

# THE TWO-DIMENSIONAL TOPOGRAPHIC DISTRIBUTION OF H-2 HISTOCOMPATIBILITY ALLOANTIGENS ON MOUSE RED BLOOD CELL MEMBRANES

GARTH L. NICOLSON, ROBERT HYMAN, and S. J. SINGER. From the Department of Biology, University of California at San Diego, La Jolla, California 92037, and the Armand Hammer Cancer Institute of the Salk Institute for Biological Studies, La Jolla, California 92037. Dr. Nicolson's present address is The Armand Hammer Cancer Institute of The Salk Institute for Biological Studies, La Jolla, California 92037.

## INTRODUCTION

In connection with studies of the molecular organization of biological membranes (13) we have been interested in determining the two-dimensional topographical distribution of specific components on membranes. Electron microscopy with ferritin-antibody (12) and ferritin-plant agglutinin (9) conjugates can be used to localize specific antigens and oligosaccharide components, respectively, with a resolution of about 300 Å. Labeled-antibody techniques have previously been used to study specific surface antigens of various cell types (cf. Refs. 1, 4-7). In earlier studies, however, the labeled cells had been embedded and sectioned before examination in the electron microscope; in such electron micrographs, essentially only the one-dimensional distribution of an antigenic component is revealed. In order to determine the two-dimensional surface distribution, we have developed an alternative method of specimen preparation (9). Cells are lysed at an air-water interface, which causes the entire cell membrane to spread out flat at the interface. The flattened-out membranes are then picked up on an electron microscope grid, the ferritin-antibody stain is applied to the membrane on the grid, and the grid is then examined by direct transmission electron microscopy.

In this note, we demonstrate the use of this technique in conjunction with the indirect ferritin-antibody staining method to visualize the two-dimensional distribution of the H-2 histocompatibility alloantigens (2) over large areas of the mouse red blood cell membrane.

## MATERIALS AND METHODS

### *Reagents and Antisera*

Horse spleen ferritin (6 × recrystallized, Miles Labs, Inc., Kankakee, Ill.) was further purified by cadmium sulfate crystallization, ammonium sulfate precipitation, and ultracentrifugation (8). Rabbit

anti-mouse 7S- $\gamma$ -globulin antiserum was obtained from Meloy Labs Inc., Springfield, Va., and the  $\gamma$ -globulin was isolated by precipitation with 50% saturated ammonium sulfate. This rabbit  $\gamma$ -globulin fraction was then absorbed with an equal volume of washed mouse red blood cells for 2 hr at 5°C. This absorbed rabbit  $\gamma$ -globulin was subsequently conjugated to ferritin for use as the indirect stain. As a control, rabbit  $\gamma$ -globulin containing anti-(human) spectrin antibodies (a gift of Dr. V. T. Marchesi) was used.

The mouse anti-H-2<sup>d</sup> antiserum was prepared by injecting C57BL/6J mice (of H-2<sup>b</sup> specificity) with the BALB/c tumor, Meth. A (of H-2<sup>d</sup> specificity), while the mouse anti-H-2<sup>b</sup> antiserum was prepared by injecting A/J mice (H-2<sup>a</sup>) with the C57BL tumor EL4 (H-2<sup>b</sup>). Mice were bled after 6-7 intraperitoneal injections of tumor had been given at 7-10 day intervals. The sera were absorbed at 0°C for 2 hr with 1/10 their volume of mouse red blood cells of the same strain as the mice immunized.

### *Preparation of Ferritin-conjugated Antibodies*

Ferritin conjugates of rabbit  $\gamma$ -globulin with antibody specificity for mouse 7S- $\gamma$ -globulin (Fer-MGG), and of rabbit  $\gamma$ -globulin with antibody specificity for human spectrin (Fer-HSp) were prepared by the toluene-2,4-diisocyanate coupling method (14). The ferritin-conjugates were separated from unconjugated proteins (8) by gel filtration on Agarose A-1.5 m (Bio-Rad Labs, Richmond, Calif.) and were concentrated to 2-5 mg protein/ml. The fractionated Fer-MGG was then absorbed three times at 0°C for 2 hr each with an equal volume of mouse myeloma, lymphoma, and red blood cells, respectively. After the last absorption, the solution was centrifuged at 10,000 *g* for 10 min to remove any residual cells, and was stored in sterile vials.

