relative enrichment of four IgG isotypes eluted from protein A columns. Isotype concentrations were measured using RID and two sets of subclass-specific antisera from different sources. Relative enrichment at each pH is expressed as the ratio of the amount of isotype in each fraction of IgG over the amount of isotype in unfractionated normal A/J IgG.

§ Anti-arsonate Ig was affinity purified from pooled ascites fluid from A/J mice hyperimmune to ABA-KLH on an ABA-tyraminyl-affigel column and chaotropically eluted.

‖ Normal A/J IgG was isolated from pooled ascites fluid induced by multiple intraperitoneal injections of 0.3 ml CFA/PBS (9:1) emulsion. Ascites fluid was run through an ABA-BSA column before isolation of the IgG by Na_2SO_4 precipitation, DEAE fractionation, and gel chromatography.

¶ Percent of total IgG that was purified at each pH. Protein content was determined using OD_{280} and $\epsilon_{280\,nM}^{1\,mg/ml} = 1.4$.

** Percent of total CRI^{AD8} that was purified at each pH. CRI^{AD8} content was determined using ELISA as in Table II.

ABA-BSA-Sepharose to remove any possible anti-arsonate antibody before IgG purification. No anti-ABA antibody could be eluted. The two IgG preparations were each fractionated into subclasses by differential pH elution from staplylococcus protein A-Sepharose. The efficiency of the protein A fractionation step was monitored by RID by using two sets of subclass-specific antisera from different sources. Significant amounts of antibody were eluted at three pH values: 8.6, 5.5, and 4.5. These three fractions accounted for 93% of the total anti-ABA antibody adsorbed to the column and for 96% of the normal Ig. Insignificant amounts of protein desorbed at pH 7.0 (data not shown). Neither IgM nor IgA detectable by RID was found in any of the three fractions. The values of the different subclasses relative to the values of normal IgG before fractionation are shown for each elution pH. The pH 8.6 fraction was enriched 1.4-fold for IgG_1 and depleted 10-fold of IgG_{2b}, whereas neither IgG_{2a} nor IgG_3 was detected in this pH fraction. The pH 5.5 fraction was enriched twofold for IgG_{2a}, contained significant levels of IgG_{2b} (0.6 × normal levels), and was depleted fivefold of IgG_1 and IgG_3. The pH 4.5 fraction was enriched fourfold for IgG_3, contained significant levels of IgG_{2a} and IgG_{2b} (1.0 and 0.6, respectively) and was virtually depleted of IgG_1. Although the subclass fractionation was not absolute, it was sufficient to differentiate IgG_1 from IgG_2 and IgG_3.

The percentage of total IgG purified at each pH for both Ig preparations was calculated and compared with the percentage of idiotype contained in the same pH

fractions. A striking difference was observed in the isotype association of CRI^{AD8} in ABA^{+} IgG compared with CRI^{AD8} in ABA^{-} IgG. For anti-ABA IgG, CRI^{AD8} was uniformly distributed across all three pH fractions (CRI^{AD8}/IgG ratios from 1.0 to 1.2). However, a quite different pattern was observed in the ABA^{-} IgG: the IgG_1-rich (pH 8.6) fraction was depleted 80% of CRI^{AD8}, whereas the IgG_3-IgG_{2a}-rich (pH 4.5) fraction was enriched 230% for CRI^{AD8}. This suggests that the CRI^{AD8} found in normal IgG is preferentially associated with the IgG_3 and IgG_2 subclasses. This subclass distribution is similar to that seen in TI-1 antibody responses (35). On the other hand, after immunization with ABA-KLH, a strong thymus-dependent antigen, CRI^{AD8}, is expressed as frequently on IgG_1 as on the other subclasses. The bottom row of Table V lists the percentage of total IgG that contains CRI^{AD8}, and shows that CRI^{AD8} is enriched >200-fold in the ABA-specific antibody preparation compared with the normal Ig preparation.

CRI^{AD8} is Found on at Least 80% of the Molecules Expressing CRI^{α} in ABA-KLH-induced Ascites. Because the expression of CRI^{AD8} closely parallels that of CRI^{a} (Table II) (28), it was important to determine the degree of association between the two idiotypes in ABA-KLH hyperimmune ascites. For this experiment, hyperimmune anti-ABA-KLH ascites fluid was absorbed with either AD8-Sepharose 4B or normal rat Ig-Sepharose 4B. After absorption, the amounts of CRI^{AD8} and CRI^{a} remaining in the filtrate were determined. The results of this experiment are shown in Fig. 1. Absorption on AD8-Sepharose removed >99% (Fig. 1 A) of the CRI^{AD8} and ~80% (Fig. 1 B) of the CRI^{a} from the hyperimmune anti-ABA-KLH ascites. On the other hand, the control normal rat Ig immunoadsorbant did not significantly remove any CRI^{AD8} from the preparation. Additional assays (not shown) indicated that >99% of CRI^{5Ci} was removed by absorption on AD8-Sepharose. This experiment shows that, in an idiotype-rich anti-ABA-KLH ascites fluid, CRI^{AD8} and CRI^{a} are expressed on the same molecules at least 80% of the time. Under these conditions, CRI^{5Ci} is practically always coexpressed on molecules containing the CRI^{AD8} marker.
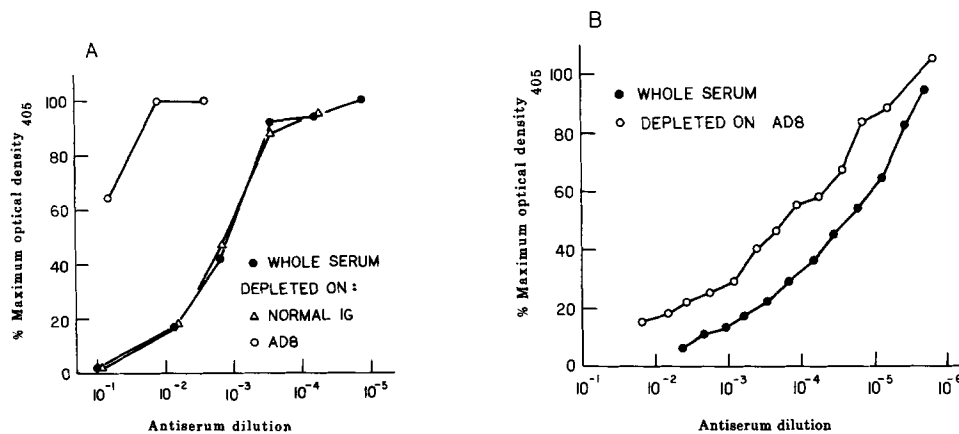

