ANTI-SELF RECEPTORS

I. Direct Detection of *H-2L* Region-restricted Receptors on Murine Thymocytes

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There have been numerous reports in the past that substantial subpopulations of murine lymphocytes can form rosettes with both autologous and allogeneic erythrocytes (1–5). In the case of thymocytes, as high as 30% of the cells can rosette with autologous erythrocytes (2, 5). However, the nature of this interaction and its functional significance has remained a mystery.

In recent years it has become increasingly apparent that products of the major histocompatibility complex (*MHC*)[1] of mice and other species play a critical role in immune induction. For example, self-H-2K and H-2L/H-2D antigens play a vital part in the recognition of foreign antigens by cytotoxic T cells (6, 7). In a similar manner, it appears that self structures mapping to the *I* region are recognized in association with foreign antigen by the T cells that express help (8), delayed-type hypersensitivity (9), and antigen-specific proliferation in vitro (10, 11). Although the mechanism of recognition of self plus foreign antigen in these systems is still uncertain, frequently it has been suggested that two receptors are involved, one that recognizes foreign antigen, the other that recognizes a self-*MHC* product (12–15).

With these concepts in mind, it appeared possible that the reaction between lymphocytes and autologous erythrocytes might be mediated by an anti-self receptor on lymphocytes that had syngeneic preference for a *MHC* gene product on erythrocytes. This anti-self receptor would probably weakly cross-react with other haplotypes, and thus multipoint binding, via a low-affinity receptor, would explain the interaction of lymphocytes with both allogeneic and syngeneic erythrocytes.

This paper describes our attempts to test this hypothesis. The experimental protocol was to measure the ability of different erythrocyte sonicates to inhibit the rosetting of thymocytes with autologous erythrocytes. With this method it was possible to demonstrate that autorosette formation is, indeed, mediated by an *H-2*-restricted receptor. In fact, the receptor appears to be primarily directed against an *H-2L* region-controlled molecule.

Materials and Methods

Animals. Table I lists the mouse strains used in this study together with the haplotype origin of their *H-2* regions. The mice and Lewis rats were bred at the John Curtin School of Medical Research (Canberra, Australia), the majority under specific pathogen-free conditions. Mice were used as donors of erythrocytes and/or thymocytes from 8–20 wk of age.

[1] *Abbreviations used in this paper:* FCS, fetal calf serum; *MHC*, major histocompatibility complex.

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TABLE I

Haplotype Origin of H-2 Regions Carried by Mouse Strains Used in this Study

Strain	Haplotype	Region*								
		K	*A*	*B*	*J*	*E*	*C*	*S*	*G*	*L/D*
C57BL/6, C57BL/10	b	b	b	b	b	b	b	b	b	b
BALB/c, DBA/2	d	d	d	d	d	d	d	d	d	d
B10.D2 (M504)	dm1	d	d	d	d	d	d	d	d	d‡
BALB/c (C55)	dm2	d	d	d	d	d	d	d	d	d‡
B10.A (2R)	h2	k	k	k	k	k	d	d	?	b
B10.A (4R)	h4	k	k	b	b	b	b	b	b	b
B10.A (3R)	i3	b	b	b	b	k	d	d	d	d
CBA/H, B10.BR	k	k	k	k	k	k	k	k	k	k
DBA/1, B10.G	q	q	q	q	q	q	q	q	q	q
SJL/J, A.SW	s	s	s	s	s	s	s	s	s	s
A.TH	t2	s	s	s	s	s	s	s	s	d
B10.AQR	y1	q	k	k	k	k	d	d	d	d
B10.T(6R)	y2	q	q	q	q	q	q	q	?	d

* Haplotype origin of regions according to Klein et al. (16).

‡ The *dm1* mutant carries modified *H-2L* and *H-2D*, whereas the *dm2* mutant lacks *H-2L* (20).

Antisera. We are indebted to Dr. I. F. C. McKenzie (Department of Medicine, Austin Hospital, Heidelberg, Victoria, Australia) for providing an anti-H-$2L^d$ serum that was raised in (BALB/c-H-2^{dm2} × A.SW)F_1 mice against BALB/c lymphoid cells (AS 207). We also thank Dr. G. Hämmerling (Institut für Genetik, 5000 Köln, Federal Republic of Germany) for supplying a monoclonal anti-H-2.25 antibody (H100-27.R9). By microcytotoxicity the anti-H-$2L^d$ serum had a titer of between 1/16 and 1/32 on BALB/c spleen, whereas the anti-H-2.25 antibody gave a titer of 1/20,000 on CBA/H spleen.

Preparation of Cell Suspensions. Thymocyte cell suspensions were prepared by gently pressing thymus fragments through wire sieves into cold Hank's balanced salt solution that contained 0.5% (vol/vol) fetal calf serum (FCS). Higher concentrations of FCS in media were avoided because FCS tended to inhibit autologous rosetting. The cell suspensions were gently pipetted, cell clumps removed by low-speed centrifugation (100 *g* for 30 s), and the dispersed cells washed twice with cold medium. The thymocytes were then counted and cell viability assessed by trypan blue exclusion. Viability usually exceeded 90%. Cells were then diluted to a concentration of 2×10^6/ml in medium.

Blood was collected from mice in Alsever's solution and washed four times in normal saline. Packed or 50% suspensions of erythrocytes in medium were then disrupted by sonication for 10 s at 30 W with a Branson probe-type sonicator (model B12, Branson Sonic Power Co., Danbury, Conn.). The erythrocyte suspensions were completely transparent after this treatment. Sonicates were usually prepared on the day of use or stored for no longer than 24 h at 4°C.

