

## IDIOTYPES OF ANTI-Ia ANTIBODIES

### I. Expression of the 14-4-4S Idiotypic in Humoral Immune Responses

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Antigens encoded by the I region of the major histocompatibility complex (MHC)<sup>1</sup> play unique roles in immune responses. Allogeneic I region antigens elicit strong responses in mixed lymphocyte reactions, whereas syngeneic I region antigens appear to be recognized in macrophage T cell interactions during T cell proliferative and T-dependent antibody responses (1, 2). The nature of immune receptors recognizing I region antigens is therefore of particular interest, and characterization of these receptors should provide important insights concerning the generation and regulation of immune responses. In addition, the generation of anti-idiotypic reagents against such receptors may permit specific manipulation of the immune response to I region antigens, and may thus have implications for the modification of transplantation immunity.

Previous studies of immune receptor idiotypes directed against MHC antigens have used either heterogeneous conventional alloantibodies (3) or alloreactive cell populations as sources of receptors (4, 5). However, anti-idiotypic reagents raised against such receptor materials have proved difficult to prepare reproducibly (6; N. Shinohara and D. H. Sachs, unpublished data). The use of hybridoma technology (7) now permits a more direct approach to such studies because, by the use of monoclonal anti-MHC antibodies, both idiotypic and anti-idiotypic can be reliably prepared in large quantities.

Because a monoclonal antibody is used as idiotypic in these studies, it should be noted that antibodies to MHC antigens may be very heterogeneous idiotypically. Thus, the idiotypic of a single hybridoma clone may represent only a small proportion of the overall response to the same antigen. This has been our experience in the study of several anti-H-2K<sup>k</sup> idiotypes (8).

In the present work, the idiotypic of an anti-Ia monoclonal antibody, 14-4-4S, has been studied. Heterologous anti-idiotypic antibodies were produced, purified, and characterized for specificity. The anti-idiotypic was then used to analyze alloantisera for expression of the idiotypic. In contrast to our previous results for anti-H-2K<sup>k</sup>

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<sup>1</sup> *Abbreviations used in this paper:* ELISA, enzyme-linked immunosorbent assay; HA, hemagglutination assay; HAI, hemagglutination inhibition assay; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PBS-Tween, PBS containing 0.025% Tween 20; RAPIg, rabbit anti-pig immunoglobulin; (T,G)-A--L, (poly-L-tyrosine, L-glutamic acid)-poly-DL-alanine-poly-L-lysine.

idiotypes (8), the 14-4-4S idiotypic is shown here to be readily detectable in alloantisera produced in the same immunization combination as that from which the hybridoma arose. We report studies of idiotypic expression in antisera from other responding strains, and also an examination of genetic factors influencing such expression.

### Materials and Methods

*Animals.* CWB-13 mice, derived from the 13th backcross to C3H.SW, were kindly provided by Dr. Melvin Bosma, Institute for Cancer Research, Philadelphia, Pa. C3H.SW and C3H/HeJ mice were either purchased from The Jackson Laboratory, Bar Harbor, Maine, or bred in our own colony. Miniature swine were bred and housed at the National Institutes of Health Animal Center, Poolesville, Md.

*Monoclonal Antibodies.* The derivation and specificity of monoclonal antibodies 14-4-4S and 17-3-3S have been described previously (9). Both are IgG2a, kappa antibodies reacting with I-E<sup>k</sup>, but with different determinants. Specificity of 14-4-4S for Ia.7 was confirmed by testing of high-titered ascites fluid, as shown in Table I. The 14-4-4S protein used for xenoinmunization was purified from culture supernate by affinity chromatography on protein A coupled to Sepharose 4B and elution with 3 M KSCN. The purified antibody at 1.2 mg/ml had a cytotoxic titer of 1:128 on C3H spleen cells, and was pure as evidenced by a single band in immunoelectrophoresis. Antibody for coupling to Sepharose and for use in enzyme-linked immunosorbent assays (ELISA) was purified from ascites fluid on protein-A Sepharose. Cells of the BALB/c myeloma, LPC-1, were obtained from Dr. Michael Potter, National Institutes of Health, Bethesda, Md., and the myeloma protein was purified from ascites, as described above.

*Anti-Idiotypic Immunization.* An adult miniature swine was immunized by intramuscular injection of 200  $\mu$ g of purified 14-4-4S protein in 1 ml of saline emulsified with an equal volume of complete Freund's adjuvant. The pig was boosted with an identical antigen preparation after 3 wk, then bled at weekly intervals. Bleedings were tested for activity against mouse IgG2a, and kappa proteins were tested by passive hemagglutination (HA) (see below). After a total of 5 immunizations and 10 test bleedings, the pig was exsanguinated. The eighth bleeding was chosen for purification of anti-idiotypic because of the high titer and large volume obtained.

