IMMOBILIZATION OF MEMBRANE H-2 ANTIGENS
BY PARAFORMALDEHYDE FIXATION

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INTRODUCTION

The use of specific antibodies coupled to visual markers such as ferritin or fluorescein to study the distribution of cell surface membrane antigens has resulted in the recognition that these membrane components are free to move in the plane of the membrane and that the antibodies used for labeling them may induce changes in their distribution (Frye and Edidin, 1970; Taylor et al., 1971; Davis, 1972). Up to the present, labeling studies have been carried out only on fresh, or unfixed, cell membranes. On fixed cells the surface membrane antigens might be immobilized and no longer susceptible to the alterations of their distribution that can be caused by antibodies. We recently demonstrated (Parr and Oei, 1973) that mouse cell membranes fixed with paraformaldehyde were able to bind anti-H-2 antibodies to the same extent as fresh membranes. The purpose of the present work was to determine whether paraformaldehyde fixation immobilizes mouse H-2 antigens on the surface membrane.

MATERIALS AND METHODS

Congenic resistant mice, C3H/HeDiSn (H-2k) and C3H.JK/Sn (H-2j), were obtained initially from the Jackson Laboratory (Bar Harbor, Maine) and subsequently bred in our laboratory. Details of immunization, collection of antiserum, and assay of antiserum titer by hemolysis have been described (Parr and Oei, 1973).

Preparation of Mouse Antiserum γ-Globulin-Ferritin Conjugates (MγG-F)

Mouse antiserum γ-globulin was purified as suggested by Frye and Edidin (1970). Ferritin (Nutritional Biochemicals Corporation, Cleveland, Ohio, crystallized two times) was purified by centrifugation at 45,000 g for 4 h, as suggested by Davis and Silverman (1968). The concentration of ferritin was determined from its optical density at 310 nm as described by Siess et al. (1971).

Equal weights of ferritin and mouse γ-globulin, usually about 40 mg of each, were combined at a final concentration of 15-20 mg/ml in a dialysis bag, which was then immersed in 0.10 M phosphate buffer, pH 7.5. An amount of glutaraldehyde equal to 2 or 3% of the weight of the total protein was added to the solution outside the dialysis bag, and conjugation was allowed to proceed for about 24 h at 4°C. The conjugate in the dialysis bag was then layered over 2.2 M sucrose and centrifuged at 15,000 g for 16 h in a swinging bucket rotor. After centrifugation, the clear supernate above the sucrose layer was removed, while the sucrose layer, containing both free and conjugated ferritin, was diluted and centrifuged at 45,000 g for 4 h. The resulting pellet, essentially free of unconjugated γ-globulin, was dissolved in phosphate-buffered saline (PBS) (Stimpfling, 1961), about 5 ml if 40 mg of ferritin was used initially.

Paraformaldehyde-Fixed Cells

Washed spleen and peritoneal cells were suspended in 1.0 ml of Hanks' solution at 0°C, and 9.0 ml of 1% paraformaldehyde (MC&B Manufacturing Chemists, Norwood, Ohio) in 0.9% NaCl solution at 0°C was added slowly with stirring. Fixation continued for 1 h at 0°C. The fixed cells were washed once briefly by centrifugation and resuspension in Hanks' solution, after which they were incubated in 10% nonimmune C3H.JK/Sn serum for 30 min before labeling.

Labeling of Cells

All of the cell labeling experiments reported here were done with a single, 3% glutaraldehyde, conjugate. Spleen and peritoneal cavity cells, both fresh and fixed, from both strains of mice, were incubated for 30 min at 0°C in undiluted MγG-F. This concentration of the conjugate appeared to saturate all of the H-2 antigens on the cells, since diminished labeling density was not noted until dilutions of MγG-F greater than 0.25 were used. Labeled cells were washed on sucrose gradients and either fixed for electron microscopy or further incubated in goat anti-mouse 7S-γ-globulin (Meloy Laboratories Inc., Springfield, Va.) as described with the results. After washing to remove the unbound anti-mouse γ-globulin, the cells were fixed for electron microscopy.
RESULTS

The Conjugate, MyG-F

Conjugates made with 2 or 3% glutaraldehyde seemed to label cells equally well, and their activity was retained indefinitely in sterile solution at 4°C; we kept two conjugates for 60 days and both seemed fully active at the end of that period.

