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HYBRIDOMA CELL LINES SECRETING MONOCLONAL ANTIBODIES TO MOUSE H-2 AND Ia ANTIGENS

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Hybridoma cell lines secreting antibodies to mouse H-2 or Ia antigens have been generated by fusing mouse immune lymphocytes with appropriate myeloma lines. Among the 11 established clones reported here, nine produce anti-H-2 antibodies and two produce anti-Ia antibodies. The specificities and cross-reactions of these monoclonal antibodies have been studied in detail. One hybridoma antibody reacted only to K^k antigens without any detectable cross-reactions, thus suggesting reaction to a private specificity of the K^k molecule. All other anti-H-2 hybridoma antibodies appeared to detect public specificities as defined either by reactions with products of more than one H-2 locus or with different alleles at one or more loci. The two anti-Ia antibodies both reacted with I-E/C products, but exhibited different cross-reactivity patterns.

Strain distribution analyses so far indicate that the public specificities detected by these monoclonal antibodies are considerably different from those that had been established by traditional serology. Since public specificities defined by the hybridoma antibodies must by definition represent cross-reactions, these findings may have important implications relating to the structure and evolution of MHC gene products.

The murine major histocompatibility complex (MHC)[1] encodes at least two series of cell surface antigens, the H-2 antigens (K, D, L) and the Ia antigens (I-A, I-E/C). Both the *H-2* and the *Ia* loci exhibit unusually high levels of polymorphism as determined from studies of the complex antigenic nature of the cell surface products. The existence of similar polymorphic systems of antigens in all other mammalian species so far examined suggests that these antigens are of physiologic importance, although the natural functions of these molecules have only recently begun to be studied and are still uncertain. Clearly the capacity to study these antigens both in terms of *H-2* genetics and in terms of functional and structural parameters is highly dependent on the availability and purity of anti-H-2 and anti-Ia reagents.

A new approach to the production of such reagents has recently been made possible through hybridoma technology developed by Köhler and Milstein (1). This procedure involves cell fusion between immune lymphocytes and myeloma cells and allows the production of permanently proliferating cell lines that secrete large amounts of monoclonal antibodies. Production of anti-H-2 hybridomas was at first surprisingly difficult relative to other antigenic specificities, although over the past 2 years several laboratories, including our own, have succeeded in producing such hybridomas (2, 3). We report here the properties of the first 11 anti-H-2 and anti-Ia monoclonal antibodies that we have produced.

MATERIALS AND METHODS

Animals. Adult male and female mice of the strains C3H, C3H.SW, BALB/c, B10, and B10.BR used for immunizations and fusions were either purchased from The Jackson Laboratory (Bar Harbor, Maine) or were produced in our own animal colonies. All other strains and (C3H.SW × BALB/c)F_1 animals used for specificity determination and for collection of ascites antibody were produced in our own colonies.

Immunizations. The primary immunization was performed either by grafting tail skin onto the dorsal thorax of the recipients (4) or by i.p. injection of 2 to 4 × 10^7 live spleen cells. Boosts with 2 × 10^7 live lymphocytes were given either i.p. or i.v. at a variety of intervals as indicated in Table I. Fusion was performed at 2, 3, 5, or 12 days after the final boost, also as indicated in Table I.

Parental myeloma cells. Three myeloma variant cell lines have been used in these studies. P3 × 63AgU1 (P3U1) (5), a nonsecretor, synthesizing κ-chains and SP2/0 Ag14 (SP2/0), a hybridoma variant not synthesizing Ig components (6) were both kindly provided by Dr. M. Scharff (Albert Einstein College of Medicine, Bronx, N. Y.). NS-1 (7), a nonsecreting, κ-chain synthesizing cell line was kindly provided by Dr. J. Minna (NCI-VA Medical Oncology Branch, NIH, Bethesda, Maryland). All cell lines were maintained in Dulbecco's modified Eagle's medium (DME; GIBCO, Grand Island, N. Y.) plus 15% fetal bovine serum (FCS, GIBCO) supplemented with 0.03% glutamine and 50 μg/ml gentamicin in 10% CO_2 at 37°C. Cultures were exposed to 6-thioguanine (P3U1), or 8-azaguanine (SP2/0 or NS-1) for 1 week every 3 to 4 weeks to suppress revertants.

Cell fusion. Previously published methods of Köhler and Milstein (1) and of Gefter *et al.* (8) were employed with small modifications. Lymphocyte suspensions from spleens and mesenteric lymph nodes of immunized mice were prepared by gentle teasing. Removal of red blood cells was effected by NH_4Cl lysis (9), and cells were suspended in cold DME at about 2 × 10^7 cells/ml. Exponentially proliferating myeloma variant cells were washed three times with chilled serum-free DME and resuspended in DME at about 1 × 10^7 cells/ml. 10^8 immune

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[1] Abbreviations used in this paper: MHC, major histocompatibility complex; DME, Dulbecco's modified Eagle's medium; PEG, polyethylene glycol; HAT, hypoxanthine, aminopterin, thymidine; P3U1, P3 × 63AgU1; SP2/0, SP2/0Ag14; 2(R), B10.A(2R); 3(R), B10.A(3R); 4(R), B10.A(4R); 5(R), B10.A(5R).

