MONOCLONAL ANTIBODY AGAINST AN Ir GENE PRODUCT?*

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Immune response (Ir) loci that map in the I region of the H-2 gene complex regulate the capacity to generate an immune response against numerous foreign antigens (1). Loci that map in the I region also control polymorphic cell surface antigens that are expressed in immunocompetent cells (I-region-associated antigens or Ia loci) (2) and antigens that stimulate T cell proliferative responses between I region disparate strains (lymphocyte-activating determinants or Lad loci) (3).

Genetic recombination studies have shown that the I region can be divided into subregions, yet no crossover has yet been detected that allows separation of Ir loci from Ia loci or Lad loci. For example, antisera prepared between I-A-subregion-incompatible strains react with Ia-1 glycoproteins (4), Lad-1 -controlled antigens that stimulate T cell proliferative responses (5), and specifically block T cell responses to antigens under control of the Ir-1A locus (6). These observations have led to the tentative conclusion that the same locus controls all three traits. However, because the I-A subregion represents a segment of chromosome potentially comprised of several loci, it could be argued that conventional anti-I-A-subregion sera contain multiple specificities. More recent studies with an I-A-subregion mutant strain suggest that the Ia-1, Lad-1, and H-2A loci are identical (7).

Biochemical and functional studies have significantly advanced our understanding of the genetic control of Ia glycoproteins and support the notion that Ia loci, Lad loci, and Ir loci are equivalent. Antisera prepared between I-E-subregion-incompatible strains precipitate three glycoproteins, designated E_α (~33,000 mol wt), E_β (~30,000 mol wt), and I_i (~31,000 mol wt) (8). The E_α chain is controlled by a locus that maps in the I-E subregion, whereas the E_β chain is controlled by a locus that maps in the I-A subregion (9). We therefore prefer the designation A_e rather than E_β for this lower molecular weight chain. Whether the invariant I_i chain is controlled by a locus mapping in the I region or elsewhere in the genome remains to be determined. Two forms of the A_e chain have been detected biochemically (9). One form is found in the cytoplasm of lymphocytes from strains in which no E_α chain is detected. The second
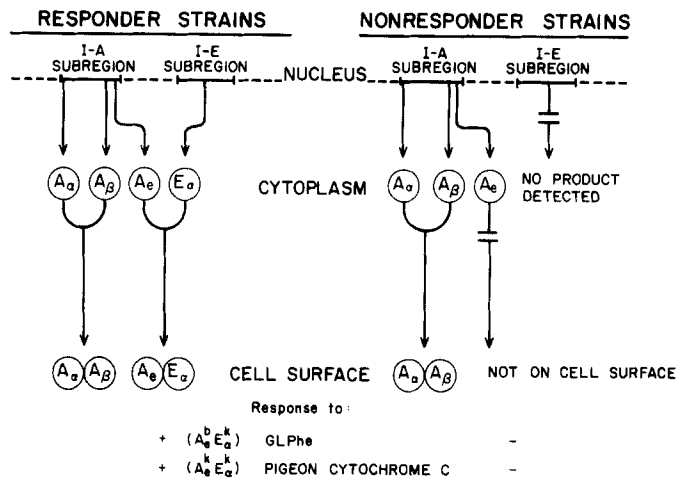
Fig. 1. Biochemically described products from the I-region of the H-2 complex. A_α and A_β represent glycoproteins that are encoded in the I-A subregion and are associated on the surface of B lymphocytes of all mouse strains. The A_e and E_α glycoproteins are encoded in the I-A and I-E subregions, respectively. Most strains that produce an E_α chain express this product in association with the A_e chain on the cell surface. Most strains that lack an E_α chain produce an A_e chain which can be found in the cell cytoplasm but not on the cell surface. Strains which have a surface A_e^b chain respond to GLPhe, whereas strains with a surface A_e^k chain respond to pigeon cytochrome c (responder strains). Strains which lack surface A_e chains do not respond to these antigens (nonresponder strains).

form is found on the cell surface of lymphocytes from strains which synthesize an E_α chain, and is associated with the E_α chain. These findings are summarized in Fig. 1.

In some cases, both *Ir* and *Lad* phenotypes are associated with the surface appearance of the $A_e:E_\alpha$ complex (9–11). Immune responses to the synthetic polypeptide antigen poly(L-glutamic acid,L-alanine,L-phenylalanine) (GLPHe)[1] correlate with the cell surface appearance of the A_e^b chain, whereas immune responses to pigeon cytochrome c correlate with the cell surface appearance of the A_e^k chain (10, 11). In addition, certain clones of alloreactive T cells appear to recognize and are stimulated by the surface form of the A_e chain (12). Taken together, these observations provide additional support for the notion that *Ia* antigens in general, and the $A_e:E_\alpha$ complex in particular, are products of *Ir* loci and serve as *Lad* antigens. Evidence that bolsters this hypothesis is presented here.

We have produced a monoclonal antibody that recognizes a conformational or combinatorial determinant formed by certain $A_e:E_\alpha$ complexes. This monoclonal antibody blocks mixed lymphocyte cultures (MLC) directed at the $A_e:E_\alpha$ complex and blocks T cell responses to antigens (GLPhe and pigeon cytochrome c) where the response is dependent on interaction between *Ir* loci that map in the I-A and the I-E

subregions. Thus, these experiments constitute the strongest evidence to date that at least some *Ia* antigens are products of *Lad* and *Ir* loci, and that the *Ia* loci, *Ir* loci, and *Lad* loci are identical.

Materials and Methods

Mice. (BALB/c × C57BL/6)F$_1$ (CB6F$_1$) mice and the B10.A, B10.A(5R) and (B10 × B10.A)F$_1$ mice used in the peritoneal-exudate-T lymphocyte-enriched subpopulation (PETLES) experiments were obtained from The Jackson Laboratory, Bar Harbor, Maine. All other strains were bred and maintained in our own animal facilities at the Yale University School of Medicine, New Haven, Conn.

Antisera. Rabbit anti-mouse immunoglobulin (MIg) was prepared by injecting rabbits with DEAE-cellulose purified MIg in complete Freund's adjuvant intraperitoneally followed by intravenous boosting with alum-precipitated MIg. Anti-Thy-1.2 was prepared as previously described (13). Anti-I-E^k, I-C^{d}S^{d}G^d serum was produced by hyperimmunizing (B10 × HTI)F$_1$ recipients with a mixture of B10.A(3R) lymph node and spleen cells. Monoclonal anti-I-A^k was obtained from the hybridoma cell line 10-2.16 (Oi et al. [14]) provided by the Cell Distribution Center at the Salk Institute, La Jolla, Calif.

