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Immunology

Surface mapping of mouse thymocytes

(blocking assay/H-2, TL, and Ly alloantigens/supramolecular repatterning/paraformaldehyde fixation)

LORRAINE FLAHERTY AND DEBRA ZIMMERMAN

Division of Laboratories and Research, New York State Department of Health, Albany, New York 12201

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ABSTRACT **The blocking method used previously for determining the relative positions of different components of the cell surface was modified by first fixing the cells with paraformaldehyde. This technique was applied to the H-2K (K), H-2D (D), TL, Lyt-1, and Lyt-2 surface components of mouse thymocytes, and the results were compared in parallel with data obtained with the original technique with unfixed cells. Previous mapping data with unfixed cells, indicating the positions of these molecules relative to one another, were confirmed with paraformaldehyde-fixed cells, with one exception. On unfixed cells, D and TL appeared sufficiently adjacent to produce mutual interference in the attachment of anti-D and anti-TL antibodies. With paraformaldehyde-fixed cells this was not so, D and TL appearing sufficiently separated from one another to obviate interference in the attachment of anti-D and anti-TL antibodies. The previously reported close association of K with Lyt-1 and of D with Lyt-2 were demonstrable equally with unfixed and paraformaldehyde-fixed thymocytes. It is suggested that activation of D sites, and alternatively of TL sites, by antibody in the present experiments brings these two molecules into apposition and that this movement may exemplify a mechanism concerned in immunological recognition and response.**

One of the focal points of modern immunogenetics is the study of mouse alloantigens, their inheritance, biochemistry, and selective representation on functionally distinct sets of lymphoid cells. Relatively little, however, has been determined about their topographical arrangement on the cell surface. In 1968, by use of an antibody blocking assay, Boyse *et al.* (1) demonstrated that there was a close physical association between several alloantigens on the mouse thymocyte. Presumably, in this assay, if two antigens are sufficiently close, the attachment of antibody to one site will interfere with attachment of antibody to the other site; thus the impediment to absorption of the second antibody can be used as a measure of the proximity of any two antigens. By this means, Boyse *et al.* determined that there was a supramolecular patterning of the thymocyte surface and that certain alloantigens were adjacent to one another, notably, Lyt-2 to H-2D (D), Lyt-1 to H-2K (K), and D to TL.

In this report, we have reexamined the topographical relationships among D, K, TL, Lyt-1, and Lyt-2 by use of the same blocking assay as Boyse *et al.* with one modification: Prior to blocking, we fixed the thymocytes with paraformaldehyde to prevent movement of cell surface components during the assay and then tested these fixed cells in parallel with unfixed cells. Our results confirm the original observations of Boyse *et al.* with one notable exception, which may have important theoretical implications.

MATERIALS AND METHODS

Mice. Female B6-*Tla*^{a} or B6 mice were used as a source of thymocytes throughout these blocking studies. Mice used for producing antisera and ones used for sources of thymocytes or lymphocytes were maintained by brother-sister matings in our colony at the New York State Department of Health. The B6.AK1 (*H-2*^{ozl}) strain is an H-2 recombinant congenic inbred strain which was derived from a (B6 × B6-*H-2*^{k})F_1. It has the phenotype K^b:Ia^b:Ss^h:D^k:Qa-1^-:Qa-2^-:Qa-3^-:TL^-.

Antisera. See Table 1 for list of antisera and their specificities. Immunizations were performed according to Shen *et al.* (2). The anti-D and anti-K sera were produced in our laboratory. The other sera were the generous gifts of F-W. Shen and E. A. Boyse (Memorial Sloan-Kettering Cancer Center).

Fixation of Cells. Cells were fixed with paraformaldehyde according to Parr and Oei (3, 4). Briefly, thymocytes were incubated with 1% paraformaldehyde made in isotonic medium for 1 hr on ice. They were then washed three times in M-199 containing 10% gamma globulin-free fetal calf serum (GIBCO) and used in the blocking assay.

Blocking Assay. The blocking assay was performed according to Boyse *et al.* (1) with fixed and unfixed cells. Briefly, the assay consists of three basic steps.

Step 1. Viable thymocytes were washed and divided into two portions. One portion remained on ice while the other was fixed with paraformaldehyde as described above.