FIG. 1. Anti-ABA-KLH ascites fluid from A/J mice, passed through an AD8 immunoabsorbent, was analysed for CRI^{AD8} content (A) and for CRI^{a} content (B). Whole ascites fluid (●); passed through AD8-Sepharose (○); and passed through normal rat IgG-Sepharose (△). CRI^{AD8} levels were measured with an ELISA in which the inhibition of the binding of AD8 to serum CRI-coated plates was measured with a goat anti-rat IgG enzyme probe; CRI^{a} was measured by inhibiting the binding of rabbit anti-CRI-alkaline phosphatase conjugates.

CRI^{AD8} *Can Be Expressed on A/J Hybridoma Proteins Independently of Either* CRI^a *or Anti-ABA Reactivity.* The apparent expression of CRI^{AD8} on ABA^- normal mouse Ig contrasts with the finding that most of the CRI^{AD8} in ABA-immune sera is associated with arsonate-binding molecules (see Summary of Preliminary Observations, above). These results suggest that CRI^{AD8} is a new major CRI related to the CRI^a family and, paradoxically, that the expression of this idiotope is not diagnostic for anti-ABA activity.

To further characterize the relationships between CRI^{AD8}, CRI^a, CRI^{5Ci}, and anti-ABA activity, we compared four CRI^{a+} hybridoma proteins (Fig. 2). We modified the ELISA for idiotype by coating microtiter plates with hybridoma protein R16.7 instead of serum idiotype, and by inhibiting two additional antiidiotype preparations, rabbit anti-CRI^a and 5Ci. Two of the proteins studied, 36-65 (26) and R16.7 (27), are prototypic ABA^+ CRI^{a+} Ig that contain most, if not all, of the idiotypic determinants expressed on A/J CRI^{a+} antibodies. When compared with one another using the rabbit anti-CRI^a probe (Fig. 2A), both 36-65 and R16.7 were excellent inhibitors. On the other hand, the ABA^- proteins 5H5 and 3D10 were 100- and 200-fold less effective
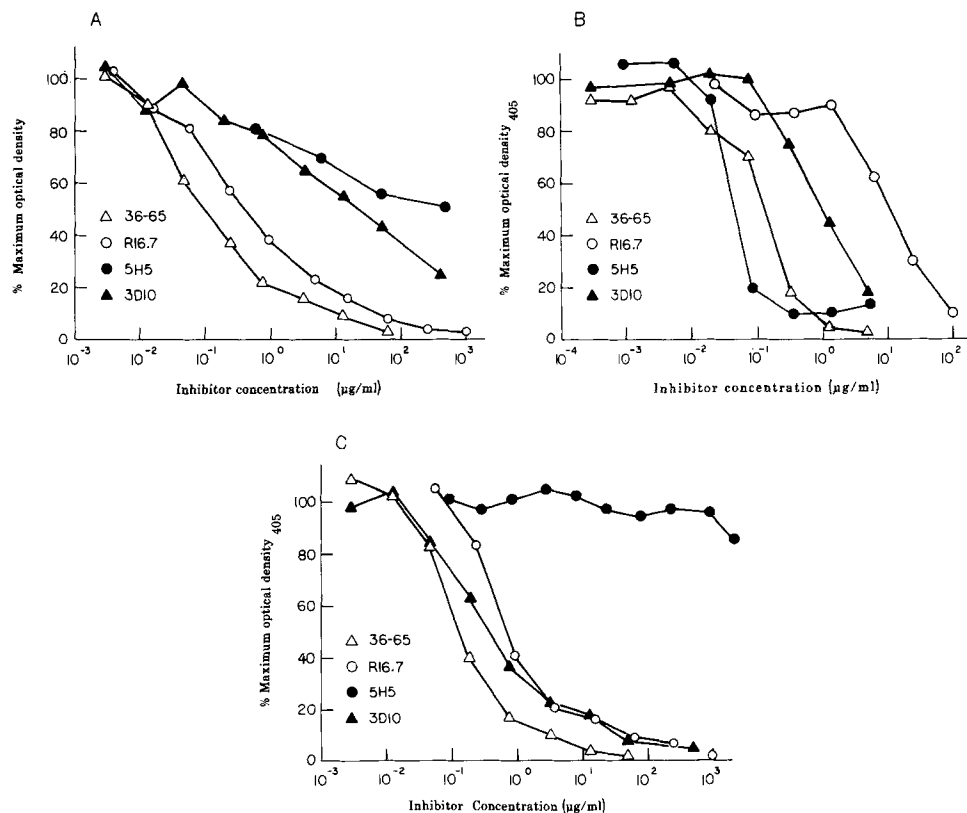

FIG. 2. Comparison of the idiotypic composition of four different CRI^+ hybridoma proteins using three different antiidiotypic probes: rabbit anti-CRI^a (A), AD8 (B), and 5Ci (C). Microtiter plates were coated with 2 µg protein R16.7 ml^{-1}, constant concentrations of the antiidiotypic reagents were inhibited by dilutions of purified hybridoma proteins, and the amount of antiidiotype bound to the plates was determined using ELISA. The inhibitors include proteins 36-65 (△), R16.7 (○), 5H5 (●), and 3D10 (▲).

than 36-65 in inhibiting rabbit anti-CRI^a, respectively. This same order of reactivity was observed when serum idiotype rather than R16.7 was used on the solid phase.