*Purification of Anti-Idiotypic Antibody.* Pig anti-14-4-4S serum was first extensively absorbed with LPC-1 coupled to Sepharose, to remove activity against IgG2a and kappa constant region determinants, and was tested for residual activity in HA using LPC-1 coupled to sheep erythrocytes. The antibody was then adsorbed to 14-4-4S Sepharose, eluted with 4 M guanidine HCl, pH 7.0, and dialyzed into phosphate-buffered saline (PBS). The yield at each step and the activity in HA are given in Table II. The final material was passed over LPC-1 Sepharose once again to assure removal of all antibody directed against constant region determinants, but no further change in specificity was observed.

*Alloimmunization of Mice.* Mice were immunized by full-thickness grafts of tail skin followed by intraperitoneal boosting with spleen cells, as described previously (10). Test bleeds were performed individually 1 wk after each immunization and were tested for cytotoxic activity by trypan blue dye exclusion assays (10). When titers reached plateau levels, Ehrlich ascites tumor cells were injected intraperitoneally into the mice, and after fluid accumulation, were tapped at ~3-d intervals. Sera and ascites were decomplexed at 56°C for 30 min.

*Inhibition ELISA.* The ELISA technique was adapted from Voller (11). MicroELISA plates (Dynatech Laboratories, Inc., Dynatech Corp., Alexandria, Va.) were coated overnight at 4°C with 200  $\mu$ l/well of a solution of purified 14-4-4S protein in PBS, at 1  $\mu$ g/ml. In subsequent steps, 0.025% Tween 20 (P-1379, Sigma Chemical Co., St. Louis, Mo.) in PBS (PBS-Tween) was used as diluent. All incubations were at room temperature, and plates were washed five times with PBS-Tween between steps.

The binding of pig anti-idiotypic to coated plates was titrated in preliminary experiments, and an amount was chosen (0.1  $\mu$ g/well) that was on the steep slope of the titration curve. Inhibitors were serially diluted in 25- or 50- $\mu$ l volumes in round-bottomed microtiter plates using microdiluters (Dynatech Laboratories). A 1:8 dilution of C3H.SW normal mouse serum in PBS-Tween was used as diluent to confine detection to 14-4-4S idiotypic determinants that are not shared with normal serum immunoglobulin. This method has been described in the

arsonate idiotypic system (12). When normal serum was omitted from the diluent, a low-level inhibition by normal serum was generally observed in the ELISA.

After addition of anti-idiotypic, plates were incubated for 30 min, and the mixtures were then brought up to 200  $\mu$ l per well with PBS-Tween, transferred to the 14-4-4S-coated ELISA plates, and incubated a further 30 min. Rabbit anti-pig immunoglobulin (RAPIg), purified and conjugated to horseradish peroxidase, was purchased from Miles-Yeda, Ltd., Rehovot, Israel. It was adsorbed with glutaraldehyde-fixed normal mouse serum (13) to reduce background reactivity on mouse immunoglobulin, and was used at a 1:300 dilution. Substrate solution was prepared as follows: *o*-phenylene diamine (P2,393-8; Aldrich Chemical Co., Inc., Milwaukee, Wis.) was dissolved in absolute methanol at 10 mg/ml. 1 ml of this solution was added to 99 ml of distilled water, followed by 250  $\mu$ l of 30% hydrogen peroxide (Fisher Scientific Co., Pittsburgh, Pa.). Substrate solution, 200  $\mu$ l/well, was added to plates and incubated for 15–30 min. The reaction was stopped by addition of 50  $\mu$ l/well of 8 N H<sub>2</sub>SO<sub>4</sub>. Absorbance was read at 492 nm in a Titertek multiskan spectrophotometer (Flow Laboratories, Inc., Rockville, Md.). Results were expressed as

$$\text{Percent inhibition} = \frac{\text{uninhibited } A_{492} - \text{experimental } A_{492}}{\text{uninhibited } A_{492} - \text{background } A_{492}},$$

in which background refers to values for RAPIg-enzyme conjugate in 14-4-4S-coated wells, in the absence of pig anti-idiotypic. All dilutions refer to concentration after volume per well was adjusted to 200  $\mu$ l.

*Immunodiffusion.* Allotyping of C3H.SW and CWB mice was performed by Ouchterlony double diffusion, in 1.5% agar gels. The anti-Igh-C<sup>a</sup> and Igh-C<sup>b</sup> sera used were produced by immunization with pertussis-anti-pertussis complexes, as described (14). Typing was unambiguous in each case.

*Absorption and Cytotoxic Assays.* In vivo absorptions were performed by intraperitoneal injection of 0.2 or 0.5 ml antibody per mouse into weight-matched mice (all between 23 and 25 g). After 4 h the mice were exsanguinated. In vitro screening absorptions were performed by adding  $2 \times 10^6$  absorbing cells per well to serially diluted antisera and then testing the residual activity of the supernates in two-stage trypan blue cytotoxicity assays, as described previously (10).

*Hemagglutination.* Hemagglutination (HA) and hemagglutination inhibition (HAI) assays were performed by published methods (15, 16).

## Results

*Preparation of Anti-Idiotypic to the 14-4-4S Monoclonal Antibody.* The monoclonal anti-Ia antibody 14-4-4S, previously reported by this laboratory (9), was chosen for these idiotype studies. As seen in Table I, the reactivity pattern of 14-4-4S ascites in cytotoxic tests on a panel of mouse strains indicated detection of the product of a locus mapping to the *I-E* subregion. All *H-2* haplotypes were positive except *b*, *s*, *f*, and *q*, which do not express I-E antigens on their cell surfaces (17, 18). Thus, the specificity of 14-4-4S corresponded to Ia.7 (19).