Fluorescent Reagents. Fluorescein isothiocyanate (FITC) conjugation of the appropriate antibodies was performed as described by Johnson et al. (15). To conjugate monoclonal antibody Y-17 with biotin (J. W. Goding. Personal communication.), the antibody was adjusted to 1 mg/ml in 0.1 M NaHCO$_3$, pH 8.4. Biotin N-hydroxy-succinimide ester was dissolved in DMSO at 1 mg/ml and mixed with Y-17 in a ratio of 1:8 for 4 h. Uncoupled biotin was removed by dialysis against phosphate-buffered saline (PBS). Fluorescein- and rhodamine-coupled avidin, the second-step reagents for biotin-labeled antibodies, were obtained from Vector Labs, Burlingame, Calif.

Cell Sorting. A fluorescence-activated cell sorter (FACS II; BD FACS Systems, Mt. View, Calif.) was used to examine antigens on spleen cell surfaces.

Preparation of Lymphocyte Subpopulation. T lymphocytes were prepared by passage over Ig anti-Ig columns (16). They were >90% Thy-1.2 positive and <3% Ig positive by direct immunofluorescence. The B cells used in the FACS analysis were obtained by treating spleen cells with anti-Thy-1.2 and guinea pig complement to remove the T cells.

Immunization and Fusion Protocol. B10.A(5R) spleen cells, suspended at 5 × 10^6 cells/ml in EHAA medium (17) supplemented with 5% fetal calf serum (FCS), 50 IU/ml penicillin, and 50 µg/ml streptomycin, were stimulated with 5 µg/ml concanavalin A (Con A) (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) and 1 × 10^7 cells in complete Freund's adjuvant were injected into the foot pads and peritoneal cavity of BALB/cByJ mice. 3 wk later, the mice were given an intravenous boost of 2 × 10^7 spleen cells that had been similarly stimulated with Con A in medium that contained 0.5% normal mouse serum (NMS) in place of FCS. 3 d after boosting, their spleens were removed and the cells fused with the nonsecreting myeloma SP 2/0 Ag14 (18) by the procedures of Oi and Herzenberg (19) and Kennett et al. (20). Spleen and myeloma cells were mixed in a ratio of 1:3 and fused with 50% polyethylene glycol 1500 (PEG). After washing to remove the PEG, the pellet was resuspended to a concentration of 1 × 10^7 total cells/ml in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/liter glucose (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% NCTC 109 (Microbiological Associates, Walkersville, Md.), 15% FCS, 10 mM Hepes, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin, 0.25 µg/ml amphotericin, and Littlefield's (21) concentrations of hypoxanthine (1.0 × 10^{-4} M) and thymidine (1.6 × 10^{-5} M). 100 µl of the suspension was added to wells of flat-bottomed microtiter plates (3040; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). The hybrids were fed the above media supplemented with aminopterin, 8 × 10^{-7} M, the day after fusion and then every 3–4 d until the clones were screened.

Screening Procedures. Supernates from wells with macroscopic clones were screened using a microcytotoxicity assay described previously (22) and by a radioimmunoassay (23). In the latter method, the binding sites of flexible polyvinyl microtiter plates (Cooke Engineering Co., Alexandria, Va.) were blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at 4°C.

The plates were washed three times with PBS and 50 μl of supernate from the corresponding wells of the culture plate were added. Spleens from the appropriate test strains were removed and the erythrocytes lysed by hypotonic shock. 10 μl of spleen cells suspended at 5×10^7 cells/ ml in PBS with 5% FCS and 0.1% sodium azide were layered over the supernates and allowed to incubate for 1 h at 4°C. The plates were washed by centrifugation for 2 min at 400 g, flicking out the supernate, filling the wells with PBS followed by a second spin and flick. The pellets were resuspended by vortexing and 25,000 cpm of ^{125}I-protein A (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) in 50 μl of PBS was then added. After an additional 1 h at 4°C, the plates were washed six times as above and dried under a heat lamp. Radioautography (24) was performed with Kodak RPR x-ray film (Eastman Kodak Co., Rochester, N. Y.) and an intensifier screen (Cronex Lightning Plus, E. I. DuPont de Nemours & Co., Wilmington, Del.) mounted on the bottom of the plate. The films were developed after a 12-h exposure at −70°C. Positive wells appeared as dark spots against a faint background. To obtain more quantitative information, the wells were cut out and counted in a gamma counter (Ultrogamma 1280; LKB Produkter, Bromma, Sweden).

Purification of Monoclonal Antibody. Cells from one of the wells, termed Y-17, whose supernate was active in both the cytotoxicity and cell-binding screening procedures, were cloned by limiting-dilution. Supernates from all of the wells with subsequent growth showed activity. Cultures were expanded, and cells were frozen for future use or were injected into the peritoneal cavities of Pristane-treated BALB/c mice to generate ascites. Antibody from the ascites fluid was isolated with two successive precipitations using 45% saturated ammonium sulfate followed by dialysis against PBS. (Precipitation with sodium sulfate resulted in the complete loss of antibody activity.)

Determination of Immunoglobulin Class. The antibody fraction obtained above was further purified by IgG class specific elution from a Protein A-Sepharose column (25). The fractions were monitored with a Uvicord spectrophotometer (LKB Produckter) and collected. They were assayed for activity as described and subjected to Ouchterlony analysis against class-specific goat anti-MIg (Meloy Laboratories Inc., Springfield, Va.). The antibody was found to be complement fixing, protein A binding, and of the IgG2b glass by both protein A-Sepharose chromatography and Ouchterlony analysis.

Radiolabeling, Detergent Extraction, Immunoprecipitation, and Two-Dimensional Polyacrylamide Gel Electrophoresis (2-D PAGE) of Ia Antigens. Proteins were immunoprecipitated from Nonidet-P40 detergent extracts of [^{35}S]methionine-labeled spleen cells according to published procedures (8). Extract from 10^7 cells was mixed with 100 μl of Y-17 culture supernate or with 30 μl of one of the following sera: BALB/c normal mouse serum, C3H anti-CSW (*anti-H-2^b*), or B10.A(18R) anti-B10.A(5R) (*anti-J^{k}E^{k}C^{d}S^d*). Antigen-antibody complexes were isolated using *Staphylococcus aureus* bacterial adsorbent (IgG Sorb; The Enzyme Center, Boston, Mass.). Immunoprecipitated proteins were separated by 2-D PAGE, using nonequilibrium pH gradient electrophoresis (8, 26) for the charge separation in the first-dimension gels. After the second dimension separation by sodium dodecyl sulfate (SDS) slab gel electrophoresis, the gels were processed for radioautography using Kodak NS-2T No Screen x-ray film.

