SEROLOGIC CROSS-REACTIVITY BETWEEN CLASS I MHC MOLECULES AND AN H-2-LINKED DIFFERENTIATION ANTIGEN AS DETECTED BY MONOCLONAL ANTIBODIES

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Class I antigens consist of a 45,000-dalton glycoprotein heavy chain associated noncovalently with beta-2 microglobulin and found on the surface of all cells. In the mouse, four such Class I antigens have been defined to date, one (H-2K) encoded in the K region, and three (D, L, R) encoded in the D region of the major histocompatibility complex (MHC)[1] (1, 2). However, it is now clear from DNA hybridization studies with Class I DNA probes that there are many more copies of DNA sequences homologous to Class I–like genes in the mouse genome than have been demonstrated to be expressed in any haplotype (3, 4). Two of these Class I–like[2] genes have been identified by their determination of TL antigens (5), antigens that are expressed only on thymus and leukemia cells, and that are encoded by genes located ~1.5 centimorgans to the right of the H-2 complex along chromosome 17 (6, 7). Another of these genes has been reported to encode for the Qa 2 antigen (5), which is expressed only on certain subpopulations of peripheral lymphoid cells (8) and which was recently defined by monoclonal antibodies (9–11). However, there remain numerous other Class I–like sequences, the products of which are either not expressed or have not yet been detected.

In addition to DNA sequence homology between the genes encoding H-2 products and those encoding TL and Qa antigens, unique similarities exist between their gene products. Analogous to H-2 antigens, TL and Qa antigens are glycoproteins of ~40–45,000 daltons associated with beta-2 microglobulin (12). In addition, Qa antigens, like H-2K and H-2D function as direct targets for cytotoxic T cells (13–15). However, in contrast to the H-2 response, in vivo priming is required to elicit anti-Qa responses (13–15). There are at least two other major differences between H-2 antigens and Qa/TL antigens. As noted

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[1] Abbreviations used in this paper: CR, cross-reactive; FITC, fluorescein isothiocyanate–conjugated; FMF, flow microfluorometry; IF, immunofluorescence; Ig, immunoglobulin; MAb, monoclonal antibody; MHC, major histocompatibility complex; TRA, Texas-red conjugated avidin.

[2] The products of H-2D–linked loci, Qa and Tla, are sometimes considered Class I antigens due to their biochemical similarities to the products of H-2K and D. However, the definition of Class I antigens originally included ubiquitous tissue distribution, which along with serologic and functional properties clearly distinguishes Qa and TL antigens from Class I. Since it is important to the nature of this study to both compare and contrast products of the classical MHC subregions with those of the Qa subregion, we have chosen in this report to use the traditional definitions and refer to the H-K,D,L, and R antigens as Class I and to the products of Qa and TL loci as Class I–like.

above, the first of these is dissimilar tissue distributions. Equally striking is the fact that while H-2 antigens display extraordinary structural polymorphism (16–18), Qa antigens exhibit extremely low polymorphism (12, 19).

One of the first lines of evidence indicating that H-2K and H-2D antigens were derived from a common primordial gene was the detection of cross-reactive or "public" H-2 specificities shared by K and D region antigens (20). This hypothesis has subsequently been substantiated by both biochemical and molecular genetic data (reviewed in reference 21). It might therefore seem reasonable to expect that if other Class I–like gene products are expressed on cell surfaces, either as differentiation antigens during ontogeny or on subpopulations of adult cells, they might be detected by cross-reactions with anti–Class I (MHC) antibodies.

In this laboratory a series of ~50 monoclonal antibodies (MAb) reacting with a variety of Class I antigens has been produced (22–25). During the routine use of one of these antibodies as a negative control on a cell population to which it was not expected to react, reactivity with subpopulations of splenic lymphocytes was observed. As shown in this report, further study of this cross-reaction indicated that the antigen detected was determined by gene(s) mapping to the right of the H-2D region, and that the antigen was expressed preferentially on a subpopulation of peripheral lymphoid cells. Subsequent studies have shown that at least one other Class I specific monoclonal antibody also cross-reacts with this new antigen. In this report we present data characterizing the expression of this antigen at both genetic and cellular levels, and discuss the implications of these findings for the evolution of MHC systems.

Materials and Methods

Animals. Adult male and female mice used were either bred in our own colonies or purchased from the Jackson Laboratory (Bar Harbor, ME). B6.K3 (L. Flaherty) and BALB.A11 (D. H. Sachs) are newly derived recombinant congenic strains that will be described in the Results. C3H.KBR mice are recombinants derived from F_2 between C3H/HeJ and C3H.SW (26). B6-H-2^k mice were provided by Dr. R. H. Schwartz, NIH and were bred from stock originally supplied by Dr. E. A. Boyse.

Reagents. The anti-MHC monoclonal antibodies used have been previously described (23–25, 27, 28). D3.262 is an IgM monoclonal anti-Qa 2 antibody described elsewhere (9). Fluorescein-labeled Rabbit F(ab)$'_2$ anti-mouse IgG2a was produced under contract NCI-CB-53912-31 and was provided by Dr. H. B. Dickler, NIH. Fluorescein-conjugated (FITC) Goat F(ab)$'_2$ was the gift of B. J. Fowlkes and Dr. R. Asofsky, NIH. Biotin-labeled anti-I-A^k monoclonal antibody 10-2.16 (27) and biotin-labeled anti-I-A^b monoclonal antibody 25-9-17 (24) were kindly provided by J. Titus, NIH. Biotin-labeled anti-mouse Thy-1.2 (Clone 30-H-12) was purchased (Cat# 1331) from Becton-Dickinson FACS Systems (Sunnyvale, CA). Texas-red conjugated avidin (TRA) was prepared as described previously (29), and was the gift of J. Titus, NIH.

Two monoclonal anti-MHC antibodies were purified by protein A column separation and were fluorescein conjugated. These were 20-8-4, an IgG2a, kappa product of a C3H anti-C3H.SW immunization and fusion to SP2/0 Ag14, and 34-1-2, an IgG2a (kappa) product of a C3H anti-BDF$_1$ immunization. For some experiments, 0.2-ml aliquots of a 1:8 dilution of monoclonal antibody 20-8-4 were absorbed with 6×10^8 normal spleen cells of various strains for 60 min at 4°C.

Immunofluorescence Staining. Spleen, lymph node, and thymus cells were prepared by gentle teasing of the organs with forceps followed by passage through nylon mesh to remove clumps. Bone marrow single cell suspensions were prepared by flushing of femurs

and tibias using a 25-gauge needle followed by resuspension through a 25-gauge needle and passage through nylon mesh to remove clumps. Cell suspensions were prepared in Hanks' Balanced Salt Solution containing 10% fetal calf serum, and all further procedures were performed using Hanks' Balanced Salt Solution without phenol red containing 0.1% bovine serum albumin and 0.1% sodium azide.