Fixed Cells Labeled with MyG-F

On fixed spleen lymphocytes and peritoneal cavity cells, ferritin label was bound apparently randomly over the entire cell surface (Fig. 1). There were none of the patches that are found when living cells are labeled by an indirect procedure. In places, ferritin occurred in clumps of approximately 3-15 molecules, but otherwise there was no suggestion of nonrandomness in the labeling. The labeling of peritoneal monocytes and that of lymphocytes were very similar, and both were similar to that of spleen lymphocytes. The densities of labeling on fixed and fresh cells were indistinguishable. This is consistent with our earlier adsorption studies which showed that the antigenicity of the H-2 locus was completely preserved during paraformaldehyde fixation (Parr and Oei, 1973). Fixed control cells, C3H.JK/Sn, showed no labeling of the surface membrane in any experiment.

Fresh Cells Labeled with MyG-F

On labeled fresh spleen lymphocytes, the number of ferritin molecules per micrometer of membrane, the maximum length of the bare regions on the surface membrane, and the width of the ferritin layer above the membrane, all were essentially the same as on fixed cells. Fresh peritoneal lymphocytes also could not be distinguished from fixed peritoneal lymphocytes. Fresh peritoneal monocytes, however, were more vacuolated, contained more ferritin in intracellular vacuoles than did fixed monocytes, and generally had less label on the surface membrane. When fresh peritoneal cells were labeled with MyG-F for 30 min at 0°C and another 30 min at 25°C, many cells, both lymphocytes and monocytes, had ferritin-containing vesicles deep in their cytoplasm, and the lengths of the unlabeled regions of surface membrane increased. The label remained randomly distributed, however, and no patches of label appeared (Fig. 2). When fresh peritoneal cells were preincubated at 0°C in 1.0 mM NaF for 30 min to inhibit pinocytosis, the pattern seen after labeling with MyG-F was indistinguishable from that seen on fixed peritoneal cells. Fresh control cells, C3H.JK/Sn, showed no labeling of the surface membrane in any experiment.

Cells Labeled with MyG-F and then Incubated in Anti-Mouse γ-Globulin

To determine whether paraformaldehyde fixation immobilizes membrane H-2 antigens, we studied its effect on the redistribution of antigen that can be induced on fresh cells by anti-mouse γ-globulin. Several kinds of comparisons were made. When labeled, fresh spleen or peritoneal cells were washed and suspended in undiluted anti-mouse γ-globulin for 10 min at 37°C the cells became tightly agglutinated. Electron microscopy showed that reorganization of the label had occurred, as compared to fresh cells not exposed to anti-mouse γ-globulin. There were patches of continuous, densely packed label, and there were long stretches of unlabeled membrane. Some of the labeled regions were probably patches, while others may have been caps (Taylor et al., 1971). There was considerable evidence of pinocytosis. When labeled, fixed peritoneal cells were incubated for 30 min at 37°C in anti-mouse γ-globulin, no agglutination of the cells occurred. Electron microscopy showed that there was no reorganization of the label; the pattern observed was identical to that seen on fixed cells in the absence of anti-mouse γ-globulin. The absence of antigen patches on labeled, fixed cells after exposure to anti-mouse γ-globulin suggests that the H-2 antigens were no longer free to migrate in the plane of the membrane.

If the movement of labeled complexes on the surface membrane of fixed cells was only relatively restricted, or slowed, it might be possible that the undiluted anti-mouse γ-globulin could saturate all of the mouse γ-globulin in the labeled complexes before any cross-linking and aggregation could occur. To examine this possibility, two additional experiments were done, one using a 0.25 dilution and the other using a 0.1 dilution of anti-mouse γ-globulin. In both cases labeled, fresh cells incubated in anti-mouse γ-globulin for 30 min at 25°C agglutinated, and were observed to have heavily labeled patches of surface membrane separated by unlabeled segments (Fig. 3). Pinocytosis was extensive, and on peritoneal cells in particular there was often little label left on the surface of the cell. Cells fixed in paraformaldehyde before label-
**FIGURE 1** This peritoneal cavity monocyte (PCM) was fixed in paraformaldehyde and then labeled with MyG-F. Ferritin is distributed randomly all over the cell surface, except for occasional small clusters of label that appear at irregular intervals. The bar represents 0.1 µm in all micrographs. X 81,000.

**FIGURE 2** This peritoneal cavity cell was labeled for 30 min at 0°C and for an additional 30 min at 33°C in MyG-F without prefixation in paraformaldehyde. Numerous pinocytic vesicles (p) containing ferritin can be seen. Ferritin is distributed randomly over the cell surface, except for the occasional small clusters of label which were also present on fixed cells. X 81,000.
FIGURE 3  This spleen lymphocyte (SL) was labeled with MyG-F and additionally incubated in a 0.25 dilution of anti-mouse γ-globulin for 30 min at 25°C without prefixation in paraformaldehyde. Abundant ferritin is present in pinocytic vesicles (p). Ferritin is distributed in discrete, dense patches (arrows) separated by unlabeled regions. X 81,000.