Standard Rosetting Assay. The standard autologous rosetting assay was carried out in the following manner in 96 well U-bottomed microplates (Linbro Chemical Co., Hamden, Conn.). To 25 μl of ice-cold thymocytes (2×10^6/ml in medium) was added 25 μl of a 0.5% suspension of erythrocytes in ice-cold medium. This represented the minimum concentration of erythrocytes needed for high rosetting percentages. The erythrocyte-thymocyte mixture was then pelleted by centrifugation at 200 *g* for 1 min at 4°C. The cell pellets were incubated on ice for at least 60 min before being very gently resuspended with a short Pasteur pipette. Methyl violet staining solution (100 μl) (17) was then added to the wells, each sample transferred to a hemocytometer chamber, and the percentage of rosette-forming cells assessed. Usually a minimum of 100–200 thymocytes were scanned for rosettes, and, when the rosette frequency was <10%, up to 500 thymocytes were examined. Any thymocyte that bound four or more erythrocytes was classified as a rosette.

Rosette-Inhibition Assay. In this assay 50 μl of serial dilutions of erythrocyte sonicates were

TABLE II

Strain Specificity of Autorosette Inhibition

Thymocytes	Erythrocytes	Erythrocyte sonicate*							
		Nil	BALB/c ($H\text{-}2^d$)	DBA/2 ($H\text{-}2^d$)	CBA/H ($H\text{-}2^k$)	B10.BR ($H\text{-}2^k$)	C57BL/6 ($H\text{-}2^b$)	SJL/J ($H\text{-}2^s$)	DBA/1 ($H\text{-}2^q$)
BALB/c	BALB/c	43 ± 2.5	[9 ± 3.0‡]	[5 ± 2.0]	[11 ± 2.1]	[17 ± 1.4]	43 ± 2.1	40 ± 2.5	44 ± 2.0
DBA/2	DBA/2	33 ± 1.5	[5 ± 2.0]	[4 ± 2.0]	[5 ± 2.5]	[13 ± 1.5]	30 ± 1.6	31 ± 2.1	31 ± 2.1
CBA/H	CBA/H	34 ± 2.3	[6 ± 2.5]	[6 ± 2.0]	[6 ± 1.2]	[9 ± 2.1]	31 ± 2.1	32 ± 2.0	31 ± 1.5
B10.BR	B10.BR	57 ± 3.2	[26 ± 2.1]	[24 ± 2.1]	[19 ± 3.5]	[29 ± 0.7]	55 ± 3.5	57 ± 3.5	57 ± 0.7
C57BL/6	C57BL/6	45 ± 2.7	44 ± 2.1	45 ± 1.5	45 ± 2.1	43 ± 3.1	[14 ± 2.0]	43 ± 4.0	44 ± 2.5
SJL/J	SJL/J	21 ± 1.6	20 ± 2.4	22 ± 1.5	21 ± 3.1	21 ± 2.0	21 ± 2.6	[5 ± 1.0]	20 ± 1.5
DBA/1	DBA/1	42 ± 3.0	41 ± 2.1	43 ± 2.6	40 ± 1.5	41 ± 1.7	42 ± 2.1	43 ± 3.1	[5 ± 3.5]

* Thymocytes preincubated for 60 min at 4°C with 100% erythrocyte sonicate before rosetting with autologous erythrocytes.

‡ Results are expressed as percentage of rosetting thymocytes ± standard deviation of mean of three determinations. Values that represent significant inhibition are enclosed.

placed in the wells of microplates. Usually the highest concentration of sonicate was prepared from packed (100%) erythrocytes. 25 μl of thymocytes (2×10^6/ml in medium) were then added to each well and the mixture incubated on ice for 60 min. The wells of the microplate were then filled with 200 μl of ice-cold medium, the cells sedimented by centrifugation at 200 *g* for 1 min at 4°C, and the supernate discarded by flicking the plate. The cells in each well were washed once more with 200 μl of ice-cold medium and then resuspended, by gentle vortexing, in 25 μl of ice-cold medium. 25 μl of a 0.5% suspension of erythrocytes was added to each well, and the standard rosetting assay was then performed.

Blocking Rosette Inhibition with Antisera. In some experiments erythrocytes were preincubated with anti-H-2 sera before sonication and use in the inhibition assay. The preincubations were as follows: To 0.125 ml of packed erythrocytes was added 0.4 ml of either undiluted anti-H-$2L^d$ serum or a 1/2,000 dilution of H-2.25 monoclonal antibody. The mixtures were left on ice for 30 min, and the erythrocytes were then washed three times with medium before sonication as packed erythrocytes.

Results

Inhibition Assay for Assessing Specificity of Autorosetting. It is well established that a proportion of murine thymocytes forms rosettes with both syngeneic and allogeneic erythrocytes (1–5). However, this rosetting phenomenon may be a result of a strain-specific receptor whose specificity is masked by multipoint interaction between thymocytes and erythrocytes. To test this hypothesis an inhibition assay was developed that entailed preincubating thymocytes with different erythrocyte sonicates, washing the thymocytes, and then rosetting the thymocytes with autologous erythrocytes. With this assay it was possible to demonstrate some strain specificity in the thymocyte receptor that recognizes autologous erythrocytes.

Table II summarizes the results obtained with this inhibition assay when seven different mouse strains were tested. It was found that with C57BL/6, SJL/J, and DBA/1 thymocytes the autorosette inhibition was completely strain specific. In contrast, inhibition was strongly cross-reactive between BALB/c, DBA/2, CBA/H, and B10.BR mouse strains. This result suggested that in BALB/c, DBA/2, CBA/H, and B10.BR mice the thymocyte receptors have identical or very similar specificity. The complete inhibition data obtained with BALB/c thymocytes are depicted in Fig. 1. Again, it can be seen that BALB/c, DBA/2, CBA/H, and B10.BR erythrocyte

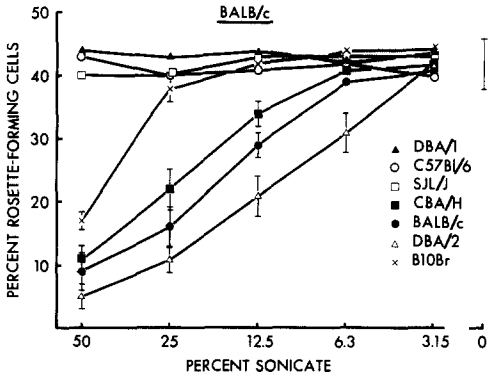

FIG. 1. Ability of erythrocyte sonicates from different mouse strains to inhibit the rosetting of BALB/c thymocytes with BALB/c erythrocytes. The erythrocyte sonicates used as inhibitors are listed on the figure. Thymocytes were preincubated with different concentrations of sonicates before rosetting. Vertical bars represent standard deviations of means. The rosetting of control thymocytes, which were not preincubated with sonicates, is indicated in the right-hand margin of the figure.

sonicates show strong inhibition, whereas C57BL/6, SJL/J, and DBA/1 sonicates are not inhibitory.

