H-2 Antigen on Cell Membranes: An Explanation for the Alteration of Distribution by Indirect Labeling Techniques

Abstract. *The distribution of H-2 isoantigens on the plasma membrane of lymphocytes and thymocytes was studied with ferritin-conjugated alloantibody. The spatial arrangement of the membrane components which possess H-2 isoantigens is markedly altered by the attachment of a second antibody, directed toward mouse gamma globulin or ferritin; the second antibody appears to cause the aggregation of the sensitized H-2 antigens into discrete zones on the cell membrane. Other membrane antigens may be equally affected by indirect labeling techniques and, thus, topographic analysis of the antigenic structure should be done with antibody that is directly conjugated.*

The application of the techniques of immunoelectron microscopy to the study of the distribution on cell surfaces of H-2 antigens in mouse and HL-A antigens in humans has given divergent results (*1–3*). Studies with indirect labeling techniques using ferritin-conjugated antibody (*3*) and hybrid antibody, with activity directed against globulin and against either ferritin or virus (*2*) have indicated that alloantigens occupy discrete areas on the cell surface of lymphocytes, thymocytes, and bone marrow. Although the indirect labeling procedures have yielded consistent results, these results have proven to be artifactual (*4*). We will show that the membrane components which possess H-2 isoantigens do not appear to have a fixed position on the cell membrane and that the addition of multiple antibodies physically alters their distribution.

Lymphocytes and thymocytes were incubated with various antiserums (Table 1). The labeling patterns on small lymphocytes obtained by the use of direct and indirect ferritin-conjugated antibody techniques (*5*) are shown in Figs. 1 and 2. Cells incubated directly with ferritin-conjugated, monospecific or polyspecific, alloantibody to H-2 exhibit extensive labeling of the cell surface. Protrusions (pseudopods), as well as indentations, of the cell surface are labeled. Thymocytes exhibit the same pattern of labeling but in a lower concentration.

Cells incubated first with alloantibody and then with ferritin-conjugated rabbit antibody to mouse globulin show extensive labeling of discrete areas on the cell surface of lymphocytes. These discrete areas may extend from several microns to half the plasma membrane (Fig. 2). All examined cells were labeled in a similar manner. Thymocytes are less extensively labeled. The label that is evident, however, is present in discrete zones on the cell surface.

The distribution of ferritin-labeled antibody, obtained with direct or indirect techniques, is unaltered by varying the procedures of incubation and of preparation of lymphocytes for electron microscopy; however, the concentration of ferritin antibody on cell membranes varies with the time of incubation and the hemagglutination titer of the conjugated antibody. The concentration of directly conjugated antibody is reduced

