ROSETTE FORMATION BETWEEN MURINE LYMPHOCYTES AND ERYTHROCYTES

A New Locus in the H-2 Region*

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Histocompatibility is necessary for many collaborative and aggressive cell interactions. The sharing of alleles at certain loci of the H-2 region on chromosome 17 of the mouse is essential for optimal interaction of lymphocytes with each other (1–7), with macrophages (8, 9), and with target cells in certain types of cytotoxic killing by T lymphocytes (10–17). This evidence indicates that lymphocytes can and must recognize products of the H-2-chromosomal region for optimal responses to take place.

Several curious observations which bear on the general question of self-recognition by lymphocytes have appeared in the recent literature. Koskimies and Mäkelä (18) reported that T-cell-deficient mice made stronger anti-hapten responses to conjugates of syngeneic erythrocytes than to conjugates of allogeneic or xenogeneic erythrocytes. These findings, together with others demonstrating that an appreciable fraction of murine lymphocytes form rosettes with autologous, but not with isologous, erythrocytes (19–20), support the existence on at least some immunocytes of a receptor for a self-marker which is expressed on erythrocytes.

Because this form of self-recognition is likely to be of significance for a complete understanding of the mechanisms of lymphocyte activation, it was of interest to further investigate the phenomenon of autologous rosette formation. The present study confirms the phenomenon, establishes that the receptor in question is expressed on both T and B lymphocytes, and that it is not immunoglobulin in nature. Moreover, the use of recombinant strains of mice indicates that this form of self-recognition is associated with a marker coded by a new locus in the H-2-region mapping between H-2G and H-2D.

Materials and Methods

Mice. Various strains of mice were purchased from Simonsen Laboratories, Gilroy, Calif. Some recombinant strains were kindly provided by Dr. J. Stobo (University of California, San Francisco) and Dr. J. D. Waterfield (University of California, San Diego).

Mitogen. Lipopolysaccharide from *Escherichia coli* (LPS)[1] was obtained from Sigma Chemical Co., St. Louis, Mo.

Culture Conditions. Cells were cultured in cluster 24 tissue culture plates at a cell concentration

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[1] *Abbreviations used in this paper:* DNP, dinitrophenyl; FCS, fetal calf serum; LPS, lipopolysaccharide; MHC, major histocompatibility complex; RBC, erythrocytes.

of 1.0×10^7/ml in 0.5 ml. Cultures were incubated in plastic boxes in an atmosphere of 10% CO_2, 83% N_2, and 7% O_2.

Medium. The medium used in these experiments was RPMI-1640 supplemented with glutamine and gentamicin.

Cell Fractionation. B cells were removed from spleen cell suspensions, as previously described, by allowing the spleen cell suspensions to settle onto plastic Petri plates precoated with affinity-purified rabbit anti-mouse Ig (21, 22). This procedure routinely yielded T-cell preparations that were >95% pure, as judged by anti-Thy-1 cytotoxicity and by staining with fluorescent anti-Ig.

Anti-Thy-1 Serum. Anti-mouse Thy-1 antiserum was generated in rabbits using mouse brain as the antigen and rendered specific as described previously (21).

Indirect Fluorescence Assay for Thy-1. $(Fab')_2$ fragments from anti-Thy-1 antibody were prepared by standard procedures and substituted with Dinitrophenyl (DNP) groups to a level which did not curtail antigen binding. The DNP-$(Fab')_2$ conjugates were reacted for 30 min at 4°C with spleen cells. The cells were washed and then treated with fluorescein-conjugated $(Fab')_2$ fragments of rabbit anti-DNP antibody prepared against DNP-ovalbumin conjugates. The immunoglobulin reagents used in this procedure were $(Fab')_2$ fragments to avoid reactions with Fc receptors on lymphoid cells.

