IMMUNE CYTOLYSIS: ELECTRON MICROSCOPIC LOCALIZATION OF CELLULAR ANTIGENS WITH FERRITIN-ANTIBODY CONJUGATES*

By JOHN M. EASTON, M.D., BURTON GOLDBERG, M.D., and HOWARD GREEN, M.D.

(From the Department of Pathology, New York University School of Medicine, New York)

Plates 24 to 31

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Electron microscopic and chemical studies (1–4) have indicated that the primary action of antibody and complement on animal cells is the production of a permeability defect in the cell membrane leading to a form of colloid osmotic lysis. By employing ferritin-labeled antibody, prepared by the method of Singer (5), the present study identifies ultrastructural sites of antibody-antigen union in intact and broken cell preparations of Krebs ascites tumor cells, and analyzes further the mechanism of action of antibodies and complement on animal cells.

Materials and Methods

Cells, Antibodies, and Complement.—Prepared by methods previously described (1, 3). Krebs-2 ascites tumor cells were maintained by successive passage through the peritoneal cavities of Swiss mice. Rabbit immune gamma globulin (RIGG) was prepared by sodium sulfate fractionation of the serum of rabbits immunized by successive injections of the ascites tumor cells. Sheep anti-rabbit gamma globulin was supplied by Dr. Jeanette Thorbecke. The source of complement (C′) was fresh, normal rabbit serum stored at −80°C until use. It contained an average of 12 hemolytic units per ml (6) and was added in excess in all experiments.

Ferritin.—The horse ferritin used in the majority of the experiments was prepared and donated by Dr. Abraham Mazur. Duplicate iron (7, 8) and nitrogen (9) determinations showed the ferritin solution to contain 14.2 mg iron/ml and 8.1 mg nitrogen/ml. Additional ferritin obtained from Nutritional Biochemicals Corporation, Cleveland, contained 11.8 mg iron/ml and 8.3 mg nitrogen/ml and did not differ in its behavior from Dr. Mazur's preparation.

Coupling of Ferritin to Gamma Globulin.—The original method of Singer was followed (5), employing *m*-xylylene diisocyanate as the coupling agent, and using the reactants in approximately the same proportions. The solutions containing the coupled products were dialyzed against balanced salt solution (BSS) (10), pH 7.4, and stored at 4°C. Any precipitate formed during storage was removed by centrifugation.

Electron Microscopy.—Cells and broken cell preparations were fixed in 1 per cent OsO_4

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(veronal-buffered pH 7.2) and carried through increasing concentrations of ethanol for dehydration. Embedding was in 8:2 (butyl:methyl) methacrylate with benzoyl peroxide as the catalyst. Polymerization was performed at 60°C for 36 hours. Sections were cut on a Porter-Blum microtome, floated on formvar-coated copper grids, and alternate sections stained with 2 per cent uranyl acetate for 15 minutes. The sections were examined in an RCA-EMU-3D electron microscope and the majority of survey plates taken at 12,500 diameters magnification. The ferritin molecules could not be seen on the fluorescent viewing screen at this magnification, and thus sampling bias with respect to localization of ferritin was minimized. Suitable photographic enlargments were made from the plates and final magnifications accompany the figures.

FINDINGS

ANALYSIS OF FERROGLOBULIN SOLUTIONS

All the preparations used in these studies were made by reacting gamma globulin and ferritin in a molecular ratio of 3.9 to 1. The final dialyzed solutions contained the coupled product, unreacted ferritin, and unreacted gamma globulin, and are referred to in the text as "ferroglobulins." Calculations from the Fe/N ratio of such preparations showed, in the majority of cases, that the final ferroglobulin had substantially the same proportion of gamma globulin to ferritin as the original reaction mixture. Sometimes, however, some precipitation of protein occurred during the reaction, and this resulted in a small shift of the proportion in one direction or the other depending on which proteins had come out of solution. The coupling reaction proceeded in the same way for mouse gamma globulin as for rabbit.

In order to estimate the amount of coupling, and in order to test the antibody reactivity of the coupled product itself, it was desirable to separate the coupled product from all unreacted antibody. This was accomplished by two cycles of ultracentrifugation at 105,000 *g* for 1 hour, according to Singer (11). The heavy iron-containing ferritin molecules and any gamma globulin molecules conjugated to them were sedimented, while the supernatant contained free gamma globulin, together with apoferritin and ferritin containing small amounts of iron ("light ferritin"), and any globulin coupled to them. The sedimented product ("purified ferroglobulin"), which was diluted to the original volume, contained 46 per cent by weight of the ferritin and 6 per cent of the gamma globulin present in the unpurified ferroglobulin. An analysis of two representative purified ferroglobulin solutions is given in Table I.