When the four proteins were compared for CRI^{AD8} expression (Fig. 2 B), quite a different pattern emerged. The ABA$^-$ protein 5H5, which was one of the least effective inhibitors of rabbit anti-CRI^a, was the most effective inhibitor of AD8. Surprisingly, R16.7 was 85-fold less effective an inhibitor than 36-65, suggesting that the AD8 idiotope is less fully expressed on R16.7 than on 36-65 or 5H5. These two sets of observations demonstrate that CRI^{AD8} expression can occur independently of the expression of either CRI^a or arsonate binding. Complete dissociation of CRI^a from CRI^{AD8} was seen with protein AB2-37.2, which strongly expresses CRI^a but which lacks CRI^{AD8} (data not shown). The independence of arsonate binding and CRI^{AD8} is further underscored by the ABA$^-$ protein 3D10, which expresses CRI^{AD8} with an antigenicity intermediate in effectiveness between proteins 36-65 and R16.7. Thus, CRI^{AD8}, like CRI^a (7), can be expressed on ABA$^-$ antibodies. Furthermore, all ABA$^-$, idiotype-positive proteins that we have examined seem to express CRI^{AD8} more effectively than they do CRI^a.

Because the relative inhibition profiles of these four hybridoma proteins varied widely between the rabbit anti-CRI^a and the AD8 probes (Figs. 2 A and B), we repeated the experiment using protein 5Ci, another monoclonal antiidiotype derived by Wysocki and Sato (Fig. 2 C) (7). The ABA$^-$ protein 3D10 as well as two prototypic proteins, 36-65 and R16.7, expressed CRI^{5Ci} very efficiently, whereas protein 5H5 completely lacks this idiotope.

Selective Stimulation of the ABA$^-$ Subpopulation of CRI^{AD8} Correlates with Limited Responsiveness of the ABA$^+$ Subpopulation of CRI^{AD8} to ABA-KLH. In the previous sections, it was shown that although CRI^{AD8} expression could be dissociated from the expression of CRI^a, CRI^{5Ci}, and ABA binding, immunization with ABA-KLH regularly elicits antibodies having all of these markers. In this section, we will show that it is possible to selectively stimulate the ABA$^-$ CRI^{AD8+} set and that such stimulation correlates with limited responsiveness of the ABA$^+$ CRI^{AD8+} set to ABA-KLH.

Selective immunization of the ABA$^-$ subpopulation of CRI^{AD8} is shown in Table VI. On the basis of preliminary dose-response experiments using both normal rat IgG and AD8 in the range of 1 ng to 100 μg per mouse, a dose of 40 μg per mouse was selected for this experiment. This dose was chosen because it stimulated the production of ABA$^-$ CRI^{AD8+} antibodies while causing only a marginal increase in anti-rat IgG antibodies. Artifactual inhibition of the CRI^{AD8} assay by mouse anti-rat IgG antibodies was prevented by modifying the ELISA as follows: antiserum dilutions from AD8- or normal rat IgG-immunized animals were mixed with 100 μg ml^{-1} normal rat IgG and incubated 20 min at 37°C; an equal volume of this mixture was added to a solution of 200 ng/ml AD8 and incubated for 30 min; this solution was added to idiotype-coated plates and assayed for the inhibition of AD8 binding as described in Materials and Methods. In control experiments, this modification removed nonspecific inhibition of the CRI^{AD8} assay produced by a standard mouse anti-rat IgG antiserum but did not reduce the apparent CRI^{AD8} content of antisera from mice immunized with AD8. Specificity of this system for CRI^{AD8} was further established in a large series of experiments (c.f., Tables VII and VIII) in which normal rat IgG was used as an immunization control for AD8. In none of these experiments did normal rat IgG

TABLE VI

Immunization with Antiidiotope AD8 Compared with Immunization with ABA-KLH: Differential Effects upon the Induction of Idiotype and Anti-Arsonate Antibodies in A/J Sera

Group	Treatment*	n	CRI^{AD8},‡ 50% Dilution	CRI^a,§ 50% Dilution	CRI^{5Ci},‖ 50% Dilution	Anti-ABA,¶ μg equivalent ml^{-1}
A	—	5	132 (1.30)	37 (1.31)	<10	0
B	40 μg AD8	5	740 (1.26)	51 (1.81)	<10	0
C	40 μg AD8, 3 ×	5	616 (1.66)	89 (1.49)	<10	2.3 (1.44)
D	40 μg ABA-KLH, 3 ×	5	616 (1.44)	776 (2.24)	47 (1.42)	25.1 (1.87)

* Immunogen injected intraperitoneally in PBS. Groups C and D received three doses at 3-d intervals. Sera were collected on day 7 after last immunization.

‡ Determined as in Table II, except that plates were coated with R16.7 instead of serum idiotype. The antiidiotypic nature of the response was confirmed by including 100 μg ml^{-1} of normal rat IgG in the incubation mix to inhibit nonidiotypic interactions.

§ Determined by ELISA, in which microtiter plates were coated with R16.7 and the antiidiotypic probe was rabbit antiidiotype alkaline phosphatase conjugates. CRI^a levels in group B were not significantly elevated when compared with group A ($P < 0.1501$), whereas both group C and D were significantly elevated ($P < 0.0018$ and $P < 0.0001$, respectively). Geometrical mean is followed by geometrical deviation.

‖ Determined by ELISA, in which plates were coated with R16.7 and probed with 5Ci-alkaline phosphatase conjugates. Only group D showed a significant increase ($P < 0.0238$) in CRI^{5Ci} titer.

¶ Determined using ELISA as in Table II. Geometrical mean is followed parenthetically by geometrical deviation.