MLC. MLC were performed as described previously (27). Briefly, responder T cells were prepared by passing spleen cells over Ig anti-Ig columns. Stimulator spleen cells were treated with mitomycin C, washed thoroughly, and incubated with Y-17 or medium alone for 1 h at 37° C. 5×10^5 or 10^6 responder and 5×10^5 stimulator cells were cultured in triplicate, with 0.2 ml of EHAA medium supplemented with 0.5% fresh NMS or 5% FCS in Falcon 3040 microtiter plates. Cultures were pulsed after 5 d with 1 μCi of [^{3}H]thymidine ([^{3}H]TdR; 50–60 Ci/mmol; New England Nuclear, Boston, Mass.) for 5 h. The cells were harvested onto glass fiber strips with a MASH II harvester (Microbiological Associates) and samples counted in a scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). The data are expressed as mean counts per minute; SEM were <10%.

Effect of Monoclonal Antibody on T Cell-proliferative Response to Antigen

ANTIGENS.   Pigeon cytochrome *c* was purchased from the Sigma Chemical Corporation, St. Louis, Mo. and, subsequently purified by column chromatography on carboxymethyl cellulose to remove contaminants such as polymeric and deamidated forms of the molecule (28).

GLPhe was originally purchased from Miles-Yeda (Rehovot, Israel) and was the generous

gift of Dr. Alan Rosenthal, Merck, Sharp and Dohme, Rahway, N. J. The branched-chain synthetic amino acid polymer poly(tyrosine, glutamic acid)-D,L-alanine--L-lysine [(T,G)-A--L] (lot MC6) was also purchased from Miles-Yeda, and was the generous gift of Drs. Howard Dickler and Alfred Singer, National Cancer Institute, National Institutes of Health, Bethesda, Md.

IMMUNIZATIONS.   Mice were immunized in the hind foot pads with 30 μg GLPhe, 50 μg (T,G)-A--L, or 30 μg pigeon cytochrome c emulsified 1:1 in complete Freund's adjuvant that contained 1 mg/ml of killed *Mycobacterium tuberculosis*, strain H37 Ra (Difco Laboratories, Detroit, Mich.) in a total vol/animal of 0.1 ml.

CELL CULTURES.   The preparation of PETLES and their in vitro culture with antigen are described in detail elsewhere (29). Briefly, 100 μl of Eagle's high amino acid (EHAA) medium supplemented with 10% FCS and that contained 1×10^6–2×10^6 PETLES were placed in each well of a sterile, U-bottomed microculture plate. To assay lymph node proliferation, draining inguinal and popliteal lymph nodes were aseptically removed from animals 8 d after immunization, teased, screened to form a single cell suspensions, and passed over nylon wool columns in medium that contained 5% FCS. Eluted cells were then plated in 100 μl at 4×10^5 cells/well in flat-bottomed 96-well microtiter plates in EHAA medium supplemented with 10% FCS.

An SAS precipitate of ascites containing Y-17 at 12 mg/ml in 0.1% sodium azide was diluted 1:20 in complete medium supplemented with 10% FCS and then exhaustively dialyzed to remove the azide. To assess blocking of antigen-induced T cell proliferation, 40 μl of the diluted and dialyzed monoclonal antibody was added to wells, which then received either antigen or medium alone, to achieve a final dilution of Y-17 of 1% (vol:vol). The antigens were then added to the cultures to achieve the following final concentrations in a total vol of 200 μl/well: GLPhe, 50 μg/ml; pigeon cytochrome c, 30 μg/ml; (T,G)-A--L, 50 μg/ml; purified protein derivative from *M. tuberculosis* (PPD) (Connaught Medical Research Labs, Willowdale, Ontario, Canada), 20 μg/ml. Medium without antigen served as a nonstimulated control.

The cultures were then incubated at 37° for 5 d. Stimulation was assessed by measuring the incorporation of a 1-μCi pulse of [^{3}H]TdR 16–18 h before harvesting the cultures. Determinations were done in triplicate and the data expressed as the difference between antigen-stimulated and nonstimulated responses (Δcpm). The standard errors for the Δcpm values were derived from the square root of the sum of the squares of the standard errors of the mean antigen-stimulated and nonstimulated responses. Percent inhibition by Y-17 was calculated by dividing the Δcpm in the presence of Y-17 by the Δcpm in its absence, subtracting the ratio from 1 and multiplying by 100.

To assess the effect of Y-17-plus-complement-induced killing on subsequent antigen-induced proliferation, nylon wool-purified, antigen-primed lymph node cells were incubated in a 1:20 dilution of the monoclone at 10^8 cells/ml for 30 min at 4°C. The cells were then washed twice and incubated at the same concentration in a 1:10 dilution of rabbit complement at 37°C for another 30 min. The cells were washed twice again, resuspended in EHAA medium with 10% FCS, and added to microtiter plates at 4×10^5 living cells (assessed by trypan blue dye exclusion) per well, either in the presence of antigen or of medium alone.

Results

Y-17 Reacts with an H-2-controlled Antigen (Table I).   As judged by both cytotoxicity and radioimmunoassay, Y-17 reacts with immunizing strain B10.A(5R) spleen cells (50% titer, $\sim 10^5$) but not recipient strain BALB/c spleen cells (Table I). Roughly 55% of splenic lymphocytes are lysed in the presence of complement. To determine whether Y-17 reacts with an H-2- or non-H-2-controlled antigen, strain B10.D2 was tested. This strain shares the H-2^d haplotype with BALB/c and the non-H-2 B10 background with B10.A(5R). Failure of Y-17 to react with B10.D2 maps the locus that controls the determinant to the H-2 complex. (Table I).

Y-17 Reacts with an I-Region-controlled Determinant (Table II).   As shown in Table II, Y-17 reacts with only two (H-2^k and H-2^r) of the eight independent H-2 haplotypes tested. Positive reactions with recombinant strains B10.A, A.TL, and B10.AQR, and

TABLE I

Y-17 Reacts with an H-2-controlled Antigen

Strain	H-2 haplotype	50% titer*	
		Cytotoxic	RIA
BALB/c	d	0	0
B10.A(5R)	i5	10⁵	10⁵
B10.D2	d	0	0

* Spleen cells tested with Y-17 using assays described in Materials and Methods.

