INDEPENDENCE OF *H-2K* AND *H-2D* ANTIGENIC DETERMINANTS ON THE SURFACE OF MOUSE LYMPHOCYTES*

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Our concept of the fine structure of the complex *H-2* region of linkage group IX in the mouse has continued to evolve almost constantly since the initial discovery by Gorer of serologically detectable alloantigens associated with a gene responsible for graft rejection (1). It became apparent by the 1950's that this gene, *H-2*, far from being a single gene, was subdivisible into antigen-determining subregions which were separable by recombination at a very low frequency (2). A concept gradually emerged according to which the *H-2* complex included from four to six closely linked but genetically separable subloci, each represented in various strains of mice by alleles governing different antigen specificities (3, 4). An *H-2* "allele" thus represented a series of several adjacent histocompatibility loci, each of which resulted in the appearance on cell surfaces of one or more serologically detectable antigen specificities.

In the 1960's, several other genes, not obviously related in a biological sense to the *H-2* alloantigens, were detected and found to map in close association with these antigens. It became clear that some of these genes were actually interspersed among the *H-2* determinants, so that these latter could no longer be supposed to lie immediately adjacent to each other (5). During this same period of time, it also became possible to simplify the multiple-sublocus hypothesis by reinterpreting the previous observations upon which it was based. Assuming that, in a few cases, a single *H-2* specificity might map in either one of two different places within the *H-2* region, then one could construct an *H-2* map consisting of only two antigen-determining subloci, rather than several.

Fig. 1 represents our current concept of this complex genetic region. Two genes, *H-2K* and *H-2D*, appear to be adequate to account for all the serologically detectable antigen determinants (6). These genes are separable by recombination at a frequency of about 0.5%, and the short chromosomal segment separating them includes the determinants of the *Ir-1* (7) and *Ss-Slp* (8) phenomena. The *Tla* gene(s) (9) map a short distance to the right of *H-2D*, and *Rgv-1* (10), a gene influencing susceptibility to viral leukemogenesis, is located in the *H-2K-Ir-1* vicinity. Thus the $H\text{-}2^{b}$ haplo-

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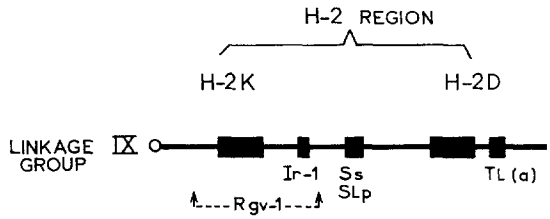

FIG. 1. Schematic representation of the present concept of the genetic map of the *H-2* region.

type,[1] for instance, is equivalent to the phenotype of the $H\text{-}2K^b$ allele plus that of the separate, but closely linked, $H\text{-}2D^b$ allele.

An important piece of supporting evidence for this two-gene concept (*H-2* = *H-2K* + *H-2D*) is the recent finding (11) that the major antigens governed by *H-2K* and *H-2D* are separable on different molecules in detergent-solubilized cell membrane fractions, by specific reaction with the corresponding antibody. Thus, in solubilized material from heterozygotes ($H\text{-}2^b/H\text{-}2^d$), four different molecules can be demonstrated, corresponding to the two *H-2K* alleles and the two *H-2D* alleles. Although it remains to be proved that all known *H-2* specificities will be associated with these same molecules, it is now possible to ask a number of questions concerning the spatial relations of these various molecules *in situ* on living cells.

Approaches to this problem of cell surface mapping have already been made by means of blocking-absorption experiments (12–14). After saturating cells with antibodies of a certain specificity (e.g. anti-*H-2K*), the capacity of the cells to absorb further antibodies of another specificity (e.g. anti-*H-2D*) can be determined. Using this method, Boyse et al. (12) suggested that *H-2K* and *H-2D* molecules are not located in close proximity to each other *in situ* on the membrane surface.

The experiments which we now report confirm and expand these findings, and take in account the dynamic state of the living cell membranes. It was recently noted that, under certain experimental conditions, at 37°C, bivalent antibodies specifically induce, at the surface of lymphocytes, a redistribution of histocompatibility antigens, which cluster in "spots," and sometimes in "caps" restricted to one pole of the cell, visible in immunofluorescence (15). This redistribution phenomenon, inhibited at 0°C, and accelerated by addition of anti-IgG to the antihistocompatibility antibodies, is very similar to that described for surface Ig determinants (16–18). Current interpretation of this phenomenon would imply antibody-mediated cross-linking of mobile antigenic sites on the cell surface, and might be compatible with a fluid model of the cell membranes (19). This antibody-induced redistribution process can provide a useful tool for the study of the relationships between two antigenic molecules at the cell surface, and is applied here to the study of *H-2K* and *H-2D* antigens. The principle is that, if these two antigens are expressed on the same molecule,

[1] It has recently become customary to refer to the serological phenotype conferred by a given haploid *H-2* region as the *H-2* haplotype.

or structure, at the cell surface, the redistribution of *H-2K* determinants induced by anti-*H-2K* antibodies should also involve the redistribution of *H-2D* determinants and vice versa. Our experiments indicate that, on the contrary, *H-2K* and *H-2D* antigens are displaced separately on the cell surface, and we therefore suggest that these molecules are not appreciably bound to each other *in situ*.

Materials and Methods

Histocompatibility-2 Antigens.—The *H-2* antigens selected for this study are private specificities governed by the D and the K end of the *H-2* locus in the $H\text{-}2^b$ and $H\text{-}2^k$ alleles. In the $H\text{-}2^b$ haplotype these specificities are H-2.33 ($H\text{-}2K^b$) and H-2.2 ($H\text{-}2D^b$). In the $H\text{-}2^k$ haplotype the specificities are H-2.23 ($H\text{-}2K^k$) and H-2.32 ($H\text{-}2D^k$).