Step 2. Unfixed and fixed cells were resuspended in an excess of the selected antibody and, for control, the same concentration of normal mouse serum (NMS), and incubated for 1 hr either on ice or at room temperature. (Incubations with anti-TL were all performed on ice to prevent antigenic modulation.) The cells were then washed, counted, and adjusted to equal cell concentrations.

Step 3. The absorption capacities of the "blocked" cells (fixed and unfixed) and the NMS-treated control cells (fixed and unfixed) were then determined quantitatively for at least three antibodies: (*i*) the same antibody used for blocking in *Step 1;* (*ii*) a second antibody whose attachment had been found by Boyse *et al.* (1) to be impeded by previous saturation with the first antibody; and (*iii*) a third antibody whose attachment had been found by Boyse *et al.* not to be impeded by previous saturation with the first antibody. The quantitative absorption procedure consisted in absorbing aliquots of antiserum, diluted to an appropriate predetermined concentration with graded numbers of thymocytes for 30 min on ice. Each aliquot was then tested for residual cytotoxicity in a one-stage cytotoxicity test against the appropriate test cell. Absorbed rabbit serum prepared according to Boyse *et al.* (5) was used as a source of complement.

The percent of blocking was then calculated according to the formula:

$$\frac{N_{50}\text{ (blocked thymocytes)} - N_{50}\text{ (NMS-treated thymocytes)}}{N_{50}\text{ (NMS-treated thymocytes)}} \times 100,$$

in which N_{50} is the number of thymocytes that will reduce the

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Abbreviations: D, H-2D; K, H-2K; NMS, normal mouse serum; B6, C57BL/6.

Table 1. List of antisera

Antiserum	Immunization	Detected specificities	Dilution for quantitative absorptions
Anti-TL	B6 × A-*Tla*^{b} anti-A strain leukemia, ASL1	TL, Qa-1*	1/1600–1/3200
Anti-D	B6.AK1 anti-B6.K1	H-2D, (H-2L)†	1/100
Anti-K	B6-*H-2*^{k} anti-B6.AK1	H-2K, Ia‡	1/400
Anti-Lyt-1	C3H anti-CE	Lyt-1	1/30–1/80
Anti-Lyt-2	C3H × B6-*Lyt-1*^{a} anti-B6 leukemia, ERLD	Lyt-2	1/50

* At the dilutions used, 1/1600–1/3200, there was no detectable Qa-1 activity in the direct cytotoxic test. To ensure specificity of this antiserum when used for blocking, it was preabsorbed with either B6-*Tla*^{a} lymph node cells or B6 thymocytes (see Table 2).

† This antiserum probably has activity against the second D-end molecule, H-2L, but we have made no attempt in these present studies to distinguish blocking of H-2D as opposed to H-2L; this is not reactive with either Qa or TL antigens.

‡ This antiserum contains Ia antibody, but because our blocking assays were performed on thymocytes, on which the level of Ia is below the level of detection under our experimental conditions, this anti-Ia contamination is not a significant factor. Furthermore we have observed that under the conditions applicable to the blocking assay, absorption of anti-Ia sera with thymocytes does not demonstrably lower subsequent reaction of Ia antisera with peripheral lymph node cells.

cytotoxic index by 50%. The cytotoxic index is calculated by the formula (A − B)/(100 − B), in which A is the percent lysis with antibody plus complement and B is the percent lysis with complement alone.

RESULTS

Relative Distances of Alloantigens on Unfixed Cells. As shown in Figs. 1 and 2 (*left*) and in Table 2, we have confirmed the original data of Boyse *et al.* (1) on unfixed cells. There was a reciprocal interference between anti-TL and anti-D (Figs. 1 and 2), between anti-Lyt-1 and anti-K (Table 2), and between anti-Lyt-2 and anti-D (Table 2). This was indicated by the reduced absorption capacity of the test antibody after the thymocytes were treated with the first "blocking" antibody. In all other combinations there was no blocking.