It should be noted that the data in Table II and Fig. 1 tend to implicate the *H-2* complex in autorosette inhibition. Thus, B10.BR ($H\text{-}2^k$) and C57BL/6 ($H\text{-}2^b$) thymocytes exhibit strain-specific inhibition even though these two strains have very similar genetic backgrounds. Conversely, both $H\text{-}2^d$ (BALB/c and DBA/2) and $H\text{-}2^k$ (CBA/H and B10.BR) strains tested yielded comparable results even though these strains have very different genetic backgrounds.

In subsequent experiments it was found that the erythrocyte sonicates used in the inhibition assays contained very small membrane vesicles that can only be sedimented by centrifugation at between 5,000 and 20,000 *g*. When erythrocyte ghosts were used as inhibitors in the assay, nonspecific inhibition of rosetting was observed. On the other hand, detergent-dissociated erythrocytes membranes were not inhibitory.

Genetic Mapping of Autorosetting. In the next series of experiments we attempted to map the gene(s) responsible for the strain-specific inhibition of autorosetting. Figs. 2 and 3 depict the complete inhibition data obtained with the seven strains examined in Table II. Virtually identical inhibition data was obtained with BALB/c ($H\text{-}2^d$), DBA/2 ($H\text{-}2^d$), CBA/H ($H\text{-}2^k$), and B10.BR ($H\text{-}2^k$) thymocytes (Fig. 2). Inhibition of rosetting was only obtained with sonicates from mice that expressed either the *d* (i.e., BALB/c, DBA/2, and A.TH) or *k* (i.e., B10.BR and CBA/H) haplotype in the *H-2L/H-2D* region of the *MHC*. However, the most striking result was that the erythrocyte sonicate from the BALB/c-$H\text{-}2^{dm2}$ mutant mouse (C55 strain) was not inhibitory. This strain carries an $H\text{-}2L^d$ deletion mutation (18–20) and, therefore, this finding strongly suggests that BALB/c, DBA/2, CBA/H, and B10.BR thymocytes are recognizing an *H-2L* region-controlled structure on mouse erythrocytes. Further analysis of this mutant strain will be presented later in this paper. An additional implication of these results is that, in this autorosetting test, thymocytes cannot distinguish between the H-2L molecules carried by *d* and *k* haplotypes.

Inhibition results obtained with SJL/J, DBA/1, and C57BL/6 thymocytes were also consistent with an *H-2L/H-2D* region-controlled molecule being recognized in

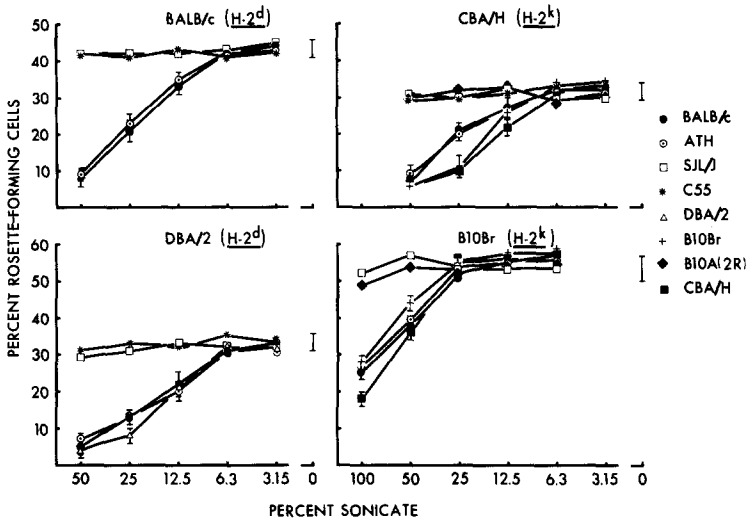

FIG. 2. Genetic mapping of inhibition of autorosetting by erythrocyte sonicates. Each graph is headed with the strain and *H-2* haplotype of the thymocytes considered. The erythrocyte sonicates used as inhibitors are listed on the right-hand side of the figure. Vertical bars represent standard deviations of means. The autorosetting of thymocytes, in absence of inhibitor, is indicated in the right-hand margin of each graph.

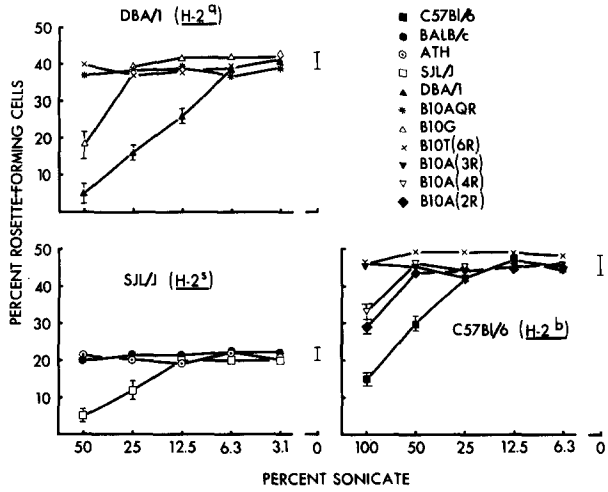

FIG. 3. Genetic mapping of inhibition of autorosetting by erythrocyte sonicates. Legend as in Fig. 2.