The genetic combinations studied on ($H\text{-}2^k \times H\text{-}2^b$)$F_1$ hybrid cells are illustrated in Fig. 2. The pairs of *H-2* antigens tested are governed by genes located either on the same parental

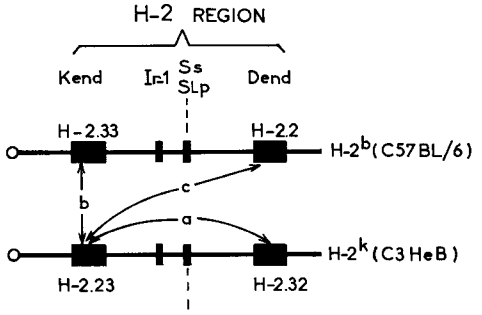

FIG. 2. Schematic representation of the types of pairs of genetic combinations tested in the study of *H-2* antigens on (C57BL/6 × C3HeB)F_1 hybrid cells. (*a*) = *cis*; (*b*) = *trans*, homologous; (*c*) = *trans*, nonhomologous.

chromosome (*cis* position) or in *trans* position in homologous location ($H\text{-}2K^b$–$H\text{-}2K^k$) or in nonhomologous location ($H\text{-}2K^k$–$H\text{-}2D^b$).

Target Cells.—Cells were obtained from 2-mo old inbred mice C57BL/6 ($H\text{-}2^b$), C3HeB ($H\text{-}2^k$), and from the corresponding (C57BL/6 × C3HeB)F_1 hybrids ($H\text{-}2^b/H\text{-}2^k$). The mesenteric lymph nodes were teased in undiluted fetal calf serum (FCS)[2] and filtered through a stainless steel grid. The cell suspensions were washed three times at 0°C and resuspended in Eagle's minimal essential medium (MEM) supplemented with 20% FCS. The final preparations contained 95–98% of mononucleated cells, as determined on smears stained with May-Grünwald-Giemsa, and fewer than 10% dead cells, as checked by trypan blue exclusion test. These preparations are referred to as "lymphocyte suspensions."

Antisera.—Antisera were prepared by hyperimmunization of young adult recipient mice with allogeneic lymphoid tissues. The donor-recipient combinations were chosen to minimize the probability of obtaining antibodies other than those recognizing the desired *H-2* specificities. Antibodies to specificities of the $H\text{-}2^b$ haplotype were elicited with cells of EL.4, a chemically induced C57BL/6 leukemia. Antibodies to $H\text{-}2^k$ specificities were elicited with cells of normal lymphoid tissues (spleen, thymus, lymph nodes) from C3H/An mice. The first small

[2] *Abbreviations used in this paper:* FCS, fetal calf serum; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; TRITC, tetramethyl rhodamine isothiocyanate.

immunizing dose, of about 10^6 cells, was administered subcutaneously; thereafter increasing doses, up to 25 × 10^6 cells, were given intraperitoneally at 2–3-wk intervals. Serum was collected, beginning after the third or fourth immunization, by tail bleeding twice after each immunization, generally on days 12 and 14. The sera were stored frozen. Table I describes the four antisera used in these studies. The *H-2* specificities recognized by these sera represent the *H-2K* and *H-2D* regions of the *H-2*^{b} and *H-2*^{k} haplotypes. The cytotoxic titer (50% end point) was determined by the ^{51}Cr assay (20) on homozygous spleen cells as targets.

BALB/c anti-C57BL/6 antisera (*H-2*^{d} anti-*H-2*^{b}), with a cytotoxic titer of 1/200, were similarly prepared. Rabbit anti-mouse IgG antiserum was used in indirect tests.

Conjugates.—Globulin fractions were obtained by precipitation of 3–4 ml of each antiserum with 40% saturated ammonium sulfate and aliquots were conjugated to fluorescein isothiocyanate, Isomer 1 (FITC) (Sigma Chemical Corp., St. Louis, Mo.) and to tetramethyl rhodamine isothiocyanate (TRITC) (Baltimore Biological Laboratories, Cockeysville, Md.). The conjugation procedure was carried out for 18 h at 4°C in sodium borate buffer, pH 9.2, at a final protein concentration of 4.5 mg/ml, using 15 μg of FITC or 40 μg of TRITC/mg protein.

TABLE I

Anti-H-2 Antisera

Recipient mice		Donor		Target cell		Specificity detected	Cytotoxic titer
Strain	*H-2* type	Tissue	*H-2* type	Strain	*H-2* type		
(BALB/c × HTI)F_1	*H-2*^{d}/*H-2*^{i}	EL.4	*H-2*^{b}	C57BL/6	*H-2*^{b}	H-2.2 (*H-2D*^{b})	1:82
(B10.A × HTG)F_1	*H-2*^{a}/*H-2*^{g}	EL.4	*H-2*^{b}	C57BL/6	*H-2*^{b}	H-2.33 (*H-2K*^{b})	1:160
A	*H-2*^{a}	C3H/An	*H-2*^{k}	C3HeB	*H-2*^{k}	H-2.32 (*H-2D*^{k})	1:98*
C3H.OH	*H-2*^{o}	C3H/An	*H-2*^{k}	C3HeB	*H-2*^{k}	H-2.11, 23 (*H-2K*^{k})‡	1:130*

* Determined using B10.BR/Sn (*H-2*^{k}) as target cells.

‡ Designated anti-H-2.23.

After conjugation, free FITC or TRITC were eliminated by chromatography through Sephadex G-25 or G-50, respectively, in phosphate-buffered saline (PBS), pH 7.2. Conjugates were further dialyzed for 24 h against PBS and stored frozen at −70°C. Several FITC and TRITC conjugates have been prepared from each antiserum, and the best conjugates, with respect to the antibody titer, were selected.

The characteristics of the conjugates used are listed in Table II. In order to verify their specificity, each conjugate was absorbed on the irrelevant cells: *H-2*^{k} cells for anti-*H-2*^{b} conjugates, and *H-2*^{b} cells for anti-*H-2*^{k} conjugates. 1 ml of the conjugate was mixed with 5–10 × 10^7 cells and incubated for 1 h at 37°C and 1 h at 4°C. The absorbed conjugates were clarified by centrifugations at 200 *g* and 10,000 *g*. Controls for nonspecific immunofluorescence staining were made using the irrelevant target cells, and in all cases fewer than 2% of cells were stained.