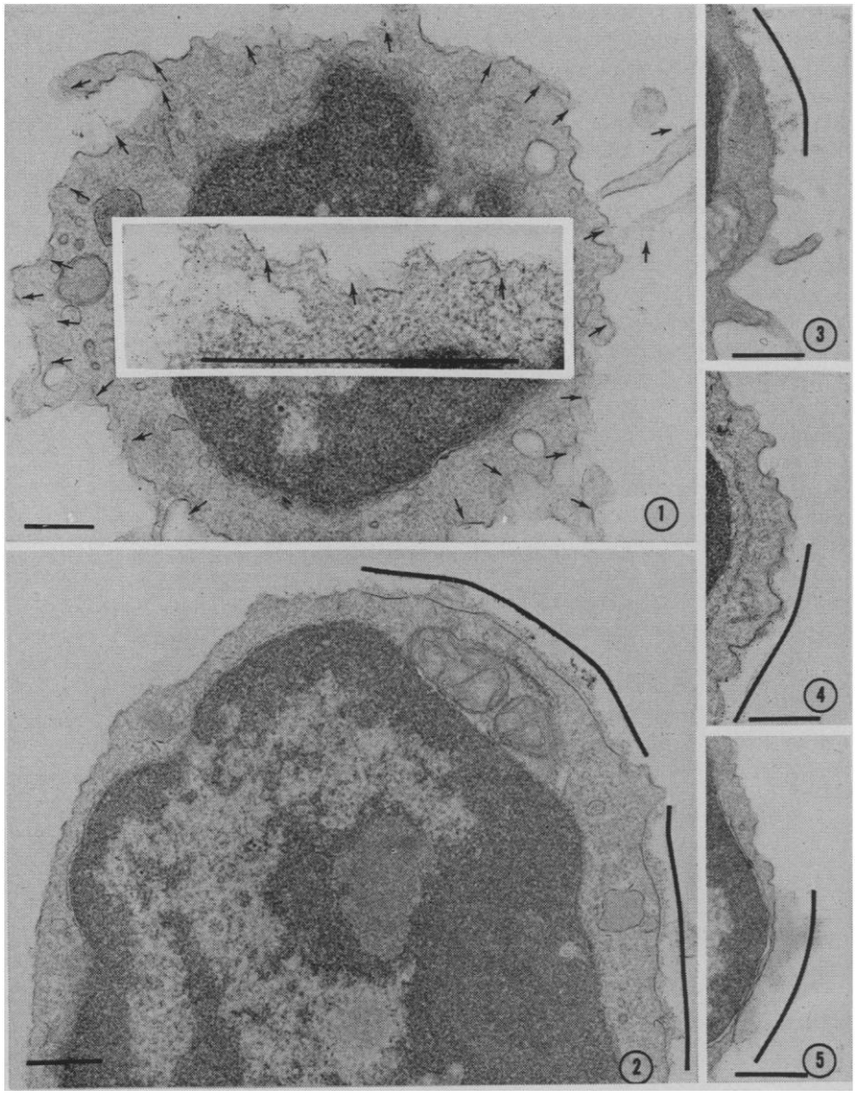

Fig. 1 and inset. Lymphocytes labeled with ferritin-conjugated alloantibody (H-2^f versus H-2^a). Ferritin marker is present over the entire cell surface (arrows point to only some of the ferritin marker). Micron marker inserted (× 35,000; in inset × 72,000). Fig. 2. Lymphocyte labeled with antibody to ferritin by the indirect technique. Ferritin (under bar) occupies only part of the cell membrane. Micron marker inserted (× 35,000). Figs. 3–5. Portions of cell membranes from lymphocytes labeled directly with ferritin-conjugated alloantibody and then mixed with rabbit antibody to ferritin (Fig. 3) or rabbit antibody to mouse γ-globulin (Fig. 4), or reacted sequentially with alloantibody, rabbit antibody to mouse γ-globulin, goat antibody to rabbit γ-globulin, purified rabbit antibody to ferritin, and ferritin (Fig. 5). The ferritin marker (under bar) occupies only part of the cell membrane. The fuzzy appearance associated with ferritin in Fig. 5 is a result of stained antibody. Micron marker inserted (× 35,000).

if the titer of the conjugate is low or if the time of incubation is shortened. The zonal distribution obtained with indirect labeling becomes more pronounced if the time of incubation is increased from 15 to 60 minutes. No difference in the two patterns of labeling is noted in the presence of antibody to one or more H-2 specificities; B10M antibody to B10A (ten possible H-2 specificities), B10A antibody to B10M (two possible H-2 specificities), and (B10S × $B10A_{F_1}$ antibody to B10M (one possible specificity).

Control cells, incubated with ferritin-conjugated alloantibodies (B10M antibody to B10A incubated with B10M cells), were unlabeled. However, occasional cells of the control that were employed in the indirect ferritin-labeling procedures exhibited small discrete zones of label. Since absorption of the antiserums with liver powder or live cells did not eliminate this labeling, it is possible that it may represent the detection of some surface-associated alloantibody.

Table 1. Results obtained from labeling mouse H-2 alloantigens with ferritin-conjugated antibody; L, lymphocytes; T, thymocytes.

Donor strain	Method	Cells examined	Distribution of ferritin marker on cell membrane
	Direct: Ferritin-conjugated alloantibody		
B10A	$H\text{-}2^f$ versus $H\text{-}2^a$	L, T	Continuous
B10M	$H\text{-}2^a$ versus $H\text{-}2^f$	L, T	
B10M	$(H\text{-}2^a \times H\text{-}2^a)_{F_1}$ versus $H\text{-}2^f$	L, T	
	Indirect: Ferritin-conjugated rabbit antibody to mouse globulin		
B10A	$H\text{-}2^f$ versus $H\text{-}2^a$ + rabbit antibody to mouse γ-globulin	L	Discontinuous
B10M	$H\text{-}2^a$ versus $H\text{-}2^f$ + rabbit antibody to mouse γ-globulin	L	
B10M	$(H\text{-}2^a \times H\text{-}2^a)_{F_1}$ versus $H\text{-}2^f$ + rabbit antibody to mouse γ-globulin	L	
	Ferritin-conjugated alloantibody plus unlabeled antibody		
B10A	$H\text{-}2^f$ versus $H\text{-}2^a$ + rabbit antibody to ferritin	L, T	Discontinuous
B10A	$H\text{-}2^f$ versus $H\text{-}2^a$ + rabbit antibody to mouse γ-globulin	L, T	
	Unlabeled antibody		
B10A	$H\text{-}2^f$ versus $H\text{-}2^a$ rabbit antibody to mouse γ-globulin + goat antibody to rabbit γ-globulin + purified rabbit antibody to ferritin + ferritin	L	Discontinuous