A pig antiserum against 14-4-4S was raised, and the anti-idiotypic antibodies were purified from it, as described in Materials and Methods. Table II shows the yields of anti-idiotypic antibodies at several stages in the purification, and also their specificity as assessed by hemagglutination. An inhibition ELISA was then established in which samples were tested for their ability to inhibit the binding of anti-idiotypic to idiotype-coated plates. To exclude detection of variable region determinants widely shared with normal immunoglobulins, normal mouse serum was included in the diluent (12; see Materials and Methods).

As shown in Fig. 1, 14-4-4S itself was able to inhibit the binding of pig anti-idiotypic to 14-4-4S-coated plates, whereas 17-3-3S and LPC-1 could not. Because 17-3-3S and

TABLE I  
Cytotoxic Titer of 14-4-4S Ascites on a Panel of Mouse Haplotypes

Mouse strains	Haplotype of origin								Cytotoxic titer*
	I								
	K	A	B	J	E	C	S	D	
Independent haplotypes									
C3H	k	k	k	k	k	k	k	k	32,000
B10	b	b	b	b	b	b	b	b	<2
B10.D2	d	d	d	d	d	d	d	d	128,000
B10.M	f	f	f	f	f	f	f	f	<2
B10.P	p	p	p	p	p	p	p	p	64,000
DBA/1	q	q	q	q	q	q	q	q	<2
B10.RIII	r	r	r	r	r	r	r	r	64,000
B10.S	s	s	s	s	s	s	s	s	<2
Recombinant haplotypes									
B10.A	k	k	k	k	k	d	d	d	16,000
C3H.OL	d	d	d	d	d	d	k	k	16,000
D2.GD	d	d	b	b	b	b	b	b	<2
B10.A(2R)	k	k	k	k	k	d	d	b	4,000
B10.A(4R)	k	k	b	b	b	b	b	b	<2
B10.A(5R)	b	b	b	k	k	d	d	d	4,000
A.TL	s	k	k	k	k	k	k	d	16,000
A.TH	s	s	s	s	s	s	s	d	<2
B10.MBR	b	k	k	k	k	k	k	q	16,000

\* Ia-type cytotoxic titer defined as last dilution giving:

$$\% \text{ dead cells} \geq \frac{(\text{maximum \% dead} - \% \text{ dead in C' control})}{2} + (\% \text{ dead in C' control}).$$

TABLE II  
Fractionation of Pig Anti-Idiotypic Serum

Antiserum fraction	Volume*	A <sub>280</sub>	Total protein	Hemagglutination titer (log <sub>2</sub> ) on SRBC coated with			
				LPC-1	14-4-4S	17-3-3S	FBS‡
	<i>ml</i>		<i>mg</i>				
5620, pig anti-14-4-4S	50	ND§	ND	19-20	>22	6-18	6
5620(LPC-1)I	100	ND	ND	6-7	>11	6-7	5-6
5620(LPC-1)II	100	1.594	114	15-16	19-20	15-16	1
5620(LPC-1)I(14-4-4S)I	120	ND	ND	7-8	15-16	5	6-7
5620(LPC-1)I(14-4-4S)II¶	14	1.363	14	5-6	>22	1	2

\* After concentration in some cases.

‡ Fetal bovine serum.

§ Not determined.

|| Proteins shown in parentheses were covalently attached to Sepharose affinity columns. After parentheses, I indicates pass-through material, and II indicates bound and eluted material.

¶ An additional adsorption and elution of 5620(LPC-1)I(14-4-4S)I to 14-4-4S Sepharose yielded a further 3.8 mg of antibody, also showing specificity for 14-4-4S and not for 17-3-3S-coated cells.

LPC-1 are both IgG2a, kappa proteins, the anti-idiotypic was not contaminated with detectable antibody against constant-region determinants. The controls indicated that the ELISA could reliably detect idiotype concentrations as low as 50 ng/ml. This heterologous anti-idiotypic preparation is presumably heterogeneous, and may contain antibody directed against multiple idiotypic determinants (idiotopes) of 14-4-4S.

*Detection of the 14-4-4S Idiotype in Alloantisera.* The 14-4-4S monoclonal antibody was derived from a C3H.SW anti-C3H immunization. Thus, C3H.SW anti-C3H sera might contain the idiotype, although other monoclonal anti-MHC idiotypes have not been detectable in alloantisera (see Introduction). A representative ELISA test of a C3H.SW anti-C3H reagent and several controls is shown in Fig. 1. The 14-4-4S idiotype was clearly detectable in C3H.SW anti-C3H product 1080. Little or no idiotype was detected in C3H.SW normal serum, or in serum of C3H.SW mice immunized to the irrelevant antigen, (poly-L-tyrosine, L-glutamic acid)-poly-DL-alanine-poly-L-lysine (T,G)-A-L (data not shown). In other tests, C3H anti-C3H.SW sera were also found to be negative (data not shown). Detection of the idiotype in C3H.SW anti-C3H immune serum and ascites was highly reproducible both in the ELISA assay and in HAI (data not shown) for all pooled blood samples tested. A purified rabbit anti-idiotypic to 14-4-4S prepared in the same way could also detect the 14-4-4S idiotype in the C3H.SW anti-C3H reagent.