From the analytical data it was calculated that approximately 13 per cent of the gamma globulin added to the original reaction mixture had coupled to ferritin; and if one assumed a predominance of one-to-one coupling, as has been suggested by Singer (11), about half of the ferritin molecules had coupled to gamma globulin molecules.

Similar results were arrived at independently by specifically precipitating the gamma globulin of a purified ferroglobulin preparation with antibody and observing the amount of ferritin taken out of solution. An excess of sheep antiserum to rabbit gamma globulin was added to a purified ferroglobulin solution and to a mixture containing the same quantities of gamma globulin (0.51 mg) and of ferritin (0.83 mg iron), respectively. The tubes were kept at 4°C for 24 hours, and the precipitates centrifuged and washed twice with cold isotonic sodium chloride. The precipitates and

combined supernatants were analyzed in triplicate for iron. The results are given in Table II. It can be seen that while virtually no coprecipitation of ferritin occurred in control tube 1, in tube 2 half of the ferritin was precipitated with the antigen-antibody complex and therefore was conjugated to the rabbit gamma globulin.

Using a ferritin conjugate as an antibody, it was found that unpurified anti-ovalbumin ferroglobulins could be precipitated at equivalence with ovalbumin, demon-

TABLE I

Chemical Data for Purified Ferroglobulins

	Preparation 1*	Preparation 2*
Total nitrogen, *mg/ml*	0.359	0.364
Iron, *mg/ml*	0.857	0.835
‡ Ferritin N (assuming Fe/N ratio of 2.97), *mg/ml*	0.289	0.281
Gamma globulin N (total N − ferritin N), *mg/ml*	0.070	0.083
§ Ferritin protein (Fe × 3.78), *mg/ml*	3.24	3.16
Gamma globulin protein (gamma globulin N × 6.25), *mg/ml*	0.44	0.52
‖ Fraction of gamma globulin added to original reaction mixture which coupled	0.12	0.15
Fraction of ferritin added to original reaction mixture which coupled (assuming 1:1 coupling)	0.47	0.59

* Initial reactants—8.0 mg/ml of gamma globulin, 8.5 mg/ml of ferritin.

‡ Ultracentrifuged ferritin is enriched with respect to iron, as apoferritin is left in solution. 2.97 is the experimentally determined Fe/N ratio for twice ultracentrifuged ferritin. This contrasts with the Fe/N ratio of 1.75 for the same ferritin before ultracentrifugation.

§ Ultracentrifuged ferritin had an iron concentration of 26.5 per cent. The ferritin before ultracentrifugation had an iron concentration of 17.5 per cent.

‖ Corrected, assuming that apoferritin is equally likely to couple, and is left in solution during ultracentrifugation. Correction factors are 2.12 and 2.30 for Preparations 1 and 2, respectively.

TABLE II

Quantitative Precipitation of Purified Ferroglobulin with Sheep Antiserum to Rabbit Gamma Globulin

Tube	1 Control	2
Contents	Rabbit gamma globulin plus ferritin plus sheep antiserum to rabbit gamma globulin	Purified rabbit ferroglobulin plus sheep antiserum to rabbit gamma globulin
Precipitate		
Color	White	Rusty
Fe, *mg*	0.01	0.43
Combined supernatants		
Fe, *mg*	0.79	0.41

strating that the conjugates retained immunologic specificity for pure antigens. Analyses of the precipitates revealed that 10 to 15 per cent of the antibody in unpurified ferroglobulins was coupled to ferritin, and 44 to 54 per cent of the ferritin was coupled to gamma globulin.

Purified anti-mouse cell ferroglobulin was tested for its ability to agglutinate mouse erythrocytes, and it was found to have approximately the same agglutination titer as the gamma globulin from which it was prepared. Thus, ferroglobulins were shown to retain the immunologic reactivity of the antibody moiety, whether the latter was directed to soluble or cellular antigens.

ELECTRON MICROSCOPIC LOCALIZATION OF IMMUNE FERROGLOBULIN ON KREBS MOUSE ASCITES TUMOR CELLS

Ferroglobulin solutions prepared from anti-ascites tumor cell antisera were incubated with the cells *in vitro* in the presence and absence of complement.