The alteration, by indirect labeling techniques, of the distribution of antigen and antibody on the cell surface is associated with the application of one or more additional antibodies. The results of these studies are summarized (Table 1, Figs. 3–5). The B10A lymphocytes and thymocytes that were incubated with directly conjugated alloantibody and then subsequently incubated with either unconjugated rabbit antibody to ferritin (Fig. 3) or unconjugated rabbit antibody to mouse γ-globulin (Fig. 4) exhibited large discontinuties in the surface distribution of ferritin antibody marker. Since the ferritin on the cell surface was present on the mouse globulin, the change in distribution must be a change in the position of the labeled H-2 antigen. Control B10A cells, incubated only with ferritin-conjugated B10M antibody to B10A cells, exhibited a continuous label. When B10A cells were incubated, sequentially, with alloantibody, rabbit antibody to mouse γ-globulin, goat antibody to rabbit γ-globulin, rabbit antibody to ferritin, and ferritin, they exhibited discontinuities in the distribution of ferritin label (Fig. 5). The alteration in antigen distribution is thus not associated with any change in the physical properties of antibodies conjugated to ferritin.

These data confirm the original observations (*1*) that H-2 isoantigens are distributed over the entire cell membrane of lymphocytes and thymocytes, and demonstrate conclusively that the discrete zoning of H-2 isoantigens on cell membranes is an artifact which can be produced by four, separate, indirect labeling techniques. It appears then that the data obtained with hybrid antibodies on H-2 and other mouse alloantigens also must be reevaluated (*2, 3*). Although, theoretically, synthetic univalent antibodies to globulin and to either ferritin or virus should yield results comparable to those obtained with ferritin-conjugated alloantibody, the observations suggest that this is not the case. However, the presence of any residual divalent antibody (to globulin, to ferritin, or to virus) could mask the primary reaction of the hybrid antibody and give spurious results.

The alteration of the distribution of H-2 antigen by antibody suggests that the molecule which bears the H-2 isoantigens [and possibly other alloantigens (*2*)] possesses a unit character and has no fixed position within the infrastructure of the cell membrane. If this is true, it would partly explain the release of H-2 antigen from cell membranes by enzymes and in vitro "autolysis" (*6*) without cell destruction. It would also provide indirect support for the existence of large-molecular-weight forms of mouse H-2 (*7*) and human HL-A isoantigens (*8*). Moreover, it would provide a explanation for why the addition of antibody to globulin or ferritin causes a shift of ferritin antibody–labeled antigen to discrete zones; that is, H-2 antigen–antibody bodies drifting over the surface of the cell membrane become trapped in an antigen-antibody lattice when another antibody is added.

This report calls attention to the artifactual results obtained by the use of indirect labeling techniques at the ultrastructural level (*2, 3*) and points out their implications on the physical composition of cell membranes (*7–9*).

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5. The alloantiserums used in this study were prepared in congenic resistant strains of mice (*1, 4*). The strains of mice were B10A ($H\text{-}2^a$), B10M ($H\text{-}2^f$), [B10S ($H\text{-}2^a$) × B10A ($H\text{-}2^a$)]$_{F_1}$. Ferritin antibody conjugates were prepared with glutaraldehyde as the coupling agent (*4*). Goat antibody to rabbit γ-globulin, rabbit

antibody to mouse γ-globulin, and rabbit antibodies to ferritin were prepared by conventional procedures [C. A. Williams and M. W. Chase, *Methods in Immunology and Immunochemistry* (Academic Press, New York, 1967), pp. 371–72]. Purified antibody to ferritin was prepared by acid elution from glutaraldehyde-aggregated ferritin [C. A. Williams and M. W. Chase, *ibid.*; S. Avrameas and T. Ternyneck, *Immunochemistry* **6**, 53 (1969)]. Peripheral blood leukocytes were isolated by NH_4Cl lysis of whole blood [K. Hummler, T. N. Harris, N. Tomassini, M. Hechtel, M. B. Farber, *J. Exp. Med.* **124**, 255 (1966)].
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10. Supported by funds provided for biological and medical research by the State of Washington initiative measure No. 171, and PHS general research support grant FR 5465. Electron micrographs were taken in the Electron Microscope Facility of the Animal Disease and Parasite Research Division, U.S. Department of Agriculture, College of Veterinary Medicine, Washington State University. We thank Donald Gayman for technical assistance.

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Lateral Interactions at Inner Plexiform Layer of Vertebrate Retina: Antagonistic Responses to Change

Abstract. *Lateral interactions at the inner plexiform layer of the retina of the mudpuppy were studied intracellularly after they were isolated from interactions at the outer plexiform layer with a special stimulus. The isolation was confirmed by recording no surround effect at bipolar cells under conditions that elicited a strong surround effect at ganglion cells. It appears that amacrine cells, which respond to spatiotemporal change at one retinal region, inhibit the response to change in on-off ganglion cells at adjacent sites.*

As the visual message passes through the retina it is modified by systems of lateral interneurons that relate activity elicited at adjacent regions of the visual field. Investigations at the outer plexiform layer of the retina have revealed a significant functional role for the horizontal cells as lateral interneurons; when driven by receptors at one region they act to reduce the efficacy of transmission at adjacent receptors (*1*). As a result, the receptive field for each receptor (*2*) and for each bipolar cell, which is driven by a small group of receptors (*3*), seems to be embedded in an antagonistic field mediated by laterally oriented horizontal cells.