TABLE II

Y-17 Reacts with an I-region-controlled Antigen

A. Independent H-2 haplotypes

Strain	H-2 haplotype	K	A	B	J	E	C	S	G	D	50% titer	Ae:Eα complex expressed on surface
B10, A.BY, BALB.B	b	b	b	b	b	b	b	b	b	b	0	—
B10.D2, BALB/c	d	d	d	d	d	d	d	d	d	d	0	d:d
B10.M, A.CA	f	f	f	f	f	f	f	f	f	f	0	—
B10.BR, BALB.K, CBA/N	k	k	k	k	k	k	k	k	k	k	10⁵	k:k *
B10.P	p	p	p	p	p	p	p	p	p	p	0	p:p
B10.G	q	q	q	q	q	q	q	q	q	q	0	—
B10.RIII	r	r	r	r	r	r	r	r	r	r	10⁵	r:r
B10.S, A.SW	s	s	s	s	s	s	s	s	s	s	0	—

B. Recombinant H-2 haplotypes

Strain	H-2 haplotype	K	A	B	J	E	C	S	G	D	50% titer	Ae:Eα complex expressed on surface
B10.A, A/Sn	a	k	k	k	k	k	d	d	d	d	10⁵	k:k
A.AL	a1	k	k	k	k	k	k	k	k	d	10⁵	k:k
C3H.OL	o1	d	d	d	d	d	d	k	k	k	0	d:d
C3H.OH	o2	d	d	d	d	d	d	d	k	k	0	d:d
B10.A(2R)	h2	k	k	k	k	k	d	d	d	b	10⁵	k:k
B10.A(4R)	h4	k	k	b	b	b	b	b	b	b	0	—
B10.A(3R)	i3	b	b	b	b	k	d	d	d	d	10⁵	b:k
B10.A(5R)	i5	b	b	b	k	k	d	d	d	d	10⁵	b:k
B10.A(18R)	i18	b	b	b	b	b	b	b	?	d	0	—
A.TL	t1	s	k	k	k	k	k	k	k	d	10⁵	s:k
B10.S(7R), A.TH	t2	s	s	s	s	s	s	s	s	d	0	—
B10.HTT	t3	s	s	s	s	k	k	k	k	d	10⁴-10⁵	s:k
B10.S(9R)	t4	s	s	.	k	k	d	d	d	d	10⁴-10⁵	s:k
B10.S(8R)	as1	k	k	.	.	s	s	s	s	s	0	—
B10.AQR	y1	q	k	k	k	k	d	d	d	d	10⁵	k:k
B10.T(6R)	y2	q	q	q	q	q	q	q	.	d	0	—
B10.F(13R)	?	n	n	n	n	n	n	b	b	b	0	n:n
A.TFR5	ap5	f	f	.	.	k	k	k	k	d	0	-:k

* Boxed Ae:Eα complexes associated with killing of cells with Y-17 plus complement.

negative reactions with recombinant strains A.TH, B10.S(7R), and B10.T(6R), localize control of this determinant between the K region and I-C subregion. Based on current Ia specificity charts with independent H-2 haplotypes, the specificity detected by Y-17 is similar to Ia.19 (30). However, because the Ia.19-positive recom-

binant strains B10.A(4R) and B10.S(8R) do not react with Y-17, we designate this new specificity Ia.m.44 (P. Demant. Personal communication.).

Y-17 Reacts with an Antigen That is Under Two-Gene Control (Table III). At first glance, data presented in Table II suggested that Y-17 reacts with an I-E^k-subregion-controlled antigen. However, the observation that strain A.TFR5, which carries the I-E^k subregion and expresses low but detectable levels of E$_\alpha^k$ chains (31), failed to react with our monoclonal antibody raised the possibility that Y-17 was specific for a hybrid antigen. To test this possibility, we tested Y-17 on cells from several F$_1$ hybrid crosses prepared between strains, neither of which reacted with Y-17.

For example, strains B6 and BALB/c both failed to react with Y-17. In contrast, a strong reaction was observed with CB6F$_1$ hybrids (Table III). A similar result was obtained with several other F$_1$ hybrids, including (B10.S[7R] × A.TFR5)F$_1$. However, (B10.T[6R] × A.TFR5)F$_1$ animals did not react with Y-17, demonstrating that gene interaction between non-H-2-linked genes can not account for expression of the determinant detected by Y-17. We conclude that Y-17 reacts with a determinant whose expression is generated by interaction between two *I* region loci.

Evidence That Y-17 Reacts with Certain A$_e$:E$_\alpha$ Complexes (Fig. 2). To determine the biochemical nature of the determinant detected by Y-17, we labeled B6, BALB/c and CB6F$_1$ spleen cells biosynthetically with [^{35}S]methionine, and examined the molecules

TABLE III

Y-236 Reacts with an Antigen That Is Under Two-Gene Control

Strain	Allele expression*				50% titer	A$_e$:E$_\alpha$ complex detected
	A$_e$ chain		E$_\alpha$ chain			
B6	b	Cytoplasm	—	None	0	—
BALB/c	d	Surface	d	Surface	0	—
F$_1$	b,d	Surface	d	Surface	10^5	b:d
B10.S	s	Cytoplasm	—	None	0	—
B10.D2	d	Surface	d	Surface	0	—
F$_1$	s,d	Surface	d	Surface	10^5	s:d
B10.A(4R)	k	Cytoplasm	—	None	0	—
A.TFR5	—	None	k	Surface	0	—
F$_1$	k	Surface	k	Surface	10^5	k:k
B10	b	Cytoplasm	—	None	0	—
A.TFR5	—	None	k	Surface	0	—
F$_1$	b	Surface	k	Surface	10^5	b:k
B10.S(7R)	s	Cytoplasm	—	None	0	—
A.TFR5	—	None	k	Surface	0	—
F$_1$	s	Surface	k	Surface	10^5	s:k
B10.T(6R)	—	None	—	None	0	—
A.TFR5	—	None	k	Surface	0	—
F$_1$	—	None	k	Surface	0	—

