ANTIGENIC STRUCTURE OF CELL SURFACES

AN IMMUNOFERRITIN STUDY OF THE OCCURRENCE AND TOPOGRAPHY OF H-2α, THETA, AND TL ALLOANTIGENS ON MOUSE CELLS

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Of various labels that have been attached to antibody, ferritin is the most useful visual marker for locating antigens at the high degree of resolution permitted by electron microscopy, and ferritin-labeled antibody has been used extensively to study the surface structure of viruses, bacteria, and cells of various sorts (1).

The technique developed by Singer involves the coupling of ferritin to the antibody by metaxylylene diisocyanate or toluene 2,4-diisocyanate (2, 3). Alternatively, ferritin may be coupled to an anti-γ-globulin antibody, providing an "indirect" immunoferritin method based on the same principle as the indirect immunofluorescence test; in both instances there is the advantage that a single labeled antiserum suffices for the recognition of more than one antigen. Recently, Hammerling et al. (4) devised a new method of ferritin labeling that avoids undesirable consequences of coupling ferritin to antibody chemically. Hybrid F(ab')2 antibody is prepared according to Nisonoff and Rivers (5) from two sera, rabbit anti-mouse γG and rabbit anti-ferritin, each hybrid molecule having two different combining sites, one for mouse γG and the other for ferritin. To locate, for instance, a cell surface alloantigen, the cells are first exposed to the relevant alloantibody (mouse γG), then to the hybrid antibody anti-mouse γG/anti-ferritin, and finally to ferritin.

The new method has been used so far only in this indirect form, although it is equally applicable in the direct form (e.g. with anti-alloantigen/antiferritin hybrid antibody). Its outstanding advantage is that it opens the way to the use of a variety of visual markers, such as viruses, the only requirements being that these be morphologically distinguishable, of suitable size, and immunogenic in the species in which the hybrid partner antibody is to be prepared.

Exemplifying the application of immunoferritin methods to the study of cell surface structure, ABO group alloantigens have thus been found to cover all or most of the human erythrocyte, while Rh alloantigens are confined to limited areas (6, 7).

Among nucleated cells, many surface antigens of mouse lymphoid cells, both normal

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and leukemic, have been delineated during the past decade, mainly by analysis of cytotoxic antisera (8, 9). These include a variety of alloantigens, as well as species antigens and leukemia-related antigens. Aoki et al. (10) investigated the representation of alloantigens belonging to three systems, H-2, TL, and θ, on mouse thymocytes and lymphocytes, using the indirect method with anti-γG chemically coupled to ferritin.

**H-2 antigens** occur on most cells of the mouse and are the major blood group and histocompatibility determinants of this species (11). More than 20 specificities are determined by the H-2 locus (which appears to comprise a series of closely linked genes in the 9th linkage group), and mice can be classified into 16 or more phenotypes on the basis of their constellations of H-2 antigens. The H-2 locus comprises at least two genetic regions separable by crossing over (the “D” and the “K” end) and determining antigens that are on different molecules (12, 13), and are situated on different sites on the surface membrane (14, 15).

**θ antigens** (15): All mice have one or the other allelic form of this alloantigen. It is found in higher concentration on thymocytes than on lymphocytes, in contrast to H-2, and is present in adult brain but not elsewhere. θ is not linked with H-2 (17).

**TL (thymus leukemia) antigens**, in normal mice, are present on thymocytes only, the phenotypes being TL−, TL.2, or TL.1,2,3 (18). TL antigens are determined by a series of genes (Tla locus) linked to H-2. A remarkable feature of this system is that TL antigens appear on leukemias of TL− mice, indicating that genes for TL synthesis are present in these animals but are normally repressed.

Aoki et al. (10) found θ alloantigen to occupy almost the entire surface of thymocytes, whereas H-2 and TL alloantigens occupied only small areas. These observations have now been extended, with the use of the newer method of ferritin labeling, adding weight to our previous conclusion that distinctive patterns of alloantigen representation on cell surfaces are a prominent feature of cellular differentiation.