Difference between Fixed and Unfixed Cells: TL vs. D. In parallel studies, there was one notable difference between untreated and paraformaldehyde-fixed cells. Whereas on unfixed cells there was mutual interference between anti-D and anti-TL, no such interference occurred when these same tests were performed on parformaldehyde-fixed cells (compare *E* and *F* in Figs. 1 and 2). The anti-TL sera, when used for blocking in step 1, was previously absorbed with either B6 thymocytes or B6-*Tla*^{a} lymphocytes, (Fig. 1 and Table 1), therefore crossreactive H-2D:TL antibody is not an explanation for the approximation of D and TL. Another possible explanation is that paraformaldehyde perturbs the cell surface in such a way as to cause TL and D to move apart. We exclude this explanation for the following reasons:

(*i*) There was no observable alteration in TL or D expression or in the attachment of anti-TL or anti-D to their respective antigens after paraformaldehyde fixation. (Compare the quantitation absorption data in *A* and *B* of Figs. 1 and 2.)

(*ii*) All other alloantigen proximities were similar on fixed and unfixed cells. Lyt-1 is sufficiently close to K, and Lyt-2 to D, to cause blocking on paraformaldehyde-treated cells (Table 2).

(*iii*) When paraformaldehyde fixation was performed *after* incubation with the blocking antibody, the results were similar to those obtained with *unfixed* cells (Fig. 3). This observation is critical in showing that paraformaldehyde does not alter the capacity of anti-D to block anti-TL attachment.

We are left with the interpretation that positions of TL and D relative to one another are altered by the attachment of specific antibody, and that this movement is prevented by paraformaldehyde fixation. This movement of TL or of D or of both TL and D must occur during the incubation with blocking antibody because fixation beforehand prevents this rearrangement while fixation after incubation does not (Fig. 3). Therefore we presume that initially TL and D are sufficiently separated from one another to obviate mutual interference in the attachment of anti-D and anti-TL antibodies. Evidently, in the absence of paraformaldehyde, binding of either anti-D or of anti-TL activates D sites or TL sites, respectively, and brings these two molecules into apposition (Fig. 4).

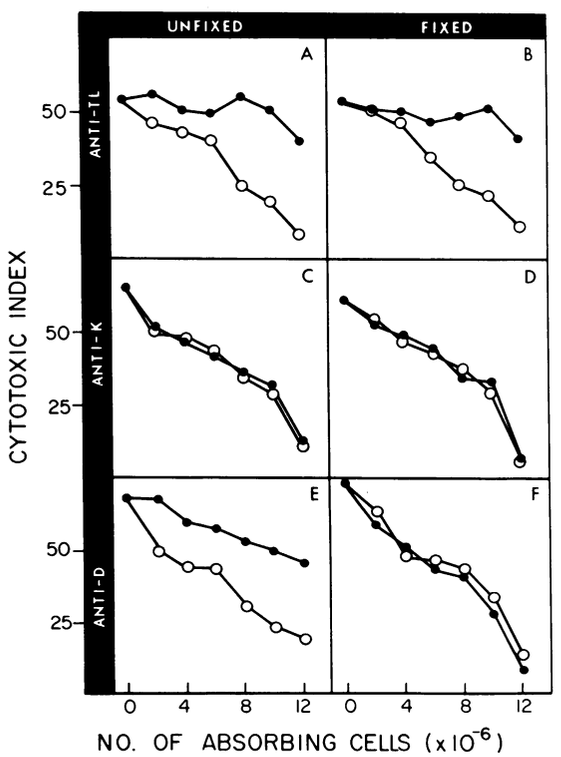

FIG. 1. Blocking with anti-TL (absorbed with B6 thymus) and testing for anti-TL, anti-K, and anti-D reactivity. (*A* and *B*) Test of absorption capacity for anti-TL; (*C* and *D*) test of absorption capacity for anti-K; (*E* and *F*) test for absorption capacity of anti-D. ○, Incubation with NMS; ●, incubation with anti-TL.