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TABLE I

Immunization, cell fusion, and the production of stable anti-MHC hybridomas

No.[a]	Mouse Strains	Schedule[b]	Parental Myeloma Cell	Total Cultures	Cell Growth Positive Cultures	Fusion Efficiency	Cytotoxicity Positive Cultures[c]		Established Hybridomas
						%		%	
1	C3H.SW α-C3H	i.p. 5 F	P3.U1	480	53	(11.0)	0	(0)	0
3	BALB/c α-C3H	i.p. 21 i.p. 3 F	P3.U1	690	101	(14.6)	6	(5.9)	1
	C3H.SW α-C3H	i.p. 21 i.p. 3 F	P3.U1	702	114	(16.2)	2	(1.7)	0
4	C3H.SW α-C3H	s.g. 22 i.p. 3 F	P3.U1	576	301	(52.3)	1	(0.3)	0
5	BALB/c α-C3H	i.p. 22 i.p. 3 F	P3.U1	572	201	(35.1)	0	(0)	0
6	C3H.SW × BALB/c α-C3H	s.g. 21 i.p. 3 F	P3.U1	716	80	(11.2)	0	(0)	0
7	B10 α-B10.Br	i.p. 21 i.p. 3 F	P3.U1	383	231	(60.3)	0	(0)	0
8	C3H·SWαC3H	i.p. 21 i.p. 2 F	P3.U1	763	299	(39.2)	0	(0)	0
9	BALB/c α-C3H	s.g. 12 F	SP2/0	238	11	(4.6)	0	(0)	0
10	C3H·SWαC3H	i.p. 24 i.v. 2 F	SP2/0	196	9	(4.6)	1	(11.0)	0
11	BALB/c α-C3H	s.g. 21 i.v. 2 F	SP2/0	480	22	(4.6)	3	(13.6)	0
12	C3H.SW α-C3H	s.g. 14 i.v. 10 i.v. 3 F	SP2/0	288	20	(6.9)	4	(20.0)	1
			P3.U1	288	23	(8.0)	2	(8.7)	0
13	C3H.SW α-C3H	s.g. 21 i.v. 3 F	SP2/0	672	91	(13.5)	2	(2.2)	0
			P3.U1	576	145	(25.2)	11	(7.5)	0
14	C3H.SW α-C3H	i.p. 14 i.v. 14 i.v. 2 F	SP2/0	624	54	(8.7)	3	(5.5)	1
			P3.U1	528	203	(38.4)	12	(5.9)	0
15	C3H.SW α-C3H	i.p. 21 i.v. 10 i.v. 3 F	SP2/0	414	28	(6.7)	3	(10.7)	2
			P3.U1	410	41	(10.0)	3	(7.3)	1
16	C3H.SW α-C3H	s.g. + i.p. 21 i.v. 3 F	SP2/0	384	117	(30.5)	8	(6.8)	1
		s.g. + i.p. 21 i.v. 3 F	NS-1	288	39	(13.5)	7	(17.9)	3
17	C3H.SW α-C3H	s.g. 21 i.v. 3 F	SP2/0	768	38	(4.9)	3	(7.9)	1

[a] Fusion 2 was a technical failure.

[b] The numbers on horizontal lines indicate the time intervals (days) between immunization(s) and cell fusion. Abbreviations are s.g., skin graft; i.p., intraperitoneal injection; i.v., intravenous injection; F, fusion.

[c] Antibodies from culture supernatants were detected by ^{51}Cr-release from C3H spleen cells in C-dependent cytotoxicity assay with facilitating reagent. Cultures showing more than 15% specific release were scored as positives.

lymphocytes were mixed with 1 to 2 × 10^7 myeloma variant cells and were pelleted in 17 × 100 mm plastic tubes (Falcon, Cockeysville, Md.). Three hundred microliters of 30% polyethylene glycol (PEG) (10), m.w. 1000 (Baker, Phillipsburg, N. J.) dissolved in DME at 39°C were added to the pellet; tubes were mixed and centrifuged at 1500 rpm for 3 min. The cells were allowed to stand so that the total exposure time to PEG was 8 min at room temperature. Pellets were resuspended in 5 ml of chilled DME, mixed, and recentrifuged to remove PEG. The pellet containing fused cells was resuspended in approximately 40 ml of DME + 10% NCTC 109 (Microbiological Associates, Bethesda, Md.), 15% FCS, 0.03% glutamine, 100 μM hypoxanthine, 10 μM aminopterin, and 30 μM thymidine (HAT selection medium) (11), and distributed into microtiter wells (96 wells per plate, flat bottom, Falcon) at a concentration of 2.5 × 10^5 immune cells in 100 μl per well and incubated at 37°C. 1% Nonessential amino acid solution 100× (GIBCO), 0.2 units/ml insulin, 1 mM oxalacetic acid, and 0.5 mM sodium pyruvate, all obtained from Sigma (St. Louis, Mo.), were added to the HAT medium at the suggestion of Dr. J. Minna, starting with fusion number 14, and have been employed routinely subsequently. Two to 3 days after fusion, 100 μl of fresh selection medium were added. Withdrawal of the selection medium was begun 7 to 8 days after fusion by replacing with 100 μl of fresh medium containing all of the ingredients described in HAT selection medium except hypoxanthine, aminopterin, and thymidine. Medium change was repeated every 3 to 4 days. Two to 3 weeks after fusion, the culture wells showing vigorous cell growth on microscopic examination were marked, fusion efficiency was calculated, and 100 μl of culture supernatant were collected for primary screening of antibody activity.