autorosetting. Thus, the rosetting of SJL/J ($H\text{-}2^s$) thymocytes (Fig. 3) was strongly inhibited by an SJL/J sonicate but not by an A.TH sonicate, A.TH mice differing from SJL/J mice at the *H-2L/H-2D* region of the *MHC*. Similarly, DBA/1 ($H\text{-}2^q$) autorosettes (Fig. 3) were inhibited with DBA/1 ($H\text{-}2^q$) and B10.G ($H\text{-}2^q$) sonicates but were not blocked by B10.AQR and B10.T(6R) sonicates. B10.AQR maps the inhibition effect to the right of *H-2K*, whereas B10.T(6R) maps the effect to the *D* end of the *MHC* (i.e., to the right of the *S* region). Finally, with C57BL/6 ($H\text{-}2^b$) rosetting (Fig. 3), significant inhibition was obtained with C57BL/6 ($H\text{-}2^b$),

B10.A(2R), and B10.A(4R) sonicates. B10.A(4R) maps the inhibition to the right of *I-A*, whereas B10.A(2R) maps the effect to the *D* end of the *MHC*, i.e., to the right of the *S* region. Similarly, the lack of inhibition by B10.A(3R) and B10.T(6R) sonicates maps the effect to the right of *I-J* and to the *D* end of the *MHC*, respectively. It should be emphasized that the inhibition of C57BL/6 autorosetting was not as marked as with other strains. However, the inhibition obtained with the 100% sonicates of B10.A(2R) and B10.A(4R) erythrocytes was statistically significant ($P < 0.05$).

The autorosette inhibition of two additional mouse strains has also been genetically mapped (data not shown), namely the B10.G ($H\text{-}2^q$) and ASW ($H\text{-}2^s$) strains. Inhibition of B10.G rosetting followed the same genetics as that obtained with DBA/1 ($H\text{-}2^q$) thymocytes (Fig. 3). Similarly, ASW autorosette-inhibition corresponded to the genetics observed with *H-2*-compatible SJL/J thymocytes (Fig. 3).

During these inhibition studies it became apparent that erythrocyte sonicates from mouse strains carrying the B10 background tended to be substantially less inhibitory than sonicates from other strains, e.g., CBA/H compared with B10.BR in Fig. 1 and Table I, DBA/1 compared with B10.G in Fig. 3, and C57BL/6 compared with B10 recombinants in Fig. 3. This effect may reflect a lower density of H-2 molecules on the surface of erythrocytes from mice with the B10 background. Preliminary alloantibody absorption studies are consistent with this interpretation.

Genetic Mapping of Rosetting with Allogeneic and Xenogeneic Erythrocytes. It was important to determine whether the same thymocyte receptors recognize syngeneic, allogeneic, and xenogeneic erythrocytes. To answer this question, we examined the genetics of rosette inhibition when either allogeneic or xenogeneic (rat) erythrocytes were used as rosetting targets.

When CBA/H ($H\text{-}2^k$) thymocytes were reacted with allogeneic C57BL/6 ($H\text{-}2^b$) erythrocytes this rosetting showed the same genetics of inhibition as when autologous (CBA/H) erythrocytes were used (compare Figs. 2 and 4). Thus, CBA/H ($H\text{-}2^k$), BALB/c ($H\text{-}2^d$), and A.TH erythrocyte sonicates were inhibitory, whereas C57BL/6 ($H\text{-}2^b$) and C55 ($H\text{-}2^{dm2}$) sonicates did not inhibit (Fig. 4). In a similar manner, when CBA/H ($H\text{-}2^k$) thymocytes were rosetted with xenogeneic Lewis rat erythrocytes the rosette inhibition was identical to that observed with autorosetting. Again, CBA/H, BALB/c, and A.TH sonicates inhibited, but Lewis rat and C55 mutant sonicates lacked inhibitory activity (Fig. 4).

Collectively these results indicate that the same thymocyte receptor (probably *H-2L*-region specific) interacts with syngeneic, allogeneic, and xenogeneic (rat) erythrocytes.

Analysis of Autorosetting in H-2L/H-2D-Region Mutant Mice. Experiments described earlier in this paper demonstrated that erythrocyte sonicates from BALB/c-$H\text{-}2^{dm2}$ mutant mice (C55 strain) were unable to inhibit the autorosetting of BALB/c thymocytes (Fig. 2). We then tested the ability of thymocytes from the BALB/c-$H\text{-}2^{dm2}$ mice to rosette with autologous, allogeneic, or xenogeneic (rat) erythrocytes (Table III). The mutant thymocytes were almost devoid of rosetting activity (1–3% rosettes) with all erythrocytes tested. In contrast, the wild-type BALB/c strain strongly rosetted with all the target erythrocytes used (18–55% rosettes). In fact, BALB/c thymocytes rosetted well with the mutant erythrocytes even though these erythrocytes lack *H-2L* region-controlled antigens. Presumably the receptors on BALB/c thymocytes primarily recognize *H-2L* antigens but weakly cross-react with *H-2K* and *H-2D*

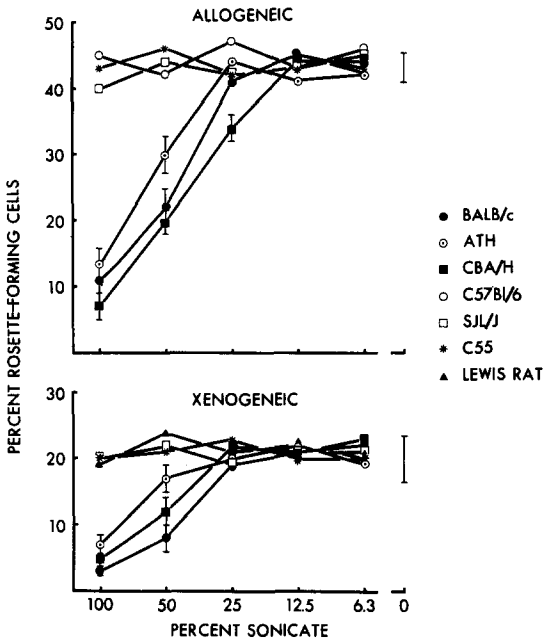

FIG. 4. Ability of erythrocyte sonicates from different mouse strains to inhibit the rosetting of CBA/H thymocytes with allogeneic (C57BL/6) and xenogeneic (Lewis rat) erythrocytes. The erythrocyte sonicates used as inhibitors are listed on the right-hand side of the figure. Vertical bars represent standard deviations of means. The rosetting of control thymocytes, which were not preincubated with sonicates, is indicated in the right-hand margin of each graph.