The antibody activity of each selected conjugate was estimated by immunofluorescence titration. For this purpose, 10^6 relevant homozygous cells were incubated for 30 min at 37°C with 0.05 ml of several dilutions of each conjugate and washed three times in PBS at room temperature, and the percentages of cells stained were ascertained. Most conjugates were found to be already on the decreasing phase of the titration curve at a 1:1 dilution. This relatively low activity of the conjugates was presumably due to the fragility of anti-*H-2* antibodies with regard to the coupling procedure, and to the low fluorochrome/protein molar ratios used to minimize the loss of antibody activity. The efficiency of the conjugates was still lower when the cells staining was performed in the cold and when hybrid target cells

TABLE II

Characteristics of the Conjugates Used

Designation	F/P ratio*	Protein concentration	Absorbed with	Tests on reactive cells			Negative controls	
				Target cells	Labeled cells‡	Labeled cells§	Cells	Labeled cells‡
		mg/ml			%	%		%
FITC anti-33	1.33	2.00	C3HeB	C57BL/6	78	95	C3HeB	2
				(C57BL/6 × C3HeB)F_1	44	91		
TRITC anti-33	0.30	2.40	C3HeB	C57BL/6	57	49	C3HeB	1
				(C57BL/6 × C3HeB)F_1	0	48		
FITC anti-23	1.42	1.88	C57BL/6	C3HeB	33	90	C57BL/6	1
				(C57BL/6 × C3HeB)F_1	29	62		
TRITC anti-23	0.70	0.94	C57BL/6	C3HeB	38	51	C57BL/6	2
				(C57BL/6 × C3HeB)F_1	19	64		
FITC anti-2	1.18	1.90	C3HeB	C57BL/6	48	69	C3HeB	2
				(C57BL/6 × C3HeB)F_1	22	22		
TRITC anti-2	0.61	1.31	C3HeB	C57BL/6	55	38	C3HeB	2
				(C57BL/6 × C3HeB)F_1	2	34		
FITC anti-32	1.41	2.00	C57BL/6	C3HeB	29	98	C57BL/6	1
				(C57BL/6 × C3HeB)F_1	1	9		
TRITC anti-32	0.80	1.66	C57BL/6	C3HeB	0	36	C57BL/6	2
				(C57BL/6 × C3HeB)F_1	0	11		

* Fluorochrome/protein molar ratio as determined as in reference 21.

‡ Immunofluorescence staining performed at 0°C.

§ Immunofluorescence staining performed at 37°C.

were used instead of homozygous cells. This introduced a limiting technical factor in some experiments.

Immunofluorescence Procedures.—The procedure for determining the kinetics of the redistribution of anti-*H-2* antibodies was as follows: 1.5×10^6 lymphoid cells were distributed in 1-ml plastic microtubes and washed two times at 0°C with MEM containing 20% FCS. After centrifugation in the cold, the cell pellet was resuspended in 0.05 ml of the undiluted proper conjugate, incubated for 30 min at 0°C, washed three times, and resuspended in 0.05 ml of medium at 0°C. The cell suspension was then incubated at 37°C for 2 h. Microsamples of cells were aspirated in the tube at time intervals, and examined directly in suspension for immunofluorescence or dried-fixed before examination. In this case, the microdroplet of cell suspension was spread and dried on a glass slide, fixed with pure methanol for 5 min at room temperature, and mounted under a cover slip in buffered glycerol, pH 7.4.

In order to test if the redistribution of a given *H-2* specificity induced by the corresponding conjugated antibody had modified the distribution of another *H-2* specificity, two-tone successive immunofluorescence labeling was performed in the following way: washed lymphoid cells were stained for 30 min at 37°C in 0.05 ml of the first conjugate, then washed three times in medium, resuspended in 0.5 ml of medium, and further incubated for 45 min at 37°C in order to induce redistribution of antibody-bound *H-2* antigens. At the end of the incubation period, the cells were cooled to 0°C, incubated for 30 min at 0°C with 0.05 ml of the second conjugate labeled with the opposite fluorochrome, and washed three times at 0°C before

immunofluorescence examination. This second stage of the procedure was performed in the cold, in order to minimize the redistribution phenomenon.

The preparations were examined under a Leitz Ortholux fluorescence microscope (E. Leitz, Inc., Rockleigh, N. J.), equipped with a Ploem-type epi-illumination system, using a HBO 200 mercury lamp as UV source. For FITC, the following combination of filters was used: for excitation, 4 mm BG 38, 3 mm Schott GG 480, and two interferential filters KP 490 with dichroic mirror TK 510, and barrier filters K 515 and K 530, For TRITC, excitation filters were 4 mm BG 38, 2 mm BG 36, and interferential filter S 546 nm, with dichroic mirror TK 580 and barrier filter K 580.

RESULTS

Redistribution of H-2 Antigens at 37°C after the Labeling with Fluorescent Antibodies.—Immediately after lymphoid cells were incubated at 0°C with anti-*H-2* conjugates, the pattern of fluorescence was observed to be dispersed over the entire surface of the cell. During the incubation at 37°C, this diffuse surface fluorescence clustered rapidly, forming large "spots" or "patches" of fluorescence and leaving zones of the cell surface totally unlabeled. On some cells the clustering resulted in the formation of a single crescent or cap at one pole of the cell. The lymphocytes remained viable during these events, as judged by trypan blue exclusion. The clustering of fluorescent antibodies was strongly inhibited at 0°C.