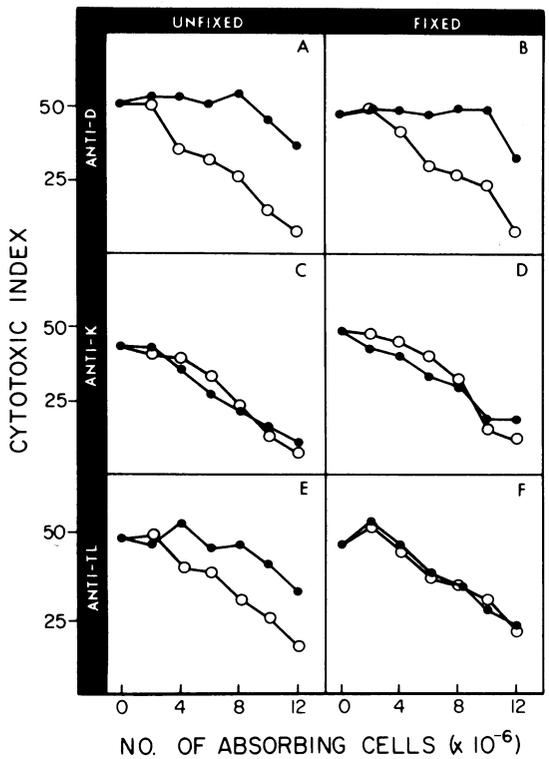

FIG. 2. Blocking with anti-D and testing for anti-D, anti-K, and anti-TL reactivity. (*A* and *B*) Test of absorption capacity for anti-D; (*C* and *D*) test of absorption capacity for anti-K; (*E* and *F*) test of absorption capacity for anti-TL. ○, Incubation with NMS; ●, incubation with anti-D.

DISCUSSION

The dual blocking assay, on unfixed and on paraformaldehyde-fixed cells, confirms in all respects the original thymocyte surface "map" of Boyse *et al.* (1) and adds a critical finding which may imply processes whereby sets of cell surface molecules assume new patterns.

This new finding is that reaction of unfixed thymocytes with H-2D antibody or with TL antibody evidently triggers a response that brings these two components of the plasma membrane together. Since trivial explanations, such as crosslinking by an unidentified crossreactive H-2D:TL antibody, have been excluded, and since rearrangements of other components of the map were not observed, the migration bringing H-2D and TL into adjacent positions evidently exemplifies specific repatterning. Thus, presumably cells can specifically change their surface phenotypes without addition or subtraction of molecules.

The apparent example of specific migration uncovered in this study concerns TL. If this were the only instance of specific repatterning consequent on binding of a ligand, then its interest would be greatly diminished because many mouse strains (TL^- strains) do not express TL, except on leukemia cells. But we surmise that this is not an isolated instance and that other examples of specific repatterning will come to light with further study. In this respect we shall be particularly concerned with the several Qa components, determined by genes in the *Tla* region, which have recently come to light (6–10) and which may be alternative or additional components with properties similar to TL.

The purpose of such repatterning is perhaps to create the sort of supramolecular assembly envisaged as necessary for reactions of hormones with the cell surface (11) and for cellular interactions more generally (12–14). For T cells, one thinks especially of the antigen-receptor itself as an element in the supramolecular pattern, and the process of repatterning might be viewed as the mechanism that primes the cell for appropriate reaction and response to antigen or other immunologically related signals. Current hypotheses that the molecular dispositions of the plasma membrane are governed by submembranous cytoskeletal elements such as microtubules and microfilaments (15–18) are particularly cogent in this context.

It is noteworthy that the instance of repatterning that we have observed involves TL, which is already known to be peculiarly responsive to the external stimulus of antibody, is closely associated with D, and exhibits other features not yet known for other surface components. Thus, TL, which is confined to thymocytes and leukemia cells (19), undergoes the process known as antigenic modulation whereby anti-TL or its Fab fragment can induce the phenotypic loss of TL from the cell surface (20–23). Stackpole *et al.* (24) suggest that this process

Table 2. Blocking studies on unfixed and paraformaldehyde-fixed cells

Exp.	Specificity of blocking antibody	Specificity of test antibody	% blocking on* Unfixed cells	% blocking on* Fixed cells
1	TL†	TL	>80	>80
		K	3	1
		D	52	0
2	TL‡	TL	>80	>80
		K	4	0
		D	>80	0
3	D	D	74	42
		K	2	2
		TL	40	0
4	K	K	>80	>80
		D	0	4
		TL	0	5
5	D	D	60	62
		Lyt-1	2	0
		Lyt-2	25	44
6	K	K	74	>80
		Lyt-1	66	32
		Lyt-2	0	0
7	Lyt-1	Lyt-1	35	52
		K	41	26
		D	0	0
8	Lyt-1	Lyt-1	NT§	52
		K	NT	14
		D	NT	3
		Lyt-2	NT	1
		TL	NT	5
9	Lyt-2	Lyt-2	NT	53
		K	NT	0
		D	NT	72
		Lyt-1	NT	0
		TL	NT	5