TABLE VII

Comparison of the Idiotype Levels and Anti-ABA Activity in A/J Antisera to ABA-KLH in Mice Pretreated with AD8 or Normal Rat Ig

Group	n	Pretreatment	Antiserum dilution at 50% inhibition‡			Anti-ABA,§ μg equivalent ml
			CRI^{AD8}	CRI^{Rbt}	CRI^{5Ci}	
A	5	20 μg normal rat Ig	13,600 (2.60)	102,000 (1.65)	81,300 (11.4)	135 (2.93)
B	5	20 μg AD8	2,300 (4.02)	15,800 (4.29)	832 (2.65)	228 (8.42)
		t test, B vs. A	$P < 0.033$	$P < 0.023$	$P < 0.006$	$P < 0.321$

* Mice were pretreated with 20 μg of AD8 or nRatIg in PBS by intraperitoneal injection. 7 d later they were primed with 150 μg ABA-KLH/CFA i.p. and 3 wk later were boosted with 150 μg ABA-KLH/IFA i.p. Antisera were collected 7 d after boosting.

‡ Antiserum dilutions that inhibit 50% of the binding of three different antiidiotypes in ELISA. Microtiter plates were coated with 2 μg R16.7 ml^{-1}. Constant concentrations of antiidiotypic reagents (AD8, rabbit antiserum idiotype-alkaline phosphatase, or 5Ci-alkaline phosphatase) were inhibited by serial dilutions of n different antisera, and the amount of antiidiotype bound to the plates was determined using ELISA.

§ Determined using ELISA as in Table II.

elicit an apparent increase in CRI^{AD8}. For this reason, normal rat IgG immunization controls were omitted from some experiments (c.f., Table VI).

In the experiment shown in Table VI, a single 40-μg dose of AD8 stimulated a fivefold increase in CRI^{AD8} titer compared with nonimmunized mice (Table VI, group B vs. group A) without any apparent increase in CRI^a, CRI^{5Ci}, or anti-ABA antibody activity. This result suggests that giving a single 40-μg dose of AD8 selectively stimulates the $ABA^- CRI^{AD8+}$ set. Repeated inoculations of AD8 were required to increase the CRI^a and anti-ABA titers (Table VI, group C); however, in this group

TABLE VIII

*Dose-Response Correlation of the Appearance of CRI^{AD8} after
AD8 Treatment with the Appearance of Idiotype Suppression Induced by AD8
followed by ABA-KLH**

| Group | n | Pretreatment | CRI^{AD8}, 50% dilution‡ | |
			Before ABA-KLH§	After ABA-KLH‖
A	6	1.1 µg AD8	179 (1.69)	21,460 (1.60)
B	6	3.3 µg AD8	87 (1.53)	13,022 (1.68)
C	6	10 µg AD8	182 (2.00)	9,651 (2.58)
D	6	20 µg AD8	993 (1.54)	3,685 (2.51)
E	6	20 µg nRatIg	100 (1.65)	16,482 (1.74)
F	6	—	ND	14,981 (1.72)

* Same protocol as in Table VII, except that the IgG pretreatment doses are varied.

‡ Determined using ELISA as in Table II, except plates were coated with hybridoma protein R16.7 instead of serum idiotype. Values expressed as geometrical means followed in parentheses by geometrical deviations.

§ Sera from animals that were bled 7 d after pretreatment. Students' t test between the nRatIg control group and the AD8 experimental groups indicated that a significant increase in CRI^{AD8} content ($P < 0.0001$) occurred only in the mice that received 20 µg AD8.

‖ Sera from animals that were immunized with 150 µg ABA-KLH/CFA 7 d after pretreatment, boosted with 150 µg ABA-KLH/IFA 3 wk later, and bled 7 d after boost. Significant suppression in the AD8-treated groups relative to the nRatIg control group appeared only in the group pretreated with 20 µg AD8 ($P < 0.0033$).

the titers of CRI^{AD8} were not increased relative to those found for mice that received a single 40-µg dose of AD8. Immunization with ABA-KLH in PBS caused significant increases in CRI^{5Ci} as well as in CRI^a, CRI^{AD8}, and anti-ABA activity. To further document the selectivity of low doses of AD8 for stimulating the ABA^- CRI^{AD8+} set, hybridomas were made from mice immunized with 40 µg of AD8. None of the hybridomas were ABA^+ or CRI^{5Ci+}. However, all of the CRI^{AD8+} hybridomas were cross-reactive in the CRI^a assay (Fig. 2). Like the ABA^- CRI^{AD8+} antibodies found in normal sera, the ABA^- CRI^{AD8+} hybridomas were either of the IgG_2 or IgG_3 subclasses. The ABA^- CRI^{AD8+} hybridomas 2E2 and 5H5 cross-reacted in the CRI^a assay, giving relatively shallow inhibition curves when compared with the prototypic CRI^{a+} hybridomas 36-65 and R16.7 (Fig. 2, and unpublished observations). Because both serum and hybridoma ABA^- CRI^{AD8+} antibodies give shallow inhibition curves in the CRI^a assay, it is difficult to detect less than threefold changes in their concentrations. It is possible that this is the reason we failed to detect a significant increase in CRI^a when 40 µg of AD8 was used as a stimulus. In all of our experiments, giving 20 µg or more of AD8 has resulted in a 5-10-fold increase in CRI^{AD8} concentrations.

Together, the data from immunizations with AD8 contrast with the responses to ABA-protein conjugates. By using a single low dose of AD8, it is possible to selectively stimulate the ABA^- CRI^{AD8+} set. This subpopulation is also CRI^{5Ci-} and is possibly cross-reactive in the CRI^a assay. By contrast, immunization with ABA-KLH selectively stimulates the ABA^+ CRI^{AD8+} set (Table VI and below) which is CRI^{5Ci+} and

fully cross-reactive in the CRI^a assay. Because each of the CRI^{AD8} subpopulations could be selectively immunized, it was possible to determine if stimulating the ABA^- subpopulation of CRI^{AD8} affected subsequent responses of the ABA^+ subpopulation to ABA-KLH. If responsiveness of the ABA^+ CRI^{AD8+} set remains unaffected by prior stimulation of the ABA^- CRI^{AD8+} set, it is unlikely that idiotopes such as CRI^{AD8} are used as regulatory idiotopes. On the other hand, if responsiveness of the ABA^+ CRI^{AD8+} set is affected, it is possible that CRI^{AD8} correlates with a regulatory idiotope.