For indirect one-color staining, 1×10^6 cells were incubated at 4°C for 45 min with unconjugated antibody, washed twice by centrifugation, incubated at 4°C for 45 min with a fluorescein-conjugated anti-mouse immunoglobulin reagent, washed twice again, and finally resuspended and analyzed for fluorescence. For two-color immunofluorescence staining, 1×10^6 cells were incubated at 4°C for 45 min with fluorescein-conjugated antibody, washed twice, incubated at 4°C for 45 min with biotin-conjugated antibody, washed twice, and incubated at 4°C for 20 min with Texas-red avidin. Following this incubation, cells were washed twice, resuspended, and analyzed for fluorescence. Controls were performed to show that the resulting fluorescence using this staining method was identical by FMF analysis with cells were reacted with either set of reagents in separate samples or with both sets of reagents in a single sample. These controls demonstrated that there were no competitions or cross-reactions between the reagents used for green (fluorescein) fluorescence and those for red (Texas-red) fluorescence.

For inhibition experiments, 1×10^6 cells were incubated at 4°C for 60 min with antibody being tested for blocking activity. After 60 min, fluorescein-conjugated antibody was added in limiting amounts and the incubation was continued at 4°C for 20 min. Following this incubation, cells were washed twice, resuspended, and analyzed for fluorescence.

Flow Microfluorometry (FMF) Analysis. FMF analysis was performed as previously described (30) using a B-D Dual Laser FACS II (Becton-Dickinson FACS Systems, Sunnyvale, CA). Fluorescein isothiocyanate (FITC) was excited at 488 nm and Texas-red was excited at 568.2 nm. Data on individual cells were collected, stored, displayed, and analzyed using a PDP 11/34 computer (Digital Equipment Corporation, Maynard, MA) interfaced to the FACS II (31). Fluorescence data were collected using either linear or logarithmic amplification, on 50,000 viable cells as determined by forward light scatter intensity. Logarithmic amplification was provided by a 3-decade logarithmic amplifier constructed from an NIH-modified design of R. Hiebert, LASL (Los Alamos, NM). One-color fluorescence data are displayed as cell frequency histograms or immunofluorescence (IF) profiles in which logarithmically increasing fluorescence intensity is plotted in 1024 channels on the x-axis and cell number is shown on the y-axis. Two-color immunofluorescence data are displayed as contour diagrams in which increasing intensities of green (FITC) fluorescence were plotted in 64 channels on the x-axis and increasing intensities of red (Texas-red) fluorescence were plotted on the y-axis. Arbitrary levels were selected on the z-axis which contained the number of cells at any intersection of x and y values. These levels resulted in rings or contours around the peaks of cells correlating red and green fluorescence.

Data from inhibition experiments were calculated by the formula:

$$\% \text{ Inhibition} = 100 - (FI_{exp} - FI_{bkg})/(FI_{con} - FI_{bkg}),$$

where FI = linear units of fluorescence intensity; exp = cells stained with FITC-Antibody in the presence of a potential inhibitor; con = cells stained with FITC-Antibody in the presence of media; and bkg = unstained cells.

Results

Unexpected Reactivity of MAb 20-8-4. MAb 20-8-4, an IgG2a product of a C3H anti-C3H.SW immunization, was originally characterized by antibody and complement cytotoxicity on a panel of inbred and recombinant inbred mouse strains. On the basis of that analysis, the MAb 20-8-4 was described to have anti–H-2 reactivity to K^b, D^b, K^d, $H-2^r$, and $H-2^s$ (24). However, when 20-8-4 was subsequently tested by immunofluorescence and FMF analysis, unexpected reactivity

patterns were observed on both B10 (K^b,D^b, control positive) and B10.A (K^k,D^d, expected negative) spleen cells. As shown in Fig. 1, the reaction of a control positive MAb 28-8-6 (anti-K^b,D^b) with B10 spleen cells resulted in a unimodal IF profile (Fig. 1a) characteristic of spleen cells stained with anti–Class I MHC antibodies (32, 33), a pattern of reactivity which suggested that all spleen cells bore equivalent amounts of Class I antigens. In contrast, reaction of 20-8-4 with the same B10 spleen cells unexpectedly gave a bimodal IF profile (Fig. 1b), which was of a similar intensity level to that observed for 28-8-6. Fig. 1c demonstrates that when 20-8-4 was used to stain B10.A (used as negative control) spleen cells, the resulting IF profile was not only unexpectedly positive but also clearly bimodal with both the dull positive and bright positive (relative to the fluorescent reagent control) peaks of much lower intensity than those seen with B10 spleen cells (Fig. 1, c vs. b). B10.BR and C3H spleen cells did not react with 20-8-4 since these reactions resulted in IF profiles identical to those from cells
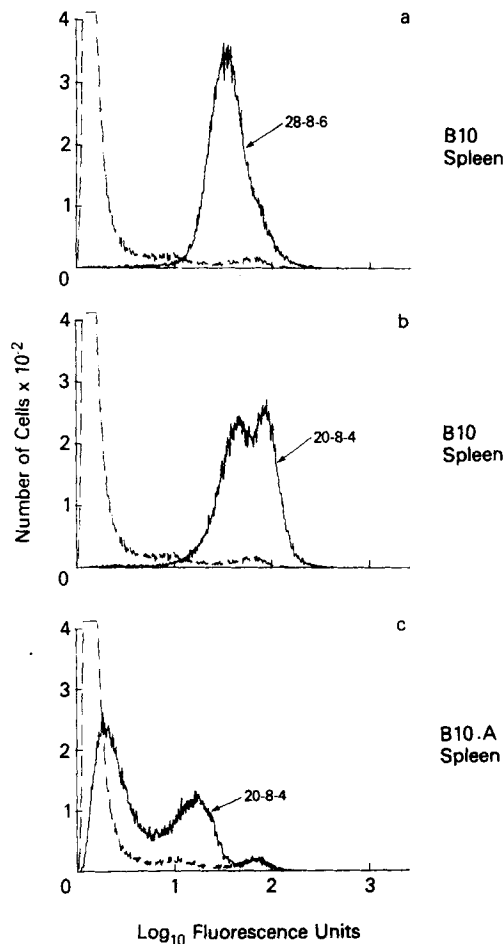

FIGURE 1. IF profiles of spleen cells reacted with monoclonal anti-MHC antibody (MAb) plus FITC anti-IgG2 (———) or FITC anti-IgG2 only (— —); (a) B10 cells with MAb 28-8-6 (anti-K^bD^b); (b) B10 cells with MAb 20-8-4; (c) B10.A cells with MAb 20-8-4.

incubated with the fluorescent reagent alone (data not shown), confirming that alloreactivity of 20-8-4 was H-2 linked.

There were several possible explanations for the unexpected IF profiles seen following 20-8-4 staining on B10 and B10.A spleen cells. It was possible that MAb 20-8-4 culture supernatant, although derived from a cloned cell line, contained two different antibodies, one that reacted with H-2 molecules on B10 cells and one that reacted with another H-2–linked antigen expressed differentially on spleen cell subpopulations from both B10 and B10.A. Alternatively, the determinant(s) detected by 20-8-4 might be physically located such that although present on Class I molecules, (K, D, L), these determinants were differentially accessible to antibody on spleen cell subpopulations, and a previously undetected cross-reaction on D^d or L^d was being detected on B10.A cells. Finally, it was possible that 20-8-4 was indeed a single antibody that reacted with conventional Class I MHC antigens but also cross-reacted with a MHC-linked antigen expressed differentially on spleen cell subpopulations. These alternatives were investigated.