### Materials and Methods

**Sero logical Test Systems.**—See Tables I and II. Diluent and suspending medium was Medium 199.

**Ferritin Labeling.—**

**Conventional method:** Mouse γG was isolated by filtration of C57BL/6 serum through Sephadex G-200, followed by chromatography on DEAE-Sephadex A-50 in 0.08 M Tris-HCl buffer at pH 8.0. The front of the γG peak was used (with Freund’s adjuvant) for immunization of rabbits, and this yielded a highly specific anti-mouse γG. The γG fraction of this rabbit anti-mouse γG was isolated similarly; on immunoelectrophoresis the product gave a single precipitation line with goat anti-rabbit serum.

The anti-mouse γG was coupled to horse ferritin (six times crystallized) with metaxylylene disiocyanate essentially according to Singer (2) and Rifkind, Hsu and Morgan (19). The use of a bivalent chemical reagent for ferritin coupling frequently resulted in a complex mixture of reaction products containing a relatively small proportion of the desired ferritin-conjugated anti-mouse γG. Immunoelectrophoresis revealed four major components (listed in decreasing order of their electrophoretic mobility): (a) altered ferritin, (b) rabbit γG conjugated with ferritin, (c) rabbit γG conjugated with breakdown products of ferritin, and (d) rabbit γG. Chromatography of the crude reaction mixture on Sepharose 4B equilibrated with phosphate-buffered saline was found to be a convenient and effective method of purification (see Fig. 1).
### TABLE I

**Test Systems Used for H-2 Antigens**

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Target cell</th>
<th>Cytotoxic titer</th>
<th>H-2 specificities</th>
</tr>
</thead>
<tbody>
<tr>
<td>A anti-C57BL leukemia EL4 (H-2* vs. H-2*)</td>
<td>C57BL/6(TL+) thymocytes</td>
<td>1:160</td>
<td>2, 22, 33</td>
</tr>
<tr>
<td></td>
<td>C57BL/6(TL+) lymphocytes</td>
<td>1:2560</td>
<td>2, 22, 23</td>
</tr>
<tr>
<td></td>
<td>C57BL/6(TL+) spleen cells</td>
<td>1:640</td>
<td>2, 22, 23</td>
</tr>
<tr>
<td></td>
<td>C57BL leukemia EL4</td>
<td>1:600</td>
<td>2, 22, 23</td>
</tr>
<tr>
<td></td>
<td>C57BL/6 peritoneal cells</td>
<td>1:256</td>
<td>2, 22, 23</td>
</tr>
<tr>
<td>C57BL/6 anti-BALB/c ascites sarcoma Meth A (H-2* vs. H-2*)</td>
<td>A thymocytes</td>
<td>1:1280</td>
<td>3, 4, 8, 10, 13, 31</td>
</tr>
<tr>
<td></td>
<td>A lymphocytes</td>
<td>1:2560</td>
<td>3, 4, 8, 10, 13, 31</td>
</tr>
<tr>
<td></td>
<td>C3H/Bl lymphocytes</td>
<td>1:512</td>
<td>3, 8</td>
</tr>
<tr>
<td>C57BL/6 anti-A strain leukemia ASL5 (TL--) (H-2* vs. H-2*) (I)</td>
<td>A thymocytes</td>
<td>1:164</td>
<td>1, 3, 4, 8, 10, 11, 13, 25</td>
</tr>
<tr>
<td></td>
<td>A lymphocytes</td>
<td>1:1280</td>
<td>1, 3, 4, 8, 10, 11, 13, 25</td>
</tr>
<tr>
<td></td>
<td>A spleen cells</td>
<td>1:640</td>
<td>1, 3, 4, 8, 10, 11, 13, 25</td>
</tr>
<tr>
<td>C57BL/6 anti-A strain leukemia ASL1 (H-2* vs. H-2*) (II)**</td>
<td>A(TL--) thymocytes</td>
<td>1:80</td>
<td>1, 3, 4, 8, 10, 11, 13, 25</td>
</tr>
<tr>
<td></td>
<td>A(TL--) lymphocytes</td>
<td>1:2560</td>
<td>1, 3, 4, 8, 10, 11, 13, 25</td>
</tr>
<tr>
<td></td>
<td>A(TL--) spleen cells</td>
<td>1:640</td>
<td>1, 3, 4, 8, 10, 11, 13, 25</td>
</tr>
<tr>
<td></td>
<td>A thymocytes</td>
<td>1:2560</td>
<td>1, 3, 4, 8, 10, 11, 13, 25</td>
</tr>
<tr>
<td>C57BL/6(H-2*)§§ anti-C57BL leukemia ELA (H-2* vs. H-2*)</td>
<td>C57BL/6 thymocytes</td>
<td>1:160</td>
<td>2, 6, 14, 22, 27, 28, 29, 33</td>
</tr>
<tr>
<td></td>
<td>C57BL/6 lymphocytes</td>
<td>1:640</td>
<td>2, 6, 14, 22, 27, 28, 29, 33</td>
</tr>
<tr>
<td></td>
<td>C57BL/6 spleen cells</td>
<td>1:320</td>
<td>2, 6, 14, 22, 27, 28, 29, 33</td>
</tr>
<tr>
<td>(C57BL/6 × BALB/c)F1 anti-C3H ascites sarcoma BP8 (H-2* vs. H-2*)</td>
<td>A thymocytes</td>
<td>1:160</td>
<td>1, 11, 25</td>
</tr>
<tr>
<td></td>
<td>A lymphocytes</td>
<td>1:1280</td>
<td>1, 11, 25</td>
</tr>
</tbody>
</table>