Indirect Fluorescence Assay for Ig-Bearing Cells. Rabbit anti-mouse Ig serum was prepared by standard procedures. The anti-Ig immunoglobulins were reacted for 30 min at 4°C or 37°C with spleen cells. The cells were washed and then treated with fluorescent protein A for 30 min at 4°C.

Rosette Test. Lymphoid cells were harvested and washed twice in balanced salt solution and once in Tris-NH_4Cl to eliminate erythrocytes (RBC). Thereafter, lymphoid cells and RBC were resuspended in phosphate-buffered saline containing 1% fetal calf serum (FCS) and 500 μg/ml of sodium azide.

Lymphocytes and erythrocytes were mixed in a ratio of 1:10 in a 0.1-ml total volume. The cell suspensions were centrifuged for 2 min at 200 *g*. The pellet was incubated on ice for 30 min and gently resuspended for 2 min on a roller. Rosettes, defined by the binding of at least four RBC per lymphocyte, were enumerated using a hemocytometer.

Results

Optimal Conditions for Autologous Rosette Formation. To establish the optimal conditions for obtaining autologous rosettes, spleen cells from BDF_1 mice were rosetted either immediately or after varying periods of time in culture with or without FCS and 50 μg/ml of LPS. Maximum numbers of rosette-forming cells (12–18% of total lymphocytes) were obtained when cells were cultured for 24 h (Fig. 1). FCS or LPS had no apparent effect on the expression of this activity. In the following experiments, therefore, spleen cells were routinely cultured for 24 h in serum-free medium before assay.

Nature of the Rosetting Cells. To determine whether T cells, B cells, or both cell types were rosetting with autologous erythrocytes, spleen cells were either treated with anti-Thy-1 serum and complement before and after culturing to eliminate T cells or depleted of B cells by adherence to plastic surfaces coated with rabbit anti-mouse-Ig antibody. The nonadherent population contained <5% Ig-positive cells by immunofluorescence and was compared with the anti-Thy-1-treated population, with normal thymocytes and with spleen cells of nude mice for rosette formation with syngeneic erythrocytes. The results indicate that both cell types include rosette-formers, with B cells comprising ≅70% of the rosetting population (Fig. 2).

These findings were confirmed by staining unfractionated spleen cells, B-cell-depleted spleen cells, and thymocytes with fluorescein-conjugated anti-Thy-1 antibody before rosetting. Thy-1-positive cells accounted for ≅30% of the rosette-forming cells (Table I).