The inner plexiform layer also contains a system of lateral interneurons, the amacrine cells, but their functional role is not yet clear. Like horizontal cells the processes of each amacrine cell extend laterally for a few hundred microns (*4*) and appear capable of reciprocal feedback synapses with the cells that drive them (see Fig. 1). Unlike horizontal cells, which respond with sustained hyperpolarizing potentials that are graded with intensity, amacrine cells depolarize and respond only to changing stimuli. A major difficulty in studying lateral interactions at the inner plexiform layer alone is that light-evoked activity there has already been affected by horizontal cell interactions. In this report a special stimulus was used to isolate and characterize lateral interactions at the inner plexiform layer and to determine the role of these interactions in processing the visual message.

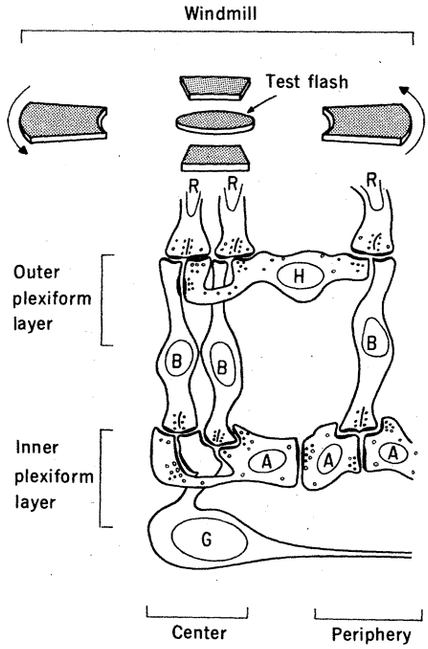

Fig. 1. Synaptic sites for lateral interactions in the retina. Structures are abstracted from electron microscopic study of the mudpuppy retina. Synaptic regions are indicated by thickening of membranes and clustering of synaptic vesicles in the presynaptic cytoplasm. Cell types are indicated by initials: *R*, receptors; *H*, horizontal cells; *B*, bipolar cells; *A*, amacrine cells; *G*, ganglion cells. The stippled areas represent the lighted parts of the stimulus. The four square elements represent the spinning vanes, inside diameter is 1 mm, outside diameter is 1.5 mm. The central disk represents the locus of the central test flash, diameter 300 μm. Windmill spins at ¼ rev/sec, so the vanes have a mean tangential velocity of about 1 mm/sec. Intensities were within 2 log units of ganglion cell threshold.

The retina of the mudpuppy, *Necturus maculosus*, was used because each cell type can be studied intracellularly (*1*), and because the synaptic structures found in the mudpuppy retina (*4*) are characteristic of most other vertebrates (*5*). The greatest variation in retinal structures between different vertebrates lies in the relative number of synaptic contacts by amacrine cells in the inner plexiform layer; the number in the mudpuppy lies intermediate between the very complex retina of the frog and the relatively simple retina of the cat (*6*). Techniques for recording, identifying, and stimulating cells have been reported previously (*1*).

Lateral interactions at the inner plexiform layer were isolated by taking advantage of the difference in response properties for horizontal and amacrine cells; horizontal cells respond to steady levels of illumination with sustained potentials whereas amacrine cells respond only to change. To illustrate this difference, the activities were recorded in each cell type as a vane-shaped stimulus was moved across the receptive fields at 1 mm/sec (Fig. 2). While the stimulus was moving the horizontal cell hyperpolarized and the amacrine cell depolarized over a region spanning about 1 mm. When the vane was stopped within the receptive field of these cells the horizontal cell remained polarized, but the amacrine cell activity was lost and the membrane potential returned to the resting state. This indicates that horizontal cells are polarized by the presence of the vane, but amacrine cells respond only to its movement. To isolate activity at the inner plexiform layer, four vanelike segments were incorporated into a "windmill" stimulus. Its presence activated horizontal cells, but only its spin activated amacrine cells. Activity elicited by the vanes was measured in cells lying at the center of the windmill because lateral effects from all four vanes tend to converge there, and the effects of lateral interactions were tested by a disk of illumination flashed at the center (see Fig. 1). The intensities of the disk and windmill were not critical as long as they were below saturating levels for the cells studied.

It is first necessary to show that there