*Specific Absorption of the Idiotype from Alloantisera by Ia.7-positive Cells.* The C3H.SW anti-C3H alloantisera would be expected to contain antibody to multiple antigens of the *H-2<sup>k</sup>* haplotype, and suitable recombinants are not available on the C3H background to permit immunization to I-E in the absence of additional antigenic differences. Thus, it was necessary to determine whether the idiotype detected serologically was expressed on antibodies specific for I-E antigens.

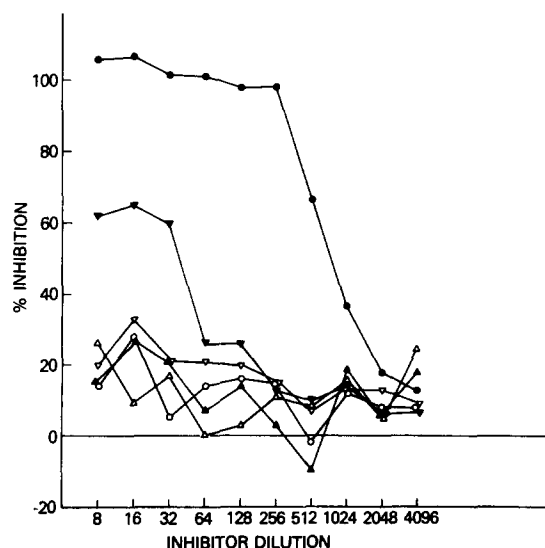


FIG. 1. Inhibition ELISA for the 14-4-4S idiotype and detection of the 14-4-4S idiotype in C3H.SW anti-C3H alloantibody. Percent inhibition is defined in Materials and Methods. ●, purified 14-4-4S; ○, purified 17-3-3S; △ and ▲, C3H.SW normal serum, two different pools; ▽, LPC-1 ascites fluid; ▼, C3H.SW anti-C3H immune ascites 1080. Dilutions of antibodies purified from ascites fluids were started at 50  $\mu$ g/ml. Diluent, 1:8 dilution of C3H.SW normal serum.

TABLE III  
*Cytotoxic Activity of In Vivo Absorbed Sera: Completeness of Absorption*

Mouse strain used for absorption	H-2 haplotype I								Antibody absorbed*	Postabsorption cytotoxic titer on		
	K	A	B	J	E	C	S	D		B10.A(4R) (H-2 titer)‡	B10.A(5R) (Ia-titer)§	B10.A(2R) (H-2 titer)
B10.A(4R)	k	k	b	b	b	b	b	b	C3H.SW	<2	32	NT
									anti-C3H	<2	32	
									14-4-4S	<2	32	NT
B10.A(5R)	b	b	b	k	k	d	d	d	C3H.SW	64	<2	NT
									anti-C3H	64	<2	
									14-4-4S	<2	<2	NT
B10.A(2R)	k	k	k	k	k	d	d	b	C3H.SW	NT	<2	<2
									anti-C3H			
									14-4-4S		<2	<2
B10	b	b	b	b	b	b	b	b	C3H.SW	64	32	NT
									anti-C3H			
									14-4-4S	NT	8	

\* Undiluted C3H.SW anti-C3H ascites 1080 was absorbed by injection of 0.5 ml per animal. Hybridoma 14-4-4S ascites was absorbed by injection of 0.2 ml per animal of a 1:100 dilution. Two values for a sample indicate two independent absorptions.

‡ H-2 titer defined as last dilution giving % dead  $\geq$  50%.

§ Ia titer, see note to Table I.

|| Not tested.

Product 1080 was absorbed in vivo in B10, B10.A(2R), B10.A(4R), and B10.A(5R) mice (Table III), and 14-4-4S ascites was similarly absorbed as a positive control. In B10.A(2R) and B10.A(5R) mice, one would expect anti-I-E<sup>k</sup> antibodies to be absorbed; whereas in B10.A(4R) mice, anti-H-2K<sup>k</sup> and anti-I-A<sup>k</sup> antibodies, but not anti-I-E<sup>k</sup> antibodies, should be absorbed (Table III). The B10 strain, which expresses no antigens recognized by C3H.SW anti-C3H serum, was used as a dilution control. The resulting absorbed sera were then tested in a complement-mediated cytotoxicity assay for residual activity, and in the ELISA for residual 14-4-4S idiotypic activity. In each case, no residual cytotoxic activity was detected against spleen cells from mice of the same strain as the mice used for absorption (Table III), indicating that the absorptions were complete. As shown in Fig. 2, absorption of 14-4-4S in B10.A(5R) or B10.A(2R) removed the idiotypic activity detected in the ELISA, and absorption in B10.A(4R) or B10 did not, as was expected. Similarly, with conventional C3H.SW anti-C3H antibodies, absorption in B10.A(5R) or B10.A(2R) removed the idiotypic activity, whereas absorption in B10.A(4R) or B10 did not. These results are consistent with the presence of the idiotypic activity detected in this serum on specific anti-I-E antibodies.<sup>2</sup> The control absorption of 1080 in C57BL/10 mice showed a fourfold to eightfold dilution of idiotypic activity detected in the ELISA, as compared to unabsorbed 1080 (data not shown). This dilution was comparable to that observed in B10.A(4R) mice.