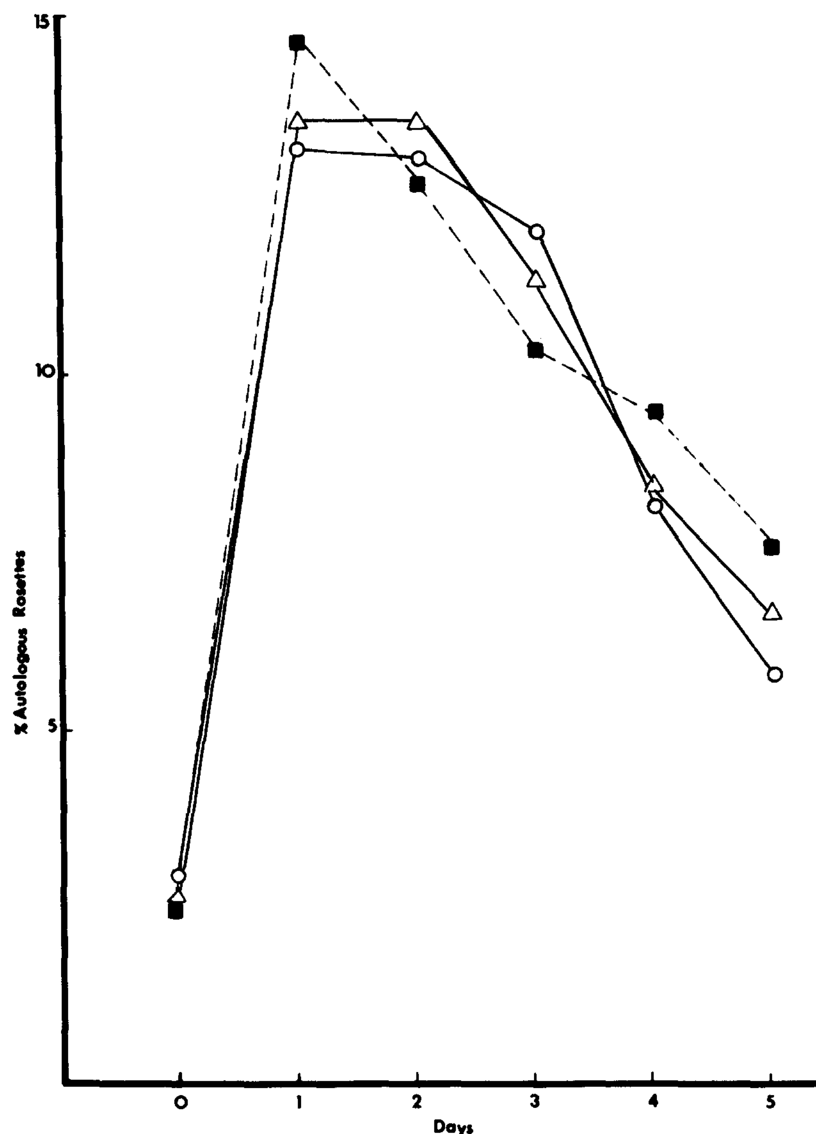

FIG. 1. Spleen cells from BDF_1 mice were cultured for up to 5 d without serum (○), in 5% FCS (△), or in 5% FCS with 50 μg of LPS (■). Autologous rosette forming ability was assayed before culturing and at each day of the culture period.

The Lymphocyte Receptor for Syngeneic RBC Is Not Immunoglobulin. The question of the possible immunoglobulin nature of the lymphocyte receptor for syngeneic RBC was investigated in the following way. Unfractionated spleen cells and cells which did not adhere to anti-Ig-coated plates (enriched T cells) were treated with fluorescent rabbit anti-mouse Ig antibody either at 4°C for 30 min (noncapping conditions) or at 37°C for 60 min (capping conditions). The data in Table II clearly show that capping and internalization of surface Ig did not diminish the number of rosette-forming cells (RFC), which apparently excludes the involvement of surface Ig in rosette formation. Furthermore, the figures at 4°C show that ≅70% of the rosette-forming spleen cells

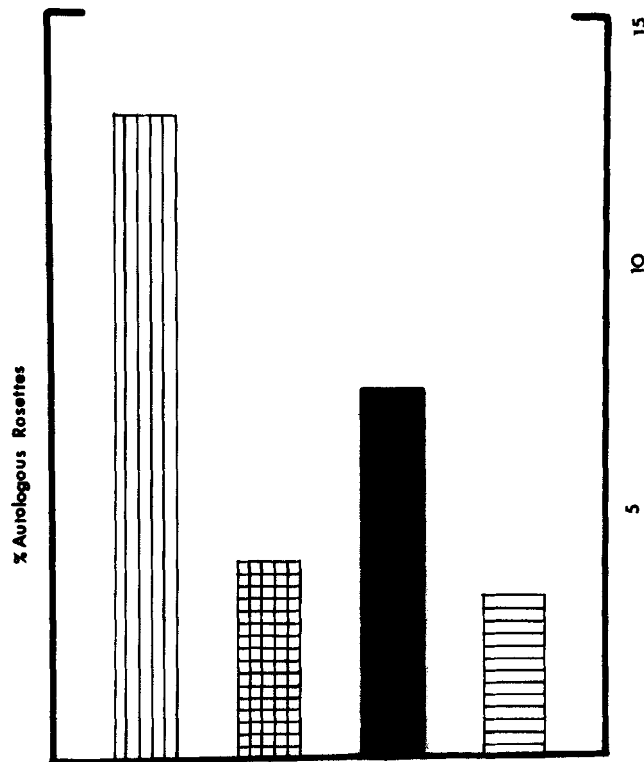

FIG. 2. Autologous rosette formation by various cell populations from BDF_1 mice. Spleen cells (▥), anti-Ig-nonadherent lymphocytes (▦), anti-thy-1 treated spleen cells (■) and thymocytes ▤.