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* This table includes systems that are not illustrated in the text but gave results similar to those dealt with in the text; they are included to indicate the extent of the material on which this study is based.

‡ According to Snell and Stimpfling (11).

§ Congenic stock derived from 8th back-cross (from A to C57BL/6) of cross-over H-2/TL 1,2,3.

§§ Congenic stock derived from 9th back-cross (from C57BL/6 to A) of cross-over H-2/TL-.

** This antiserum contains TL antibodies as well as H-2 antibodies, but these are irrelevant because the test cells listed are TL-negative.

†† Congenic stock derived from 9th back-cross (from C57BL/6 to A) of cross-over H-2/TL-.

§§ Congenic stock derived from 15th back-cross from AKR, typed by hemagglutination.
TABLE II
Test Systems Used for TL and \( \theta \) Antigens

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Target cell</th>
<th>Cytotoxic titer</th>
<th>Specificities</th>
</tr>
</thead>
<tbody>
<tr>
<td>(BALB/c × C3H/An)F1 anti-A strain leukemia ASL1</td>
<td>Thymocytes of TL.1,2,3 strains (18)</td>
<td>1:2000</td>
<td>TL.1,3 (18)</td>
</tr>
<tr>
<td></td>
<td>Thymocytes of TL- strains and lymphocytes of all strains</td>
<td>&lt; 1:2</td>
<td>None</td>
</tr>
<tr>
<td>C57BL/6 anti-A strain leukemia ASL1</td>
<td>C57BL/6(TL+) thymocytes</td>
<td>1:4000</td>
<td>TL.1,2,3 (18)</td>
</tr>
<tr>
<td></td>
<td>C57BL/6(TL+) lymphocytes</td>
<td>&lt; 1:1</td>
<td>None</td>
</tr>
<tr>
<td>AKR anti-C3H/Bi thymocytes</td>
<td>C57BL/6(TL+) or A thymocytes</td>
<td>1:256</td>
<td>( \theta )-C3H (16)</td>
</tr>
<tr>
<td></td>
<td>C57BL/6(TL+) lymphocytes</td>
<td>1:32</td>
<td>( \theta )-C3H (16)</td>
</tr>
<tr>
<td>C3H/An anti-AKR thymocytes</td>
<td>A(( \theta )-AKR)* thymocytes</td>
<td>1:320</td>
<td>( \theta )-AKR</td>
</tr>
<tr>
<td></td>
<td>A(( \theta )-AKR) lymphocytes</td>
<td>1:32</td>
<td>( \theta )-AKR</td>
</tr>
</tbody>
</table>

* Congenic stock differing from A at \( \theta \) locus.