Data presented in Tables VII and VIII suggest that boosting a prior response in the ABA^- CRI^{AD8+} set is connected with reduced responsiveness in the ABA^+ CRI^{AD8+} set to ABA-KLH. The experiment shown in Table VII shows that AD8 can induce idiotype suppression in the anti-ABA-KLH response. A/J mice received 20 μg of either AD8 or normal rat IgG followed by priming and boosting with ABA-KLH. 7 d after boosting the mice were bled and their sera assayed for anti-ABA antibodies, CRI^a, CRI^{5Ci}, and CRI^{AD8}. All three idiotopes were suppressed (83% for CRI^{AD8}, 85% for CRI^a, and 99% for CRI^{5Ci}). Anti-ABA activity remained unchanged. These results are similar to those previously reported for idiotype suppression in the CRI^a system (36, 37). Like the CRI^a system (37), idiotype suppression in the CRI^{AD8} system can be transferred with cells (unpublished observations), which suggests an active mechanism and rules out possible effects of residual AD8 on the CRI^{AD8} assay. Along with idiotype depletion experiments (see below), this experiment suggests that AD8 priming causes a significant reduction in the responsiveness of the ABA^+ CRI^{AD8+} set to ABA-KLH.

To correlate stimulation of the ABA^- CRI^{AD8+} set with reduced responsiveness of the ABA^+ CRI^{AD8+} set to ABA-KLH, an experiment was performed in which the dose of AD8 was varied from 1 μg per mouse to 20 μg per mouse in narrow increments. 7 d later and immediately before immunization with ABA-KLH, the mice were bled. The mice were bled again 7 d after priming and boosting with ABA-KLH, and both sets of sera were analyzed for CRI^{AD8}. The results are shown in Table VIII. Doses of 10 μg or less of AD8 (groups A, B, and C) failed to reveal either significant stimulation of CRI^{AD8} or significant suppression of CRI^{AD8} after immunization with ABA-KLH. By contrast, doubling the dose of AD8 to 20 μg per mouse (group D vs. E) stimulated an ~10-fold increase in CRI^{AD8} relative to the normal rat IgG control group (before immunization with ABA-KLH) and primed for 77% suppression of CRI^{AD8} after immunization with ABA-KLH. Limited responsiveness of the ABA^+ CRI^{AD8+} set was verified by exhaustively depleting each of the individual anti-ABA-KLH antisera from groups D and E on ABA immunosorbents. Residual CRI^{AD8} titers for the two groups were statistically indistinguishable (338 (x/÷ 1.82) for group D and 324 (x/ ÷ 2.10) for group E). These data show that in mice receiving 20 μg AD8, the ABA^+ CRI^{AD8+} response to ABA-KLH is reduced by ~80%. Selectivity of ABA-KLH toward the ABA^+ CRI^{AD8+} set was shown by depleting sera from group E, both before and after ABA-KLH, on ABA immunosorbents and analyzing for residual CRI^{AD8}. After depletion, the pre-ABA-KLH titer of CRI^{AD8} was 100 (x/÷ 1.65) whereas that for the post-ABA-KLH was 324 (x/÷ 2.10), which indicates that ABA-KLH does not significantly stimulate the ABA^- CRI^{AD8+} set.

Discussion

The experimental results described above suggest that shared idiotypy can functionally connect immune responses directed toward structurally dissimilar epitopes.

Furthermore, these data are consistent with the argument favoring a connection between idiotype dominance in induced antihapten responses and the prior occurrence of idiotypically related responses directed toward internal or environmental stimuli.

Our experiments were possible because of the serendipitous discovery of a rat monoclonal antiidiotype antibody detecting a new cross-reactive idiotope CRI^{AD8}. In A strain or related mice, CRI^{AD8} is found on both induced anti-ABA antibodies and naturally occurring antibodies not binding ABA. Furthermore, division of the CRI^{AD8} idiotope family into two dominant subpopulations is supported by the lack of CRI^{5Ci} expression in the naturally occurring $ABA^- CRI^{AD8+}$ set in contrast to the induced $ABA^+ CRI^{AD8+}$ set that expresses CRI^{5Ci}. Each of these CRI^{AD8} subpopulations could be selectively immunized. A single low dose of antiidiotype AD8 selectively stimulated the $ABA^- CRI^{AD8+}$ set. In contrast, immunization with ABA-KLH selectively stimulated the $ABA^+ CRI^{AD8+}$ set. Because the CRI^{AD8} subpopulations could each be selectively immunized, it was possible to study the effect of stimulating one subpopulation on subsequent responsiveness of the reciprocal subpopulation. If each subpopulation responds independently of the other after sequential stimuli, it is unlikely that sharing of CRI^{AD8} by the two subpopulations is biologically important. If, however, shared idiotypy functionally connects these two responses, stimulating one subpopulation might modify subsequent responses of the reciprocal subpopulation. In principle, the experiment could be done by giving either ABA-KLH or AD8 first and then immunizing with the reciprocal stimulus. We chose to first give AD8 followed by ABA-KLH because this immunization schedule has been shown to elicit idiotype-specific suppression in the CRI^a system (36). Using this immunization schedule, the mice were bled before being given ABA-KLH to correlate responses in the $ABA^- CRI^{AD8+}$ set with the subsequent responsiveness of the $ABA^+ CRI^{AD8+}$ set to ABA-KLH. These experiments suggest that stimulating the $ABA^- CRI^{AD8+}$ set dramatically limits the subsequent responsiveness of the $ABA^+ CRI^{AD8+}$ set to ABA-KLH and that CRI^{AD8} expression links the $ABA^- CRI^{AD8+}$ set with the $ABA^+ CRI^{AD8+}$ at the regulatory level. Based on these observations, Jerne's original concept (13) of dissimilar immune responses being linked via unspecific parallel sets appears to be correct.