Screening for anti-MHC antibody. Anti-MHC antibodies in hybridoma culture supernatants were detected by a two-stage C-mediated cytotoxicity assay (12) using ^{51}Cr-labeled spleen cells as targets and using purified pig anti-mouse Ig as a facilitating reagent to enhance the sensitivity of detection (13). Briefly 25 μl target cells at 1 × 10^6 cells/ml were exposed to 25 μl culture supernatant for 15 min at 37°C. Cells were washed and mixed with 25 μl of facilitating antibody for 2 to 3 min. Twenty-five microliters of an appropriate dilution of rabbit complement (C) were added, and cells were mixed well and incubated for 30 min at 37°C.

Cytotoxicity was calculated as

$$\frac{^{51}\text{Cr-release by hybridoma supernatant} - {}^{51}\text{Cr-release from C control}}{^{51}\text{Cr-complete release in detergent} - {}^{51}\text{Cr-release from C control}} \times 100.$$

Cultures producing greater than 15% cytotoxicity were scored as positive.

Cloning and establishment of hybridoma cell lines. Antibody-producing hybridoma cultures were cloned by limiting dilution on 2000 R irradiated (Gammator, M., Isomedic, Parsippany, N. J.) feeder layer rat fibroblasts (1 to 5 × 10^2 cells/well) (01-542, Flow Laboratory, McLean, Va.). Cells were counted, diluted appropriately, and seeded into microtiter wells (96 wells/plate) at 0.3 to 1 cell per 100 μl per well. Seven to 8 days after the cloning, 50 μl of the fresh medium was added to each culture. Ten to 14 days later, the culture supernatants from 5 to 10 clones were tested for cytotoxic activity. One to three clones with positive cytotoxicity were then propagated for large-

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scale culture. Cloned cells and uncloned cells were frozen in a mixture of fresh culture medium and cryoprotective medium (Microbiological Associates) (1:1 ratio) at 5×10^6 cells/ml and stored in liquid nitrogen.

Production of hybridoma antibody. 1) Large-scale culture: Cloned hybridoma cells were propagated in six-well cluster plates (3 to 4 ml/well) (Costar, Cambridge, Mass.). Cells were cultured at a concentration range of 3 to 10×10^5 cells/ml and passaged every 2 to 4 days. At every passage, culture supernatants were collected until the total volume of supernatant collected from each clone was 500 to 1000 ml.

2) Ascites fluid: In order to obtain larger amounts of antibody, hybridoma cells were injected into appropriate F_1 hybrid animals primed with 0.5 ml tetramethylpentadecane (Pristane, Aldrich Chemicals, Milwaukee, Wis.) 3 to 5 days before injection of 1 to 2×10^6 cells per mouse. For example, C3H.SW anti-C3H clones were propagated in (C3H.SW × BALB/c)F_1 animals. Animals were observed daily and were tapped successively every 2 to 3 days after appearance of ascites tumors (generally 7 to 14 days after inoculation). Cytotoxic titers of ascites were generally several orders of magnitude greater than those of culture supernatants. Other properties and use of ascites will be described in a subsequent communication.

Determination of serologic specificity of monoclonal antibodies. Culture supernatants from each hybridoma were assayed on splenic lymphocytes from a panel of strains representing the major H-2 haplotypes and recombinants thereof. For these assays a microcytotoxicity assay was used, as previously described (12). Briefly, 25 μl of 5×10^6 cells/ml and 25 μl of serially diluted culture supernatant were mixed and incubated for 15 min at 37°C. Cells were washed and then incubated with 25 μl of rabbit C for 30 min at 37°C. Percent dead cells was determined by trypan blue uptake.

Determination of Ig class. Immunoglobulin chains secreted by hybridoma cells were determined by Ouchterlony double diffusion in agar on culture supernatants concentrated approximately 3- to 4-fold by vacuum dialysis. Reagents specific for mouse γ-1, γ-2a, and γ-2b were generous gifts of Dr. R. Asofsky, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Maryland. Rabbit anti-mouse μ- and rabbit anti-mouse κ-reagents were purchased from Litton Bionetics (Kensington, Md.). Rabbit anti-mouse λ-reagent was purchased from Miles Laboratories (Elkhart, Ind.).

RESULTS

Cell fusion and establishment of hybridoma clones. A variety of different immunization schedules were attempted for obtaining hybridomas with anti-MHC antibody activity. Table I shows immunization schedules of each cell fusion and the results of the fusion; i.e., the total wells prepared, fusion efficiency, the number of cytotoxicity positive cultures at the initial screening, and finally the number of hybridoma cell lines that could be established from each cell fusion. Although our experience with each parameter to date is too small to make firm conclusions, several possible trends were noted: 1) Good fusion efficiency was observed irrespective of immunization scheme; i.e., recipient strains, routes, or time course; 2) fusion with P3U1 generally gave higher fusion efficiency than either of the other two myeloma cells. However, the number of cytotoxicity positive cultures did not appear to correlate with the fusion efficiency; 3) immunization protocols involving i.p. boosting (e.g., i.p. *3 to 4 weeks*, i.p. *3 days*, F), which had generally been used by others to obtain anti-H-2 or anti-Ia hybridomas (2, 3), gave poor results in our experience. As seen in fusions 3 to 8, the cytotoxicity positive cultures at the primary screening averaged less than 1.5%, and only one hybridoma cell line was established by this protocol. When the last immunization was given by the i.v. route, the number of cytotoxicity-positive hybridomas detected at the first screening appeared to increase (see fusions 12 through 17). Thus, 10 hybridomas were obtained from the immune lymphocytes after a single or two immunization(s) followed by an i.v. boost; 4) there seemed to be little difference in generation of antibody-positive hybrid cells between the two modes of priming tested (skin graft *vs* i.p. spleen cells).