* E$_\alpha^{d,k,p,r}$ chains, but not E$_\alpha^{b,f,q,s}$ chains detected serologically and biochemically. A$_e^{b,d,k,p,r,s}$ chains, but not A$_e^{f,q}$ chains detected biochemically. A$_e^{b,d,k,p,r,s}$ chains found in the cytoplasm of cells from strains carrying I-E^{b,f,q,s} and on the cell surface of strains carrying I-E^{d,k,p,r}. Quantitatively less E$_\alpha^k$ chains found on the cell surface in strains carrying I-A^{f,q}.
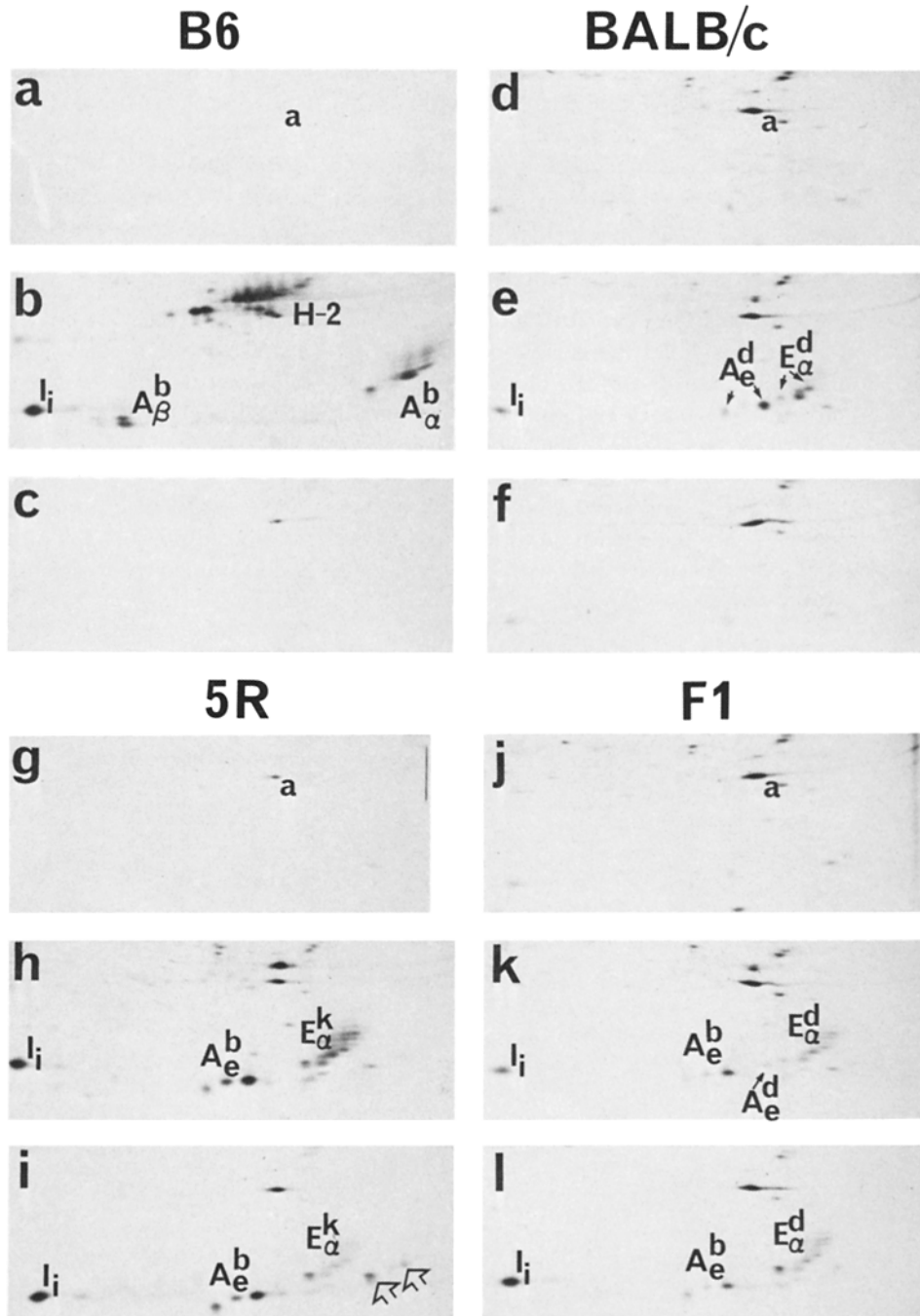
Fig. 2.   2-D PAGE analysis of *Ia* immunoprecipitates. Extracts from [^{35}S]methionine-labeled spleen cells were immunoprecipitated with normal mouse serum (a, d, g, and j), C3H anti-CSW (anti-H-2^b) (b), B10.A(18R) anti-B10.A(5R) (anti-J^{k}E^{k}C^{d}S^d) (e, h, and k), and Y-17 supernate (c, f, i, and l). Each panel represents the immunoprecipitate from the extract of 4 × 10^6 cells. Radioautographic exposures of the gels were for 21 d. The position of actin is indicated by the letter "a" in the upper panels. The identity of the polypeptide chains is indicated, based on previous findings (8, 9). The significance of the spots marked by the large arrows in i is discussed in the Results.

precipitated from cell lysates with Y-17 by 2-D PAGE (Fig. 2). No molecules were precipitated from B6 or BALB/c lysates; thus, Y-17 does not react with A_α^b, A_β^b, or the cytoplasmic form of the A_e^b chain (B6), or the cell surface form of the A_e^d chain associated with the E_α^d chain (BALB/c), or I_i chains from either strain. In contrast, the E_α^d chain, the cell surface form of the A_e^b chain, and the I_i chain were precipitated from CB6F$_1$ lysates. Because I_i chains were found in all Ia precipitates (8), these results demonstrate that Y-17 reacts with a determinant formed by the association of A_e^b and E_α^d.

In sum, Y-17 reacts with a conformational or combinatorial determinant formed by the following A_e:E_α complexes: A_e^b:E_α^d (e.g., CB6F$_1$); A_e^b:E_α^k (e.g., B10.A[5R] and [B10 × A.TFR5]F$_1$); A_e^k:E_α^k (e.g., B10.A and [B10.A(4R) × A.TFR5]F$_1$); A_e^r:E_α^r (e.g., B10.R111); A_e^s:E_α^k (e.g., B10.HTT, [B10.S(7R) × A.TFR5]F$_1$); and A_e^s:E_α^d (e.g., [B10.S × B10.D2]F$_1$). Y-17 does not react with the cytoplasmic form of the A_e^b (e.g., B10), A_e^k (e.g., B10.A[4R]), or A_e^s (e.g., B10.S[7R]) chains, the cell surface form of free E_α^k chains (e.g., A.TFR5), or complexes formed by A_e^d:E_α^d (e.g., BALB/c and B10.D2), or A_e^p:E_α^p (e.g., B10.P) chains.