The strict interpretation of our findings should be qualified in two ways. First, the data connecting a response in the $ABA^- CRI^{AD8+}$ set with reduced responsiveness in the $ABA^+ CRI^{AD8+}$ set were correlative, and as such, do not establish causality. Second, the selectivity of a single low dose of AD8 toward the $ABA^- CRI^{AD8+}$ set may be more apparent than real. Although repeated innoculations of AD8 were required to induce even small ABA^+ responses, it is possible that AD8 stimulates a cryptic response in the $ABA^+ CRI^{AD8+}$ set which leads to idiotype suppression after immunization with ABA-KLH. Without formally overcoming these objections, the experiment in which the dose of AD8 was varied in narrow dose increments argues against these interpretations. In this experiment, giving doses of 10 μg or less of AD8 failed to either reproducibly stimulate the $ABA^- CRI^{AD8+}$ set or to alter the responsiveness of the $ABA^+ CRI^{AD8+}$ set to ABA-KLH. Doubling the dose of AD8 to 20 μg both boosted the $ABA^- CRI^{AD8+}$ set and reduced the responsiveness of the $ABA^+ CRI^{AD8+}$ set to ABA-KLH. Although these data are correlative, the repeated appearance of both boosting in the $ABA^- CRI^{AD8+}$ set and reduced responsiveness of the $ABA^+ CRI^{AD8+}$ set, at exactly the same dose of AD8, suggests that the two phenomena are causally related. If a single low dose of AD8 induces a cryptic response in the $ABA^+ CRI^{AD8+}$

subpopulation, leading to suppression after immunization with ABA-KLH, it seems unlikely that the dose response for this putative cryptic response would be the same as that found for boosting the ABA^- CRI^{AD8+} set. The importance of immunizing with AD8 is further underscored by other experiments (data not shown) in which the ABA^- CRI^{5Ci-} CRI^{AD8+} hybridoma 5H5 was infused into normal A/J mice to levels approximating those elicited by AD8 before immunization with ABA-KLH. This manipulation had no effect on the subsequent responsiveness of the ABA^+ CRI^{AD8+} set to ABA-KLH despite the similarity of protein 5H5 to the antibodies found in mice immunized with AD8 (5H5 was raised by immunization with AD8 before fusion).

Assuming that the reduced responsiveness of the ABA^+ CRI^{AD8+} set is due to boosting by AD8 of the ABA^- CRI^{AD8+} set, the mechanism by which this boosting reduces the responsiveness of the ABA^+ CRI^{AD8+} set is a fertile area for new experiments. In form, our protocol is similar to that originally devised by Hart et al. (36) for suppressing CRI^a, and because both CRI^{AD8} and CRI^a are found on ABA^- antibodies in the sera of unprimed mice, it is tempting to speculate that in both instances, idiotype suppression in the anti-ABA-KLH response is initiated by boosting the endogenous response with antiidiotype. If this is true, then antiidiotype antibodies that do not react with naturally occurring antibodies might not induce idiotype suppression using the present protocol since they would not boost an endogenous response. This interpretation could also apply to the recent studies of Nelles et. al. (38), who made three monoclonal anti-CRI^a antibodies and found that two would induce suppression whereas the third would not. We would predict that the former two antibodies can boost the endogenous CRI^a response but that the third cannot. This interpretation could also be extended to the anti-staphylococcal nuclease system (Nase). Here, priming with an antiidiotype antibody induces serum idiotype with no specificity for Nase. Immunization with Nase after giving antiidiotype antibody fails to reveal suppression (11). The CRI^{AD8} and Nase systems are similar in that giving antiidiotype calls up idiotype with unknown antigen-binding specificities (12). However, we would postulate that in the Nase system, the antiidiotype antibody is not boosting an endogenous response. Here the antiidiotype would be stimulating a *de novo* response. The possible connection between endogenous immune responses and idiotype dominance in induced antihapten responses has been made before in other systems (8, 15). The experiments shown above lend further credence to this notion. To date, we have been unable to identify the stimulus responsible for the endogenous CRI^{AD8}. However, the IgG subclass restriction to IgG_3 and IgG_2 suggests that the naturally occurring CRI^{AD8} is the result of a TI-1 antibody response (35). Such responses are very often elicited by bacterial antigens that may be implicated in the endogenous response reported here. Our findings are similar to those in the MOPC-460 system where the endogenous idiotype was dramatically reduced in germ-free mice, implicating the endogenous flora as the relevant antigen (8). Unfortunately, no germ-free strain A mice are available to do a similar experiment. We are now searching for the stimulus for the endogenous response.

At first glance, it would seem that only those idiotopes originally called unspecific parallel sets by Jerne (13) should function as regulatory idiotopes. By definition, the unspecific parallel sets are idiotopes that are distinct from the antigen-binding sites, and therefore, can be found on antibodies having dissimilar antigen-binding specificities. The usual criterion for antigen-binding site-associated idiotopes is the hapten