<sup>2</sup> Although these studies do not formally rule out anti-I-J or anti-I-C antibodies, only I-A and I-E have been demonstrated to encode Ia antigens readily detectable in cytotoxic tests on spleen cells.

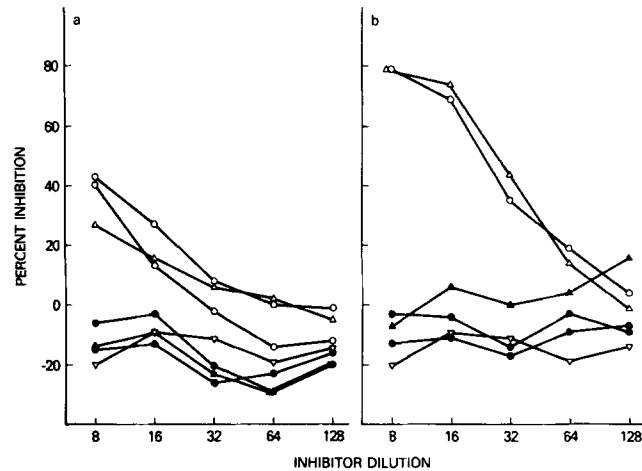


FIG. 2. Depletion of 14-4-4S idiotypic in alloantisera by absorption in Ia.7-positive mice. (a) absorptions of C3H.SW anti-C3H 1080; (b) absorptions of 14-4-4S ascites. Absorptions in vivo as follows: ● in B10.A(5R), two independent absorptions of each antibody; ○ in B10.A(4R), two independent absorptions of 1080; ▲ in B10.A(2R); △ in B10. ▽, B10.A(4R) normal serum.

TABLE IV

*Expression of the 14-4-4S Idiotypic in Responses of C3H.SW Mice to B10.A(2R) and B10.A(4R) Cells*

Serum	Percent inhibition in ELISA for 14-4-4S idiotypic*	Mean $\pm$ SD	Significance of difference (Student's two-tailed <i>t</i> test)‡
C3H.SW anti-B10.A(2R)	84, 80, 77, 48	72 $\pm$ 16	} $P < 0.002$
C3H.SW anti-B10.A(4R)	4, 16, 1, 25, 14, 11	12 $\pm$ 9	
Controls			
C3H.SW normal serum pool		15 $\pm$ 1 (triplicates)	
C3H.SW anti-C3H pool, 1080		72 $\pm$ 2 (duplicates)	
14-4-4S, purified, 0.8 $\mu$ g/ml		90 $\pm$ 5 (duplicates)	

\* All inhibitions shown for 1:8 dilution of test serum. Values are given for individual animals.

‡ Comparison is between bracketed groups.

Confirmation of I-E specificity was obtained by another approach. In tests of additional sera, the idiotypic was detectable in C3H.SW anti-B10.A(2R) but not in C3H.SW anti-B10.A(4R) sera (Table IV), with the two groups differing significantly ( $P < 0.002$ ). These results indicate that the antigen responsible for eliciting idiotypic was determined by genes that map to *I-E*.<sup>2</sup> (For relevant haplotypes, see Table I.)

*Penetrance of Idiotypic Expression in C3H.SW Mice.* The alloantisera tested above were pools obtained from 20 mice, and the idiotypic detected could have been due to high levels of expression in rare individuals. Results for testing of individual immune sera are shown in Table V. Nine individual animals tested expressed detectable amounts of the 14-4-4S idiotypic, as evidenced by inhibition values more than two standard deviations above the mean for nonimmune sera. One animal was found to be idiotypic negative, although its serum contained anti-H-2<sup>k</sup> cytotoxic activity. The response of this animal did include anti-I-E antibodies, as seen in cytotoxic assays using absorbed serum (data not shown). This finding will be discussed below.

TABLE V  
*Expression of the 14-4-4S Idiotypic in C3H.SW and CWB Anti-C3H Sera*

Serum	Igh-C	Percent inhibition in ELISA for 14-4-4S idiotype*	Mean $\pm$ SD	Significance of differences (Student's two-tailed <i>t</i> test)‡
C3H.SW anti-C3H	a	45, 31, 38, 46, 38, 73, 65, 53, 47, 9	44 $\pm$ 18	$P < 0.001$ } $P > 0.1$ } $P < 0.001$
CWB anti-C3H	b	18, 13, 28, 16, 8, 38, 29, 23, 12, 23, 5, 8, 0, 12	17 $\pm$ 10	
C3H.SW nonimmune	a	24, 12, 9, 14, 14, 6, 0, 22, 14, 10	12 $\pm$ 7	
Controls				
C3H.SW normal serum pool			7 $\pm$ 3 (triplicates)	
C3H.SW anti-C3H pool, 1080			56 $\pm$ 6 (duplicates)	
14-4-4S, purified, 0.8 $\mu$ g/ml			94 $\pm$ 1 (duplicates)	

\* All inhibitions shown for 1:8 dilution of test serum. Values are given for individual animals.