The kinetics of the modification of the pattern of fluorescence labeling was studied at time intervals during the incubation at 37°C. As shown in Fig. 3, using a fluorescein-conjugated, monospecific anti-*H-2* antiserum, the percentage of cells with dispersed fluorescence decreased progressively, being replaced by cells with patchy or polar fluorescence; within 90 min, no more

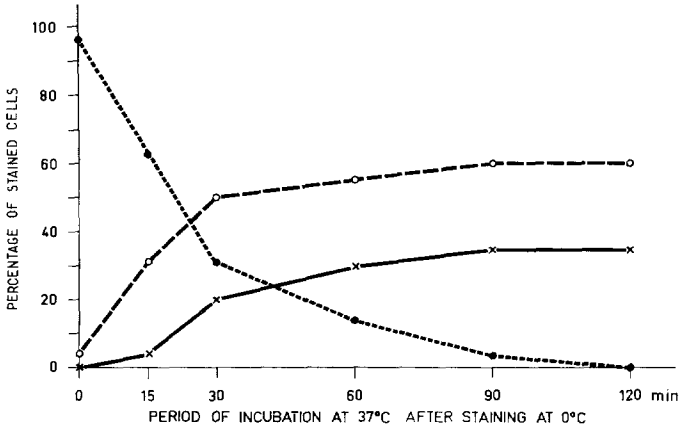

FIG. 3. Evolution of the surface immunofluorescence pattern of (C57BL/6 × C3HeB)F_1 mouse lymphocytes labeled with FITC anti-H-2.23 at 0°C, washed, and examined after warming at 37°C over a 2 h incubation period. (●----●) Cells with dispersed fluorescence; (O--O) cells with patchy fluorescence; (✕—✕) cells with polar fluorescence.

cells with diffuse fluorescence were seen, and the pattern of fluorescence was aggregated on all cells, i.e., patchy on 65% of cells and forming caps on 35% of cells. A similar evolution of the fluorescence pattern of bound anti-*H-2* antibodies was observed on both homozygous cells (*H-2*^{b} and *H-2*^{k}) and on (*H-2*^{b} × *H-2*^{k})F_1 hybrid cells similarily stained with monospecific or polyspecific anti-*H-2*-conjugated antisera. The addition of nonconjugated rabbit antimouse IgG antiserum at dilutions varying from 1:3 to 1:27, to cells coated with fluorescent anti-*H-2* antibodies induced a significant increase in the intensity of the fluorescent spots and caps observed.

In order to check if the *H-2* antigens were displaced together with the corresponding antibodies, double labeling experiments were performed. *H-2*^{b} lymphocytes were stained with FITC anti-H-2.33 conjugated, incubated 45 min at 37°C in order to allow the aggregation of fluorescence to occur, and then relabeled at 0°C with the same antiserum, conjugated with TRITC. Under these conditions, no red labeling was observed outside the green clusters already formed, indicating therefore that the *H-2* antigens are displaced by the redistribution of antibodies. Identical results are obtained with other antisera and cells. In all cases no remaining *H-2* antigen was detected on the cell surface by the second conjugate, outside the clusters already formed by the first anti-*H-2* conjugate. This strongly suggests that the redistribution phenomenon, observed in our experimental conditions, involved the totality of detectable *H-2* antigenic sites bound to antibody. Similar observations concerning the redistribution of human histocompatibility antigens have been made (15).

Comparative Study of the Redistribution of H-2K and H-2D Antigens on Homozygous Cells.—The study of redistribution of different *H-2* antigens on homozygous cells was made with monospecific anti-*H-2* antisera conjugated to FITC or TRITC. Generally, after the first labeling of one antigen and incubation at 37°C, the clustering of fluorescence occurred (patches and/or caps) on 80–100% of cells. The second labeling was performed with another antiserum conjugated with the opposite fluorochrome, and incubated in the cold in order to minimize the antibody-induced aggregation of antigens. Both patterns of surface fluorescence for each fluorochrome were scored on 100–200 cells individually examined for red and green labeling. Under these conditions the second fluorescence labeling was diffuse on most double-stained cells. The pattern of fluorescence labeling for both conjugates was aggregated or remain diffuse on few cells. The cells of the preparation which were stained only with one conjugate and not the other were not taken in account in the tables.

This type of experiment has been performed on cells from homozygous mice, C57BL/6 (*H-2*^{b}) and C3HeB (*H-2*^{k}).

Experiments on C57BL/6 (H-2^b*) cells:* The results of a typical experiment are given in Table III a. The clustering of H-2.33 antigen (K end) was induced by incubating at 37°C the lymphocytes previously coated with TRITC anti-H-2.33 conjugate. The cells were then incubated in the cold with the

TABLE III a

Pattern of FITC and TRITC Labeling on C57BL/6 (H-2^b) Cells

TRITC anti-H-2.33	FITC anti-H-2.2		
	Diffuse	Patchy	Polar
Diffuse	5	0	0
Patchy	57	2	0
Polar	36	0	0

First labeling with TRITC anti-H-2.33 30 min at 37°C followed by 45 min incubation at 37°C in MEM 20% FCS.

Second labeling with FITC anti-H-2.2 30 min at 0°C.

Prior redistribution of H-2.33 antigens fails to modify the diffuse redistribution of H-2.2 antigens.

TABLE III b

Pattern of FITC and TRITC Labeling on C3HeB (H-2^k) Cells

TRITC anti-H-2.23	FITC anti-H-2.32		
	Diffuse	Patchy	Polar
Diffuse	4	35	58
Patchy	0	1	0
Polar	0	0	2

First labeling with FITC anti-H-2.32 30 min at 37°C followed by 45 min incubation at 37°C in MEM 20% FCS.

Second labeling with TRITC anti-H-2.23 30 min at 0°C.

Prior redistribution of H-2.32 antigens fails to modify the diffuse distribution of H-2.23.

TABLE III c

Pattern of FITC and TRITC Labeling on (C57BL/6 × C3HeB)F$_1$ (H-2^b/H-2^k) Cells

TRITC anti-H-2.33	FITC anti-H-2.23		
	Diffuse	Patchy	Polar
Diffuse	4	0	0
Patchy	36	0	0
Polar	56	1	3

First labeling with TRITC anti-H-2.33 30 min at 37°C followed by 45 min incubation at 37°C in MEM 20% FCS.

Second labeling with FITC anti-H-2.23 30 min at 0°C.