Absorption Analysis of 20-8-4. To eliminate the possibility that the 20-8-4 reagent contained two antibodies, the absorption experiment shown in Fig. 2 was performed. If there existed two antibodies, both of which reacted with B10 but only one of which reacted with B10.A, then B10 but not B10.A cells would be expected to absorb all B10 reactivity from the 20-8-4 reagent. As can be seen in Fig. 2, absorption with B10.A spleen cells removed all detectable reactivity of 20-8-4, as did the absorption with B10 spleen cells. In contrast, the control absorption with negative B10.BR (K^k,D^k) spleen cells did not remove 20-8-4 reactivity on either B10 or B10.A spleen cells, although the titer of the absorbed reagent was slightly lower than the untreated control, presumably due to dilution of the antibody. These results ruled out contamination of 20-8-4 with an additional antibody as an explanation for the unexpected B10.A reactivity, and
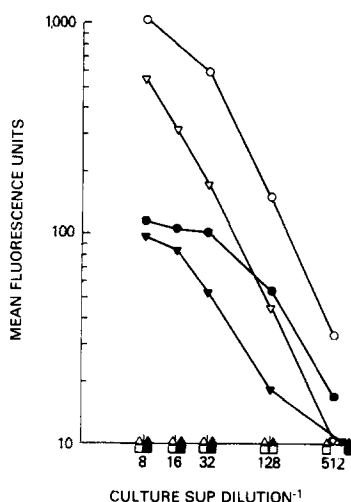

FIGURE 2. Analysis of 20-8-4 reactivity after absorption (See Materials and Methods) with nothing (O, ●); B10.BR spleen cells (∇, ▼); B10 spleen cells (△, ▲); or B10.A spleen cells (□, ■). B10 (open symbols) or B10.A (closed symbols) spleen cells were incubated with absorbed 20-8-4 followed by FITC anti-IgG2.

suggested instead that 20-8-4 is a single antibody that reacts strongly with B10 cells, as previously described (24), but also cross-reacts with a previously unde-tected determinant on B10.A spleen cells.

Mapping Studies and Strain Distribution. The cross-reaction (CR) of 20-8-4 was studied with a panel of inbred and H-2 recombinant inbred mouse strains in order to map the locus (loci) which controlled expression of the previously undetected determinant(s) on B10.A spleen cells. Because 20-8-4 also reacted with some H-2 antigens, only strains negative for 20-8-4 H-2 reactivity could be used. As summarized in Table I, spleen cells from B10.M (H-2^f), B10.P (H-2p), and B10.BR (H-2^k) animals did not react at all with 20-8-4. Because B10.A (H-2^a) and B10.Q (H-2^q) spleen cells were positive (with reactions indistinguish-able from that shown in Fig. 1c), the antigen detected by the 20-8-4 H-2 CR was

TABLE 1

Mapping and Strain Distribution of H-2 Cross-Reactive Antigen Detected by Monoclonal Antibody 20-8-4

Mouse subline	H-2*			Qa‡				T1a‡		H-2 CR§
	K	I	D	Qa2(4)$^‖$	Qa 5	Qa 7	Qa 8	Qa 1	T1a	20-8-4
B10	b	b	b	a	a	a	a	b	b	++¶
B10.A	k	k	d	a	b	b	b	a	a	+
A/J	k	k	d	a	b	b	b	a	a	+
A.AL	k	k	d	a		b	b		c	+
A.TL	s	k	d	a	b	b	b	b	c	+
B10.AQR	q	k	d	a						+
B10.T6R	q	q	d	a						+
B10.Q	q	q	q	a		a	b			+
B10.AKM	k	k	q	a				a	a	+
B10.BR	k	k	k	b	b	b	b	a	a	−
C3H/HeJ	k	k	k	b	b	b	b	b	b	−
C58	k	k	k	b	b	b	b	a	a	−
AKR/J	k	k	k	b	b	b	b	b	b	−
B10.M	f	f	f	b		b	b	d		−
B10.P	p	p	p	b		b	b	a		−
BALB/cKh	d	d	d	b	b	b	b	b	c	ND**
BALB.K	k	k	k							ND
‡‡BALB.A11	k	k	d							−
B6-T1a^a	b	b	b	a	a	a	a	a	a	ND
B6-H-2^k	k	k	k	b	b			b	b	−
‡‡B6.K3	k	k	k	a				a	a	++
B10.A(2R)	k	k	b	a	a	a		b	b	++

* See references 34 and 26.
‡ See references 11, 12, 34–38.
§ Reactivity of 20-8-4 with non-H-2 antigen(s) as determined by FMF analysis. High (++) or low (+) reactivity was determined by intensity of 20-8-4 staining. See Figs. 1 and 5.
‖ Qa specificities 2 and 4 have recently been combined to define a single specificity based upon antibody inhibition of H-2 unrestricted cytotoxic T cells (39). Because Qa 3 and Qa 6 have the same strain distribution as Qa 2/4 for all sublines tested in this study and shown in this Table, they have not been listed separately.
¶ 20-8-4 also has specificity for H-2K^b on B10) spleen cells. Positive reactivity with H-2CR is presumed because of bimodal IF profile (see Fig. 1), and because of positive reactivity of 20-8-4 with B10.A(2R) cells which carry genes telomeric to H-2D derived from B10. All other strains for which H-2CR typing is shown are do not bear H-2K or D antigens reactive with 20-8-4.
** ND, not determined.
‡‡ New recombinant inbred mouse subline. See text.

controlled by H-2–linked genes. Spleen cells from two other H-2^a (K^k,D^d) strains, A/J and A.AL, as well as B10.AKM (K^k,D^q) also reacted with 20-8-4 in an identical fashion to B10.A spleen cells. This suggested that the reaction mapped in or to the right of the H-2D subregion. As can be seen in Table I, the strain distribution correlated with that of the Qa 2^a(Qa 4^a), Qa 3 and Qa 6 alleles (see footnote $^{||}$, Table I), but not with the distribution of Qa 1, Tla, or Qa 5, 7, 8 alleles.