We have observed a number of apparently positive cultures that showed cytotoxic activity lower than 15%, but higher than two standard deviations above background. However, these cultures invariably lost antibody activity within 1 to 3 weeks of subsequent cultures despite the fact that many of such cultures grew well. The operational demarcation between positives and negatives was made because of this observation. In some cases even those cultures exhibiting cytotoxicity higher than 15% lost the antibody activity in subsequent culture without diminishing proliferative activity. In these situations, early cloning performed within 1 week of detection did not yield positive clones. The cause of the loss of antibody activity is not clear. It might be due to chromosomal segregation, overgrowth of nonproducers, or some cultures may represent false positives due to residual antibodies secreted by unfused spleen cells. Hybrid cells produced with P3U1 appeared to be more prone to antibody loss in subsequent culture than did hybridoma cell lines produced with SP2/0 cells. In addition to antibody loss from proliferating clones, there were a significant number of hybridomas lost due to lack of proliferation.

Cloning was performed within a week after the antibody was detected. Cells were inoculated into microtiter wells at 0.5 to 1 cell/well containing a feeder layer of x-ray irradiated rat fibroblasts. The remaining cells were allowed to expand in mass culture. Clone efficiency as detected 10 to 14 days later varied from 5 to 30%. Culture supernatants of 5 to 10 clones from each original hybridoma were then retested for antibody activity. The vast majority of cloned cells have remained cytotoxic (although for clone 14-4-4S, only three of five clones were positive). The clone with the highest antibody activity was selected from each cloning and allowed to expand. As soon as sufficient cell growth was obtained, some cells were frozen and stored for future use. A large amount of culture supernatant from each selected clone was then collected, and tests for H-2 and Ia specificities and Ig class were performed.

Specificities of monoclonal antibodies. For the cytotoxicity assay, culture supernatants were serially diluted, and C-mediated cytolysis was examined on various haplotypes. The concentration of monoclonal antibodies in culture supernatants has generally been found to be 20 to 50 μg/ml for those antibodies we have purified so far. Figure 1 and Figure 2 show representative reactivity patterns of four H-2 and two Ia hybridoma culture supernatants on several essential haplotypes. The complete list of reactivity to the panel is described in terms of cytotoxic titers in Table II.

Assignment of reactivity of nine antibodies as anti-H-2 and of two antibodies as anti-Ia was based predominantly on patterns of cytotoxicity and on mapping of reactivity with intra-H-2 recombinant strains. In the following paragraphs we present an analysis of the features of each of these 11 hybridomas, a summary of which is shown in Table III. Table III also includes the immunoglobulin class of each hybridoma antibody.

3-83P. This hybridoma antibody reacted with products of all

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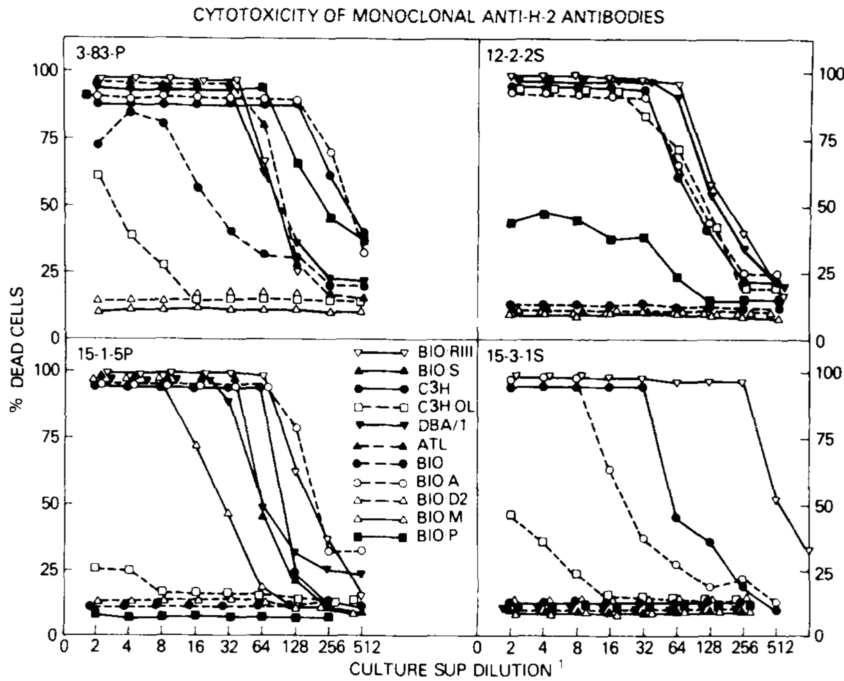

Figure 1. C-mediated cytotoxicity patterns of four anti-H-2 hybridoma antibodies on a panel of haplotypes. The incomplete lysis of B10.P splenic target cells by 12-2-2S was reproducible in two experiments. The basis for this pattern of lysis is not clear, but is under further investigation by immunoprecipitation analyses.