TABLE III

Rosetting of BALB/c Mutant ($H\text{-}2^{dm2}$) and BALB/c ($H\text{-}2^{d}$) Thymocytes With Different Erythrocytes*

Thymocyte	Rosetting erythrocyte									
	BALB/c ($H\text{-}2^{dm2}$)	*BALB/c* ($H\text{-}2^{d}$)	DBA/2 ($H\text{-}2^{d}$)	B10.BR ($H\text{-}2^{k}$)	CBA/H ($H\text{-}2^{k}$)	C57BL/6 ($H\text{-}2^{b}$)	SJL/J ($H\text{-}2^{s}$)	DBA/1 ($H\text{-}2^{q}$)	B10.G ($H\text{-}2^{q}$)	Lewis rat
BALB/c ($H\text{-}2^{dm2}$)	1 ± 1.7‡	2 ± 2	2 ± 2	2 ± 2	1 ± 1	2 ± 1	1 ± 1	2 ± 0.6	2 ± 1.5	3 ± 1.4
BALB/c ($H\text{-}2^{d}$)	48 ± 4.2	40 ± 2.1	36 ± 2.1	55 ± 2.1	32 ± 3.5	47 ± 2.1	27 ± 4.2	46 ± 2.8	53 ± 2.1	18 ± 1.4

* BALB/c-$H\text{-}2^{dm2}$ strain also designated as C55 strain (Table I).

‡ Results are expressed as percentage of rosetting thymocytes ± standard deviation of at least three determinations.

antigens. This weak cross-reaction, combined with multipoint binding, would allow rosette formation to occur with the mutant erythrocytes.

Although the results above are consistent with the *H-2L* region playing a key role in the autorosetting phenomenon it could be argued that a mutation in some other unrelated gene has modified the rosetting behavior of the $H\text{-}2^{dm2}$ mutant. To rule out this possibility another mutant mouse was examined, namely the B10.D2-$H\text{-}2^{dm1}$ (M504) strain. This mutant has substantially modified *H-2L* and *H-2D* antigens (21). When this mutant was tested in the rosette inhibition assay it was found that the $H\text{-}2^{dm1}$ erythrocyte sonicate was unable to inhibit the autorosetting of $H\text{-}2^{d}$ (BALB/c) thymocytes (Table IV). Conversely, $H\text{-}2^{d}$ sonicates were unable to inhibit the autorosetting of $H\text{-}2^{dm1}$ thymocytes. This same specificity of inhibition was maintained when either wild-type ($H\text{-}2^{d}$) thymocytes were rosetted with mutant erythrocytes or mutant thymocytes were rosetted with wild-type erythrocytes (Table IV). Thus, these

TABLE IV

Specificity of Autorosette Inhibition of B10.D2 Mutant ($H\text{-}2^{dm1}$) Thymocytes*

Thymocyte	Erythrocyte	Erythrocyte sonicate‡		
		Nil	$H\text{-}2^{d}$	$H\text{-}2^{dm1}$
$H\text{-}2^{d}$	$H\text{-}2^{d}$	39 ± 3.1§	6 ± 2.7	37 ± 2.1
$H\text{-}2^{dm1}$	$H\text{-}2^{dm1}$	57 ± 3.4	54 ± 1	31 ± 3.1
$H\text{-}2^{d}$	$H\text{-}2^{dm1}$	55 ± 2.9	21 ± 3.5	51 ± 2.6
$H\text{-}2^{dm1}$	$H\text{-}2^{d}$	37 ± 2.8	36 ± 2.5	5 ± 1.7

* B10.D2-$H\text{-}2^{dm1}$ strain also designated as M504 strain (Table I). $H\text{-}2^{d}$ refers to BALB/c strain, $H\text{-}2^{dm1}$ refers to B10.D2 mutant ($H\text{-}2^{dm1}$) strain.

‡ Thymocytes preincubated for 60 min at 4°C with 100% erythrocyte sonicates before rosetting with appropriate erythrocytes.

§ Results are expressed as percentage of rosetting thymocytes ± standard deviation of three determinations. Values that represent significant inhibition are enclosed.

TABLE V

*Blocking of Autorosette Inhibition by Anti-H-2L Antibodies**

Antiserum treatment of sonicate‡	Erythrocyte sonicate§		
	None	BALB/c	CBA/H
Nil	46 ± 4.0	9 ± 3.0	6 ± 2.1
Anti-H-2L^{d}	—	43 ± 2.1	20.5 ± 2.5
Anti-H-2K^{k}	—	8.5 ± 1.8	8.5 ± 2.3

* Inhibition of autorosetting between BALB/c thymocytes and BALB/c erythrocytes.

‡ Erythrocytes preincubated with antisera before sonication and use as inhibitors. Anti-H-2L^{d} = (BALB/c-$H\text{-}2^{dm2}$ × ASW)F_1 anti-BALB/c serum. Anti-H-2K^{k} = monoclonal H-2.25 antibody (H100-27.R9).

§ Thymocytes preincubated for 60 min at 4°C with 100% erythrocyte sonicates before rosetting. Results are expressed as percentage of rosetting thymocytes ± standard deviation of three determinations.

data are consistent with the notion that the thymocyte receptors primarily recognize H-2L antigens on erythrocytes.

It should be noted from the experiment presented in Table IV that the $H\text{-}2^{dm1}$ mutant erythrocytes rosetted more effectively with thymocytes (either $H\text{-}2^{d}$ or $H\text{-}2^{dm1}$) than $H\text{-}2^{d}$ erythrocytes. The explanation of this effect is unclear. Presumably it is a result of the different genetic background of BALB/c and B10.D2 mice.