In order to further map this CR antigen, two new recombinants were employed. The first of these was the BALB.A11, a new H-2^a recombinant derived from a backcross of (BALB.K × BALB/cKh)F$_1$ to BALB/cKh. The BALB/cKh subline, as described previously (35), is Qa 2^b (negative) and might therefore be useful for correlation of the CR with Qa 2. However, since BALB/cKh is K^d positive, it could not be tested directly due to the reactivity of 20-8-4 with K^d. Thus the BALB.A11, which has K^k,I^k from BALB.K and D^d and genes to the right of D from BALB/cKh (Sachs et al., unpublished data), permitted testing of H-2D and linked genes from a Qa 2–negative subline. As can be seen in Fig. 3 a, spleen cells from the BALB.A11 expressed K^k and D^d. These same cells were also positive with anti–I-A^k (10-2.16, [27]) and negative with anti–I-A^d (MKD-6, [28]) (data not shown), demonstrating that the BALB.A11 is indeed K^k,D^d. As
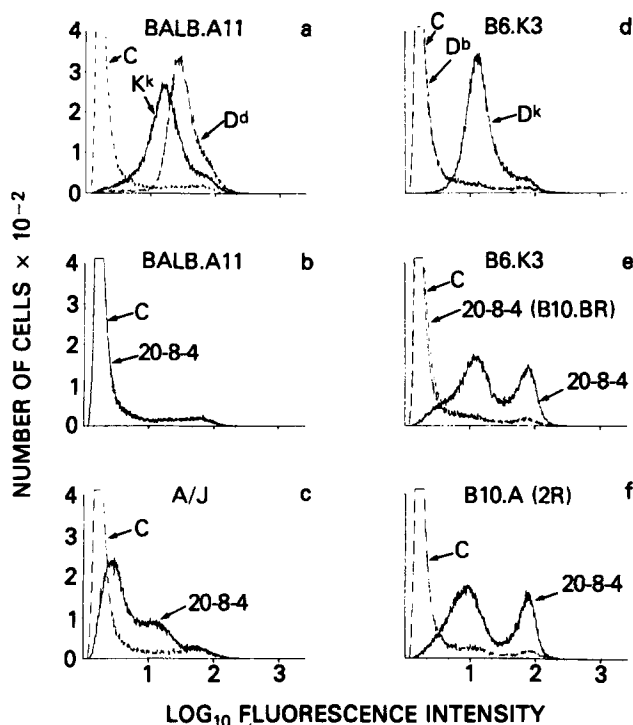

FIGURE 3. IF profiles of spleen cells incubated with MAb followed by FITC anti-IgG2. (a) BALB.A11 with 11-4.1, anti-K^k (———), 34-2-12, anti-D^d (— —) or no MAb (-----); (b) BALB.A11 with 20-8-4 (———) or no MAb (— —); (c) A/J cells with 20-8-4 (———) or no MAb (— —); (d) B6.K3 cells with 15-5-5, anti-D^k (———), 34-2-12, anti-D^d (----) or no MAb (— —); (e) B6.K3 cells with 20-8-4 (———) or no MAb (— —), B10.BR cells with 20-8-4 (-----); (f) B10.A(2R) cells with 20-8-4 (———) or no MAb (— —).

seen in Fig. 3 b, the BALB.A11 spleen cells did not react at all with 20-8-4, while A/J cells did react to give the characteristic bimodal IF profile. The lack of reactivity of 20-8-4 with BALB.A11 therefore demonstrated that 20-8-4 does not react with D^d and that the antigen detected by the 20-8-4 CR is not expressed when the H-2D–linked genes are derived from a BALB subline that is Qa 2 negative.

To confirm that expression of the 20-8-4 CR antigen was indeed controlled by loci to the right of H-2D, another newly derived recombinant, the B6.K3, was used. The B6.K3 is a recombinant between B6-H-2^k (H-2^k from AKR) and B6-Tla^a, itself a recombinant in the Tla region between B6 and A/J. The B6.K3 expresses the H-2 phenotype of B6-H-2^k (K^k,D^k) and the Qa,Tla phenotype of the B-6-Tla^a (Qa 2,3,4,5 positive, Tla^a) (Flaherty et al., unpublished results). The results of analysis of spleen cells from the B6.K3 are shown in Fig. 3, c–f. As can be seen in Fig. 3 d, B6.K3 spleen cells were indeed D^k positive and D^b negative. The same cells were also positive with anti-K^k (11-4.1, [27]), negative with anti-K^b,D^b (28-8-6, [28]) and positive with an anti-Qa 2 MAb D3.262 (9) (data not shown), confirming that B6.K3 is K^k,D^k and Qa 2 positive. When B6.K3 cells were stained with 20-8-4, the IF profile shown in Fig. 3 e was obtained. Most (>90%) spleen cells were positive when compared with control B10.BR cells and the B6.K3 IF profile showed the two distinct peaks of fluorescence intensity characteristic of the new CR antigen. These results demonstrated that the new antigen is determined by genes to the right of H-2D.

The overall intensity of staining of 20-8-4 on the B6.K3 cells (Fig. 3 e) was significantly higher than that seen when 20-8-4 reacted with A/J cells (Fig. 3 c). Fig. 3 f shows the IF profile of 20-8-4 with B10.A (2R) (K^k,D^b) spleen cells. It was expected that this IF profile would resemble that of B10 cells (Fig. 1 b), since 20-8-4 was thought to contain reactivity to D^b. However, as the data in Fig. 3 f demonstrate, the reactivity of 20-8-4 on a D^b positive strain, B10.A(2R), was similar to that on B6.K3 cells. This pattern of reactivity of 20-8-4 with B10.A(2R) cells suggested that 20-8-4 did not react with D^b on these cells, as previously presumed, but rather with the CR antigen. It is significant in this regard that the reactivity of 20-8-4 with B10.A(2R) spleen as measured by cytotoxicity, previously thought to define D^b reactivity, was of lower magnitude than seen with all other positive strains (24).

The mapping studies and strain distribution analysis summarized in Table I therefore showed that the 20-8-4 MAb not only reacted with H-2 Class I antigens (K^b,K^d, D^s,r) as previously demonstrated, but also cross-reacted with an antigen not controlled by genes within the H-2 region, but rather by linked genes outside of H-2. Furthermore, these studies showed that the expression of this antigen was correlated with expression of Qa 2.

Inhibition of 20-8-4 Reactivity. In order to analyze further the determinants detected by the 20-8-4 CR, a variety of antibodies were analyzed for their ability to block 20-8-4 FITC reactivity. The antibodies tested were either anti–Class I MHC antibodies or antibodies with no specificity for H-2 antigens, but reactive to Qa region antigens. The results of these studies are shown in Table II. As shown in Expt. 1 of this table, one of thirteen anti–Class I MHC MAbs tested, 34-1-2, was able to block completely the reaction of 20-8-4-FITC with B10.A

TABLE II

Analysis of Determinants Detected by 20-8-4 by Blocking Studies

Expt.	Target* cells	Blocking reagent		Test anti-body	% Inhibi-tion[‡]	Block[§]
		Antibody	Specificity			
1	B10.A	28-8-6	K^b,D^b	20-8-4[∥]	14.7	
		28-11-5	D^b,D^d,q,p		31.5	
		28-13-3	K^b,f		16.8	
		27-11-13	D^b,D^d,q		21.0	
		28-14-8	L^d,D^b,q		12.6	
		34-1-2	K^d,D^d,K^b,s,r,q,p		103.0	+
		34-2-12	D^d		29.4	
		34-4-21	D^d		23.1	
		34-5-8	D^d		−21.0	
		15-5-5	D^k,K^d,f		14.7	
		23-10-1	L^d,q		14.7	
		30-5-7	L^d,D^q,L^q,L^b		10.5	
		31-3-4	K^d		−6.2	
2	B10.A(2R)	20-8-4	K^b,K^d,r,s	20-8-4[∥]	98.9	+
		34-1-2	K^d,D^d,K^b,r,s,q,p		96.4	+
		11-4.1	K^k		−12.1	
		D3.262	Qa 2		18.1	
		K1 anti-B6	Qa 2, Qa 4, ?		99.4	+
	B10.A	20-8-4			94.8	+
		34-1-2			90.4	+
		11-4.1			−44.0	
		D3.262			26.4	
		K1 anti-B6			97.5	+
	B10.A(2R)	20-8-4		34-1-2[∥]	96.8	+
		34-1-2			96.8	
		11-4.1			−31.0	
		D3.262			−1.0	
		K1 anti-B6			98.7	+
3	B10.A(2R)	20-8-4		D3.262[¶]	1.4	
		34-1-2			24.8	
		11-4.1			−11.7	