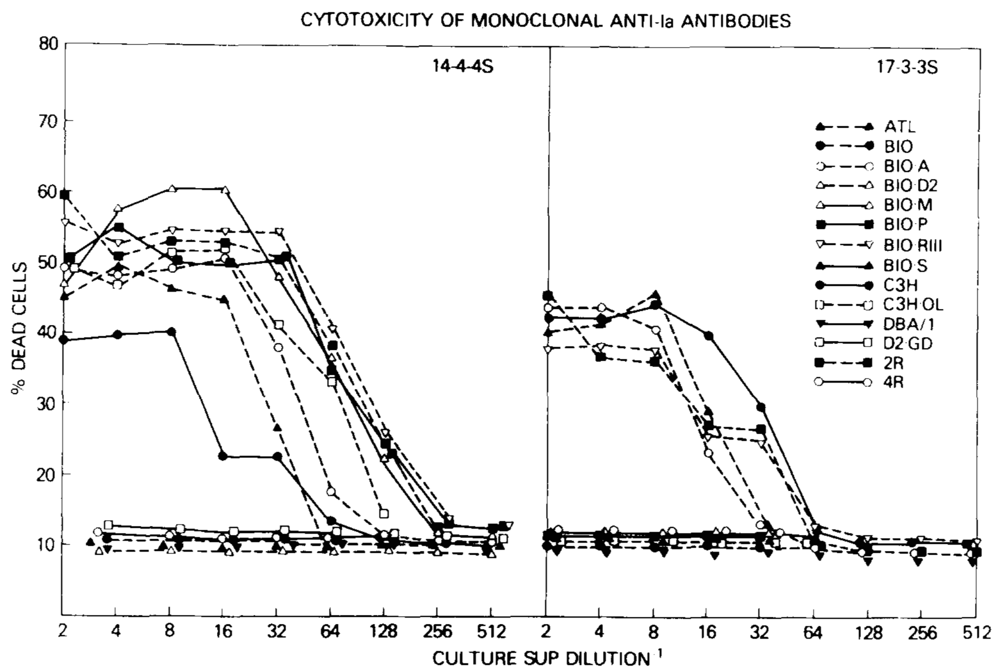

Figure 2. C-mediated cytotoxicity patterns of two anti-Ia hybridoma antibodies on a panel of haplotypes.

independent haplotypes tested, except H-2^d and H-2^f. The reactivity with H-2^b was much lower than with the other haplotypes, probably indicating a relatively weak cross-reaction, but all activity could be absorbed with H-2^b lymphocytes, as would be expected for a monoclonal product (this absorption required more than 10 times as many B10 lymphocytes as B10.A lymphocytes). Lack of reactivity of this antibody with strain D2.GD indicates that the cross-reaction with H-2^b detected a specificity of the K^b product.

This reactivity pattern corresponds with that of H-2.5 in the classical *H-2* chart (14), although more extensive panel testing and testing on wild mouse strains will be required before it would be reasonable to make such a designation (see *Discussion*). Another hybridoma cell line with the same apparent specificity has also been reported by others (2). Relative binding constants for the different cross-reactive haplotypes may help to determine whether these two monoclonal antibodies (and indeed standard anti-H-2.5 antibodies) react with the same or different antigenic determinants.

12-2-2S. Strong reactivity of this antibody with H-2^a and lack of reactivity with A.TL indicates specificity for H-$2K^k$ antigens. This was also substantiated by lack of reactivity with B10.MBR. This lack of reactivity with B10.MBR in contrast to strong reactivity with DBA-1 (H-2^q) suggests that the reactivity with H-2^q was likewise to an antigen of the K^q molecule. The negative reaction with H-2^d and positive reactivity with C3H.OL indicated reactivity with the D^k molecule as well. Reactivities with the products of H-2^r and H-2^p haplotypes were also observed,

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although the recombinants tested did not permit assignment to K or D molecules of these haplotypes. The overall reactivity pattern of this clone does not correspond to any known public H-2 specificity.

14-4-4S. As seen in Figure 2, this antibody lysed about 50% of spleen cells of various haplotypes at plateau lysis, representing a typical anti-Ia cytotoxicity pattern. The assignment of this antibody to the anti-I-E/C category was obtained from the following comparisons on intra-MHC recombinants. B10.A, B10.A(2R) (2R), B10.A(3R) (3R), and B10.A(5R) (5R) were lysed by the antibody, whereas no cytolysis was observed on B10.A(4R) (4R). In addition, the antibody reacted with B10.D2 but not with D2.GD. The antibody exhibited a broad cross-reactivity; i.e., H-2^k, H-2^d, H-2^p and H-2^r were recognized by the antibody. The specificity of this antibody thus appears to be identical to Ia-7 in the Ia chart (14).