In Addition to the A_e and E_α chains, Y-17 Precipitates a Third I-Region-controlled Molecule (Fig. 2). Studies of precipitates obtained from CB6F$_1$ hybrids suggested that a third I-region-controlled molecule was precipitated by Y-17. Because relatively low amounts of this molecule were precipitated in heterozygous animals, we examined lysates from homozygous B10.A(5R) and B10.A animals. A_e^b, E_α^k, and molecules that exhibited a 2-D PAGE pattern indistinguishable from A_α^b were precipitated with B10.A(5R) lysates (Fig. 2). A_e^k, E_α^k, and molecules that exhibited a 2-D PAGE pattern indistinguishable from A_α^k, were precipitated with B10.A lysates (data not shown). No A_β chains were detected in either precipitated lysate. Note that conventionally prepared anti-I-E-subregion sera precipitated only the A_e and E_α chains. The third chain, therefore, does not nonspecifically associate with the A_e:E_α complex during the extraction procedure. Although it is clear that this third chain is controlled by an H-2-linked locus (2-D PAGE patterns from B10.A(5R) and B10.A differ), we are not sure whether the third chain is the A_α chain or a new Ia molecule (X).

Y-17 Reacts with a Subset of B Lymphocytes and with Non-T and Non-B Cells (Table IV and Fig. 3). Previous studies have shown that anti-I-A or anti-I-E sera react with >95% of splenic B lymphocytes (32), and that the A_α, A_β, E_α, and A_e chains are synthesized by these B lymphocytes (33). In contrast, the maximum percent of spleen cells killed or stained by Y-17 was always less than the number of cells killed by conventional anti-I-E reagents, a monoclonal anti-I-A antibody, or stained with FITC-anti-MIg. Representative data for spleen cells from individual mice are shown in Table IV. Furthermore, spleen cells from 5R mice stained with both tetramethyl rhodamine isothiocyanate (TRITC)-anti-mouse μ-chain antibody and biotin-conjugated Y-17 plus FITC-conjugated avidin revealed ~20% of μ-chain-bearing cells that did not bind Y-17, and occasional (~5%) cells that were Y-17 positive, μ-chain negative.

In addition to cytotoxicity and conventional fluorescence microscopy, a FACS was used to examine anti-Thy-1.2-and-complement-treated spleen cells from a pool of five CB6F$_1$ mice. As shown in Fig. 3, cells to the right of the arrow were positive by FACS analysis. By these criteria, 3% of the unstained cells had fluorescent intensities above background levels and 82% of the cells labeled with anti-MIg were positive. Only 54% of these same cells that had been stained with Y-17 fell above this fluorescence

TABLE IV

Y-17 Reacts with a Subset of B Cells

Spleen cells from*	Percent spleen cells killed by‡				Percent spleen cells stained by	
	Y-17	Anti-I-A§	Anti-I-E‖	C' alone	Anti-MIg	Y-17
CB6F₁	40	—	63	6	70	55
	38	—	64	6	65	48
B10.A(5R)	60	—	68	5	70	60
	52	—	64	6	73	54
B10.A	60	72	72	6	71	62
	52	58	61	6	61	46

* Spleen cells from individual mice centrifuged on Ficoll-Hypaque.
‡ Plateau cytotoxic kill using rabbit complement (C').
§ Hybrid 10-2.16, anti-I-A^k (anti-Ia-17).
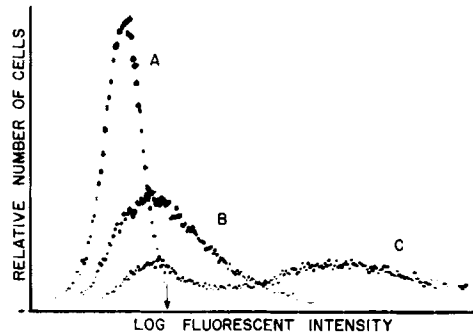‖ (B10 × HTI)F₁ anti-B10.A(3R) (anti-E^{k}C^{d}S^{d}G^d).

FIG. 3. FACS profile of Y-17 fluorescent anti-Thy-1.2-treated spleen cells. Viable anti-Thy-1.2 + C'-treated CB6F₁ spleen cells were examined unstained (A), stained with biotin-Y-17 followed by FITC-avidin (B), or stained with FITC-anti-MIg (C). Arrow gives threshold selected to discriminate positive from negative cells.

threshold, although no clear separation of Y-17-positive from Y-17-negative B cells was seen using the FACS. All of these results taken together support the conclusion that the determinant detected by Y-17 is expressed on a subset of B lymphocytes. An alternate interpretation is that all B cells bear the determinant detected by Y-17, but as indicated by the FACS profile, some of these cells have too little antigen on their surface to be killed or visually stained by Y-17.

It is known that some T cells (34–36) and some spleen cells (which lack both B and T cell markers) (37) react with anti-I-A or anti-I-E subregion sera (38). In two experiments, column-purified T cells were examined with FITC-anti-Thy-1.2, FITC-anti-MIg, and biotin-conjugated Y-17 followed by the addition of FITC- or rhodamine-labeled avidin. In the first experiment, 94% of the cells were Thy-1.2 positive, <1% Ig positive, and 7% of the total cells were stained with the monoclonal antibody. In the second experiment, in which B cell contamination was 3%, the cells were double-labeled with FITC-anti-Thy-1.2 and a rhodamine-avidin-biotin-Y-17 complex. These cells showed 91% stained only with anti-Thy-1.2, 6% only with Y-17, and

none with both conjugates. Y-17 therefore appears to react with a Thy-1.2-negative and Ig-negative set of cells as well as with B cells. We have not detected the antigen on T cells.