FIGURE 4  This spleen lymphocyte (SL) was fixed in paraformaldehyde before labeling with MyG-F and subsequent incubation in a 0.25 dilution of anti-mouse γ-globulin for 30 min at 25°C. Ferritin is distributed randomly over the entire cell surface. Anti-mouse γ-globulin has failed to induce a redistribution of label into patches such as those seen in Fig. 3. X 81,000.
ing and exposure to diluted anti-mouse γ-globulin had label randomly distributed over the entire surface of the cell (Fig. 4). Thus, the concentration of anti-mouse γ-globulin does not seem to be an important limitation on the reorganization of labeled complexes on fixed cells. Rather, it would appear that the labeled complexes on fixed cells are quite immobile.

Since pinocytosis by fresh peritoneal cells during a 30-min incubation in anti-mouse γ-globulin at room temperature was so extensive that much of the label was removed from the surface membrane, we thought that this experiment should be repeated with pinocytosis inhibited. Accordingly, peritoneal cells were preincubated 0°C in 1.0 mM NaF for 30 min; all subsequent reagents and washes also contained NaF. There was little evidence of pinocytosis by fresh cells under these conditions. Label on the fresh cells was organized into discrete patches, separated by unlabeled stretches of membrane, and the amount of label present on the surface membrane was much greater than on cells not preincubated in NaF. The differences in the labeling patterns between fresh and fixed cells were obvious.

Two additional experiments were done to compare directly the labeling pattern on fixed cells labeled with M7G-F alone with the pattern on fixed cells incubated additionally for 30 min at 25°C in undiluted anti-mouse γ-globulin. In both experiments, we found it impossible to detect any differences in the labeling patterns.

DISCUSSION

The effects of formaldehyde fixation on mouse H-2 antigens were first investigated by Brent et al. (1961). They showed that homogenates of strain A tissues fixed for 19 h at 3°C in 0.1 M (0.3%) formaldehyde retained essentially full capacity to sensitize other mouse strains against strain A antigens, and that such homogenates retained 25–50% of their capacity to adsorb anti-strain A antibodies from antisera. Davis and Silverman (1968) fixed mouse cells for 10–20 min in 4% Formalin and found that the labeling of such cells with an immunoferritin technique gave nonspecific results. Bradley and Barnes (1972) studied the effect of formaldehyde on mouse thymocyte antigens, and similar studies have been carried out on human erythrocyte alloantigens by Moscowitz and Carb (1957).

We recently demonstrated (Parr and Oei, 1973) that mouse erythrocyte membranes fixed for 1 h at 0°C in 0.9% paraformaldehyde retained 100% of their ability to adsorb antibodies from antiserum and that this adsorption was specific. In the present study, we found that the distribution of ferritin-labeled H-2 antigens on fixed and fresh cells appeared to be random except for occasional small clumps of ferritin molecules. We suspect that the clumps of ferritin were due to aggregation in the conjugate itself, since similar clumps were found in the conjugate by direct examination after agar embedding. The distribution of ferritin on fresh cells remained indistinguishable from that on fixed cells, even after prolonged incubation, except that pinocytosis progressively removed label from the surface of fresh cells. The labeling reagent did not induce the formation of patches of label such as are seen after indirect labeling procedures. Labeled, fresh cells that were further incubated in anti-mouse γ-globulin were found to be covered with discrete patches of label separated by unlabelled regions. This reorganization of the labeling pattern induced by anti-mouse γ-globulin was prevented by paraformaldehyde fixation; fixed cells always showed a random distribution of label. Our results indicate that the natural distribution of H-2 antigens on fixed cells is random. Previous observations of patches of antigen on labeled cells (Cerottini and Brunner, 1967; Aoki et al., 1969; Nicolson et al., 1971) appear to result primarily from the interaction between mouse γ-globulin and anti-mouse γ-globulin, as already concluded by Davis (1972) for H-2 antigens and Taylor et al. (1971) for lymphocyte IgG.

Although our observations indicate that formaldehyde immobilizes mouse H-2 antigens in the membrane, we have no knowledge of the molecular basis of the effect. Formaldehyde does not appreciably form cross-links between protein molecules (Hopwood, 1969). It is intriguing that formaldehyde preserves so many, and possibly all, of the H-2 specificities without denaturation. Glutaraldehyde, on the other hand, rapidly inactivated all specificities tested (Parr and Oei, 1973). This suggests that the many H-2 antigen specificities are chemically very similar.

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