### *Sensitization of Mouse Red Cells with Anti-H-2<sup>b</sup> or with Anti-H-2<sup>d</sup> Alloantisera*

The red blood cells from freshly drawn C57BL/6 or BALB/c mouse blood in Alsever's solution were washed six times by centrifugation and resuspension in isotonic 0.01 M sodium phosphate-NaCl buffer, pH

7.4 (PBS). The white cell layer was removed after each centrifugation by aspiration. Samples of 0.1 ml of washed mouse red blood cells were centrifuged into pellets in 15 ml conical centrifuge tubes and the cells were resuspended in 0.25 ml of the appropriate absorbed mouse antiserum, diluted 1:10 in PBS. After incubation with mixing at 37°C for 45 min, the mixtures were centrifuged to pellet the cells, and the supernatants were removed for cytotoxic testing. The sensitized cells were then washed 4 times at 0°C in PBS containing 0.1% crystalline bovine serum albumin (Armour Pharmaceutical Co., Kankakee, Ill.).

#### Cytotoxic Testing

Direct cytotoxic tests were carried out essentially by the method of Boyse and Old (3, 10) except that Dulbecco's modified Eagle's medium (15) was used as the diluent. BALB/c and C57BL/6 spleen cells were used as indicator cells and guinea pig serum diluted 1:3 was used as the source of complement.

#### Ferritin-Antibody Staining of Sensitized Red Blood Cell Membranes

The mouse red blood cells sensitized with mouse anti-H-2 antibodies were lysed on distilled water surfaces as described by Nicolson and Singer (9). The spread-out cell membranes were then picked up on carbon-coated collodion films on electron microscope grids. Without drying, they were then conditioned with a 4% solution of bovine serum albumin in PBS, and a large drop of Fer-MGG was applied for 3-5 min at room temperature. The grids were then washed by floating them face down on several fresh PBS solutions and finally on distilled water. After air drying, the specimens were examined in a Philips Model 300 electron microscope.

#### RESULTS

The results in Table I show that little cytotoxic activity was removed from the anti-H-2 sera upon sensitization of the red blood cells. This demonstrates that the anti-H-2 antibodies were in great excess, and makes it likely that the H-2 antigenic sites on the red blood cell membranes were saturated with the mouse anti-H-2 antibodies.

The method of preparing membrane specimens by lysing sensitized red blood cells at an air-water interface produces flattened-out membranes, large areas of whose outer surface can be examined (Fig. 1, insert). With BALB/c red blood cell membranes sensitized with the H-2<sup>d</sup> antibodies and then stained with Fer-MGG, the ferritin molecules were found in discrete and irregularly arranged patches on the membrane surface (Fig. 1). Closely similar results were obtained with H-2<sup>b</sup>

TABLE I  
*Antisera Used for the Localization of H-2  
Alloantigens on Mouse Red Blood Cells*

Antiserum	H-2 Allele Detected	Red Cell Used for Sensitization	Cytotoxic Titer*
C57BL/6 anti-Meth. A	H-2 <sup>d</sup>	None	320
		C57BL/6 (H-2 <sup>b</sup> )	320
		BALB/c (H-2 <sup>d</sup> )	160
A/J anti-EL4	H-2 <sup>b</sup>	None	160
		BALB/c (H-2 <sup>d</sup> )	160
		C57BL/6 (H-2 <sup>b</sup> )	160