15-1-5P. This antibody showed strong reactivity to the K^k antigen, as evidenced by the strong reaction against B10.A(4R) and a negative reaction with B10. A unique feature of the reactivity pattern of this antibody was a positive reaction with H-2^d. Lack of reactivity with D2.GD mapped this H-2^d reactivity to the D region. BALB/c and BALB/c-H-2^{dm-2} showed similar levels of cytotoxicity, suggesting that this reactivity was probably with D^d rather than with L^d (15), although sequential precipitation studies will be necessary to determine whether the antibody also cross-reacts with L^d.

A low level of reactivity with C3H.OH indicated that this antibody also crossreacts with the D^k molecule. Likewise, a low level of reactivity with B10.MBR indicates that at least part of the crossreactivity of this antibody with H-2^q is directed towards the D^q molecule. The reactivity pattern of this antibody was not that of any known public H-2 specificity.

15-3-1S. This antibody showed a limited distribution of positive strains, consistent with reactivity to K^k, as evidenced, for

TABLE II

Cytotoxic titer^{-1} of each hybridoma culture supernatant on a panel of H-2 haplotypes[a]

Strain	Haplotype of Origin								Hybridoma Antibody										
	K	A	B	J	E	C	S	D	3-83P	12-2-2S	15-1-5P	15-3-1S	15-5-5S	16-1-2N	16-1-11N	16-3-1N	16-3-22S	14-4-4S	17-3-3S
C3H	k	k	k	k	k	k	k	k	256	64	64	32	8	128	16	128	16	64	64
B10	b	b	b	b	b	b	b	b	16	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
B10.A	k	k	k	k	k	d	d	d	256	64	128	16	<2	16	128	128	128	64	16
B10.A(2R)	k	k	k	k	k	d	d	b	128	256	64	8	<2	4	128	256	64	128	32
B10.A(3R)	b	b	b	b	k	d	d	d	NT	NT	NT	NT	NT	NT	NT	NT	NT	64	16
B10.A(4R)	k	k	b	b	b	b	b	b	128	256	64	32	2	16	16	128	64	<2	<2
B10.(5R)	b	b	b	k	k	d	d	d	8	<2	16	<2	<2	<2	<2	<2	<2	32	32
C3H.OL	d	d	d	d	d	d	k	k	8	64	4	4	16	16	<2	<2	<2	64	<2
B10.D2	d	d	d	d	d	d	d	d	<2	<2	16	<2	32	<2	<2	<2	<2	64	<2
D2.GD	d	d	b	b	b	b	b	b	<2	≤2	<2	<2	4	<2	<2	<2	<2	<2	<2
B10.S	s	s	s	s	s	s	s	s	64	<2	32	<2	<2	<2	<2	<2	<2	<2	<2
A.TL	s	k	k	k	k	k	k	d	64	NT	32	<2	<2	<2	<2	<2	<2	64	16
A.TH	s	s	s	s	s	s	s	d	64	<2	64	<2	2	<2	<2	<2	<2	<2	<2
B10.M	f	f	f	f	f	f	f	f	<2	<2	<2	<2	4	<2	<2	<2	<2	<2	<2
B10.P	p	p	p	p	p	p	p	p	128	32	<2	<2	<2	8	64	16	<2	64	<2
DBA/1	q	q	q	q	q	q	q	q	64	128	32	<2	<2	16	8	32	<2	<2	<2
B10.RIII	r	r	r	r	r	r	r	r	64	128	128	512	≤2	8	128	256	<2	64	32
B10.MBR	b	k	k	k	k	k	k	q	NT	<2	8	<2	NT	<2	<2	<2	<2	NT	NT

[a] C-mediated cytotoxicity of each culture supernatant was measured by the two-stage Trypan blue assay as described in *Materials and Methods.* Titers are reported as reciprocal of the highest 2-fold dilution producing more than 50% of maximum specific lysis obtained for the supernatant being assayed.

TABLE III

Hybridoma clones secreting antibodies to H-2 or Ia antigens

Hybridoma Cell	Fusion: Source of immune cells × Myeloma variant	Immunoglobulin Class: H	Immunoglobulin Class: L	Specificity	Cross-reactions	Remarks
3-83P	BALB/c × P3U1	γ2a	κ	K^k, D^k	K^h, s, p, q, r	Specificity 5 in H-2 chart
12-2-2S	C3H.SW × SP2/0	μ	κ	K^k, D^k	K^q, p, r	Previously unidentified public
14-4-4S	C3H.SW × SP2/0	γ2a	κ	I-E^k/C^k	d, p, r	Specificity 7 in Ia chart
15-1-5P	C3H.SW × P3U1	γ2b	κ	K^k, D^k	D^d, s, q, r	Previously unidentified public; autoreactive (?)
15-3-1S	C3H.SW × SP2/0	γ2a	κ	K^k, D^k	r	Previously unidentified public; heteroclitic
15-5-5S	C3H.SW × SP2/0	γ2a	κ	D^k	K^d, f	Previously unidentified public; autoreactive (?)
16-1-2N	C3H.SW × NS-1	γ2a	κ	K^k, D^k	K^q, p, r	The same specificity as 12-2-2S
16-1-11N	C3H.SW × NS-1	γ2a	κ	K^k	K^q, p, r	Previously unidentified public
16-3-1N	C3H.SW × NS-1	γ2a	κ	K^k	K^q, p, r	Previously unidentified public
16-3-22S	C3H.SW × SP2/0	γ2a	κ	K^k	none	Private
17-3-3S	C3H.SW × SP2/0	γ2a	κ	I-E^k	r	Previously unidentified public

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example, by positive reactivity on H-2^a strains and negative reactivity on A.TL and on B10.MBR. It also showed weak cross-reactivity with D^k as indicated by negative reaction with B10.D2 and positive reaction with C3H.OL. All other H-2K and D alleles appeared to be negative, with the remarkable exception of H-2^r, on which this antibody showed a very strong reactivity, much greater than the activity on H-2^k. This reactivity may thus represent a heteroclitic pattern (16). The strain distribution of this specificity is again unlike that of any known public H-2 specificity.