TABLE I
Autologous Rosette Formation by Various Cell Populations from BDF_1 Mice

Cell source	Fluorescent anti-thy-treatment	Autologous rosettes	Fluorescent rosettes	Fluorescent lymphocytes
		%	%	%
Spleen cells	−	13	—	—
Spleen cells	+	12	25	44
Nonadherent SC*	−	4	—	—
Nonadherent SC	+	4	70	82
Thymocytes	−	3	—	—
Thymocytes	+	3	86	79

* Spleen cells incubated on anti-mouse-Ig-coated plates to remove B cells.

are surface Ig-positive, confirming the findings in the previous section.

Allogeneic Rosette Formation. To determine whether syngeneic rosette formation is associated with the H-2 complex on chromosome 17 of the mouse, as are other cell interactions involving self-recognition, lymphocytes and erythrocytes from various congenic and recombinant strains of mice were tested bidirectionally for rosette formation. The data in Table III show results in only one direction, but it should be understood that reversing the donors of spleen cells and RBC was always carried out and invariably confirmed the complementary experiment.

TABLE II
Autologous Rosette Formation by Spleen Cells from BDF_1 Mice Treated with Fluorescent Anti-Ig under Capping and Noncapping Conditions

Cell source	Anti-Ig treatment	Autologous rosettes	Fluorescent rosettes	Fluorescent lymphocytes
	°C	%	%	%
Spleen	37	12	30	21
Spleen	4	14	60	51
Spleen	—	13	—	—
Nonadherent*	37	3	15	1
Nonadherent	4	4	12	3
Nonadherent	—	3	—	—

* Spleen cells incubated on anti-mouse-Ig-coated plates to remove B cells.

It can be seen (Table III) that sharing some part of the H-2 haplotype by the donors of lymphocytes and erythrocytes was required for rosette formation. Thus, lymphocytes from B10 mice (H-2^b) did not rosette with RBC from the congenic strain B10.BR (H-2^k) or vice versa (reverse direction not shown). These two strains have identical genetic backgrounds, but differ across the entire H-2 region. On the other hand, positive results with strain combinations which have different genetic backgrounds, such as B10.D2 with A/J and A.TL with BDF_1, minimize the probability that loci in other chromosomal regions are involved.

The results obtained with other strain combinations permit more precise mapping of the responsible locus or loci. The combination of A.TL and C3H (different only at the K and D loci) was negative, indicating that the I, S, and G regions are not involved. A/J and C3H, which are identical at K but different at D, also failed to rosette at high frequency, indicating that the K locus is not involved. Because the combination of A.TL and BDF_1 cells (identity at the D locus only) gave positive results, it was tempting to conclude that identity at the D locus between the interacting cells was necessary and sufficient for rosette formation. However, A/J and A.TH cells (both D^d) failed to form rosettes, raising the possibility that the responsible locus or loci might be closely linked to H-2D either within or outside the H-2 region.

Experiments to assess the involvement of regions that are telomeric to H-2D are summarized in Table IV. The most useful strain combination for this purpose proved to be A.TH and A/J. Because cells from these strains did not rosette each other, and because they are considered to be identical at all loci that are telomeric to H-2D, it is unlikely that the telomeric segment is involved in the control of rosette formation. Moreover, involvement of regions centromeric to H-2K could also be excluded because strains of the same background, such as A.TL and A.TH, differed in their rosetting with BALB/c cells (Table V).