inhibitability of the idiotope antiidiotope interaction. Our data on the hapten inhibitability of the AD8-CRI^{AD8} interaction demonstrate that the criterion of hapten inhibitability should be used with caution for defining binding site-associated idiotopes. In our experiments, the 50% hapten-inhibition point for AD8 binding to CRI^{AD8} varied dramatically with the source of CRI^{AD8}. When hybridoma protein R16.7 was used as the source of CRI^{AD8}, its interaction with AD8 was inhibited by relatively low concentrations of free hapten. On the other hand, when serum CRI or hybridoma protein 36-65 were used as a source of CRI^{AD8}, their interactions with AD8 were inhibited by only very high concentrations of free hapten. In other experiments, the same concentrations of free hapten inhibited the interaction between normal Ig and a monoclonal antibody against IgG-Fc, suggesting that these hapten concentrations nonspecifically inhibit the AD8-CRI^{AD8} interaction. Thus, depending on the source of CRI^{AD8}, the AD8-CRI^{AD8} interaction can appear to be more or less binding-site specific. Within the limits imposed by our relatively small sample size, it appears that the strength of the AD8-CRI^{AD8} interaction varies inversely with the ease of hapten inhibition of this interaction. This statement is based on the observations that hybridoma protein R16.7 interacted with AD8 very poorly when compared with either serum idiotype or 36-65, whereas the interaction of AD8 and R16.7 was inhibited by relatively low concentrations of hapten when compared with the high hapten necessary to compete the interaction of AD8 with either serum CRI or 36-65. These findings agree with other reports (7, 8) showing that a binding site-related idiotope can be found on antibodies that have no demonstrable affinity for the original hapten. Furthermore, such an idiotope can be correlated with the cross regulation of two immune responses, each being specific for a different epitope. At the present, it is only safe to say that CRI^{AD8} is correlated with the presence of a regulatory idiotope. It is not possible to say that CRI^{AD8} is a regulatory idiotope since we have no direct data that the actual idiotope recognized by AD8 is, in fact, the same structure recognized by the regulatory elements of the immune system. Further studies using antiidiotypic T cells, autologous antiidiotype antibodies, and our panel of CRI^{AD8+} and CRI^{AD8-} hybridoma proteins will be required to answer this question.

Summary

A new cross-reactive idiotope family (CRI^{AD8}) is described that contains subpopulations of antibodies binding to different epitopes. One subpopulation occurs naturally in normal sera from strain A mice, is found mainly on IgG$_2$ and IgG$_3$ subclasses, does not bind p-azobenzenearsonate (ABA)$^+$, does not express CRI^{5Ci}, and can be selectively stimulated by low doses of antiidiotype antibody (AD8). The second subpopulation is not found in normal serum, binds ABA, is found on all IgG subclasses, expresses CRI^{5Ci}, and is selectively stimulated by ABA-conjugated proteins. Since CRI^{AD8} was found on both subpopulations of antibody, and since each subpopulation could be selectively expanded, it was possible to study the effect that expansion of the ABA$^-$ CRI^{AD8+} set had on subsequent responses elicited by ABA-keyhole limpet hemocyanin (KLH) in the ABA$^+$ CRI^{AD8+} set. In these experiments, prior immunization with AD8 restricted the subsequent response of the ABA$^+$ CRI^{AD8+} set to ABA-KLH. Furthermore, only those doses of AD8 that stimulated the ABA$^-$ CRI^{AD8+} set reduced the responsiveness of the ABA$^+$ CRI^{AD8+} set to ABA-KLH, suggesting that the two phenomena are causally related. These findings argue that CRI^{AD8} correlates well

with a regulatory idiotope and that immune responses by lymphocyte clones that have different antigen-binding specificities can affect one another as a result of their sharing such an idiotope. These results strongly favor a network organization of the immune system.

References

1. Oudin, J., and P. A. Cazenave. 1971. Similar idiotypic specificities in immunoglobulin fractions with different antibody functions or even without detectable antibody function. *Proc. Natl. Acad. Sci. USA.* **68:**2616.
2. Metzgar, D., A. Miller, and E. Sercarz. 1980. Sharing of an idiotypic marker by monoclonal antibodies specific for distinct regions of hen lysozyme. *Nature (Lond.).* **287:**540.
3. Adorini, L., M. Harvey, and E. Sercarz. 1979. The fine specificity of regulatory T cells. IV. Idiotypic complementarity and antigen-bridging interactions in the anti-lysozyme response. *Eur. J. Immunol.* **9:**906.
4. Ju, S. T., B. Benacerraf, and M. Dorf. 1980. Genetic control of a shared idiotype among antibodies directed to distinct specificities. *J. Exp. Med.* **152:**170.
5. Urbain, J., C. Collignon, J. D. Franssen, B. Mariame, O. Leo, G. Urbain-Vansanten, P. Van de Walle, M. Wikler, and C. Wuilmart. 1979. Idiotypic networks and self-recognition in the immune system. *Ann. Immunol. (Paris).* **130C:**281.
6. Enghofer, E., C. P. J. Glaudemans, and M. J. Bosma. 1979. Immunoglobulins with different specificities have similar idiotypes. *Mol. Immunol.* **16:**1103.
7. Wysocki, L. J., and V. L. Sato. 1981. The strain A anti-*p*-azobenzene-arsonate major cross-reactive idiotypic family includes members with no reactivity toward *p*-azophenyl arsonate. *Eur. J. Immunol.* **11:**832.
8. Dzierzak, E. A., and C. A. Janeway. 1981. Expression of an idiotype (Id-460) during in vivo anti-dinitrophenyl antibody responses. III. Detection of Id-460 in normal serum that does not bind dinitrophenyl. *J. Exp. Med.* **154:**1442.
9. Eichmann, K., A. Coutinho, and F. Melchers. 1977. Absolute frequencies of lipopolysac-charide-reactive B cells producing A5A idiotype in unprimed, streptococcal A carbohydrate-primed, anti-A5A idiotype-sensitized and anti-A5A idiotype-suppressed A/J mice. *J. Exp. Med.* **146:**1436.
10. Kohno, Y., I. Berkower, J. Minna, and J. A. Berzofsky. 1982. Idiotypes of anti-myoglobin antibodies: shared idiotypes among monoclonal antibodies to distinct determinants of sperm whale myoglobin. *J. Immunol.* **128:**1742.
11. Sachs, D. H., M. El-Gamil, and G. Miller. 1981. Genetic control of the immune response to staphylococcal nuclease. XI. Effects of *in vivo* administration of anti-idiotypic antibodies. *Eur. J. Immunol.* **12:**509.
12. Miller, G., P. I. Nadler, Y. Asano, R. J. Hodes, and D. H. Sachs. 1981. Induction of idiotype-bearing nuclease-specific helper cells by in vivo treatment with anti-idiotype. *J. Exp. Med.* **154:**24.
13. Jerne, N. K. 1974. Towards a network theory of the immune system. *Ann. Immunol. (Paris).* **125C:**373.
14. Bona, C. A., E. Heber-Katz, and W. E. Paul. 1981. Idiotype-anti-idiotype regulation. I.