Prior redistribution of H-2.33 antigens fails to modify the diffuse distribution of H-2.23 antigens.

FITC anti-H-2.2 (D end) conjugate. On most cells the green labeling, corresponding to H-2.2 antigens, was diffuse and clearly spread outside the red clusters of H-2.33 antigens, indicating therefore that redistribution of H-2.33 antigens failed to induce the redistribution of H-2.2 antigens. On few cells the

labeling patterns for FITC and TRITC conjugates were either both aggregated or both diffuse. The aggregation of both FITC- and TRITC-conjugated antisera at the same place on the surface of a few cells may have been due to nonspecific staining or to an independent aggregation of H-2.33 and H-2.2 antigens, since it is known that two independent antigens may gather at the same pole of a cell (18).

As noted above (see Results, first paragraph), if a second labeling of these cells coated with TRITC anti-H-2.33 is performed with the same anti-H-2.33 antiserum, FITC conjugated, no green labeling is detected outside the red patches and caps already formed indicating that all detectable antigenic sites have been displaced by the first conjugate. As expected, a blocking effect is exerted by the previously fixed conjugate, in these conditions (see Table IV).

TABLE IV

Double Labeling Experiments Performed on C57BL/6 ($H\text{-}2^b$) Cells

First labeling at 37°C		Second labeling at 0°C		Labeling pattern of both conjugates on 100 cells			
Conjugate	Stained cells	Conjugate	Stained cells	1st labeling aggregated 2nd labeling diffuse	1st labeling aggregated 2nd labeling aggregated	1st labeling diffuse 2nd labeling aggregated	1st labeling diffuse 2nd labeling diffuse
	%		%				
TRITC anti-H-2.33	47	FITC anti-H-2.2	48	93	2	0	5
FITC anti-H-2.33	100	TRITC anti-H-2.2	55	55	5	0	40
TRITC anti-H-2.2	36	FITC anti-H-2.33	78	85	14	0	1
TRITC anti-H-2.33	50	FITC anti-H-2.33	0				
FITC anti-H-2.33	95	TRITC anti-H-2.33	0				
TRITC anti-H-2.2	40	FITC anti-H-2.2	2				

Conversely, as shown in Table IV, prior redistribution of H-2.2 antigens with a TRITC anti-H-2.2 conjugate failed to modify the diffuse distribution of H-2.33 antigens revealed with a FITC anti-H-2.33 conjugate.

In order to eliminate possible errors due to a difference in the sensitivity of fluorochrome detection, the sequence of labeling by TRITC or FITC conjugates was reversed. This inversion did not modify the results (Table IV). These data therefore suggest that H-2.33 and H-2.2 antigens on $H\text{-}2^b$ lymphocytes are borne by molecules susceptible to independent redistribution by the specific corresponding bivalent antibodies.

Experiments on C3HeB ($H\text{-}2^k$) cells: Experiments performed with anti-H-2.32 and anti-H-2.23 conjugates are illustrated in Table III b. The results were similar to those described above for $H\text{-}2^b$ cells. After the clustering of fluorescent anti-H-2.32 antibodies induced by incubation of sensitized cells at 37°C, it was not possible to label the lymphocyte membrane outside the patches and caps with the same antiserum conjugated to the other fluorochrome. When the second incubation was performed with the anti-H-2.23 conjugate,

the labeling remained diffuse over the entire surface of the cells, indicating that the displacement of H-2.32 antigens did not provoke the apparent displacement of H-2.23 antigens, as shown in Table V. Conversely, the clustering of H-2.23 antigens did not affect the diffuse distribution of H-2.32 antigens. Similar results were obtained if the sequence of TRITC and FITC labeling is reversed. Therefore these results indicate that H-2.32 and H-2.23 antigens on $H\text{-}2^k$ lymphocytes are subject to independent redistributions.

Redistribution of Different H-2 Antigens on Hybrid Cells.—On heterozygous cells, it is possible to study separately the *H-2K* and *H-2D* antigens governed by genes known to be present on either the same haplotype (in *cis* position) or on different haplotypes (in trans position).

TABLE V

Double Labeling Experiments Performed on C3H/eB ($H\text{-}2^k$) Cells

First labeling at 37°C		Second labeling at 0°C		Labeling pattern of both conjugates on 100 cells			
Conjugate	Stained cells	Conjugate	Stained cells	1st labeling aggregated 2nd labeling diffuse	1st labeling aggregated 2nd labeling aggregated	1st labeling diffuse 2nd labeling aggregated	1st labeling diffuse 2nd labeling diffuse
	%		%				
FITC anti-H-2.32	98	TRITC anti-H-2.23	26	94	2	0	4
TRITC anti-H-2.23	54	FITC anti-H-2.32	12	62	0	0	38
TRITC anti-H-2.32	38	FITC anti-H-2.23	24	96	2	1	1
FITC anti-H-2.32	81	TRITC anti-H-2.32	4				
TRITC anti-H-2.23	48	FITC anti-H-2.23	4				
TRITC anti-H-2.32	35	FITC anti-H-2.32	5				

Study of the respective redistribution of two H-2K antigens on hybrid cells: The hybrid cells come from (C57BL/6 × C3HeB)F_1 mice ($H\text{-}2^b$ × $H\text{-}2^k$). The antigenic specificities H-2.33 and H-2.23 have been chosen. Both are coded for by the K region of the $H\text{-}2^b$ and $H\text{-}2^k$ haplotypes, respectively. As shown in Tables III c and VI, when clusters of fluorescent anti-H-2.33 antibodies were formed, it was not possible to detect the H-2.33 antigens outside these clusters, but the distribution of H-2.23 antigen remained diffuse over the entire surface of cells. The inversion of the sequence of labeling with FITC or TRITC did not modify the results. Therefore the H-2.33 and H-2.23 antigenic specificities coded by the *H-2K* gene of different haplotypes appear to have an independent redistribution on heterozygous cells. The interference between redistributions of two allelic antigen specificities coded by the *H-2D* gene could not be studied because of the weak titer of anti-H-2.2 and anti-H-2.32 conjugates which failed to react strongly enough with hybrid cells at 0°C (see Materials and Methods). These hybrid cells possess only half the quantity of each *H-2* antigen as homozygous cells.