Blocking of Autorosette Inhibition with Anti-H-2 Sera. If thymocytes primarily recognize *H-2L* antigens on erythrocytes, then erythrocyte sonicates should lose their ability to inhibit autorosetting if they are coated with anti-H-2L antibodies. To test this prediction BALB/c erythrocytes were preincubated with anti-H-2L^{d} antibodies before sonication and used as inhibitors of autorosetting by BALB/c thymocytes. Table V shows that anti-H-2L^{d} preincubation did completely block the inhibitory activity of the BALB/c sonicate. Complete blocking was achieved with 0.4 ml of anti-H-2L serum (Materials and Methods), whereas 0.1 ml of the antiserum only produced partial blocking of autorosette inhibition. Furthermore, when CBA/H erythrocytes were preincubated with the anti-H-2L^{d} serum (0.4 ml) they partially lost their inhibitory activity (Table V). In contrast, the anti-H-2L^{d} serum did not affect the ability of SJL/J erythrocyte sonicates to inhibit the autorosetting of SJL/J thymocytes

(data not shown). These results imply a serological cross-reaction between the H-2L^k and H-2L^d molecules that has not been detected previously with anti-H-2L^d sera (18, 20, 22). Perhaps noncomplement-fixing antibodies are involved. Further serological analysis of the blocking of autorosette inhibition is in progress.

To control against antibody bound to erythrocytes nonspecifically blocking autorosette inhibition we showed that a monoclonal anti-H-2.25 antibody (anti-H-2K^k) did not block inhibition by CBA/H erythrocyte sonicates (Table V). In this experiment we preincubated the CBA/H erythrocytes with a concentration of anti-H-2K^k antibody that was known, from erythrocyte-absorption experiments, to completely saturate the erythrocytes with monoclonal antibody.

Discussion

The experiments described in this paper attempted to analyze the specificity of interaction between murine thymocytes and erythrocytes by using an inhibition assay. The assay entailed incubating thymocytes with different erythrocyte sonicates before rosetting the thymocytes with autologous erythrocytes. With this assay it was demonstrated that autorosetting is mediated by a highly specific receptor on thymocytes that recognizes *H-2L* region-controlled molecules on the erythrocyte surface.

Data in favor of this conclusion can be summarized as follows. First, inhibition of autorosetting in seven different mouse strains suggested that *H-2*-linked, rather than background, genes controlled the reaction (Table II). Second, with erythrocyte sonicates from a range of recombinant mouse strains it was possible to map the inhibition to the *H-2L/H-2D* region of the *H-2* complex (Figs. 2 and 3). Third, the inability of the BALB/c-*H-2^{dm2}* mutant, an *H-2L* deletion mutant (18, 20), to inhibit the autorosetting of wild-type (BALB/c) thymocytes directly mapped the specificity of autorosetting to the *H-2L* region (Figs. 2 and 3). Similarly, the B10.D2-*H-2^{dm1}* mutant, which has substantially modified H-2L and H-2D antigens (21), was unable to inhibit wild-type *H-2^d* (BALB/c) rosetting and expressed a different specificity receptor on its thymocytes (Table IV). Fourth, anti-H-2L^d sera were able to specifically block the inhibition of BALB/c autorosetting by sonicates of BALB/c erythrocytes (Table V).

The specificity of autorosetting of five different *H-2* haplotypes, namely *b*, *d*, *k*, *q*, and *s*, were examined in this study. The inhibition data suggested complete cross-reaction between the thymocyte receptors carried by the *k* and *d* haplotypes, whereas the receptors on *b*, *q*, and *s* haplotypes were haplotype specific. This result implies that thymocytes cannot distinguish between the H-2L molecules carried by the *d* and *k* haplotypes even though there are serologically detectable differences between these H-2L molecules (18, 20, 22).

Of course, as a result of the lack of suitable recombinant mouse strains, mutant mice, and alloantisera it was not possible to directly map the specificity of the autorosetting receptors to the *H-2L* region in all five haplotypes. However, data for the *d* and *k* haplotypes clearly implicated the *H-2L* region in autorosetting. With the *s* haplotype the receptor was mapped to the *H-2L/H-2D* region, whereas with the *b* and *q* haplotypes autorosetting was mapped to the *D* end of the *H-2* complex (i.e., to the right of the *S* region).

At this point it should be noted that recently Primi et al. (23) also attempted to map the genes responsible for autorosetting. They concluded that rosetting was *H-2*

restricted, but from their data suggested that this self-recognition phenomenon is associated with a new locus mapping between *H-2G* and *H-2L/H-2D*. Our data are certainly not compatible with this interpretation, although there are substantial differences between the assay systems used in the two laboratories. First, Primi et al. (23) used cultured spleen cells whereas freshly prepared thymocytes were used in our experiments. Even so, we now have evidence that uncultured spleen cells carry *H-2L* region-restricted receptors (D. Y. Sia and C. R. Parish. Manuscript in preparation.). Second, we mapped the autorosetting receptors with mutant mice and autorosette-inhibition assays, whereas Primi et al. (23) based their genetic mapping on the observation that cultured spleen cells could only rosette with erythrocytes from certain mouse strains. This is a surprising result because uncultured thymocytes and spleen cells rosette equally effectively with erythrocytes from autologous, syngeneic, or allogeneic strains (1–5). At present, the only rational explanation of these differences is that cultured spleen cells recognize different structures on erythrocytes than their uncultured counterparts.

Another important technical aspect of this study should be highlighted. The rosette assay we employed very reproducibly detected a high proportion of thymocytes that formed rosettes (20–50%). Similarly, up to 30% of spleen cells have been found to autorosette (D. Y. Sia and C. R. Parish. Manuscript in preparation.). This represents a much higher frequency of autorosettes than reported by some groups (1, 3, 4, 23), but is in agreement with the results of Sandilands et al. (2) and Kolb (5). There are probably several reasons for this variation. Most importantly, the rosettes must be gently resuspended from the erythrocyte-lymphocyte pellet. Also, certain components of serum are very potent inhibitors (5, 24) and thus rosetting must be performed in the virtual absence of serum. Finally, for optimum autorosetting, erythrocytes and lymphocytes need to be incubated as a pellet for at least 30 min before being resuspended and examined.