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**Fig. 1.** Chromatography of ferritin conjugate on Sepharose 4B.
Fractions of 5 ml were collected and monitored for protein (Folin method), ferritin (optical density at 550 m/z), and rabbit γG [immunodiffusion method of Fahey and McKelvey (20)] with goat anti-rabbit γG. Free ferritin clearly separated from free γG. The conjugated products appeared in the eluate first, followed by ferritin and by γG. Tubes 20-25 were pooled (Fig. 1) for use as ferritin-conjugated anti-mouse γG. Immunoelectrophoresis showed this to be essentially free of unbound γG. Some ferritin not coupled to γG remained but did not interfere with the serological application; further purification was therefore unnecessary except for the removal of proteins that were altered by the conjugation procedure and so would bind to cells nonspecifically. These were absorbed out with an equal volume of packed washed viable ELA ascites leukemia cells.

5-10 × 10⁶ washed viable test cells were incubated for 20 min in the relevant alloantiserum (1 ml of a dilution giving demonstrable considerable excess antibody). After two washings, the cells were resuspended in 1 ml of the absorbed ferritin-conjugated anti-mouse γG, the concentration of this with respect to anti-mouse γG being approximately 0.2 mg/ml. After further incubation for 20 min, the cells were finally washed twice.

Peritoneal cells were processed entirely in the cold, to avoid loss of macrophages by adherence; cells to be tested for TL antigens also were processed in the cold, to prevent antigenic modulation (21, 22). With these exceptions, the procedures were carried out at room temperature.

**Hybrid antibody method (4):** Briefly, cells were prepared as above, but following sensitization with alloantibody were incubated with anti-mouse γG/anti-ferritin hybrid F(ab')₂ antibody, concentration approximately 0.05 mg/ml with respect to anti-γG, and subsequently with ferritin (0.05 mg/ml, six times crystallized).

**Electron Microscopy.**—The final pellets of viable cells were covered with 2 ml of 1% glutaraldehyde (23) for 20 min at 0°C. After fixation for 60–90 min in cold 1% buffered osmium tetroxide (24), they were kept overnight in cold 0.5% uranyl acetate (25), then dehydrated with ethanol and embedded in Epon. Thin sections were cut on a Sorvall MT-2 ultramicrotome with a diamond knife, stained with 0.5% uranyl acetate in alcoholic solution and lead citrate according to Reynolds (26), and examined with a Siemens Elmiskop 1A electron microscope.

**RESULTS**

Conventional and Hybrid Antibody Methods.—Figs. 4 and 5 show the somewhat different appearances given by the two methods of ferritin labeling. With regard to the extent of cell surface labeling, however, these methods have given similar results.

**Specificity.**—A variety of controls were used in different experiments to indicate the serological specificity of immunoferitin labeling. These included omission of the alloantisera, with substitution of normal mouse serum, or substitution of test cells with the phenotype of the strain in which the alloantisera was produced. These controls were invariably negative. As the method is "indirect," involving the use of anti-γG, labeling might be anticipated from attachment of anti-γG to cells which naturally have γG on their surfaces. In practice, however, this was not observed.

**Alloantigens on Thymocytes.**—

**H-2:** The H-2 sera used contained antibodies to multiple H-2 specificities. These sera all gave similar results with thymocytes of the relevant phenotype.
The characteristic pattern is several distinct small ferritin-marked sectors (Figs. 6 and 7).

\( \theta \): The extent of labeling was considerably greater than in the case of H-2, usually more than half the perimeter being marked with ferritin (Figs. 8 and 9).

TL: Of the three antigenic systems, TL showed the smallest areas of representation (Figs. 10 and 11), many thymocytes showing only one marked sector.

**Alloantigens on Lymphocytes from Lymph Nodes and Spleen.**—

H-2: In contrast to the thymocyte, the lymphocyte showed much larger areas of H-2 representation (Figs. 12 and 13). It appears that the larger lymphocytes have more H-2 than the small lymphocytes. Cells in mitosis, apparently lymphocytes, did not obviously differ in H-2 representation (Figs. 14 and 15).

Although representation of H-2 was extensive, we have not observed the complete coverage reported by Davis and Silverman (27).

\( \theta \): This antigen, the most widely represented on thymocytes (see above), either was absent in single sections of lymphocytes from spleen or lymph nodes or appeared on one or two small sectors.

TL: This was not demonstrable by immunoferritin on any cells other than thymocytes, in conformity with serological evidence that TL antigens are thymocyte-specific.

**Alloantigens on Nonlymphoid Cells.**—Cells other than thymocytes and lymphocytes were identified in the cell suspensions from thymus, lymph nodes, spleen, and peritoneal cavity.