Rosette Formation Defines a New H-2 Region. The data, thus far, persuasively argue for the involvement of the H-2 region in the phenomenon of rosette formation, but fail to decisively implicate any of the known loci. Because the genetic region involved appeared to be closely linked with, but not identical to, H-2D, and because the G locus and regions telomeric to H-2D were effectively excluded, it seemed plausible that a hitherto undescribed locus situated between G and D might be responsible for this cell interaction. If such was the case, then in some instances nonidentity at H-2D might give positive rosetting, whereas some combinations identical at H-2D, but with a crossover between G and D, might give negative results (as in the one case already

TABLE III

Rosette Formation between Spleen Cells and Erythrocytes from Various Strains of Mice

Spleen cells	MHC haplotype									Erythrocytes	MHC haplotype									Rosetting lymphocytes
	K	A	B	J	E	C	S	G	D		K	A	B	J	E	C	S	G	D	
																				%
BDF_1	d/b	d/b	d/b	d/b	d/b	d/b	d/b	d/b	d/b*	BDF_1	d/b	d/b	d/b	d/b	d/b	d/b	d/b	d/b	d/b	9.6
A/J	k	k	k	k	k	d	d	d	d	A/J	k	k	k	k	k	d	d	d	d	10.2
B10	b	b	b	b	b	b	b	b	b	B10.BR	k	k	k	k	k	k	k	k	k	0.4
A/J	k	k	k	k	k	d	d	d	d	BDF_1	d/b	d/b	d/b	d/b	d/b	d/b	d/b	d/b	d/b	10.0
B10.D2/o	d	d	d	d	d	d	d	d	d	A/J	k	k	k	k	k	d	d	d	d	6.9
A/J	k	k	k	k	k	d	d	d	d	C3H	k	k	k	k	k	k	k	k	k	0.1
A/J	k	k	k	k	k	d	d	d	d	C57BL/10	b	b	b	b	b	b	b	b	b	0.2
ATL	s	k	k	k	k	k	k	k	d	C57BL/10	b	b	b	b	b	b	b	b	b	0.2
ATL	s	k	k	k	k	k	k	k	d	C3H	k	k	k	k	k	k	k	k	k	0.1
ATL	s	k	k	k	k	k	k	k	d	BDF_1	d/b	d/b	d/b	d/b	d/b	d/b	d/b	d/b	d/b	11.5
A.TH	s	s	s	s	s	s	s	s	d	B10.A(2R)	k	k	k	k	k	d	d	·	b	0.7
A/J	k	k	k	k	k	d	d	d	d	A.TH	s	s	s	s	s	s	s	s	d	0.1
A.TH	s	s	s	s	s	s	s	s	d	A.TH	s	s	s	s	s	s	s	s	d	8.6

Haplotypes from Klein, J. and C. Chiang. 1978. *Immunogenetics* **6**:235; and Klein, J., L. Flaherty, J. L. Vandeberg, and D. C. Shreffler. 1978. *Immunogenetics* **6**:489.

* Regions of identity between the paired strains are enclosed.

TABLE IV

Independence of Rosette Formation with Respect to Loci Telomeric to H-2 Region

Spleen cells	Haplotype						Erythrocytes	Haplotype						Rosetting lymphocytes
	L	Qa-1	Qa-2	Qa-3	H-2T	Tla		L	Qa-1	Qa-2	Qa-3	H-2T	Tla	
														%
A/J	a	a	a	a*	b	a	B10.D2	a	a?	a	a	a	c	8.0
A/J	a	a	a	a	b	a	A.TL	a	b	a	a	a	c	9.5
A.TH	a	a	a	a	b	a	BALB/c	a	b	a	a	a	c	0.0
A.TH	a	a	a	a	b	a	A/J	a	a	a	a	b	a	0.1
B10.S(7R)	a	a	a	a	b	a	A/J	a	a	a	a	b	a	0.3

Haplotypes from Klein, J. and C. Chiang. 1978. *Immunogenetics* 6:235; and Klein, J., L. Flaherty, J. L. Vandeberg, and D. C. Shreffler. 1978. *Immunogenetics* 6:489.