Perhaps the most intriguing findings reported in this paper deal with the BALB/c-$H\text{-}2^{dm2}$ mutant. Not only were sonicates of this mutant's erythrocytes unable to inhibit autorosetting of wild-type (BALB/c) thymocytes but the mutants thymocytes were virtually unable to autorosette (Table III). Because the $H\text{-}2^{dm2}$ mouse is an *H-2L*-deletion mutant (18–20) these results could be interpreted in two separate ways. First, it could be proposed that the *H-2L* gene directly codes for the receptor on thymocytes. This seems unlikely, however, as it is difficult to envisage how the *H-2L* gene could also code for the acceptor site on erythrocytes. The second, more likely interpretation, is that the *H-2L* gene product stimulates, possibly via somatic mutation, the generation of a receptor that recognizes *H-2L*. Whether this education to recognize self-*H-2L* is dependent upon thymic epithelium (15) or some other intrathymic or extrathymic elements is currently being investigated.

Rosette-inhibition results presented in this paper also suggest that the *H-2L* region-restricted receptors mediate the rosetting of thymocytes with allogeneic and xenogeneic (rat) erythrocytes (Fig. 4). The inability of thymocytes from the *H-2L*-deletion mutant ($H\text{-}2^{dm2}$) to rosette with any erythrocyte (Table III) supports this notion. Thus the anti-H-2L receptor must weakly cross-react with other *MHC* gene products, and this weak reaction, combined with multipoint binding, results in rosette formation with allogeneic and xenogeneic cells. Further support for this concept is the ability of BALB/c thymocytes to rosette with *H-2L*-deleted ($H\text{-}2^{dm2}$) erythrocytes (Table III).

Previous studies have demonstrated autorosetting thymocytes in a range of other species, such as rats, rabbits, pigs, and humans (2, 4, 5, 25–28). It seems likely that *MHC*-restricted receptors may also mediate autorosetting in these species. Furthermore, in some mammalian species the binding of xenogeneic erythrocytes by lymphoid cells (4, 5, 29, 30) may be mediated by *MHC*-restricted receptors.

A surprising aspect of this study is the dominance of *H-2L*-restricted receptors on thymocytes. From the studies with cytotoxic T cells against foreign antigens, one would have expected to also detect receptors on thymocytes against self-H-2K and self-H-2D antigens (6, 7). It could be argued that *H-2L*-restricted receptors initially appear on most thymocytes and that subpopulations of thymocytes are subsequently selected to recognize self-H-2K and self-H-2D antigens. This seems unlikely, however, as the $H\text{-}2^{dm2}$ mouse, which lacks H-2L antigens, can readily generate *K* and *D* region-restricted killers (31). Furthermore, additional experiments have demonstrated that subpopulations of peripheral T and B lymphocytes also express *H-2L* region-restricted receptors (D. Y. Sia and C. R. Parish. Manuscript in preparation.). Thus, the functional significance of these *H-2L*-restricted receptors on lymphocytes is uncertain. These data suggest, however, that *H-2*-restricted receptors play a more fundamental and complex role in the immune system than just directing the interaction of cytotoxic T lymphocytes with target cells.

Summary

A high proportion (20–50%) of murine thymocytes form rosettes with either syngeneic or allogeneic erythrocytes. The specificity of this interaction was investigated by measuring the ability of different erythrocyte sonicates to inhibit rosette formation. With erythrocyte sonicates from recombinant mouse strains it was demonstrated that rosetting with syngeneic erythrocytes was mediated by *H-2L* and/or *H-2D* region-restricted receptors. The specificity of autorosetting was directly mapped to the *H-2L* region by the inability of erythrocyte sonicates from the BALB/c-$H\text{-}2^{dm2}$ mutant, an *H-2L*-deletion mutant, to inhibit the rosetting of wild-type (BALB/c) thymocytes. The B10.2D2-$H\text{-}2^{dm1}$ mutant, which has substantially modified H-2L and H-2D antigens, supported this conclusion. Furthermore, anti-H-2L sera were able to specifically block the inhibition of rosetting by erythrocyte sonicates.

The above procedures clearly implicated the *H-2L* region in the thymocyte rosetting of *d* and *k* haplotypes. With the *s* haplotype the rosetting receptor was mapped to the *H-2L/H-2D* region, whereas with the *b* and *q* haplotypes rosetting was only mapped to the *D* end of the *H-2* complex. This study also suggested complete cross-reaction between the thymocyte receptors carried by the *k* and *d* haplotypes, whereas the receptors of *b*, *q*, and *s* haplotypes were haplotype specific. In addition, the inhibition assay indicated that the rosetting of thymocytes with allogeneic and xenogeneic (rat) erythrocytes was mediated by a receptor primarily directed against self-*H-2L*.

Finally, the critical role played by the *H-2L* region in this rosetting phenomenon was demonstrated by the inability of thymocytes from the *H-2L*-deletion mutant ($H\text{-}2^{dm2}$) to rosette with syngeneic, allogeneic, or xenogeneic (rat) erythrocytes.