15-5-5S. This antibody showed a very unusual distribution of positive strains in the panel. It was reactive with H-2^d (B10.D2) and with H-2^k (C3H), but was negative with H-2^a (B10.A). Therefore, the positive reactions with B10.D2 and C3H were presumably due to reactivity with K^d and D^k. Positive reactivity with D2.GD and negative reaction with B10 confirmed this specificity for K^d. Other haplotypes of independent origin were negative, except for H-2^f (B10.M). This reactivity pattern is also unlike that of any known H-2 public specificity.

16-1-2N. This antibody showed the same distribution of positive strains in our panel as did clone 12-2-2S, thus indicating reactivity with K^k and D^k products and cross-reactions with H-2^q, H-2^r, and H-2^p. The relative titers against these different haplotypes were not identical, however, indicating the possibility that the actual determinant recognized was not the same. In addition, this clone produced a γ-G2a antibody, whereas clone 12-2-2S was a γ-M producer.

16-3-1N and 16-1-11N. These two hybridoma clones were both obtained from the same fusion (No. 16) and showed identical specificity patterns in our panel tests. As seen from the titers in Table II, there were slight differences in relative strengths of reactivity with different strains of the panel, but it is not clear whether this represents true differences in the clones or experimental differences in the testing of these reactivities. Since both clones produce γ-G2a-κ antibodies, it will require more selective testing to determine whether these represent the same or different clones. Strong reactivity with H-2^k and H-2^a strains and negative reactions on H-2^d strains and C3H.OL indicate predominant reactivity with the K^k product. Cross-reactions were also observed on H-2^p, H-2^q, and H-2^r. Negative reactivity of these antibodies with B10.MBR indicates that the cross-reactivity to H-2^q must be with the K^q product. It is interesting that this clone produces an antibody with the same reactivity pattern as clone 11-4.1 reported previously by Oi *et al.* (3).

16-3-22S. This antibody reacted strongly with H-2^k and H-2^a strains, but was negative with C3H.OL, indicating reactivity with the K^k product and lack of reactivity with the D^k product. It showed no cross-reactions with any other products of independent H-2 alleles in the panel. Thus, within the limits of our panel testing, this clone appears to secrete an antibody reacting to a private specificity of H-$2K^k$. From the known *H-2* chart, this distribution correlates with the private specificity H-2.23, but a larger panel test will be necessary to rule out possible differences from H-2.23.

17-3-3S. As seen in Figure 2, similar to 14-4-4S, this antibody reacted with B10.A, 2R, 3R, and 5R, whereas no reaction was observed on 4R. The antibody showed a plateau of cytotoxicity similar to that of 14-4-4S, but exhibited a more restricted reactivity pattern; that is, H-2^d and H-2^p, which were positive with 14-4-4S, were negative with this antibody. Lack of reactivity with H-2^d implies that this antibody reacts with products of I-E. The only other positive haplotype was H-2^r. There is no previously reported public Ia specificity defining this reactivity.

DISCUSSION

This paper describes the 11 hybridomas that have resulted from our initial efforts to develop hybridoma clones secreting monoclonal antibodies to H-2 and Ia antigens. Six of these clones have resulted from cell fusions with nonproducer variant SP2/0 cells, two from fusions with the κ-producer P3U1, and three from a single fusion with the κ-producer NS-1. Future work will involve the SP2/0 line exclusively because of the obvious advantages of producing homogeneous immunoglobulins without the need to remove possible hybrid molecules. Although it seems unlikely, such hybrid molecules among our monoclonal antibodies produced with P3U1 and NS-1 could have affected specificity results (17).

Most of the immunizations reported here employed *H-2* congenic strains, and all of the hybridoma cell lines we have obtained produce antibodies directed toward H-2 or Ia antigens as determined by cytotoxicity patterns on a panel of appropriate strains. Confirmation of this specificity has also been obtained for four anti-H-2 antibodies and one anti-Ia antibody by immunoprecipitation studies in which glycoprotein antigens of appropriate molecular sizes have been found (unpublished data). It should be noted, however, that specificity determination always depends on the concentration of antibody tested. As can be seen in Figures 1 and 2, there are situations in which weak cross-reactions are detectable only at higher antibody concentrations. Therefore, at very high antibody concentrations (such as ascites fluids), weak cross-reactions that had not been observed with the use of culture supernatants will probably be found. Thus, it will be important to define the concentration of monoclonal antibodies in order to designate specificities.