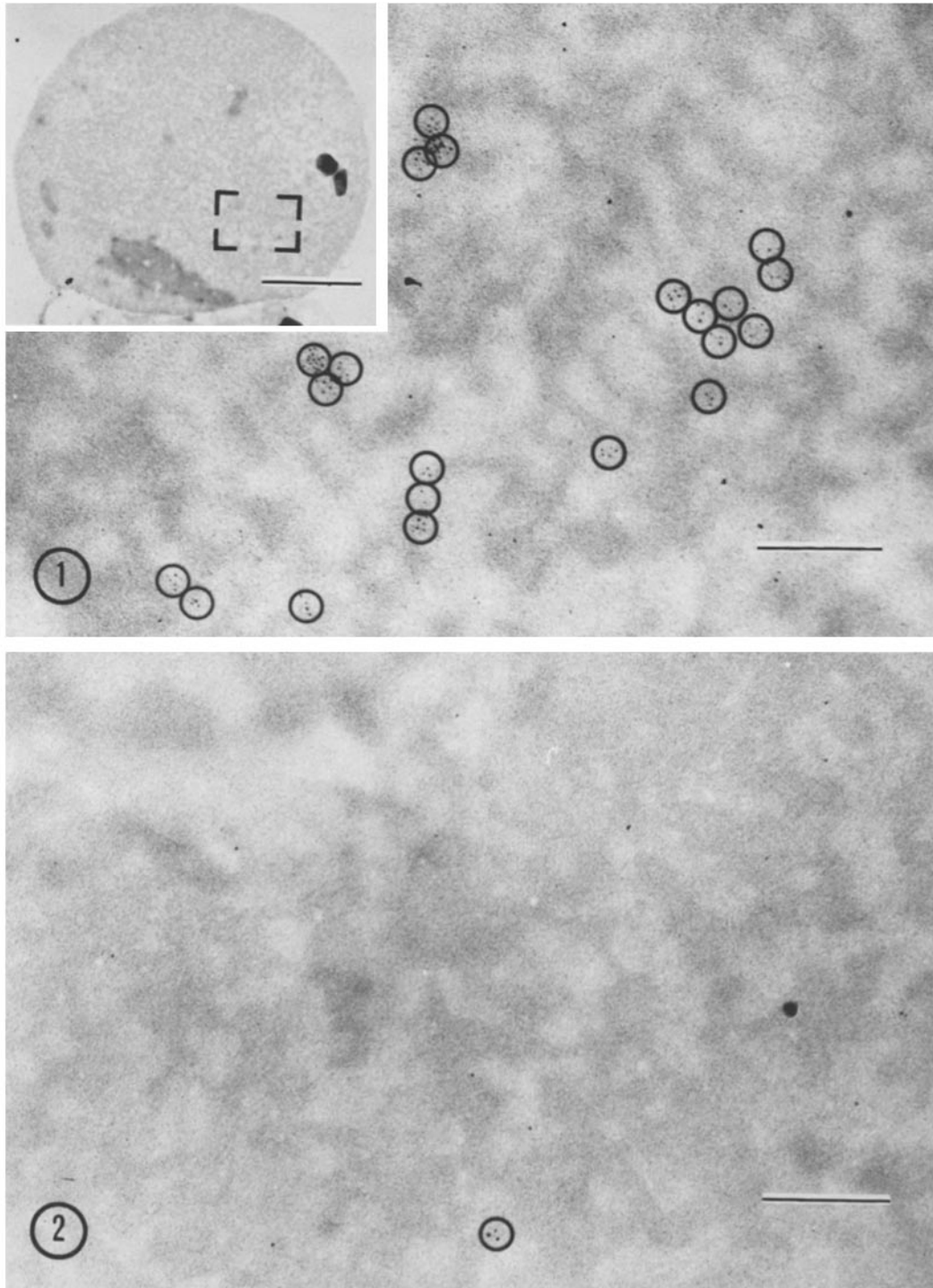
\* Cytotoxic titers were determined by titrating sera before and after sensitization with mouse red blood cells. BALB/c lymphocytes were used to detect H-2<sup>d</sup> specificities, and C57BL/6 lymphocytes were used to detect H-2<sup>b</sup> specificities (see Materials and Methods). Titers are expressed as the reciprocal of the last serum dilution showing significant (>20%) killing; the error of the method is about one serum dilution.

cells sensitized with mouse anti-H-2<sup>b</sup> antibodies and then stained with the same Fer-MGG (Fig. 3). For reasons to be given in the Discussion these patches were subdivided into circles of 300 Å radius (clusters), and the numbers of clusters per patch were measured over several cells of both types. Clusters separated by less than 1000 Å were scored as belonging to the same patch. The frequency distribution of clusters per patch is shown in Fig. 5, and applies to either the H-2<sup>b</sup> or H-2<sup>d</sup> alloantigen.