Immunization with a levan-binding myeloma protein leads to the appearance of auto-anti-(anti-idiotype) antibodies and to the activation of silent clones. *J. Exp. Med.* **153**:951.

15. Bottomly, K. 1981. Activation of the idiotypic network: environmental and regulatory influences. *ICN-UCLA Symp. Mol. Cell. Biol.* **20**:517.

16. Lieberman, R. M., M. Potter, E. B. Mushinski, W. Humphrey, and S. Rudikoff. 1974. Genetics of a new IgV$_H$ (T15 idiotype) marker in the mouse regulating natural antibody to phosphorylcholine. *J. Exp. Med.* **139**:983.

17. Tabachnik, M., and H. Sobotka. 1966. Azoproteins II. A spectrophotometric study of the coupling of diazotized arsanilic acid with proteins. *J. Biol. Chem.* **235**:1051.

18. Tung, A. S., S.-T. Ju, S. Sato, and A. Nisonoff. 1976. Production of large amounts of antibodies in individual mice. *J. Immunol.* **116**:676.

19. Oi, V. T., and L. A. Herzernberg. 1980. Immunoglobulin-producing hybrid cell lines. *In* Selected Methods in Cellular Immunology. B. B. Mishell and S. M. Shiigi, editors. W. H. Freeman & Co., San Francisco. 351–372.

20. Rousseaux, J., and H. Bazin. 1979. Rat immunoglobulins. *Vet. Immunol. Immunopathol.* **1**:61.

21. Porter, R. R. 1959. The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain. *Biochem. J.* **73**:119.

22. Olins, D. E., and G. M. Edelman. 1964. Reconstitution of 7S molecules from L and H polypeptide chains of antibodies and γ-globulins. *J. Exp. Med.* **119**:789.

23. Engvall, E., and P. Perlman. 1972. Enzyme-linked immunosorbent assay, ELISA. II. Quantification of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. *J. Immunol.* **109**:129.

24. Galfre, G., C. Milstein, and R. Wright. 1979. Rat × rat hybrid myelomas and a monoclonal anti-Fd portion of mouse IgG. *Nature (Lond.).* **277**:131.

25. de St. Groth, S. F., and D. Scheidegger. 1980. Production of monoclonal antibodies: strategy and tactics. *J. Immunol. Methods.* **35**:1.

26. Marshak-Rothstein, A., M. Siekevitz, M. N. Margolies, M. Mudgett-Hunter, and M. L. Gefter. 1980. Hybridoma proteins expressing the predominant idiotype of the anti-azophenylarsonate response of A/J mice. *Proc. Natl. Acad. Sci. USA.* **77**:1120.

27. Lamoyi, E., P. Estess, J. D. Capra, and A. Nisonoff. 1980. Heterogeneity of an intrastrain cross-reactive idiotype associated with anti-*p*-azophenylarsonate antibodies of A/J mice. *J. Immunol.* **124**:6.

28. Pawlak, L. L., and A. Nisonoff. 1973. Distribution of a cross-reactive idiotype-specificity in inbred strains of mice. *J. Exp. Med.* **137**:1442.

29. Walker, I. D., and G. Morahan. 1981. Monoclonal antiazobenzenearsonate antibodies expressing the cross-reactive idiotype: immunochemical studies show that all idiotypic determinants reside on a single molecule. *Scand. J. Immunol.* **13**:433.

30. Kresina, T. F., S. M. Rosen, and A. Nisonoff. 1982. Degree of heterogeneity of binding specificities of antibodies to the phenylarsonate group that share a common idiotype. *Mol. Immunol.* **19**:1433.

31. Laskin, J. A., A. Gray, A. Nisonoff, N. R. Klinman, and P. D. Gottlieb. 1978. Segregation at a locus determining an immunoglobulin V$_L$-region genetic marker affects inheritance of expression of an idiotype. *Proc. Natl. Acad. Sci. USA.* **74**:4600.

32. Estess, P., E. Lamoyi, A. Nisonoff, and J. D. Capra. 1980. Structural studies on induced antibodies with defined idiotypic specificities. IX. Framework differences in the heavy and light chain-variable regions of monoclonal anti-*p*-azophenylarsonate antibodies of A/J mice differing with respect to cross-reactive idiotype. *J. Exp. Med.* **151**:863.

33. Tung, A. S., and A. Nisonoff. 1975. Isolation from individual A/J mice of anti-*p*-azophenylarsonate antibodies bearing a cross-reactive idiotype. *J. Exp. Med.* **141**:112.

34. Henry, C., and A. Lucas. 1982. The relation of idiotype expression to isotype and allotype in the anti-*p*-azobenzene arsonate response. *Eur. J. Immunol.* **12**:175.

35. Slack, J., G. P. Der-Balian, M. Nahm, and J. M. Davie. 1980. Subclass restriction of murine antibodies. II. The IgG plaque-forming cell response to thymus-independent type 1 and type 2 antigens in normal mice and in mice expressing an x-linked immunodeficiency. *J. Exp. Med.* **151:**853.

36. Hart, D. A., A. C. Wang, L. Pawlak, and A. Nisonoff. 1972. Suppression of idiotypic specificities in adult mice by administration of anti-idiotypic antibody. *J. Exp. Med.* **135:**1293.

37. Owen, F. L., S. T. Ju, and A. Nisonoff. 1977. Presence on idiotype-specific suppressor T-cells of receptors that interact with molecules bearing the idiotype. *J. Exp. Med.* **145:**1559.

38. Nelles, M. J., L. A. Gill-Pazaris, and A. Nisonoff. 1981. Monoclonal anti-idiotypic antibodies reactive with a highly conserved determinant of A/J serum anti-*p*-azophenylarsonate antibodies. *J. Exp. Med.* **154:**1752.