* Spleen cells.
‡ See Materials and Methods.
§ Positive (+) blocking defined as greater than or equal to 35% inhibition.
∥ Fluorescein-conjugated antibody.
¶ D3.262 (IgM) followed by FITC-Goat F(ab')₂ anti-mouse IgM. Blocking reagents were IgG2 antibodies which in control samples did not react with fluorescent reagent.

spleen cells. 34-1-2 was derived from a different immunization and fusion than was 20-8-4 (24, 25) and has a different pattern of Class I reactivity (ie specificity) than does 20-8-4. Other anti–Class 1 MHC Abs gave minimal blocking ranging from 0 to 30% inhibition. This low level of blocking was found to be nonspecific since four of these antibodies (28-8-6, 28-14-8, 34-2-12, and 34-5-8) were unreactive with B6.K3 or B10.A(2R) cells by direct testing (data not shown). The data in Table II, Expt. 2, demonstrated that 34-1-2 was able to block 20-8-

4-FITC reactivity on B10.A(2R) as well as on B10.A spleen cells and that this blocking was comparable to that found with unlabeled 20-8-4 itself. Fig. 4a shows the IF profiles for 20-8-4-FITC staining of B10.A(2R) spleen cells following pretreatment with either 34-1-2 or media. 34-1-2 pretreatment can be seen to have reduced the 20-8-4-FITC reaction to a level of fluorescence intensity close to that of unstained cells.

The data shown in Table II, Expt. 2, also demonstrate that the anti-Qa MAb D3.262 did not block 20-8-4-FITC reactivity on either B10.A or B10.A(2R) spleen cells. In contrast, serum from a B6.K1 anti-B6 immunization, with known reactivity to Qa 2/4 and Qa 5 and with potential reactivity to other antigens controlled by the Qa and Tla subregions, completely inhibited 20-8-4-FITC reactivity on both B10.A and B10.A(2R) cells. Fig. 4b shows that B6.K1 anti-B6 pretreatment reduced the 20-8-4-FITC reaction on B10.A(2R) cells to background, while either D3.262 or 11-4.1 pretreatment did not. The same antibod-
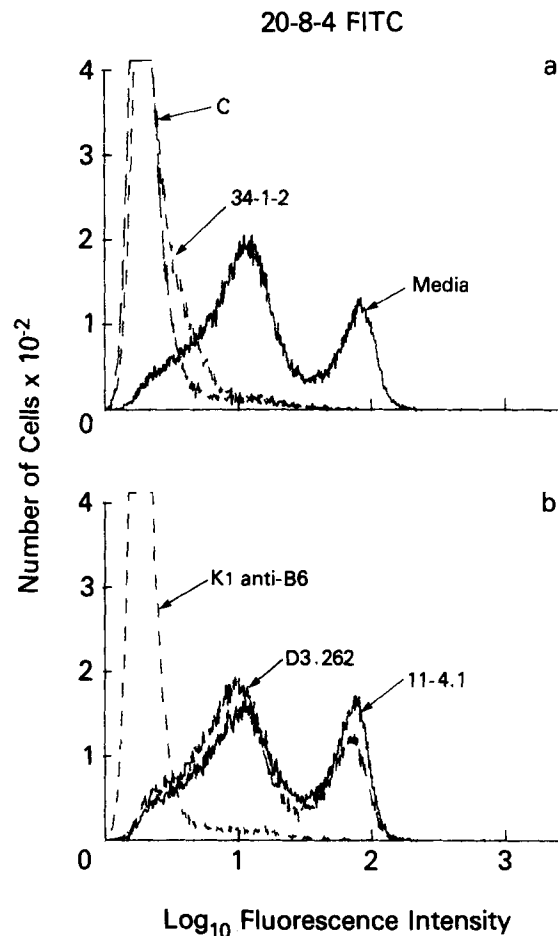

FIGURE 4.   IF profiles of B10.A(2R) spleen cells; (a) preincubated with media (———) or 34-1-2 (— —) followed by 20-8-4 FITC; C, unstained cells; (b) cells preincubated with 11-4.1 (———), D3.262 (----) or B6.K1 anti-B6 serum (— —).

ies were also analyzed for their ability to block 34-1-2 CR reactivity on B10.A(2R) spleen cells. The staining in this case was identical to that of 20-8-4 staining, showing a bimodal profile. As the data in Table II, Expt. 2, indicate, 34-1-2-FITC reactivity was inhibited by 34-1-2, 20-8-4, and B6.K1 anti-B6 serum, but not by 11-4.1 (anti-K^k, reference 27) or D3.262 (anti-Qa 2). This was the same result obtained with these reagents when they were used to block 20-8-4-FITC. In addition, the data shown in Table II, Expt. 3, demonstrate that 20-8-4 and 34-1-2 did not block the reactivity of D3.262 with B10.A(2R) spleen cells.

In summary, the inhibition experiments showed that two anti–Class I MHC MAbs, 20-8-4 and 34-1-2, also detect identical or spatially related determinants on an antigen(s) determined by genes that map to the right of the H-2 region. Antibodies present in an antiserum against products of this region also detected this determinant or spatially related determinants, while a monoclonal anti-Qa 2 antibody, D3.262 did not.