That the ferritin labeling was specific for the histocompatibility alloantigens was shown by the facts that (a) if H-2<sup>b</sup> cells were first treated with mouse-anti-H-2<sup>d</sup> antibodies and the lysed cells were then stained with Fer-MGG, very little ferritin was bound (Fig. 2). Similarly, H-2<sup>d</sup> cells exposed to mouse-anti-H-2<sup>b</sup> antibodies followed by Fer-MGG showed little ferritin staining (Fig. 4). (b) If cells of either specificity were sensitized with both anti-H-2<sup>d</sup> and anti-H-2<sup>b</sup>, and after lysis were then treated with the nonspecific Fer-HSp conjugate, no significant ferritin-staining was observed. (c) Unsensitized red blood cells of either specificity, lysed and treated with Fer-MGG, showed no ferritin staining.

#### DISCUSSION

The experiments reported here confirm and extend the observations of others (1, 4-6) that the H-2



**FIGURE 1** A mouse BALB/c ( $H-2^d$ ) red blood cell sensitized with anti- $H-2^d$  was mounted on a carbon-strengthened, collodion-coated electron microscope grid and stained with ferritin-conjugated rabbit anti-(mouse)- $\gamma$ -globulin. The ferritin clusters enclosed in circles of 300 Å radius are taken to represent single alloantigenic sites (see text). These clusters are arranged in patches with variable numbers of clusters per patch. The insert shows the entire cell ghost, and the brackets indicate the area magnified in the figure. Bar equals 0.2  $\mu$ m; insert bar equals 2  $\mu$ m.

**FIGURE 2** A control for the results shown in Fig. 1. Same legend as Fig. 1 except that mouse C57BL/6 ( $H-2^b$ ) red blood cells were sensitized with anti- $H-2^d$ .