H-2.—Erythrocytes: When it was demonstrable, H-2 appeared on small sectors separated widely from one another (Fig. 16), but in some sections of erythrocytes no H-2 was demonstrable. This is in keeping with the low content of H-2 on these cells (28).

Eosinophil leukocytes: H-2 representation (Fig. 17) was comparable with that of lymphocytes.

Plasma cells: Only a few on these were identified and examined; the majority were positive for H-2 (Fig. 18).

Reticular cells: These, whether from thymus, spleen, or lymph nodes, showed the most extensive H-2 coat of any cell so far examined (Figs. 19 and 20). In spite of this, the extremities of the microvilli were often negative (Fig. 19).

Peritoneal cells: Three cell types were observed in animals given 1 ml of a 3% starch suspension in saline 3–4 days beforehand: eosinophils, lymphoid cells, and macrophages. H-2 representation on the eosinophils and lymphoid cells was similar to comparable cells elsewhere (see above). The macrophages, however, were remarkably deficient in H-2 (Figs. 21 and 22), only very small and occasional sectors being positive. Phagocytic vacuoles within the macrophages, on the other hand, were often lined with ferritin (see Discussion).
\( \theta \) and \( TL \). \( \theta \) and TL antisera gave no immunoferritin labeling of erythrocytes, eosinophils, plasma cells, or reticular cells.

**DISCUSSION**

Our purpose in these studies, the results of which are summarized diagrammatically in Figs. 2 and 3, is a closer definition of the cell surface, with the object of finding out how the presence and configuration of its various components, recognized as antigens, are related to normal and abnormal cellular differentiation. Such detailed knowledge of cell surface antigenic structure may help to answer fundamental questions concerning selective gene action and its relation to the construction of cell surfaces.

The three systems, H-2, \( \theta \), and TL, are an illuminating trio in this context, for they have sharply contrasted properties. Thus H-2 is present on most cell types, and TL on only one, the thymocyte. Both H-2 and \( \theta \) occur on lymphocytes and thymocytes, but the former predominates on lymphocytes and the latter on thymocytes.

Visual labeling with ferritin, reported here, is entirely consistent with other
serological data. Thus, TL antibody cannot be absorbed by normal cells other than thymocytes, nor can TL antigen be detected by immunoferritin on cells other than thymocytes. The "blocking" method of mapping antigens on cell surfaces (14) has indicated that on thymocytes θ occupies extensive areas adjacent to smaller areas, like islands, occupied by several other alloantigens;

![Diagram of cell types](image)

**Fig. 3.** Occurrence of H-2 alloantigens on nonlymphoid cells.

this is precisely the picture given by immunoferritin, θ extending over most of the surface, with TL and H-2 confined to limited areas. Again, according to the results of quantitative absorption tests, erythrocytes are poor in H-2, thymocytes contain more, and lymphocytes 4-8 times as much as thymocytes (21); these proportions correspond with the fraction of the cell surface marked by immunoferritin in each case.

Because thymocytes, lymph node lymphocytes, and erythrocytes can be prepared in relatively pure suspensions, these cells lend themselves to estimation of antigen content by quantitative absorption of antibody. This is not the case with other cell types, such as eosinophils, leukocytes, and plasma cells,
that invariably occur together with other cells in mixed population; here it is more difficult to ascertain the presence and amount of surface antigens. Immunoferritin is especially suited to this question. Thus, plasma cells and eosinophils are now seen to have H-2 on their surfaces, in a patchy distribution, and to lack TL and θ antigens. Reticular cells, from thymus, lymph nodes, or spleen, also are devoid of θ and TL antigens, but are remarkable in regard to their virtually complete coat of H-2. As macrophages and reticular cells were thought to be closely related, it is surprising to find so little H-2 on the surface of peritoneal macrophages, sections of these cells only rarely showing small areas positive for H-2. Small areas of H-2 on macrophages will of course often be missed in random sections; these cells certainly possess H-2 antigen, for sera prepared in H-2 congenic strains sensitize them for lysis by complement. However, sensitization of macrophages by antibody in the absence of complement may possibly give rise to intrusion of membrane at the attachment sites, producing the ferritin-lined vacuoles in Fig. 21 and leaving the surface unmarked. Whatever the explanations, macrophages and reticular cells show very different degrees of H-2 representation, indicating either that they are not related or that differentiation of one into the other involves major revision of surface H-2 antigens.