Tissue Distribution of the CR Antigen Detected by 20-8-4. The expression of the antigen detected by the 20-8-4 CR was studied on spleen, lymph node, thymus, and bone marrow cells using immunofluorescence and FMF analysis. Fig. 5 (*a* and *b*) illustrates the IF profiles obtained when 20-8-4 was tested on B10.A(2R) and B10.A spleen cells. Both spleen cell sources showed bimodal IF profiles, with >80% of the B10.A(2R) spleen cells and 50% of the B10.A spleen cells above background. B10.A(2R) spleen cells were 6–10 times brighter than B10.A spleen cells in repeated experiments. The dull peak of B10.A spleen cells was variable in intensity from animal to animal such that only 40–80% of B10.A cells could be routinely detected as clearly positive relative to background. However
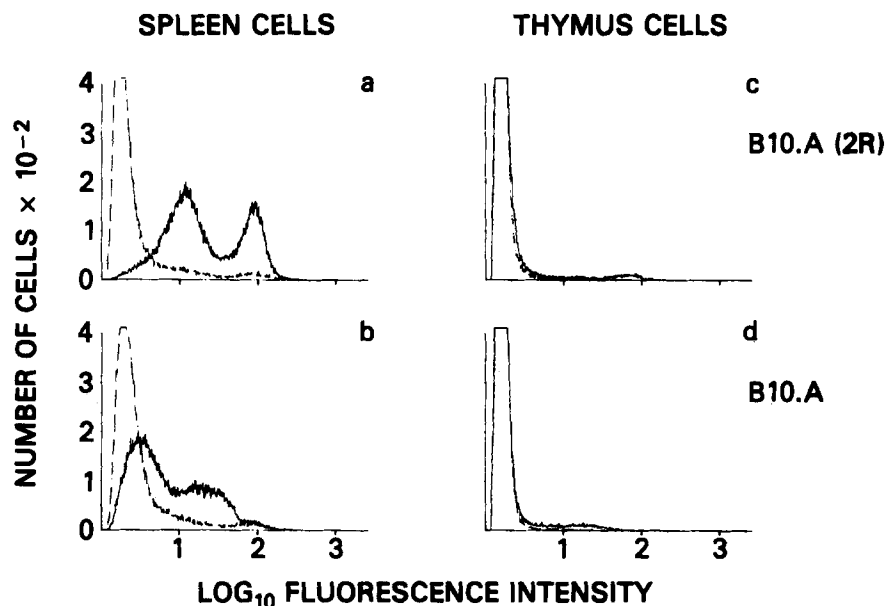

FIGURE 5. IF profiles of spleen (*a* and *b*) or thymus (*c* and *d*) cells reacted with 20-8-4 followed by FITC anti-IgG2 (———) or FITC anti-IgG2 only (— —). Cells were from B10.A(2R) (*a* and *c*) or B10.A (*b* and *d*) animals.

IF profiles such as that shown in Fig. 5b suggested that indeed most B10.A spleen cells were positive.

The IF profiles obtained for 20-8-4 on thymus cells are shown in Fig. 5, c and d. Only small subpopulations of thymocytes were positive above background. The positive B10.A(2R) thymocytes (5.7%) were much brighter than the positive B10.A thymocytes (5.6%). For both strains, the positive thymocyte peaks of cells were of the same fluorescence intensity as the bright positive spleen cells from the same animal.

To assess further the relative reactivities of 20-8-4 and D3.262 (anti-Qa 2), these MAbs were studied with B10.A(2R) and B10.A bone marrow cells (Fig. 6). The IF profile obtained with 20-8-4 on B10.A(2R) bone marrow cells (Fig. 6a) showed three subpopulations: negative cells (78% of total), dull positive cells (16%), and bright cells (6%). The small bright subpopulation was of the same intensity as the bright subpopulation of positive spleen cells and the small positive subpopulation of thymocytes from the same animal (Fig. 6a vs. Fig. 5, a and c). The B10.A bone marrow IF profile with 20-8-4 also showed a small positive subpopulation (8% of total) that correlated in intensity with the bright subpopulations in B10.A spleen and thymus (Fig. 6b vs. Fig. 5, b and d). The remainder of the B10.A bone marrow cells were not clearly above background although the IF profile shown in Fig. 6b suggested that there might be an additional weakly positive subpopulation similar to that found in B10.A (2R) bone marrow cells.
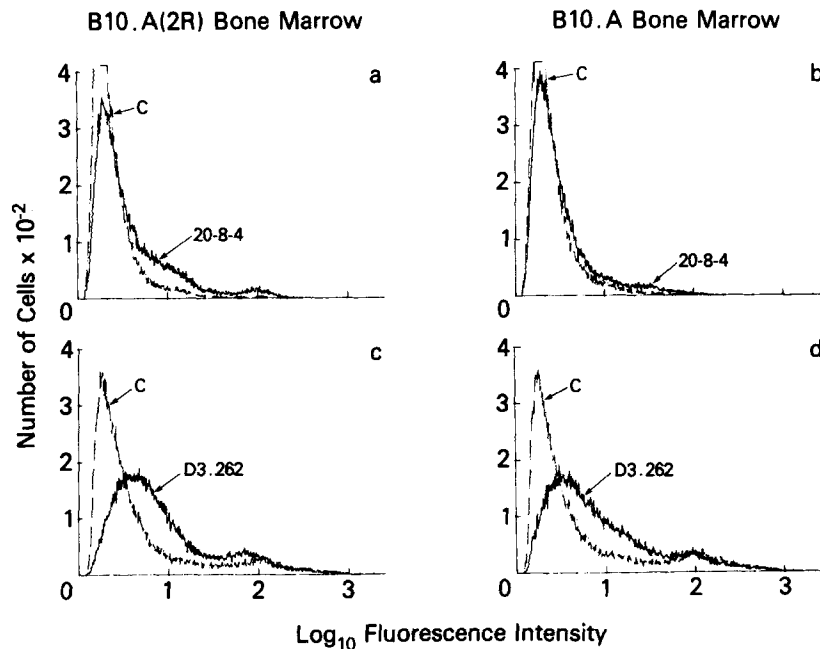

FIGURE 6.   IF profiles of B10.A(2R) (a and c) or B10.A (b and d) bone marrow cells; a and b cells reacted with 20-8-4 (——) or 25-9-17 (IgG2a, anti-I-A^b) control (C) antibody (— —) followed by FITC anti-IgG2; c and d cells reacted with D3.262 (——) or 25-5-16 (IgM, anti-I-A^b) control (C) antibody (— —) followed by FITC anti-IgM.

The reactivity of D3.262 with the same bone marrow cells (Fig. 6, c and d) was very different from that found with 20-8-4. One striking observation was that the overall intensity of staining did not differ between the two strains, such that B10.A bone marrow cells were of the same intensity as those from B10.A(2R). When spleen cells from the same animals were analyzed with D3.262, B10.A(2R) Ig⁻ cells were only 1.5 times brighter than B10.A Ig⁻ cells (data not shown). In addition, the shape of the IF profiles shown in Fig. 6 suggested that most bone marrow cells from both strains were positive with D3.262, although only 40% (B10.A(2R)) and 36% (B10.A) were clearly positive after subtraction of background.

The tissue distribution analysis of the antigen detected by the 20-8-4 CR demonstrated that this antigen was expressed on most, if not all spleen cells, but only on small subpopulations of thymus and bone marrow cells. Spleen and lymph node cells (see below) gave similar IF profiles and each clearly contained two subpopulations that differed significantly in their levels of expression of this antigen. Finally, the expresion of the CR antigen was different on bone marrow cells than that of Qa 2, as defined by the D3.262 monoclonal antibody.