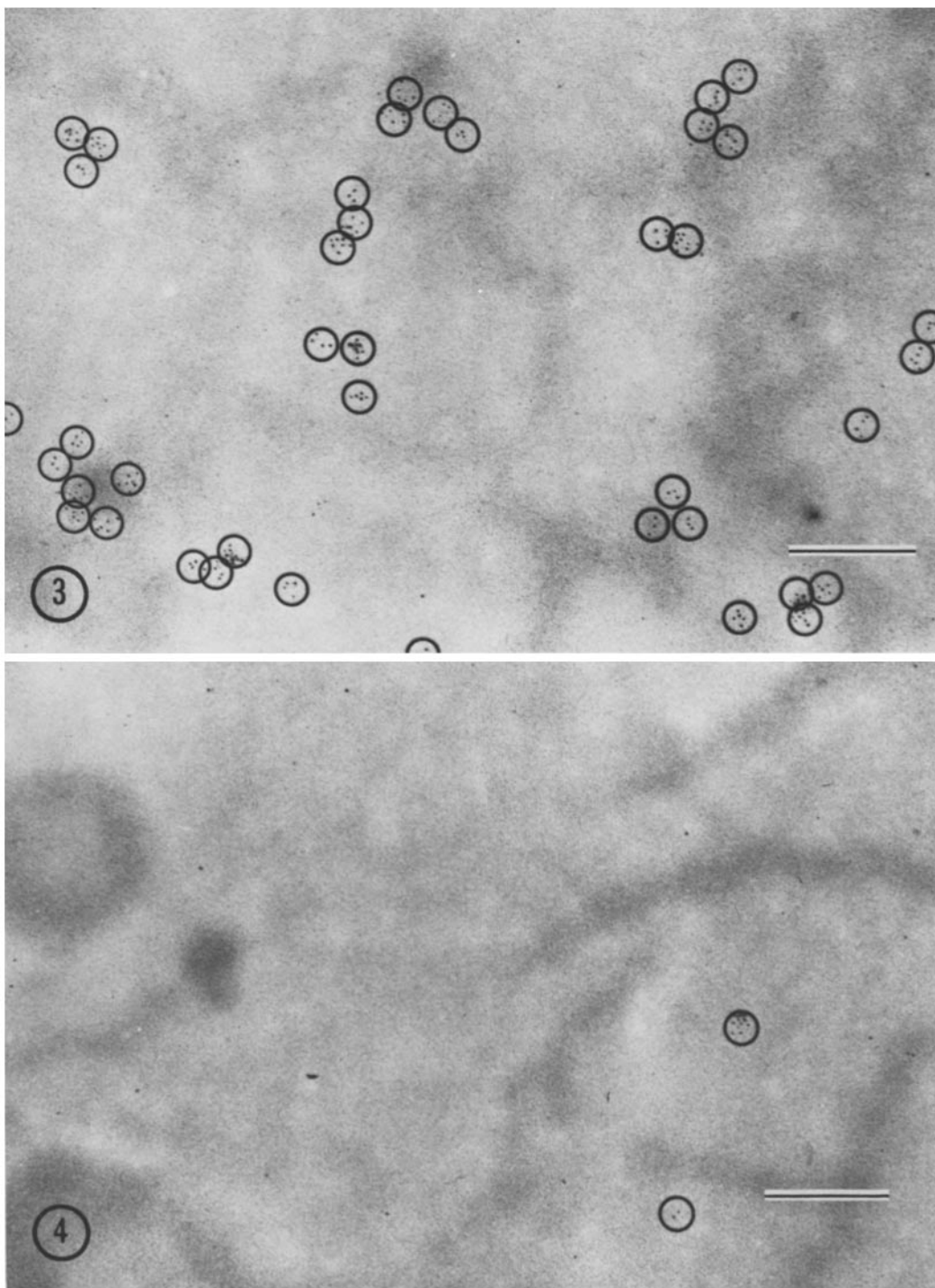


FIGURE 3 Same legend as Fig. 1 except that mouse C57BL/6 (H-2<sup>b</sup>) red blood cells were sensitized with anti-H-2<sup>b</sup>.

FIGURE 4 A control for the results shown in Fig. 3. Same legend as Fig. 1 except that mouse BALB/c (H-2<sup>d</sup>) red blood cells were sensitized with anti-H-2<sup>b</sup>.

histocompatibility alloantigens on mouse cells are present in patches on the cell surfaces. Similar results have been obtained with the HL-A histocompatibility alloantigens on human cells (11). The techniques used by these investigators, however, gave only limited one-dimensional information about the alloantigen distribution. The method of Nicolson and Singer (9) for membrane specimen preparation used in the present studies has permitted the visualization of the two-dimensional topographical distribution of the histocompatibility antigens over large areas of the red blood cell membrane surface, with the consequence that much additional information can be obtained.

The alloantigen patches were of different sizes and were irregularly dispersed over the area of the mouse red blood cell membrane. These results are different from those obtained by similar techniques with Rh<sub>0</sub>(D) antigen on human red blood cell membranes (G. L. Nicolson, S. P. Masouredis, and S. J. Singer, *Proc. Nat. Acad. Sci. U. S. A.*, In press). In the latter case, several lines of evidence strongly suggest that the D antigen is molecularly dispersed over the membrane surface. At saturation, a single human (<sup>125</sup>I)-labeled anti-D antibody was bound to each D antigenic site on the sensitized cell. When the sensitized cell was stained with ferritin-conjugated goat anti-(human)  $\gamma$ -globulin antibodies, discrete ferritin clusters containing from 2 to 8 ferritin particles were seen over the membrane surface. These clusters could be contained within circles of 300 Å radius. From the stoichiometry involved, each such cluster of ferritin was shown to be equivalent to one D antigenic site in the membrane. If as a first approximation we take such a cluster as representing a single antigenic site in the case of the indirectly-stained histocompatibility antigen as well, the results expressed in Fig. 5 then indicate that the number of antigenic sites per patch of histocompatibility antigen can vary widely, with the most frequent number being three. This variation in size is accompanied by a random distribution of patches over the two-dimensional surface of the cell membrane.

A detailed interpretation of these results must await further electron microscope studies with monospecific antisera, and would be greatly advanced by further information about the chemical structure of the histocompatibility antigens. The antisera used in the present study and in the studies of other workers which have demonstrated a patchy distribution of the H-2 antigen (1)

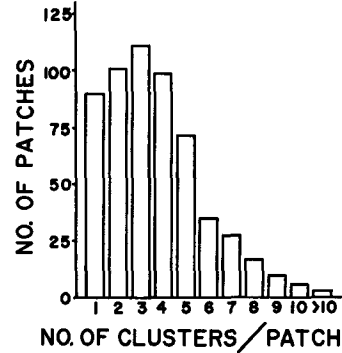


FIGURE 5 Distribution of patch sizes of H-2 alloantigenic patches on mouse red blood cell membranes. The number of clusters (Figs. 1 and 3) per patch was determined as indicated in the text.

have contained antibodies against several specificities determined by a single H-2 allele. Alloantisera which distinguish a single specificity would clearly be useful. To interpret the electron microscope results, it is also important to determine how many different specificities are present on a single molecule of a histocompatibility antigen.