‡ Comparison is between bracketed groups. Bracket furthest to the right indicates comparison of C3H.SW anti-C3H with C3H.SW nonimmune serum.

Testing of bleedings at various stages of immunization from individual animals showed that plateau levels of idiotype expression were achieved by day 42, after skin grafting and three boosts. This time-course was similar for several animals tested (data not shown).

*Expression of the 14-4-4S Idiotypic in Antibody Responses of Other Strains.* Antisera made in other inbred mouse strains in response to immunization with Ia.7-positive cells were tested for expression of the 14-4-4S idiotype to examine the genetic requirements for expression. As shown in Fig. 3, sera from a variety of responding strains of the B10 or A background inhibited much less in the ELISA than did sera from C3H.SW mice. In additional tests, [A.BY  $\times$  B10.A(4R)] anti-B10.A(2R), A.BY anti-A/J, A.TH anti-A.TL, and D2.GD anti-DBA/2 sera inhibited only marginally in the ELISA (data not shown). These immunizations would be expected (and in some cases were confirmed) to produce anti-I-E activity. The responding strains in each case differ from C3H.SW in heavy chain allotype, in addition to other background genes. Thus, further investigation of the influence of allotype-linked genes on idiotype expression was warranted.

*Idiotypic Expression in CWB Mice.* CWB mice have been developed as a congenic strain that should be identical to C3H.SW mice except for the chromosomal segment including the heavy chain allotype loci of the allele Igh-C<sup>b</sup> (20). Thus, if the genes determining expression of 14-4-4S idiotype were linked to Igh-C, CWB animals would



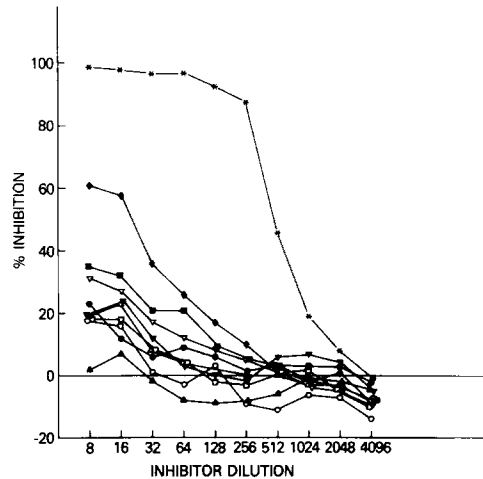


FIG. 3. Tests for idiotype in antisera from Igh-C<sup>b</sup> and Igh-C<sup>e</sup> strains. Given in parentheses after each serum is its cytotoxic titer on spleen cells of the strain used as donor in the immunization. \*, Purified 14-4-4S; ○, B10 anti-B10.D2 serum 4647 (titer 512); △, B10 anti-B10.D2 serum 4213 (256); ▽, B10 anti-B10.D2 serum 4154 (256); ▲, B10 anti-B10.D2 ascites 4791 (256); ▼, A.SW anti-A/J ascites 1862 (2048); □, B10 anti-CBA/J serum 3001 (256); ■, A.BY anti-A/J ascites 1362 (512); ◆, C3H.SW anti-C3H ascites 1080 (1024); ●, C3H.SW normal serum. Diluent, 1:8 C3H.SW normal mouse serum. Relevant allotypes are B10, Igh-C<sup>b</sup>; A.SW and A.BY, Igh-C<sup>e</sup>.

be expected to behave like B10, with which they share the Igh-C<sup>b</sup> allele, rather than like C3H.SW.

CWB and C3H.SW mice were immunized simultaneously against C3H, bled individually, and their allotypes were confirmed by Ouchterlony analysis. Idiotype testing of the same individual sera in the ELISA (Table V) showed significantly less inhibition by the CWB sera than by the C3H.SW sera when tested at the same dilution ( $P < 0.001$ ). In addition, CWB immune sera as a group did not differ significantly from values for normal serum, although certain individual CWB sera were more than two standard deviations above the mean of normal sera. Such individuals apparently express some idiotopes (see Discussion). C3H.SW and CWB normal serum pools gave similar values (data not shown).