It is clear that H-2, TL, and θ alloantigens occur discontinuously on the cell surface, and that the average size of positive areas is characteristic for cells of similar type. Whether there is variability in the size of positive areas on individual cells of the same type, or whether all differences in the size of the antigen-positive segments on similar cells are related to the plane of section, can best be decided by serial sectioning of single ferritin-labeled cells, giving a complete map for individual cells. This we hope to do. The scanning microscope may also be of value in permitting one to visualize cell surfaces vertically, rather than only transversely, a drawback of present methods.

Several factors enter into interpretations of the discontinuous representation of cell surface alloantigens indicated by ferritin labeling.

1. Blocking of sites by anti-γG that (a) has not been labeled by ferritin (in the conventional method) or (b) does not have a combining site for ferritin (in the hybrid method). This problem is solved as far as possible by purification of reagents to remove blocking antibody. It is unlikely that blocking antibody would do more than thin out the label within the areas of antigen representation.

2. Inaccessibility of regions of the cell surface to penetration by antibody by virtue of membrane folding or invagination.

3. Loss of ferritin-labeled antibody caused by repeated washing of the cells. This problem we hope to obviate in the future by carrying out the entire procedure with a single cell in a microdroplet.

4. Interposition of a natural outer coat (e.g. of sialomucin) (29, 30).

The strongest reason why these possible artifacts have not played a great
part in this investigation is that none of them accounts satisfactorily for the great reciprocal disparity in $\theta$ and H-2 antigen content on thymocytes as compared with lymphocytes. Thymocytes have an almost complete $\theta$ coat and a very sparse H-2 coat, whereas lymphocytes have an almost complete H-2 coat and a very sparse $\theta$ coat. Special considerations might apply to H-2 because of the complex nature of the locus, with many antigens shared among several strains. But although most H-2 antisera must lack specificities represented on the test cell, because of antigen sharing, this is unlikely to account for the restriction of ferritin labeling of H-2 thymocytes in contrast to lymphocytes. One could consider more complicated models, but they hardly seem called for on present evidence.

We have recently proposed a rudimentary map for the placement of alloantigens, including TL, H-2, and $\theta$, on the surface of mouse thymocytes (14). Visual confirmation of this by electron microscopy in conjunction with labeled antibody would be an important step in confirming that the cell surface has inherent supramolecular conformation. This requires the use of more than one visual marker, but until recently ferritin was the only practical marker for this purpose. To explore the possibilities of the hybrid antibody method of labeling, we prepared an $F(ab')_2$ antibody with anti-mouse $\gamma G$ and anti-southern bean mosaic virus specificities, and this we find labels H-2 on the cell surface with the virus marker in the same discontinuous fashion as with anti-mouse $\gamma G$/anti-ferritin hybrid (31). Thus, distinctive visual labeling of different antigens on the same cell should shortly be feasible.

**SUMMARY**

The representation of mouse alloantigens belonging to three systems, H-2, $\theta$, and TL, on the surface of cells from thymus, spleen, lymph nodes, and peritoneal cavity, was studied by electron microscopy with ferritin-labeled antibody. As expected from earlier serological data, TL was confined to thymocytes, $\theta$ was found on thymocytes and lymphocytes, and H-2 occurred to some extent on all cell types observed. On reticular cells, lymphocytes, plasma cells, and eosinophils, the majority of the cell surface was occupied by H-2; thymocytes had considerably less H-2, and erythrocytes and peritoneal macrophages least of all. In every instance the representation of antigen was discontinuous, the fraction of the cell surface covered being characteristic both of the antigen and of the type of cell. H-2 and $\theta$ provide a striking example of this; H-2 is present in far higher amounts on lymphocytes than on thymocytes, whereas the converse is true of $\theta$. Within areas positive for H-2 or $\theta$, protuberances of the surface membrane were often antigen-negative.