Two-Color Immunofluorescence Analysis of the 20-8-4 CR. Two-color immunofluorescence analysis was performed to determine whether or not the bimodal distribution of staining by 20-8-4 correlated with B and T cell subpopulations.
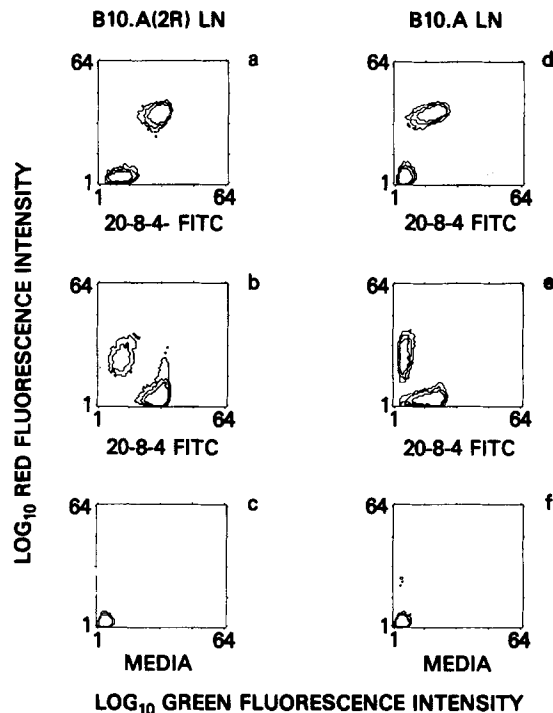

FIGURE 7. Two-color immunofluorescence analysis contour plots (see Materials and Methods). B10.A(2R) (a–c) or B10.A (d–f) lymph node cells were stained for green fluorescence with 20-8-4 FITC (a, b, d, e) and for red fluorescence with biotin-anti-Thy1.2 (a and d) or biotin anti-I-A^k, 10-2.16 (b and e) followed by TRA. c and f cells stained only with TRA.

Examples of contour analyses of the correlation between 20-8-4 CR and either Thy-1.2 or I-A expression are shown in Fig. 7. Analysis of B10.A(2R) and B10.A lymph node cells stained with 20-8-4-FITC (green fluorescence) and biotinylated-anti–Thy-1.2 plus TRA (red fluorescence) (Fig. 7, a and d) showed that in both strains all 20-8-4 bright cells were Thy-1.2 positive. Reciprocally, all Thy-1.2-positive B10.A(2R) cells and 85% of Thy-1.2-positive B10.A cells were also bright for 20-8-4 CR. Low numbers of Thy-1.2-positive, 20-8-4 dull, cells were consistently seen in both lymph node and spleen cells from B10.A animals, but were never found in B10.A(2R) spleen or lymph node cells. Fig. 7, b and c show a similar analysis between staining for 20-8-4 CR and anti-I-A^k. In agreement with the Thy-1.2 correlation, the majority of 20-8-4 dull cells from both strains were I-A positive. Fig. 7, c and f show the control contour analyses of the same cells reacted with only media (green fluorescence) and TRA (red fluorescence). Results similar to those shown in Fig. 7 were obtained with spleen cells from the same animals (data not shown). These data thus demonstrate that the H-2–linked antigen detected by 20-8-4 is preferentially expressed on peripheral T cells, although non-T cells express low amounts of the antigen.

A similar two-color immunofluorescence analysis was performed to assess the reactivity of 20-8-4 via its anti-MHC specificity (Fig. 8). In this experiment, 20-8-4 subpopulation reactivity on B10.A(2R) cells (which express only the CR antigen and not H-2K or D antigens reactive with 20-8-4) was compared to
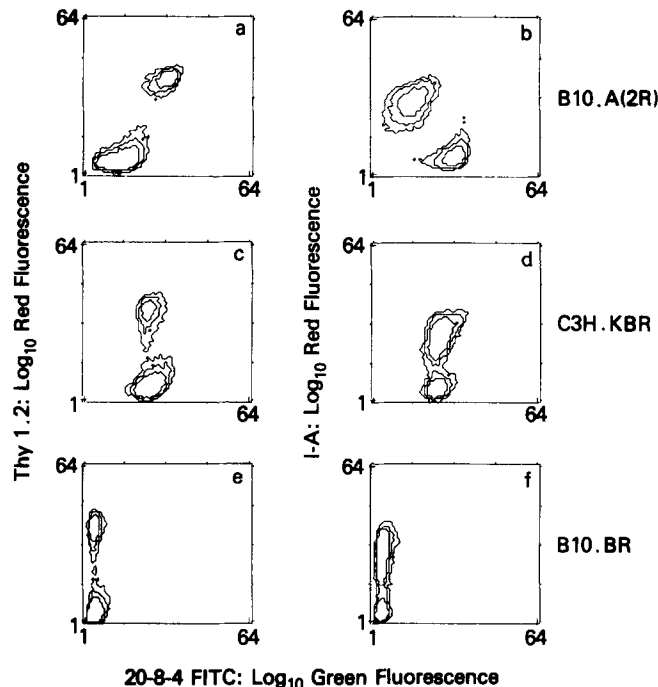

20-8-4 FITC: Log₁₀ Green Fluorescence

FIGURE 8.   Two-color immunofluorescence analysis contour plots. B10.A(2R) (a and b), C3H.KBR (c and d), or B10.BR (e and f) spleen cells were reacted with 20-8-4 FITC (a–f) as well as either biotin-anti–Thy-1.2 (a, c, and e) or biotin anti-I-A followed by TRA; b and f = 10-2.16, anti-I-A^k; d = 25-9-17, anti-I-A^b.

subpopulation reactivity on C3H.KBR (see Materials and Methods) cells. C3H.KBR expresses H-2K^b which is reactive with 20-8-4, but not the CR antigen because the H-2D and Qa/Tla regions of this animal are derived from C3H which is negative for the CR antigen (see Table I). B10.A(2R) spleen cells contained Thy-1.2–positive, 20-8-4 bright cells (Fig. 8a), and I-A^k positive, 20-8-4 dull cells (Fig. 8b), a result compatible with data shown in Fig. 7. In contrast, when subpopulation analysis of C3H.KBR (K^b,I^b,D^k,Qa 2^b) spleen cells was performed, the 20-8-4 antibody did not discriminate between T cells and B cells (Fig. 8, c and d). Thus, Thy-1.2 positive cells were of the same green fluorescence (20-8-4) intensity as Thy-1.2–negative cells (Fig. 8c). Unlike B10.A(2R) cells, I-A^b–positive C3H.KBR cells were no less intense with 20-8-4-FITC than were I-A^b–negative cells (compare Fig. 8, b vs. d). B10.BR spleen cells reacted with Thy-1.2 (Fig. 8e) and anti-I-A^k (Fig. 8f), but were, as expected, negative with 20-8-4-FITC. Therefore, two-color immunofluorescence analysis demonstrated that the single MAb, 20-8-4, reacted with Class I antigens equally on T cells and B cells, but also reacted with a different, H-2–linked, antigen predominantly expressed on T cells.