C57BL/6 Anti-B10.A(5R) and B10.A(4R) Anti-B10.A(2R) MLC Responses Are Blocked by Y-17 (Fig. 4 and Table V).   I-region-encoded determinants, especially in I-A and I-E have been shown to stimulate in MLC (3, 5). Recently, Fathman et al. (12) have observed a mixed lymphocyte reaction (MLR) apparently dependent on expression of the $A_e:E_\alpha$ complex on the stimulator cells. We have examined the ability of Y-17 to block the MLR between responding T cells from B6 mice which do not express the cell surface form of the A_e^b chain and stimulator 5R spleen cells which express the $A_e^b:E_\alpha^k$ complex on the cell surface. 5R also differs from B6 at the I-J and I-C
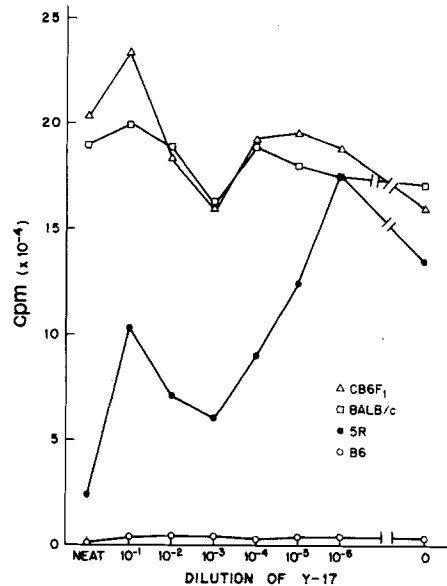

Fig. 4.   Y-17 specifically blocks B6 anti-B10.A(5R) (5R) MLC. An MLC between B6 responder T cells and mitomycin-C-treated spleen cells from CB6F₁, BALB/c, 5R, and syngeneic B6 animals was performed in the presence of various dilutions of Y-17. The response to 5R, but not that to other stimulator cells was inhibited by Y-17.

TABLE V

Y-17 Inhibits the Response of B10.A(4R) T Cells to B10.A(2R) Stimulators

Stimulator spleen cells	MHC			$A_e:E_\alpha$ complex expressed on cell surface	MLC response with Y-17 at dilution*					Block?
	I		S G D		Control	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
	K	A B J E C								
B10.A(4R)	k	k b b b b	b b b	—	(5,800)	(7,400)	(7,000)	(8,900)	(6,400)	negative control
B10.A(2R)	k	k k k k d	d d b	k:k	22,200	−800	−600	3,100	25,800	yes
B10.A(5R)	b	b b k k d	d d d	b:k	266,600	302,700	216,900	210,600	272,200	no
B10	b	b b b b b	b b b	—	101,300	179,200	152,700	121,200	165,800	no

* Control counts in groups with B10.A(4R) stimulators have been subtracted from experimental counts.

subregions and the D end of the H-2 complex. The results in Fig. 4 show that Y-17 blocks ~66% of the B6 response to 5R out to a dilution of 10^{-4}. The responses to BALB/c and to CB6F$_1$ are not affected, even though Y-17 reacts with A$_e^b$:E$_\alpha^d$ complexes on the CB6F$_1$ stimulator cells. This serves as an important control, because it suggests that Y-17 is not simply killing stimulator cells. Moreover, Y-17 completely inhibits the response of B10.A(4R) T cells to B10.A(2R) stimulators (Table V). B10.A(2R) expresses the A$_e^k$:E$_\alpha^k$ complex on the cell surface, whereas B10.A(4R) has the A$_e^k$ chain in the cytoplasmic form only. The response of B10.A(4R) to B10.A(5R) is not affected by Y-17 even though Y-17 can bind to the stimulator cell. This result could be explained by proposing that the B10.A(4R) anti-B10.A(5R) MLR is directed primarily against the A$_\alpha^b$:A$_\beta^b$ complex, whereas the B6 anti-5R MLR is directed primarily against the A$_e^b$:E$_\alpha^k$ complex. Thus, Y-17 specifically inhibits responses directed primarily at A$_e^b$:E$_\alpha^k$ complexes and not A$_\alpha^b$:A$_\beta^b$ complexes.

Y-17 Inhibits Proliferative Responses to GLPhe and Pigeon Cytochrome c. The immune responses to the synthetic polypeptide GLPhe and the protein antigen pigeon cyto-chrome *c* are controlled by interacting loci that map in the I-A and I-E subregions. The appearance of the A$_e^b$:E$_\alpha^k$ or the A$_e^k$:E$_\alpha^k$ complex on the cell surface correlates with the ability to respond to GLPhe (10) or pigeon cytochrome *c* (11), respectively. Because Y-17 recognizes both of these chain combinations, we examined the ability of Y-17 to block antigen-induced proliferation of PETLES or nylon wool-passed lymph node T cells (Table VI). The proliferative responses to GLPhe and pigeon cytochrome *c* were consistently inhibited 80–100% in the presence of 1% Y-17. Y-17 had no effect on the proliferative response to (T,G)-A--L and no significant effect on the medium controls, demonstrating that the antibody was not nonspecifically inhibitory. The proliferative response to PPD was repeatedly inhibited by 10–30% in the presence of Y-17. This result is consistent with our previous blocking studies using anti-Ia.7 sera (38) and suggests that the weak inhibition of the proliferative response to PPD represents the involvement of A$_e$:E$_\alpha$ complexes in the presentation of some determinants on this antigen. In contrast, the complete absence of any significant inhibition of proliferation to (T,G)-A--L, the response to which is under the control of an Ir gene(s) mapping in the I-A subregion, indicates that the A$_e^b$:E$_\alpha^k$ complex is not involved in the presentation of this antigen.

Pretreatment of the responding B10.A(5R) lymph node cells with a 1:20 dilution of Y-17 plus rabbit complement before culture reduced the proliferative responses to (T,G)-A--L, PPD, and GLPhe by 50–70% (data not shown), suggesting that the specific inhibition seen in the blocking experiments could not be attributed to selective elimination of an antigen-presenting cell subpopulation. Thus, Y-17 inhibits only those antigen-specific proliferative responses controlled by interacting Ir genes that map in the I-A and I-E subregions. These results are the first unequivocal demonstra-tion in the antigen-specific proliferative assay that the inhibitory component in blocking antisera is, in fact, an Ia-specific antibody rather than an antibody directed against either a closely-linked Ir gene product or against the actual T cell receptor for self-Ia (39). These results therefore strongly suggest that Ia molecules are in fact the Ir gene products.