A better definition of cell surface structure, gained from studies such as this, is necessary for further inquiry into how the cell surface is assembled, and into selective gene action in relation to cellular differentiation.
We are indebted to Mr. M. P. Lardis, Mrs. Mona Seggio, Miss S. Ono, and Miss Kimmi Fukushima for excellent technical assistance, and to Mr. Juan R. Marchese for expert photographic work.

BIBLIOGRAPHY


Figs. 4 and 5. In the conventional (indirect) immunoferritin method (Fig. 4), the ferritin is in dense aggregates at variable distances from the cell surface; in the hybrid antibody (indirect) immunoferritin method (Fig. 5), the ferritin is sparser and at a more constant distance from the cell surface. The cell is ascites leukemia EL4 (H-2b), and the antiserum II-2* vs. H-2b. To obtain better definition of the ferritin, these sections were not stained. × 54,000.
Fig. 6. H-2 antigen on C57BL/6 thymocyte. Serum: $H-2^k$ vs. $H-2^b$ 1/40. Hybrid antibody method. Five small sections are marked by ferritin. $\times$ 19,250.

Fig. 7. Higher magnification of enclosed area in Fig. 6, showing two of the discrete ferritin-marked H-2 sectors. $\times$ 31,500.
Fig. 8. θ antigen on A strain thymocytes. Serum: AKR vs. C3Hf/Bi thymocytes 1/10. Conventional method. More than half the perimeter is marked by ferritin. × 27,600.

Fig. 9. Higher magnification of enclosed area in Fig. 8. Most of the perimeter is marked by ferritin, but note three unmarked protuberances. × 46,800.
FIG. 10. TL antigen on A strain thymocytes. Serum: vs. TL, 1,1/40. Conventional method. Only two sections (indicated by the box) are marked by ferritin. × 22,000.

FIG. 11. Higher magnification of enclosed area in Fig. 10, showing two positive sectors. × 38,000.
Fig. 12. H-2 antigen on an A(TL-) lymphocyte from spleen. Serum: H-2\(^b\) vs. H-2\(^a\) (I) 1/40. Hybrid antibody method. \(\times\) 22,500.

Fig. 13. Higher magnification of enclosed area in Fig. 12. Almost all this sector is marked by ferritin. \(\times\) 37,800.
FIG. 14. H-2 antigen on an A(TL−) splenic cell in mitosis (probably metaphase).
Serum: $H^{-2b} \text{ vs. } H^{-2b'}$ (II) 1/40. Hybrid antibody method. H-2 is extensively represented, just as on the interphase cell in Figs. 12 and 13. × 23,400.

FIG. 15. Higher magnification of enclosed area in Fig. 14. × 30,400.
FIG. 16. H-2 antigen on an A strain erythrocyte. Serum: \( H-2^b \) vs. \( H-2^b \) (II) 1/40. Hybrid antibody method. There are several small ferritin-marked sectors. \( \times \) 42,000. Note: erythrocytes in section more commonly show no H-2-positive areas.
Fig. 17. H-2 antigen on an A strain eosinophil leukocyte from a lymph node. Serum: $H^{-2^a}$ vs. $H^{-2^d}$ 1/40. Hybrid antibody method. $\times 24,000$.

Fig. 18. H-2 antigen on a C57BL(TL+) plasma cell. Serum: $H^{-2^a}$ vs. $H^{-2^b}$ 1/40 Hybrid antibody method. $\times 24,000$. 

998
Fig. 19. H-2 antigen on an A strain thymic reticular cell. Serum: H-2a vs. H-2a (I) 1/40. Conventional method. Very extensive labeling of the perimeter, better seen in Fig. 20. × 15,600.

Fig. 20. Higher magnification of enclosed area in Fig. 19. × 78,000.
FIG. 21. H-2 antigen on peritoneal cells of C57BL/6 mice. Serum: $H^{-2a}$ vs. $H^{-2b}$ 1/40. Hybrid antibody method. The peritoneal lymphoid cell (left) is extensively labeled by ferritin, with several unlabeled protuberances. The peritoneal macrophage (right) shows a minute section of attached ferritin, although the lining of the vacuoles is labeled in places. $\times 37,800$ (see Fig. 22).
FIG. 22. Diagram of the distribution of ferritin on the peritoneal lymphoid cell and the peritoneal macrophage shown in Fig. 21. Note absence of H-2 on three protuberances from the lymphoid cell.