* Regions of identity between the paired strains are enclosed.

TABLE V

Rosette Formation with Cells from Recombinant Strains of Mice

Spleen cells	MHC haplotype KABJECS GD	Erythrocytes	MHC haplotype KABJECS GD	Rosetting lymphocytes
				%
ATL	s/k*k k k k k k [d]‡	BALB/c	d d d d d d d d [d]	10.9
A.TH	s s s s s s s s/[d]	BALB/c	d d d d d d d d [d]	0.7
A/J	k k k k k d d d [d]	B10.S(7R)	s s s s s s s s/[d]	0.9
B10.A(2R)	k k k k k [d d]/·/b	BALB/c	d d d d d [d d] d d	4.5
A.TH	s s s s s s s s/d	B10.A(2R)	k k k k k d d/·/b	0.7
A.TH	s s s s s s s s/d	C57BL/10	b b b b b b b b b	0.0
A.TH	s s s s s s s s/[d]	A/J	k k k k k d d d [d]	0.6

Haplotypes from Klein, J. and C. Chiang. 1978. *Immunogenetics* 6:235; and Klein, J., L. Flaherty, J. L. Vandeberg, and D. C. Shreffler. 1978. *Immunogenetics* 6:489.

* Slashes indicate positions of putative crossover events.

‡ Regions of identity between the paired strains are enclosed.

described: A/J and A.TH). These predictions are based on the possibility that in some recombinant strains the crossover between G and D would fall to the right of the new locus, with rosetting becoming dissociated from D, whereas in other strains, the crossover would be to the left of the new locus, with rosetting remaining associated with H-2D.

Selected strain combinations were tested and the results are consistent with the above considerations (Table V). Thus, B10.A(2R) lymphocytes were capable of rosetting BALB/c RBC although they differ at H-2D. In contrast, A.TH and B10.S (7R) cells, which both express the d allele at the D locus, did not rosette with A/J cells, which are also D^d. The total body of data, therefore, is only compatible with the existence of one or more loci between H-2G and H-2D which control rosette formation.

Discussion

The data presented here show that a fraction of murine B and T cells form rosettes with syngeneic and certain isogeneic erythrocytes and that rosetting is controlled by one or more loci between the G and D regions of the H-2 complex. For maximum expression of rosette formation, it was necessary to culture lymphocytes for at least 24 h before testing. The need for preculturing might be a result of an α-globulin component of mouse serum which inhibits rosette formation (20) and which might be shed from lymphocyte surfaces during the culture period. The nature of the lymphocyte receptor responsible for RBC binding has not been elucidated, but on the basis of the present findings (Table II) it is unlikely to be immunoglobulin.

The H-2 restriction of rosette formation was clearly established by the use of congenic and recombinant strains of mice. Lymphocytes and RBC from donor pairs which had different H-2 haplotypes but were otherwise genetically identical (e.g., B10 and B10.BR) failed to form rosettes (Table III). However, efforts to map the responsible locus or loci through the use of recombinant strains failed to assign it to any of the known loci in the H-2 complex, although a close association with the D region became apparent. Indeed, A.TL and BDF_1, which share only the D region of the known H-2 loci, were highly compatible for rosette formation, as were other strain combinations which expressed the same D allele (Table III). However, the correlation between identity at the D locus and rosette formation was not universal, so the possible involvement of loci telomeric to H-2D was investigated. The results of these experiments were unambiguous (as far as they went) because matching for H-2L, Qa, H-2T, and Tla was insufficient for rosette formation (Table IV). The extreme telomeric region on chromosome 17, designated Ea, codes for an erythrocyte antigen, but strains C57BL and A/J are matched at this locus and failed to rosette each other's cells.