* Percent blocking was calculated as described in *Materials and Methods*. Zero (percent blocking) indicates that antibody-treated cells absorbed at least the same quantity of test antibody as NMS-treated cells. Underlined values, % blocking is >10.

† Anti-TL was preabsorbed with B6-*Tla*^{a} lymph node cells to remove any anti-Qa-1 activity.

‡ Anti-TL was preabsorbed with B6(TL^-) thymocytes as a specificity control (see text).

§ NT, not tested.

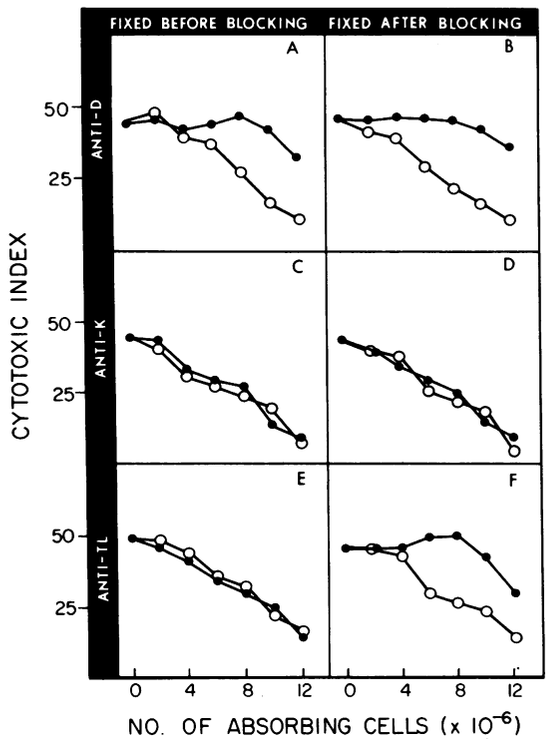

FIG. 3. Effects of fixation before and after incubation with blocking antibody, anti-D. (*A* and *B*) Test of absorption capacity for anti-D; (*C* and *D*) test of absorption capacity for anti-K; (*E* and *F*) test of absorption capacity of anti-TL. Cells in this experiment were fixed either before or after incubation with NMS and anti-D. ●, Incubation with anti-D; ○, incubation with NMS.

involves movement of the TL molecule in the plane of the plasma membrane. However, the relationship of this process to the surface rearrangement reported here is not clear. For example, anti-D causes D and TL to move into apposition but does not induce antigenic modulation (see ref. 25). Some TL antigens appear on leukemias of mice that do not ordinarily express them on their thymuses, evidently an instance of gene derepression associated with malignant transformation (19, 26, 27). There is a reciprocal quantitative relationship between TL and D such that TL^- cells express greater amounts of D than TL^+ cells (28).

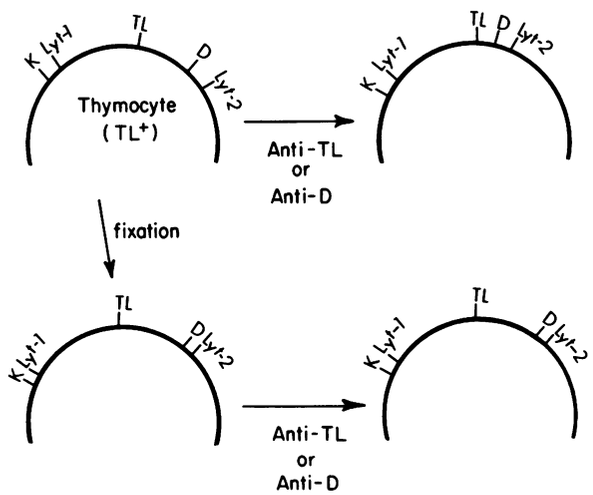

FIG. 4. Model of cell surface rearrangement.