We have recently initiated similar experiments with mouse plasmacytomas and lymphomas with basically similar experimental findings. Cells that contain larger amounts of H-2 alloantigens on their surfaces (2) have, on the average, larger patch sizes with a larger number of ferritin clusters per patch; these patches tend to be closer together but are still random in their two-dimensional arrangement.

Dr. Nicolson is a United States Public Health Service Predoctoral Fellow in the Department of Biology, University of California at San Diego. Dr. Hyman is a fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

These studies were supported by United States Public Health Service Grant GM 15971 to S. J. Singer, and by United States Public Health Service Grant AI05875 and United States Public Health Service Training Grant CA05213 to M. Cohn.

Received for publication 9 April 1971.

#### REFERENCES

1. AOKI, T., U. HÄMMERLING, E. DEHARVEN, E. A. BOYSE, and L. J. OLD. 1969. Antigenic structure of cell surfaces: An immunoferritin study of the occurrence and topology of H-2,  $\theta$  and TL alloantigens on mouse cells. *J. Exp. Med.* 130: 979.

2. BASCH, R. S., and C. A. STETSON. 1962. The relationship between hemagglutinogens and histocompatibility antigens in the mouse. *Ann. N. Y. Acad. Sci.* **97**:83.
3. BOYSE, E., L. HUBBARD, E. STOCKERT, and M. LAMM. 1970. Improved Complementation in the Cytotoxic Test. *Transplantation.* **10**:446.
4. DAVIS, W. C., and L. SILVERMAN. 1968. The localization of mouse H-2 histocompatibility antigen with ferritin-labeled antibody. *Transplantation.* **6**:536.
5. HÄMMERLING, U., T. AOKI, E. DEHARVEN, E. A. BOYSE, and L. J. OLD. 1968. Use of hybrid antibody with anti- $\gamma$ G and anti-ferritin specificities in locating cell surface antigens by electron microscopy. *J. Exp. Med.* **128**:1461.
6. HÄMMERLING, U., T. AOKI, H. A. WOOD, L. J. OLD, and E. DEHARVEN. 1969. New visual markers for electron microscopy. *Nature (London).* **223**:1158.
7. LEE, R. E., and J. C. FELDMAN. 1964. Visualization of antigenic sites of human erythrocytes with ferritin-antibody conjugates. *J. Cell Biol.* **23**:396.
8. NICOLSON, G. L., V. T. MARCHESI, and S. J. SINGER. 1971. The localization of spectrin on the inner surface of human red blood cells by ferritin-conjugated antibodies. *J. Cell Biol.* In press.
9. NICOLSON, G. L., and S. J. SINGER. 1971. Ferritin-conjugated plant agglutinins as specific saccharide stains for electron microscopy of cell membranes. *Proc. Nat. Acad. Sci. U. S. A.* **68**:942.
10. OLD, L., E. BOYSE, and E. STOCKERT. 1965. The G (Gross) leukemia antigen. *Cancer Res.* **25**:813.
11. SILVESTRE, D., F. M. KOURILSKY, M. G. NICCOLAI, and J. P. LEVY. 1970. Presence of HL.A Antigens on human reticulocytes as demonstrated by electron microscopy. *Nature (London).* **228**:67.
12. SINGER, S. J. 1959. Preparation of an electron-dense antibody conjugate. *Nature (London).* **183**:1523.
13. SINGER, S. J. 1971. The molecular organization of biological membranes. In *The Structure and Function of Biological Membranes*. L. I. Rothfield, editor. Academic Press Inc., New York. **4**:145.
14. SINGER, S. J., and A. F. SCHICK. 1961. The properties of specific stains for electron microscopy prepared by the conjugation of antibody molecules with ferritin. *J. Biophys. Biochem. Cytol.* **9**:519.
15. VOGT, M., and R. DULBECCO. 1963. Steps in the neoplastic transformation of hamster embryo cells by polyoma virus. *Proc. Nat. Acad. Sci. U. S. A.* **49**:171.