Discussion

We have produced a monoclonal antibody, Y-17, which reacts with a conforma-tional or combinatorial determinant formed by the association of the I-A-subregion-

TABLE VI

Y-17 Inhibits the T Lymphocyte Proliferative Response to Pigeon Cytochrome c and GLPhe

Stimulating antigen	PETLES proliferative response									Lymph node T cell		
	B10.A(5R)			B10.A			(B10.A × B10)F₁			B10.A(5R)		
	No Y-17	1% Y-17	Inhibition	No Y-17	1% Y-17	Inhibition	No Y-17	1% Y-17	Inhibition	No Y-17	1% Y-17	Inhibition
	Δcpm ± SEM		%	Δcpm ± SEM		%	Δcpm ± SEM		%	Δcpm ± SEM		%
Pigeon cytochrome c	—	—	—	12,900 ± 1,200	1,300 ± 300	90	19,800 ± 900	1,400 ± 900	93	—	—	—
GLPhe	118,700 ± 2,600	22,700 ± 3,200	81	—	—	—	24,000 ± 2,100	2,100 ± 1,700	91	125,500 ± 5,000	5,000 ± 800	96
PPD	31,700 ± 2,400	25,700 ± 400	20	13,800 ± 700	12,000 ± 800	13	43,700 ± 1,100	32,200 ± 880	26	112,000 ± 2,500	79,500 ± 1,500	29
(T,G)-A--L	48,900 ± 1,600	47,800 ± 1,300	2	—	—	—	8,800 ± 1,400	8,200 ± 1,600	7	29,200 ± 5,000	29,600 ± 200	0

1.5 × 10⁶ B10.A(5R), 2 × 10⁶ B10.A, or 1 × 10⁶ (B10.A × B10)F₁ PETLES or 4 × 10⁶ nylon wool-passed B10.A(5R) lymph node cells from mice primed with pigeon cytochrome c, GLPhe, or (T,G)-A--L in CFA were cultured for 5 d in the presence or absence of 1% (vol:vol) Y-17. Stimulation by antigen in culture is expressed as Δcpm [³H]TdR incorporated as described in materials and methods. The percent inhibition was calculated as described in Materials and Methods. In these experiments, the medium controls were: B10.A(5R) + no Y-17: 4,024 ± 890, + 1% Y-17: 2,257 ± 385; B10.A + no Y-17: 1,590 ± 329, + 1% Y-17: 1,093 ± 44; (B10.A × B10)F₁ + no Y-17: 2,425 ± 659, + 1% Y-17: 1,279 ± 545. For the pigeon cytochrome c stimulation of (B10.A × B10)F₁ cells, the PETLES were cultured at 2 × 10⁵/well and the medium controls were: + no Y-17: 6,878 ± 528, + 1% Y-17: 4,854 ± 756. In the experiment with B10.A(5R) lymph node cells the medium controls were: + no Y-17: 3,415 ± 411, + 1% Y-17: 3,865 ± 101.

controlled A_e chain and the I-E-subregion-controlled E_α chain (Fig. 2). Cytotoxic, conventional fluorescence, and FACS studies suggest that the determinant recognized by Y-17 is expressed on a subpopulation of splenic B cells as well as on non-T and non-B splenic cells. In functional experiments, Y-17 blocks MLC between responder T cells from strains that lack cell surface A_e:E_α complexes and stimulator cells from strains which express this complex. In addition, Y-17 completely inhibited the antigen-induced T cell proliferative response to GLPhe and pigeon cytochrome c; these responses are under dual-Ir-gene control and correlate with the expression of certain A_e:E_α complexes on antigen-presenting cell surfaces.

Two additional studies show that association of I-A- and I-E-subregion controlled products results in the expression of a serologically detected antigen. Based on strain-distribution patterns, the specificity detected by Y-17 is different from that described by Harris and Delovitch (40), and similar to that described by LaFuse et al. (Ia.22) [41]. Because preliminary data with certain F_1 hybrid combinations suggest that the specificity detected by Y-17 is not Ia.22 (D. B. Murphy. Unpublished observations.), we provisionally designated this specificity Ia.m.44. These observations, coupled with those of Fathman and Hengartner (12) in MLC reveal that numerous antigenic specificities can be generated by the association of two I-region molecules.

The observation that a third I-region-controlled chain (X) is precipitated with Y-17 is intriguing. Because conventionally prepared anti-I-E-subregion sera precipitate only the A_e and E_α chains, the X chain does not nonspecifically associate with A_e:E_α complexes. Based on 2-D-PAGE, the X chain is indistinguishable from the A_α chain. Peptide map analysis of the X chain is in progress to determine whether it is indeed the A_α chain or a new chain. Until this result is obtained, the possible hybrid Ia antigen associations include A_α:A_β, A_e:E_α, and A_e:X. These combinatorial Ia antigens (the number of which would be much amplified in heterozygotes) could have broad implications for immune response gene function.

The two functional studies presented bear on the relationship of I-region linked immune response and Lad genes to Ia antigens. Ia antigens in general, and the A_e:E_α complex in particular, are known to be recognized by T cells (5, 6, 34). Y-17 effectively blocks this recognition, both in response to allogeneic A_e:E_α complexes and in response to nominal antigen associated wih self A_e:E_α complexes. The appearance of certain A_e:E_α complexes on the cell surface correlates with the ability of T cells from a given strain of mouse to respond to pigeon cytochrome c (11) and with the ability of certain F_1 antigen-presenting cells to present GLPhe to B10.A(5R) T cells (10, 42). These results have been interpreted to indicate that Ia antigens are the Ir gene products. The blocking data in the present experiments strongly support this hypothesis.

Previous studies with radiation-induced bone marrow chimeras have suggested that anti-Ia sera inhibit antigen-presenting cell function (but not T lymphocyte function) (43), and that both Ir gene products must be expressed in the antigen-presenting cell (but neither need be expressed in the T lymphocyte) (44). The demonstration in this paper that a monoclonal antibody directed against an Ia determinant could inhibit T cell proliferation in an antigen specific manner (i.e., Y-17 blocks the GLPhe response but not the (T,G)-A--L response) suggests that Ia-bearing molecules are indeed the structures whose function is being inhibited at the antigen-presenting cell surface. However, the possibility does remain that Ir and Ia loci are distinct but closely linked in the genome and that the products of these putative two loci are also closely

associated on the cell surface, such that anti-Ia antibody sterically interferes with Ir-gene function. The final proof that Ia antigens are indeed Ir gene products must await examination of I-region point mutations in which simultaneous changes in both Ia antigens and Ir-gene function are observed.

Summary

Genetic, biochemical, and functional studies have been performed using a monoclonal antibody, Y-17, directed at a conformational or combinatorial determinant formed by certain A_e:E_α complexes. This determinant appears to be a marker present on a subset of B cells as well as on non-T and non-B spleen cells. Besides A_e and E_α chains, Y-17 precipitates a third chain that is indistinguishable from the A_α chain in two-dimensional gels. This result suggests additional combinatorial complexity in the generation of I-region encoded antigens. Y-17 can inhibit the response of T cells to A_e:E_α determinants in mixed lymphocyte cultures. Furthermore, Y-17 blocks antigen-specific T cell proliferative responses to GLPhe and pigeon cytochrome c which have been shown to require the A_e:E_α complex as a restriction element for antigen presentation. These results provide strong evidence for the molecular identity of Ia antigens, Ir-gene products and Lad antigens.

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