The lesser ability of CWB immune sera to inhibit in the ELISA could indicate that their anti-I-E response uses other idiotypes, or that they are low responders to the immunizing I-E<sup>k</sup> antigen. To distinguish between these possibilities, individual CWB immune ascites were absorbed with B10.T(6R) cells (H-2K<sup>a</sup>, I-A<sup>a</sup>, I-E<sup>a</sup>, H-2D<sup>d</sup>) to remove antibody cross-reactive with H-2D<sup>d</sup>. They were then tested for residual cytotoxic activity on B10.A(5R) cells (H-2K<sup>b</sup>, I-A<sup>b</sup>, I-E<sup>k</sup>, H-2D<sup>d</sup>) as a measure of anti-I-E<sup>k</sup> activity. Levels of anti-I-E<sup>k</sup> activity were at least as great in the CWB antibodies as in those of C3H.SW immune mice (Table VI). It should be noted that high titers of antibody cross-reactive with H-2D<sup>d</sup>, as in some of the C3H.SW anti-C3H antibodies, may obscure significant anti-I-E activity. These data would thus be insufficient to allow the conclusion that a serum had no anti-I-E activity at all. However, a higher titer on B10.A(5R) than on B10.T(6R), as with the CWB sera, is clear evidence for the presence of anti-I-E activity.

TABLE VI  
*Evidence for Anti-I-E Cytotoxic Activity in CWB Anti-C3H Antibody*

Ascites absorbed with B10.T(6R)	Cytotoxic activity of absorbed ascites*	
	H-2 titer on B10.T(6R) cells‡	Ia titer on B10.A(5R) cells‡
CWB anti-C3H		
656	<2	64
996	<2	4
661	4	32
C3H.SW anti-C3H		
667	2	32
671	4	32
673	16	16

\* In vitro screening absorptions were performed as described in Materials and Methods.

‡ Haplotypes of the strains used are as follows:

	I							
	K	A	B	J	E	C	S	D
B10.T(6R)	q	q	q	q	q	q	q	d
B10.A(5R)	b	b	b	k	k	d	d	d

### Discussion

Monoclonal anti-Ia antibody 14-4-4S, derived from a C3H.SW anti-C3H immunization, has been used to prepare a heterologous anti-idiotypic serum. Affinity chromatography was performed to remove activity against constant region determinants and to purify activity against idio type. The anti-idiotypic was then used to establish a sensitive inhibition assay for detection of this idio type. The ELISA detects variable region determinants, as demonstrated by the inability of other IgG2a, kappa immunoglobulins to inhibit. The ability of C3H.SW anti-C3H alloantisera to inhibit reflects the presence in these sera of molecules sharing variable region structural features with 14-4-4S. The 14-4-4S idio type therefore represents a shared idio type analogous to shared idiotypes expressed in responses to certain haptens, such as *p*-azophenylarsonate (12), nitrophenyl (21), and phosphorylcholine (22), and to proteins such as  $\gamma$ -globulin (23), staphylococcal nuclease (24), and lysozyme (25). To our knowledge, this is the first example of a shared idio type in the anti-I-E system, and it is also the first example of a monoclonal idio type shared in an anti-MHC system.

None of the alloantisera tested produced inhibition as complete as did 14-4-4S itself, although one C3H.SW anti-C3H individual serum showed 73% inhibition at the highest concentration tested, and had not yet reached a plateau. The incompleteness of inhibition may be due to a lower concentration of idio type in alloantisera than in the monoclonal reagent, or to the expression of some, but not all, idiotypes of 14-4-4S in alloantisera. These possibilities can be distinguished by adsorption of anti-idio type on insolubilized immunoglobulin from alloantisera to see if all reactivity against 14-4-4S can be removed. Such experiments are in progress.

The idio type detected in conventional alloantibody populations is present on

specific anti-I-E<sup>k</sup> antibody molecules, as shown by the results of *in vivo* absorptions (Fig. 2). The data indicate that very few, if any, idiotype-positive, nonantigen binding molecules are present in conventional alloantisera. This finding is similar to the original results for the cross-reactive idiotype in the arsonate system (12), but contrasts with results for ovalbumin (27) and nuclease (16), where immunization with antigen leads to production of both antigen-binding and nonantigen-binding molecules that bear idiotopes of the cross-reactive idiotype. More sensitive methods might detect some such material in the 14-4-4S system.

Penetrance of idiotype expression in the C3H.SW anti-C3H response was quite high, but one animal produced an idiotype-negative response to I-E. The penetrance of idiotype expression in the response to Ia.7 may in fact be incomplete. Alternatively, the animal producing an idiotype-negative response may have responded to determinants on I-E other than Ia.7, or to different elements of Ia.7, which is probably a family of determinants (28). Further absorption studies are in progress to determine the specificity of this individual anti-I-E response.

The failure to detect comparable idiotype expression in high-titered antisera produced in several other responding strains suggests genetic control of idiotype expression. Because these strains differ from C3H.SW in their heavy chain allotypes as well as other background genes, and because expression of idiotype is frequently linked to Igh-C (29), CWB allotype congenic mice were tested for their idiotype expression. The lower levels of idiotype detected in CWB alloantisera indicated that allotype-linked genes influence the levels of expression of at least some 14-4-4S-related idiotopes. The possible contribution of light chain genes (30) has not yet been examined.