Movement of TL relative to D fits the picture of TL as a molecule whose movement and expression are under unusual modes of control. Whether other molecules, such as those of the Qa series, will prove to have similar properties is an important question. We propose that in the experiments we describe here we are simulating a physiological process in which formation of a TL:D complex is a natural feature of thymocyte differentiation in TL^+ mouse strains.

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1. Boyse, E. A., Old, L. J. & Stockert, E. (1968) *Proc. Natl. Acad. Sci. USA* **60**, 886–893.
2. Shen, F-W., Boyse, E. A. & Cantor, H. (1976) *Immunogenetics* **2**, 591–595.
3. Parr, E. L. & Oei, J. S. (1973) *J. Cell. Biol.* **59**, 537–548.
4. Parr, E. L. & Oei, J. S. (1973) *Tissue Antigens* **3**, 99–107.
5. Boyse, E. A., Hubbard, L., Stockert, E. & Lamm, M. E. (1970) *Transplantation* **10**, 446–449.
6. Stanton, T. H. & Boyse, E. A. (1976) *Immunogenetics* **3**, 525–531.
7. Flaherty, L. (1976) *Immunogenetics* **3**, 533–539.
8. Flaherty, L., Zimmerman, D. & Hansen, T. H. (1978) *Immunogenetics* **6**, 245–251.
9. Eardley, D. D., Hugenberger, J., McVay-Boudreau, L., Shen, F-W., Gershon, R. K. & Cantor, H. (1978) *J. Exp. Med.* **147**, 1106–1115.
10. Stanton, T. H., Calkins, C. E., Jandinski, J., Schendel, D. J., Stutman, O., Cantor, H. & Boyse, E. A. (1978) *J. Exp. Med.* **148**, 963–973.
11. Cuatrecasas, P. (1974) *Annu. Rev. Biochem.* **43**, 169–214.
12. Boyse, E. A. & Old, L. J. (1970) in *Immune Surveillance*, eds. Smith, R. T. & Landy, M. (Academic, New York), pp. 5–30.
13. Boyse, E. A. & Cantor, H. (1978) *Birth Defects Orig. Artic. Ser.* **14**, 249–283.
14. Bourguigon, L. Y. W., Hyman, R., Trowbridge, I. & Singer, S. J. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2406–2410.
15. Edelman, G. M. (1976) *Science* **192**, 218–226.
16. Gabbiani, G., Chaponnier, C., Zumbe, A. & Vassalli, P. (1977) *Nature (London)* **269**, 697–698.
17. Koch, G. L. E. & Smith, M. J. (1978) *Nature (London)* **273**, 274–278.
18. Flanagan, J. & Koch, G. L. E. (1978) *Nature (London)* **273**, 278–281.
19. Boyse, E. A., Old, L. J. & Stockert, E. (1965) in *Immunopathology IV International Symposium, Monaco*, eds. Grabar, P. & Miescher, P. A. (Schwabe & Co., Basel), pp. 23–40.
20. Boyse, E. A., Old, L. J. & Luell, S. (1963) *J. Natl. Cancer Inst.* **31**, 987–995.
21. Boyse, E. A., Stockert, E. & Old, L. J. (1967) *Proc. Natl. Acad. Sci. USA* **58**, 954–957.
22. Old, L. J., Stockert, E., Boyse, E. A. & Kim, J. H. (1968) *J. Exp. Med.* **127**, 523–539.
23. Lamm, M. E., Boyse, E. A., Old, L. J., Lisowka-Bernstein, B. & Stockert, E. (1968) *J. Immunol.* **101**, 99–103.
24. Stackpole, C. W., Jacobson, J. B. & Lardis, M. P. (1974) *J. Exp. Med.* **140**, 939–953.
25. Takahaski, T. (1971) *Transplant. Proc.* **3**, 1217–1220.
26. Old, L. J., Boyse, E. A. & Stockert, E. (1963) *J. Natl. Cancer Inst.* **31**, 977–986.
27. Stockert, E. & Old, L. J. (1977) *J. Exp. Med.* **146**, 271–276.
28. Boyse, E. A., Stockert, E. & Old, L. J. (1968) *J. Exp. Med.* **128**, 85–95.