Among the anti-H-2 hybridoma antibodies reported here, we have found one (16-3-22S) that appears to detect a private specificity, in that it reacts only with K^k and shows no cross-reactions with D^k or with other independent haplotypes. Because all previously reported anti-H-2 hybridoma antibodies appeared to recognize public rather than private specificities, the question has been raised whether a "private" specificity really exists or is the result of antibody concentrations insufficient to reveal cross-reactions (18). Our results indicate that a monoclonal antibody can indeed recognize such a "private" specificity. It of course remains to be seen whether this antibody and conventional antisera detecting the K^k private specificity, anti-H-2.23, really detect the same determinant. Testing on a more extensive panel of haplotypes, perhaps including wild H-2 types, may reveal differences in distribution. Alternatively, direct binding studies, competitive binding studies, and assessment of binding site idiotypes, all of which are currently in progress in this laboratory, may help to establish or refute the "private" nature of this specificity.

Eight antibodies obtained in this work exhibit various degrees of cross-reactions. Five antibodies react with antigens shared by K and D antigen(s) in addition to cross-reactions with other haplotypes. The remaining three antibodies are specific to either K or D antigen(s), but also react with other haplotypes. This high degree of cross-reactions is consistent with the derivation of K and D from a common primordial gene, a hypothesis that was first proposed on the basis of serologic cross-reactions with conventional anti-H-2 sera (19) and has subsequently been confirmed by the discovery of marked sequence homologies (20). The preponderance of K-D crossreactions seen with monoclonal antibodies suggests also that there are fewer determinants unique to the K or to the D series of antigens than there are determinants shared between and among these series; i.e.,

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there does not appear to be "K-ness" or "D-ness" detectable at the level of antigenic determinants, a finding that is also apparent in available sequence data (20). On the other hand, this lack of K-ness or D-ness is surprising considering that duplication of the primordial *H-2* gene probably occurred before the divergence of strains or perhaps even of species. Perhaps, therefore, divergence of *K* genes by mutation has not been entirely independent of *D* gene divergence. Such dependence might also explain the maintenance of close linkage between *K* and *D* genes during evolution.

Among these cross-reactive antibodies, there was only one antibody (3-83P) the strain distribution of which corresponds to that of a known public specificity in the *H-2* chart. All of the other antibodies revealed new public H-2 specificities. There are a number of possible explanations for the discrepancy between the existing H-2 specificities and those found by monoclonal antibodies. For example, monoclonal antibodies may not represent normal clonotypes in the anti-MHC antibody response, since the process of cell fusion might select only very rare clones. This possibility seems very unlikely, since for antibodies to other antigens, idiotypic specificities of monoclonal antibodies overlap with those found in antisera (21, 22). Alternatively, there may exist an enormous number of public specificities, only a minor fraction of which has been identified by classical serology. Considering the observation that there are already several independent monoclonal antibodies with the same apparent specificity, the total number of public specificities might be more restricted than this hypothsis would permit. However, this duplication of specificities could indicate the dominant nature of certain antigenic determinants. It will be of interest to study whether such antibodies with identical nominal specificity represent identical clones or distinct clones. Among the approaches we intend to explore for this purpose are the study of idiotypic identity and isoelectric focusing patterns of these antibodies.

One antibody reacted to an unrelated haplotype far better than to the immunizing antigen (15-3-1S). The presence of such a "heteroclitic" antibody and many cross-reactive antibodies indicates that within the anti-H-2 antibody repertoire there are genes with overlapping reactivity, i.e., multispecific clones. In any case, it is certainly clear from such reactivity patterns that *monoclonality* is not synonymous with *monospecificity*. This is an important distinction that must be considered if hybridoma antibodies are to be used as typing reagents or in functional studies.

Two anti-Ia monoclonal antibodies so far obtained reacted with I-E/C antigens. 14-4-4S corresponded with Ia.7, a known serologic specificity. This monoclonal antibody, like alloantisera, exhibited a cross-reaction with other mammalian B cells (data not shown). As has been discussed elsewhere (23), Ia.7 might represent an antigenic determinant common to many mammalian species. The other anti-Ia antibody (17-3-3S) showed a more restricted specificity localized to I-E products, and of distribution not previously reported. These two monoclonal antibodies when used in sequential immunoprecipitation analysis might answer the question whether I-E/C antigens are heterogeneous molecules encoded by multiple genes or are single homogeneous molecules (24).

Two of our antibodies reacted with H-2^d antigen(s), (K^d or D^d detected by 15-5-5S or 15-1-5P, respectively) despite the fact that the myeloma parental cells used for the fusion were derived from BALB/c (H-2^d). It thus appears that these hybridoma cells represent an "autoreactive" situation. Studies are in progress to determine whether the expression of certain H-2 antigens is suppressed in such hybridoma cells or whether the co-expression of reciprocally reacting antigen and antibody is permissible. As a practical point the generation of these "autoreactive" hybridomas has encouraged us to attempt the production of additional anti-H-2^d hybridoma cell lines.

The collective effort of several laboratories in obtaining anti-MHC monoclonal antibodies will undoubtedly further our understanding of the genetics and structure of MHC antigens. In addition, it is hoped that these reagents will be helpful in delineating the repertoire of anti-MHC receptors that may be distinct from that of other antigens (25). To this end, direct binding studies and investigations of idiotypic specificities are already in progress using the monoclonal antibodies described here.

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