Although, as a group, CWB immune sera did not differ significantly from normal sera, further examination of individual CWB sera showed that the values in the ELISA reflected true differences in idiotope content and not just technical fluctuations in the assay. Specifically, isoelectric focusing of CWB anti-C3H sera followed by overlaying with <sup>125</sup>I-anti-idiotype to 14-4-4S revealed distinct bands for a serum giving high ELISA values and only faint, background labeling for a serum giving low ELISA values (S. L. Epstein, J. A. Bluestone, K. Ozato, and D. H. Sachs, manuscript in preparation). It is not yet clear whether CWB mice express only a subset of the idiotopes expressed by C3H.SW mice or lower levels of the complete set of idiotopes. A possibility is the existence of regulatory differences rather than differences in structural genes between the strains. An alternative explanation for the expression of a subset of idiotopes would be recombination between sets of V<sub>H</sub> genes during derivation of the CWB strain.

The prevalence of the 14-4-4S idiotype in the C3H.SW anti-C3H immune response contrasts with our failure to detect several monoclonal anti-H-2K<sup>k</sup> idiotypes in appropriate alloantisera by HAI (8) or by ELISA (J. A. Bluestone and D. H. Sachs, unpublished data). One possibility that might account for the difference is that the 14-4-4S clone may by chance represent one of the most common clones stimulated during the relevant normal immune response, whereas the anti-H-2K<sup>k</sup> hybridomas we have studied do not. Another possibility for the difference in levels of expression is that Ia.7, the specificity recognized by 14-4-4S, is not a true alloantigen in that it does not represent an allele at a polymorphic locus. Studies from a number of laboratories have shown that certain strains of mice, notably those bearing the *H-2<sup>b</sup>*,

*H-2<sup>s</sup>*, *H-2<sup>f</sup>*, and *H-2<sup>a</sup>* haplotypes, do not express I-E surface antigens (17, 18). Strains that do express I-E, including *H-2<sup>k</sup>*, *H-2<sup>d</sup>*, and *H-2<sup>r</sup>*, all share specificity Ia.7 and react with 14-4-4S (Table I). Thus, in an *H-2<sup>b</sup>* anti-*H-2<sup>k</sup>* response, such as C3H.SW anti-C3H, the anti-Ia.7 component may not represent a typical alloresponse. Rather, it may more closely resemble immune responses to non-MHC foreign proteins, for which there is ample precedent for cross-reactive idiotypes, as discussed above. An interesting comparison may be permitted by studies of the idiotype of monoclonal antibody 17-3-3S, which recognizes an alloantigenic determinant on I-E antigens of the *k* and *r* haplotypes (9). In preliminary studies, this idiotype was not detected in appropriate alloantisera (S. L. Epstein and D. H. Sachs, unpublished observations). Thus, the results are consistent with a more diverse repertoire of receptor idiotypes expressed in responses to alloantigenic determinants and a less diverse repertoire in response to non-MHC foreign proteins and to monomorphic MHC determinants such as Ia.7.

The availability of a shared idiotype in an anti-Ia response opens a number of important opportunities for functional studies. I-E antigens can stimulate in mixed lymphocyte reactions, and the determinant on I-E recognized by the proliferating T cells has been reported to be Ia.7 (31). Thus, T cell responses to monomorphic MHC determinants are detectable in the I-E system. In addition, I-E antigens have been reported to function as restriction elements in T-dependent antibody responses (32, 33). Studies are planned to investigate the effects of anti-idiotype on such I-E-mediated functions. Much previous evidence suggests idiotypic sharing by B and T cells reactive to a number of antigens, including MHC antigens (3-5). The 14-4-4S system should permit analysis of idiotypic sharing between B and T cells in a response to an Ia antigen.

### Summary

The idiotype of a mouse monoclonal anti-I-E antibody, 14-4-4S, has been studied using a heterologous anti-idiotypic reagent. This antibody recognizes Ia.7, an antigenic specificity present in all strains expressing a product of the *I-E* subregion. Expression of the 14-4-4S idiotype in humoral immune responses was analyzed by an idiotype-specific enzyme-linked immunosorbent assay system. The idiotype was readily detectable in C3H.SW anti-C3H alloantisera, the same immunization combination from which the hybridoma was derived. Absorption analysis demonstrated the anti-I-E specificity of the idiotype-positive molecules in these alloantisera. Penetrance of idiotype expression was high among individual C3H.SW immune mice (9 of 10 tested). To examine genetic requirements for idiotype expression, an immunization was performed using as responders CWB mice, congenic with C3H.SW but differing at the heavy chain allotype loci. Immune sera of individual CWB mice contained very little or no idiotype, demonstrating that levels of idiotype expression are influenced by allotype-linked genes, although the influence of other genes has not been ruled out. The 14-4-4S idiotype therefore represents a shared idiotype of anti-Ia antibodies and provides opportunities for analysis of the idiotypes of cellular receptors for the corresponding Ia antigen.

*Note added in proof:*

Of the anti-Ia.7 antibodies in a pool of C3H.SW anti-C3H alloantiserum, ~40%

share combining site-related idiotopes with 14-4-4S. This estimate was obtained by inhibition of antibody binding to Ia.7-positive cells by pig anti-idiotypic, followed by addition of fluoresceinated anti-mouse Ig and measurement of fluorescence intensity in the cell sorter. Thus, idiotope-positive antibodies constitute a major fraction of the response.

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