Discussion

The present study demonstrates serological cross-reactivity between Class I MHC molecules (H-2) and an H-2–linked differentiation antigen expressed predominantly on T cells and determined by genes that map to the right of H-2D. Two anti–Class I MHC-specific monoclonal antibodies (20-8-4 and 34-1-2) have been found that cross-react with this H-2 CR antigen. The cross-reactive determinant(s) is expressed on an antigen that can be tentatively classified as a Qa antigen by genetic mapping as well as strain and tissue distribution. In addition, an antiserum specific for products of the Qa/Tla region blocks reactivity of the anti–Class I MHC antibodies with the H-2 cross-reactive (CR) antigen. Although the reaction of anti–Class I MHC MAbs with this H-2 CR antigen is inhibited by B6.K1 anti-B6 serum, the determinants being detected by the serum and the MAbs must be different. Both MAbs react strongly with K^b, while the B6.K1 animal, which itself expresses K^b, would not be expected to produce detectable antibodies reactive with this antigen. Consistent with this expectation, reactivity of the MAbs to K^b itself, rather than the H-2CR antigen, is not inhibited by B6.K1 anti-B6 serum (data not shown). Therefore, it seems likely that the B6.K1 anti-B6 serum and the anti–H-2 MAbs detect distinct, but spatially related determinants on the same epitope of the H-2 CR antigen. One prediction of this analysis is that cross-reactive antibodies such as the ones described in the present study might be readily detected only in immunizations in which the responder animal and the immunizing cells differ at H-2.

The Tla region of the mouse genome located telomeric to H-2D is known to contain at least two subregions on the basis of recombinant events between Qa 2 and TL (12). Both TL antigens and the Qa 1 antigen are controlled by the TL subregion and consist of specificities distinct from the H-2 CR antigen reported in the present study on the basis of strain distribution (see Table I). Qa 6 is thought to be controlled by loci within the TL subregion (37), while Qa 2-5,7,8 are controlled by the Qa subregion (12, 38). While differences in thymus,

bone marrow, and B cell expression have been reported for these antigens, they are all expressed predominantly on peripheral T cells (12, 37, 38) as is the CR antigen described in the present study. Qa 2-8 antigens also share similar, but not identical, strain distributions distinguished by the fact that only strains that are Qa 2 positive express any of the remaining Qa 3-8 specificities (12, 37, 38). The CR antigen reported here appears to be distinct from Qa 5,7,8 on the basis of strain distribution (see Table I). While the expression of the CR antigen correlates with that of Qa 2,3,6 by strain distribution, it appears to be distinct because (a) the reactivity of 20-8-4 with the CR antigen is not inhibited by the D3.262 (anti-Qa 2) MAb; (b) the expression of this CR antigen on bone marrow cells, as shown in this study, is markedly different from that of Qa 2 as detected by D3.262; (c) two other recently described anti-Qa 2 MAbs have been reported to identify B cell subpopulations (10, 11), while the 20-8-4 reaction with the CR antigen does not; (d) Unlike Qa 3 and Qa 6 which have not been detected on thymocytes (12, 38), the CR antigen has been found on ~5% of thymocytes. Therefore, if one considers previous reports on Tla region antigens, it appears that the anti-Class I MAb cross-reactivity described here detects a previously undefined Qa antigen or specificity. However, there is sufficient ambiguity in the definition of previously defined Qa antigens to make overlap possible, and we have therefore not assigned a definite Qa specificity for the CR antigen to date. The data presented here illustrate that analysis of this type of antigen with MAbs and flow microfluoremetry permits highly reproducible and sensitive quantitation on different cellular subpopulations and mouse strains, thus providing less ambiguous definition of the antigen than has previously been described.

Recent molecular analyses of the mouse genome (reviewed in detail, reference 21) have demonstrated the existence of numerous DNA sequences that cross hybridize with H-2 Class I DNA probes, suggesting that these sequences may constitute a large gene family derived from a common ancestral gene (3, 4). It has been suggested that genes linked to H-2, even if unexpressed in normal tissues, may somehow be involved in the generation of H-2 polymorphism, possibly by a gene conversion mechanism (40–42). In addition, some or all of these genes may be expressed in a different fashion from classical Class I products. Rather than being expressed ubiquitously, they could, for example, be expressed only on specific tissues or at certain times during ontogeny. As such, these antigens could serve differentiation or tissue-specific functions.

By definition, the new Qa determinant(s) described in this study shares serological cross-reactivity with classical H-2 Class I MHC antigens. Serologically cross-reactive anti-Qa 1 alloantisera detecting H-2 Class I antigens (K^f) have previously been reported (43, 44), although anti-Qa cross-reactivity in anti-H-2 K^f alloantisera could not be detected (43). In addition, reactivity to Qa 2 by anti-K^b alloantisera has been reported (45). However, such sera are composed of complex mixtures of antibodies, and the basis of the cross-reactions is therefore more difficult to interpret. In the present study, the same MAbs shown to cross-react with a Qa-like antigen also react with known H-2 products. In fact, these two MAbs are extremely cross-reactive among H-2 Class I antigens (24, 25) and have recently been reported to detect two H-2K products in the H-2^d haplotype (46). Serologic cross-reactivity between the products of H-2 Class I MHC genes

and those of genes telomeric to H-2D suggests that these genes may share a common origin and structure. This antigen therefore fits precisely the characteristics predicted for a product of one of the many Class I-like genes defined by DNA hybridization studies but previously unrecognized as an expressed gene. Confirmation of this prediction must await identification of the gene responsible for the CR specificity, and studies using L cell transformants bearing Class-I-like genes are in progress for this purpose.

A parallel of possible significance can be drawn between the lymphoid expression of this CR antigen and of Ia antigens. Both are determined by H-2-linked genes and both show a more limited tissue distribution than do Class I H-2 antigens. By two-color immunofluorescence analysis, there is an almost completely inverse correlation between I-A antigen and H-2 CR antigen expression on spleen and lymph node cells. This inverse relationship may be fortuitous, or it may provide a clue to the possible function of the CR antigens in cellular immune interactions. The availability of MAbs which react with both Class I and Class I-like gene products also has possible practical implications. Because each of these antibodies reacts with both H-2 and non-H-2 antigens derived from genes with striking homologies, a single probe can now be used to analyze similarities in the products of these genes. These antibodies may also be useful in analysis of factors which control the differences in tissue and temporal expression of H-2 vs. H-2-linked homologous genes.

Summary

Analysis of anti-Class I major histocompatibility complex (MHC) monoclonal antibodies by immunofluorescence and flow microfluorometry demonstrated an unexpected cross-reactivity. Two of fifteen antibodies examined (20-8-4, anti-K^b,K^d,r,s and 34-1-2, antiK^d,D^d,K^b,r,s,q,p) were observed to detect an antigen determined by gene(s) mapping to the right of H-2D. Two-color immunofluorescence analysis demonstrated that this antigen, unlike classical H-2K and D antigens, was expressed in high amounts on peripheral T cells, but only weakly on Ia-positive cells and on small subpopulations of thymus and bone marrow cells. Mapping, absorption, blocking, and tissue distribution studies suggested that the cross-reactive antigen is Qa-like, but distinct from previously described Qa antigens. Thus, these data demonstrate serological cross-reactivity between a Class I MHC antigen and a differentiation antigen determined by genes linked to H-2. It seems likely that the gene responsible for this new antigen is one of the numerous Class I-like sequences detected by DNA hybridization analyses, but previously undefined in terms of tissue expression. These data suggest that many of these DNA sequences may be expressed in specific tissues and that cross-reactions of anti-Class I MAbs may provide useful probes for studying the products of such homologous genes.

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