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References

1. Micklem, H. S., and C. Asfi. 1971. Lymphoid cells reacting against "self" and their possible role in immune responses. *In* Morphological and Functional Aspects of Immunity. K. Lindahl-Kiessling, G. Alm, and M. G. Hanna, editors. Plenum Publishing Corp., New York. 57.
2. Sandilands, G., K. Gray, A. Cooney, J. D. Browning, and J. R. Anderson. 1974. Auto-rosette formation by human thymocytes and lymphocytes. *Lancet.* **I:**27.
3. Charreire, J., and J. F. Bach. 1975. Binding of autologous erythrocytes to immature T-cells. *Proc. Natl. Acad. Sci. U. S. A.* **72:**3201.
4. Braganza, C. M., G. Stathopoulos, A. J. S. Davis, E. V. Elliott, R. S. Kerbel, M. Papamichail, and E. J. Holborow. 1975. Lymphocyte: erythrocyte rosettes as indicators of the heterogeneity of lymphocytes in a variety of mammalian species. *Cell.* **4:**103.
5. Kolb, H. 1977. A receptor for "self" on lymphocytes. *Immunology.* **33:**859.
6. Doherty, P. C., R. V. Blanden, and R. M. Zinkernagel. 1976. Specificity of virus-immune effector T cells for *H-2K* or *H-2D* compatible interactions: implications for H-antigen diversity. *Transplant. Rev.* **29:**89.
7. Shearer, G. M., T. G. Rehn, and A. M. Schmitt-Verhulst. 1976. Role of the murine major histocompatibility complex in the specificity of *in vitro* T cell mediated lympholysis against chemically-modified autologous lymphocytes. *Transplant. Rev.* **29:**222.
8. Katz, D. H., and B. Benacerraf. 1975. The function and interrelationship of T cell receptors, *Ir* genes and other histocompatibility gene products. *Transplant. Rev.* **22:**175.
9. Miller, J. F. A. P., M. A. Vadas, A. Whitelaw, and J. Gamble. 1976. Role of major histocompatibility complex gene products in delayed-type hypersensitivity. *Proc. Natl. Acad. Sci. U. S. A.* **73:**2486.
10. Thomas, D. W., U. Yamashita, and E. M. Shevach. 1977. The role of Ia antigens in T cell activation. *Immunol. Rev.* **35:**97.
11. Schwartz, R. H., A. Yano, and W. E. Paul. 1978. Interaction between antigen-presenting cells and primed T lymphocytes: an assessment of *Ir* gene expression in the antigen-presenting cell. *Immunol. Rev.* **40:**153.
12. Janeway, C. A., H. Wigzell, and H. Binz. 1976. Two different V_H gene products make up the T cell receptors. *Scand. J. Immunol.* **5:**993.
13. Langman, R. E. 1977. Cell-mediated immunity and the major histocompatibility complex. *Rev. Physiol. Biochem. Pharmacol.* **81:**1.
14. Blanden, R. V., and G. L. Ada. 1978. A dual recognition model for cytotoxic T cells based on thymic selection of precursors with low affinity for self H-2 antigens. *Scand. J. Immunol.* **7:**181.
15. Zinkernagel, R. M., G. N. Callahan, A. Althage, S. Cooper, P. A. Klein, and J. Klein. 1978. On the thymus in the differentiation of H-2-self recognition by T cells. Evidence for dual recognition? *J. Exp. Med.* **147:**882.
16. Klein, J., L. Flaherty, J. L. VandeBerg, and D. C. Shreffer. 1978. *H-2* haplotypes, genes, regions, and antigens: first listing. *Immunogenetics.* **6:**489.
17. Parish, C. R., and I. F. C. McKenzie. 1978. A sensitive rosetting method for detecting subpopulations of lymphocytes which react with alloantisera. *J. Immunol. Methods.* **20:**173.
18. McKenzie, I. F. C., G. M. Morgan, R. W. Melvold, and H. I. Kohn. 1977. BALB/c-$H\text{-}2^{db}$: a new *H-2* mutant in BALB/cKh that identifies a locus associated with the *D* region. *Immunogenetics.* **4:**333.
19. Hansen, T. H., S. E. Cullen, R. Melvold, H. Kohn, L. Flaherty, and D. H. Sachs. 1977. Mutation in a new H-2-associated histocompatibility gene closely linked to *H-2D*. *J. Exp. Med.* **145:**1550.
20. Démant, P., and C. Néauport-Sautès. 1978. The *H-2L* locus and the system of *H-2* specificities. *Immunogenetics.* **7:**295.

21. Morgan, G. M., I. F. C. McKenzie, and R. W. Melvold. 1978. The relationship between the *H-2* loss mutations of $H\text{-}2^{da}$ and $H\text{-}2^{db}$ in the mouse. *Immunogenetics.* **7:**247.
22. Hansen, T. H., and D. H. Sachs. 1978. Isolation and antigenic characterization of the product of a third polymorphic *H-2* locus, *H-2L. J. Immunol.* **121:**1469.
23. Primi, D., G. K. Lewis, R. Triglia, and J. W. Goodman. 1979. Rosette formation between murine lymphocytes and erythrocytes. A new locus in the *H-2* region. *J. Exp. Med.* **149:** 1349.
24. Kolb, H. 1978. Characteristics of spontaneous rosette formation by mouse lymphocytes with autologous erythrocytes. *Cell. Immunol.* **37:**127.
25. Baxley, G., G. B. Bishop, A. G. Cooper, and H. H. Wortis. 1973. Rosetting of human red blood cells to thymocytes and thymus-derived cells. *Clin. Exp. Immunol.* **15:**385.
26. Kaplan, J. 1975. Human T lymphocytes form rosettes with autologous and allogeneic human red blood cells. *Clin. Immunol. Immunopathol.* **3:**471.
27. Gluckman, J. C., and P. Montambault. 1975. Spontaneous autorosette-forming cells in man. A marker for a subset population of T lymphocytes? *Clin. Exp. Immunol.* **22:**302.
28. Gluckman, J. C., L. Gattegno, and P. Cornillot. 1975. Significance of spontaneous autorosettes in rats. *Eur. J. Immunol.* **5:**301.
29. Jondal, M., G. Holm, and H. Wigzell. 1972. Surface markers on human T and B lymphocytes. I. A large population of lymphocytes forming nonimmune rosettes with sheep red blood cells. *J. Exp. Med.* **136:**207.
30. Stadecker, M. J., G. Bishop, and H. H. Wortis. 1973. Rosette formation by guinea pig thymocytes and thymus derived lymphocytes with rabbit red blood cells. *J. Immunol.* **111:** 1834.
31. Blanden, R. V., I. F. C. McKenzie, U. Kees, R. G. Melvold, and H. I. Kohn. 1977. Cytotoxic T-cell response to ectromelia virus-infected cells. Different *H-2* requirements for triggering precursor T-cell induction or lysis by effector T-cells defined by the BALB/c-$H\text{-}2^{db}$ mutation. *J. Exp. Med.* **146:**869.