Study of the respective redistribution of two nonhomologous antigens in trans

TABLE VI

Double Labeling Experiments Performed on (C57BL/6 × C3H/eB)F_1 Hybrid Cells ($H\text{-}2^b \times H\text{-}2^k$)

Genetic combination studied	First labeling at 37°C		Second labeling at 0°C		Labeling pattern of both conjugates on 100 cells			
	Conjugate	Stained cells	Conjugate	Stained cells	1st labeling aggregated 2nd labeling diffuse	1st labeling aggregated 2nd labeling aggregated	1st labeling diffuse 2nd labeling aggregated	1st labeling diffuse 2nd labeling diffuse
		%		%				
Antigens coded by allelic genes	TRITC anti-H-2.33	51	FITC anti-H-2.23	29	92	4	0	4
	FITC anti-H-2.33	98	TRITC anti-H-2.23	17	94	4	0	2
	TRITC anti-H-2.33	45	FITC anti-H-2.33	1				
	FITC anti-H-2.33	84	TRITC anti-H-2.33	3				
Antigens coded by nonhomologous genes in *cis* position	FITC anti-H-2.32	9	TRITC anti-H-2.23	22	92	2	0	6
	TRITC anti-H-2.32	16	FITC anti-H-2.23	28	88	9	0	3
	FITC anti-H-2.32	9	TRITC anti-H-2.32	1				
	TRITC anti-H-2.32	11	FITC anti-H-2.32	3				
Antigens coded by nonhomologous genes in *trans* position	FITC anti-H-2.2	23	TRITC anti-H-2.23	22	100	0	0	0
	TRITC anti-H-2.32	10	FITC anti-H-2.33	53	54	19	0	27
	FITC anti-H-2.2	21	TRITC anti-H-2.2	0				
	TRITC anti-H-2.32	9	FITC anti-H-2.32	3				

position: The redistribution of antigens coded by the *H-2K* and *H-2D* genes in *trans* position was also studied. As expected, the redistribution of H-2.2 antigens coded by *H-2D* gene of $H\text{-}2^b$ haplotype did not provoke the concomitant redistribution of H-2.23 antigens coded by the *H-2K* gene of the $H\text{-}2^k$ haplotype.

Study of the respective redistribution of two antigens in cis position: The hybrid ($H\text{-}2^b \times H\text{-}2^k$)$F_1$ cells provided the possibility of studying the respective redistribution of *H-2K* and *H-2D* antigens coded by genes located on the same parental chromosome. The H-2.23 and H-2.32 antigenic specificities governed by genes on the same $H\text{-}2^k$ haplotype have been chosen. In this case, the control of the redistribution of H-2.32 antigens was made by incubating the second conjugate at 37°C instead of 0°C, since this conjugate did not react at 0°C. The incubation time was not long enough to allow a total redistribution of antigens to occur during the incubation at 37°C. As shown in Table VI, the clustering of H-2.32 antigens do not apparently modify the diffuse distribution of H-2.23 antigens.

DISCUSSION

The experiments reported here take advantage of the antibody-induced redistribution of membrane antigens on living cells to analyze the relationships

between *H-2* antigenic determinants at the cell surface. It was observed that *H-2K* and *H-2D* specificities migrate separately on the surface of mouse lymphocytes, indicating that they are carried by independent structures.

The clustering of membrane antigens, induced at 37°C by bivalent antibodies, has been described for Ig determinants on the surface of mouse (16) and human (17, 18) lymphocytes, and for human HL.A histocompatibility antigens (15). It has been obtained on mouse thymocytes using anti-θ antiserum, but this requires the addition of anti-Ig antibodies reacting with the mouse alloantibodies (16). A similar observation has been made for antilymphocyte globulin antigens on human lymphocytes (15). The redistribution of *H-2* antigens by anti-*H-2* antibodies in the presence of antimouse IgG has been observed by immunofluorescence (15, 16) and immunoferritin (22) techniques. It was pointed out (15) that this phenomenon may have some bearing on the interpretation of the discontinuous distribution of these antigens at the cell surface (22, 23) observed with indirect immunoferritin techniques.

Using monospecific anti-*H-2* antisera directly coupled to FITC or TRITC, we have observed at 37°C the clustering of the fluorescence pattern, with the formation of patches and polar caps on the surface of mouse lymphocytes. The redistribution of the fluorescence affects all cells labeled, but complete cap formation occurs only on 30–50% of them. This percentage of cap-forming cells on human and mouse lymphocytes is higher for Ig molecules (16–18) than for histocompatibility antigens (15) even when cap formation with anti-HL.A antibodies is determined on the subpopulation of Ig-bearing human lymphocytes (18). This discrepancy may therefore be related to the relative density of antigenic sites on the cell membrane (24, 25) and/or to the concentration of the conjugated alloantibodies.

In our experimental conditions, the immunofluorescence antibody titer of the monospecific anti-*H-2* conjugates was relatively low, and this may be the reason why the redistribution of the corresponding antigen was readily obtained in the absence of antimouse Ig antibodies. In fact, if the cross-linking of mobile antigenic sites by divalent antibodies represents the initial event in the antibody-induced redistribution process (16, 17), then one should expect the redistribution to occur only within a certain range of antibody concentration, where free antibody valences and antigenic sites are available to insure the progressive cross-linking of mobile antigen-antibody complexes on the cell surface. It was found that the redistribution process was hampered by excess antibody (4, 15–17), but the expected inhibition of the clustering in antigen excess, i.e. at a too low concentration of antibodies where no free antibody valence is available, was not demonstrable as yet.

The redistribution with anti-*H-2* antibodies involves all the detectable *H-2* determinants concerned, since the cell surface could not be stained outside the clusters already formed by an anti-*H-2* antibody of the same specificity, whatever the titer, the specificities, and the sequence of the conjugates used. On the contrary, other *H-2* specificities were readily detected.