The most plausible conclusion to be drawn from this body of data is that the genes controlling this form of self-recognition map at a new locus closely associated with H-2D, located, most probably, between H-2G and H-2D. This hypothesis could be tested, because it predicts that some strain combinations which differ at H-2D should be compatible for rosetting, whereas others which are identical at H-2D should be incompatible, depending on recombination events which took place during the genetic history of the strains. This prediction was indeed borne out (Table V) and supports our tentative assignment of a new locus between H-2G and H-2D. Our designation for this new locus is H-2R (for rosetting).

The data in Tables III and V provide information pertaining to the probable haplotypes of some of the strains tested. Because B10.A(2R) and BALB/c were compatible for rosetting, it is likely that B10.A(2R) carries the d allele for H-2R, even though it expresses the b allele at H-2D. On the other hand, A.TH and B10.S(7R) were both incompatible with A/J, even though the three express $H\text{-}2D^d$. It is, therefore, likely that A.TH and B10.S(7R) are $H\text{-}2R^s$. The strains A.TH, B10.A(2R), and B10.S(7R) are all recombinants with a crossover between the G and D regions. Our data are consistent with this crossover occurring to the right of H-2R in all three strains and further predict that B10.A(2R) expresses the d allele at the as yet uncharacterized G locus. Furthermore, on the basis of the compatibility between A.TL and BALB/c (Table V), it can be predicted that A.TL is $H\text{-}2R^d$, a conclusion supported by the compatibility between A.TL and BDF_1, but not between A.TL and

C3H (Table III). Because A.TL carries the d haplotype at the new locus, it can also be predicted that the parental strain A.AL (kkkkkkkk/d) underwent a crossover between H-2G and H-2R.

The functional role of this form of self-recognition is obviously a paramount consideration. Is rosette formation a phenomenological oddity or has it the same order of significance of other cell interactions which require H-2-subregion compatibility? An observation which addresses this question was the demonstration that T-cell-deficient mice made stronger anti-hapten antibody responses to haptenated syngeneic erythrocytes than to allogeneic or xenogeneic conjugates (18). Presumably, dual recognition between hapten-specific lymphocytes and the syngeneic conjugates rendered them more potent thymic-independent antigens. It is well known that many haptens (e.g., drugs) may associate with erythrocytes and induce immunity as a consequence. The effect in the Koskimies-Mäkelä study was mapped to the H-2 region, but it appeared that identity at either the K or D ends of H-2 was sufficient, in contrast to our findings. Of course, the antibody response to modified erythrocytes and the rosetting of unmodified erythrocytes may prove to be unrelated phenomena, although we consider that unlikely.

Another observation which may be related to our findings was the enhanced capacity of T cells with anti-idiotypic specificity from A/J mice to form rosettes with syngeneic, as compared to allogeneic or xenogeneic, erythrocytes coated with Fab fragments bearing the idiotype (23). This property was closely linked to the H-2 complex, but did not appear to be associated with any of the known loci.

The very limited literature to date on lymphocyte-erythrocyte self-recognition may represent only the tip of a vast iceberg. The phenomenon clearly deserves extensive investigation.

Summary

More than 5% of murine splenic lymphocytes form rosettes with syngeneic erythrocytes. This property was maximally expressed when the lymphocytes were cultured for 24 h before rosetting. About 70% of the rosetting lymphocytes were B cells and 30% were T cells on the basis of surface immunoglobulin and the Thy-1-antigen. Capping surface immunoglobulin had no effect on the capacity of lymphocytes to form rosettes, indicating that the receptor in question was not immunoglobulin.

The capacity of lymphocytes to form rosettes with erythrocytes from other strains of mice was H-2 restricted. Extensive pairings of congenic and recombinant strains as donors of lymphocytes and erythrocytes showed that none of the known loci within the H-2 region-controlled rosetting. The involvement of regions on chromosome 17, telomeric or centromeric to H-2, was also excluded. The data were only compatible with the conclusion that this form of self-recognition is associated with a new locus (or loci) mapping between H-2G and H-2D.

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