The data described in the present report indicate that different *H-2* antigens move independently on the surface of the lymphocyte. A similar independence has been found among HL.A antigens coded by the first and the second locus of the HL.A region (26), and also between HL.A antigens and IgM determinants (18) on the surface of human lymphocytes. Independent movements of antigenic structures on the cell membrane recall previous work of Frye and Edidin (27) who observed an intermixing of *H-2* and human heteroantigens upon formation of mouse-human heterokaryons. Such situations are compatible with the fluid mosaic concept of cell membranes predicted by Singer and Nicolson (19). One may hypothetize that histocompatibility antigens and IgM molecules are expressed on isolated globular proteins dispersed in the fluid lipid bilayer of the membrane. These glycoproteins carrying the antigenic structures may move freely and independently on the cell membrane. The present data, according to this postulate, would suggest that the *H-2K* and *H-2D* antigens tested are expressed on different protein globules of the cell membrane. However, it is not known as yet to which extent redistribution process which takes place at the cell surface involves the membrane structures themselves.

It should be emphasized that in these studies we have examined only the strongest of the *H-2* antigen specificities among those present on $H\text{-}2^k$ and $H\text{-}2^b$ cells. It would be of interest to extend these studies to include other known *H-2* specificities, as well.

The exact relation between the antibody-induced redistribution of *H-2* antigens and the phenomenon of antigenic modulation (28) is not yet clear. Antigenic modulation was first described with respect to the TL antigens (29), which are governed by the *Tla* gene, closely linked to *H-2D*. In the presence of anti-TL antibodies, TL antigens disappear from the surface of cells bearing them, and the modulated cells become resistant to cytolysis by further anti-TL antibodies plus complement. Such modulation by alloantibodies alone could not be initially demonstrated with respect to *H-2* antigens (30), but it could be obtained by the combined use of anti-*H-2* alloantibodies plus antimouse Ig antibodies (31). A specific antibody-induced reduction of sensitivity to lysis in the presence of anti-*H-2* antibodies was observed at 37°C on ascites tumor cells (32) and more rapidly on peritoneal cells (33), where the modulation of $H\text{-}2^b$ antigens failed to involve $H\text{-}2^k$ antigens on $(H\text{-}2^b \times H\text{-}2^k)F_1$ hybrid cells. It seems likely that the first event in these antibody-induced antigenic modifications, or modulation might be the clustering of antigen molecules, as studied in our experiments. But a further step would be required for modulation to take place: the rapid removal from the cell surface of clustered antigen-antibody complexes, by pinocytosis (16, 34, 35) or elution (36). Our experiments have no bearing on this latter stage.

The antibody-induced redistribution phenomenon used here provides a useful tool for investigating whether two antigenic molecules are part of the same structure on a cell membrane. However, this method provides no in-

formation concerning the initial mapping and spatial interactions of molecules before the redistribution induced in vitro by divalent antibodies.

SUMMARY

At 37°C, fluorescein-conjugated anti-*H-2* alloantibodies specifically induce, at the surface of living mouse lymphocytes, the redistribution of the corresponding *H-2* antigens, which cluster as patches and sometimes single caps at one pole of the cell. This aggregation is inhibited at 0°C and the *H-2* antigens, stained by fluorescent antibodies in the cold, appear evenly spread over the cell surface.

This phenomenon was used to define the relationships between the membrane structures bearing the antigens coded by the *H-2K* and the *H-2D* genes of the *H-2* region. Monospecific anti-*H-2* antibodies coupled to either tetramethyl rhodamine isothiocyanate or fluorescein isothiocyanate were used to induce the redistribution of *H-2D* and *H-2K* antigens of the $H\text{-}2^b$ and $H\text{-}2^k$ haplotype at the surface of lymph node cells from homozygous and F_1 hybrid mice. It was observed that the diffuse distribution of *H-2K* antigens labeled at 0°C was not affected by the prior antibody-induced aggregation of *H-2D* antigens and vice versa. The results were the same for *H-2* antigens governed by genes located either in *cis* or in *trans* position.

These data indicate that the *H-2K* and *H-2D* antigens migrate independently at the cell surface, and suggest that the gene products from the D and the K end of the *H-2* region are expressed on independent molecules or structures at the cell membrane.

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REFERENCES

1. Gorer, P. A. 1938. The antigenic basis of tumor transplantation. *J. Pathol. Bacteriol.* **47**:231.
2. Gorer, P. A., and Z. B. Mikulska. 1959. Some further data on the H-2 system of antigen. *Proc. R. Soc. Lond. B. Biol. Sci.* **151**:57.
3. Stimpfling, J. H., and G. D. Snell. 1962. Histocompatibility genes and some immunogenetic problems. *In* Proceedings International Symposium on Tissue Transplantation, University of Chile, Santiago, Chile. A. P. Christoffanini, and G. Horecker, editors. 37.
4. Stimpfling, J. H., and A. Richardson. 1965. Recombination within the histocompatibility-2 locus of the mouse. *Genetics.* **51**:831.
5. Klein, J., and D. C. Shreffler. 1971. The H-2 model for the major histocompatibility systems. *Transplant. Rev.* **6**:3.
6. Klein, J., and D. C. Shreffler. 1972. Evidence supporting a two-gene model for the H-2 histocompatibility system of the mouse. *J. Exp. Med.* **135**:924.
7. McDevitt, H. O., B. D. Deak, D. C. Shreffler, J. Klein, J. H. Stimpfling, and G. D. Snell. 1972. Genetic control of the immune response. Mapping of the *Ir-1* locus. *J. Exp. Med.* **135**:1259.

8. Passmore, H. C., and D. C. Shreffler. 1970. A sex-limited serum protein variant in the mouse; inheritance and association with the H-2 region. *Biochem. Genet.* **4**:351.
9. Boyse, E. A., E. Stockert, and L. J. Old. 1968. Properties of four antigens specified by the *Tla* locus. Similarities and differences. *In* Proceedings International Convocation on Immunology, Buffalo, N. Y. N. R. Rose and F. Milgrom, editors. S. Karger, New York.
10. Lilly, F. 1970. The role of genetics in Gross virus leukemogenesis. *In* Comparative Leukemia Research 1969. R. M. Dutcher, editor. S. Karger, Basel. 213.
11. Cullen, S. E., B. D. Schwartz, S. G. Nathenson, and M. Cherry. 1972. The molecular basis of codominant expression of the histocompatibility-2 genetic region. *Proc. Natl. Acad. Sci. U.S.A.* **69**:1394.
12. Boyse, E. A., L. J. Old, and E. Stockert. 1968. An approach to the mapping of antigens on the cell surface. *Proc. Natl. Acad. Sci. U.S.A.* **60**:886.
13. Cresswell, P., and A. R. Sanderson. 1968. Spatial arrangement of H-2 specificities: evidence from antibody absorption and kinetic studies. *Transplantation.* **6**:996.
14. Kristofova, H., A. Lengerova, and J. Rejzkova. 1970. Indirect mapping of spatial distribution of some H-2 antigens on the cell membrane. *Folia Biol. (Praha).* **16**:81.
15. Kourilsky, F. M., D. Silvestre, C. Neauport-Sautes, Y. Loosfelt, and J. Dausset. 1972. Antibody-induced redistribution of HL.A antigens at the cell surface. *Eur. J. Immunol.* **2**:249.
16. Taylor, R. B., W. P. H. Duffus, M. C. Raff, and S. De Petris. 1971. Redistribution and pinocytosis of lymphocyte surface immunoglobulin molecules induced by anti-immunoglobulin antibody. *Nat. New Biol.* **233**:223.
17. Loor, F., L. Forni, and B. Pernis. 1972. The dynamic state of the lymphocyte membrane. Factor affecting the distribution and turnover of surface immunoglobulins. *Eur. J. Immunol.* **2**:203.
18. Preud'homme, J. L., C. Neauport-Sautes, S. Piat, D. Silvestre, and F. M. Kourilsky. 1972. Independance of HL.A antigens and immunoglobulin determinants on the surface of human lymphoid cells. *Eur. J. Immunol.* **2**:297.
19. Singer, S. J., and G. L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. *Science (Wash. D.C.).* **175**:720.
20. Wigzell, H. 1965. Quantitative titration of mouse H-2 antibodies using Cr^{51} labelled target cells. *Transplantation.* **3**:423.
21. Fothergill, J. E. 1969. Properties of conjugated serum proteins. *In* Fluorescent Protein Tracing. R. C. Nairn, editor. E. and S. Livingstone Ltd., Edinburgh. 35.
22. Davis, W. C. 1972. H-2 antigen on cell membranes and explanation for alteration of distribution by indirect labeling techniques. *Science (Wash. D.C.).* **175**:1006.
23. Aoki, T., U. Hämmerling, E. De Harven, E. A. Boyse, and L. J. Old. 1969. Antigenic structure of cell surfaces. An immunoferritin study of the occurrence and topography of H-2, θ, and TL alloantigens on mouse cells. *J. Exp. Med.* **130**:979.
24. Unanue, E. R., W. D. Perkins, and M. J. Karnovsky. 1972. Ligand-induced movement of lymphocyte membrane macromolecules. I. Analysis by immunofluorescence and ultrastructural radioautography. *J. Exp. Med.* **136**:885.
25. Karnovsky, M. J., E. R. Unanue, and M. Leventhal. 1972. Ligand-induced movement of lymphocyte membrane macromolecules. II. Mapping of surface moieties. *J. Exp. Med.* **136**:907.

26. Neauport-Sautes, C., D. Silvestre, F. M. Kourilsky, and J. Dausset. 1973. Independence of HL.A Antigens from the First and the Second Locus at the Cell Surface. Histocompatibility Testing. Munksgaard, Copenhagen.
27. Frye, L. E., and M. Edidin. 1970. The rapid intermixing of cell surface antigens after formation of mouse-human heterokaryons. *J. Cell Sci.* **7**:319.
28. Boyse, E. A., E. Stockert, and L. J. Old. 1967. Modification of the antigenic structure of the cell membrane by thymus-leukemia (TL) antibody. *Proc. Natl. Acad. Sci. U.S.A.* **58**:954.
29. Boyse, E. A., E. Stockert, and L. J. Old. 1968. Properties of four antigens specified by the *Tla* locus. Similarities and differences. *In* Proceedings International Convocation on Immunology, Buffalo, N. Y. N. R. Rose and F. Milgrom, editors. S. Karger, New York. 353.
30. Lamm, M. E., E. A. Boyse, L. J. Old, B. Lisowska-Bernstein, and E. Stockert. 1968. Modulation of TL (thymus-leukemia) antigens by Fab-fragments of TL antibody. *J. Immunol.* **101**:99.
31. Takahashi, T. 1971. Possible examples of antigenic modulation affecting H-2 antigens and cell surface immunoglobulins. *Transplant. Proc.* **3**:1217.
32. Amos, D. B., I. Cohen, and W. J. Klein. 1970. Mechanisms of immunological enhancement. *Transplant. Proc.* **2**:68.
33. Schlesinger, M., and M. Chaouat. 1972. Modulation of the H-2 antigenicity on the surface of murine peritoneal cells. *Tissue Antigens*. In press.
34. De Petris, S., and M. C. Raff. 1973. Distribution of immunoglobulin on the surface of mouse lymphoid cells as determined by immunoferritin electron microscopy. *Eur. J. Immunol.* In press.
35. Unanue, E. R., W. D. Perkins, and M. I. Karnovsky. 1972. Endocytosis by lymphocytes of complexes of anti-Ig with membrane-bound Ig. *J. Immunol.* **108**:569.
36. Chang, S., E. Stockert, E. A. Boyse, U. Hämmerling, and L. J. Old. 1971. Spontaneous release of cytotoxic alloantibody from viable cells sensitized in excess antibody. *Immunology*. **21**:829.