Ascites Cells Incubated with Immune Ferroglobulin:

One ml of ascites cells was withdrawn from the peritoneal cavity of a mouse bearing the tumor for 5 to 8 days, suspended gently in 40 ml of cold BSS, and centrifuged at 50 *g* for 5 minutes. The cells were resuspended in BSS to 3.0 ml. 0.3 ml aliquots of cell suspension, containing about 10^7 cells, were added to each of four 5 ml volumetric flasks, gassed for 1 minute with a 95 per cent O_2, 5 per cent CO_2 mixture, and incubated with gentle shaking at 37°C. The following additions were then made to each of the flasks.

Flask 1. Cells incubated with rabbit immune ferroglobulin
0.36 ml rabbit immune ferrogobulin (ferritin 5.6 mg/ml; gamma globulin 6.94 mg/ml)
0.38 ml BSS

Flask 2. (Control)—Cells incubated with ferritin
0.025 ml ferritin (81 mg/ml)
0.71 ml BSS

Flask 3. (Control)—Cells incubated with ferritin plus RIGG
0.025 ml ferritin (81 mg/ml)
0.075 ml RIGG (35 mg/ml)
0.64 ml BSS

Flask 4. (Control)—Cells incubated with mouse ferroglobulin
0.37 ml mouse ferroglobulin (ferritin 5.5 mg/ml; gamma globulin 5.94 mg/ml)
0.37 ml BSS

The amounts of iron, whether in ferritin or ferroglobulin, were equal in all of the flasks, and the amounts of gamma globulin, whether free or coupled, were approximately equal in flasks 1, 3, and 4. After the additions, incubation was continued for 30 minutes and the condition of the cells was checked at intervals in the phase microscope. At the end of incubation the contents of each flask were transferred with the aid of 5 ml of BSS to 12 ml centrifuge tubes. The suspensions were gently mixed, centrifuged at 50 *g* for 5 minutes at 0°C and the supernatants discarded. Eleven ml of cold 1 per cent OsO_4 (pH 7.2) were added to each of the tubes and the suspended cells fixed for 7 minutes at 0°C. Dehydration and embedding were performed as described under Methods.

Results.—The surface membrane change previously demonstrated to occur in cells exposed to specific antibodies (1) is illustrated in Fig. 1. The phenomenon, represented by a focal zone of evagination and invagination of the cell surface membrane, was observed in cells from flasks 1 and 3, but was absent in cells from control flasks 2 and 4.

The ferritin label, denoted by characteristic dense granules, 55 A in diameter, appeared in specific association only with the plasma membranes of cells incubated with immune ferroglobulin (flask 1). Figs. 2 to 4, taken from such cells, illustrate the characteristic array of ferritin along the cell surface membrane. The greatest amount of ferritin is seen in the zones of surface folding, but this may be partly accounted for by the great abundance of cell membranes in such zones. Fig. 5, in contrast, is representative of control cells incubated with RIGG plus free ferritin (flask 3) and it is to be noted that little or no ferritin is seen in relationship to the folded surface membrane. Cells from control flasks 2 and 4 similarly lacked the characteristic association of ferritin with the plasma membrane. It is estimated that there was less than 5 per cent as much ferritin on the surfaces of control cells as on the surfaces of cells exposed to immune ferroglobulins. Therefore, virtually all of the ferritin label in the latter instance marked antigenic sites in the cell membrane.

The ferritin label appeared in smaller amounts in the interiors of experimental and control cells, but always within smooth membrane-bounded profiles (Figs 6 and 7); it was never found free within the cytoplasmic matrix. These ferritin molecules were presumably incorporated into the cells by the process of pinocytosis (12–16), and in the case of the experimental cells, some of the ferritin was no doubt coupled to antibody.

Anti-tumor cell ferroglobulin appeared to bind specifically to antigenic sites on the cell surface membrane. The cell membrane appeared to constitute an effective permeability barrier to the direct passage of ferritin-antibody units into the cytoplasm of the cell. Those molecules entering the cell by the process of pinocytosis were excluded from other cellular compartments by the membrane barrier of the pinocytotic vesicle, for at least the 30 minute incubation period employed in these experiments.

Ascites Cells Incubated with Immune Ferroglobulin plus Complement:

To learn whether the addition of complement altered the distribution of antibody molecules, cell suspensions were prepared as above and the following additions made:

Flask 1. Cells incubated with rabbit immune ferroglobulin plus complement
0.36 ml rabbit immune ferroglobulin (ferritin 5.6 mg/ml; gamma globulin 6.94 mg/ml)
0.38 ml C′

Flask 2. (Control)—Cells incubated with ferritin plus RIGG plus complement
0.025 ml ferritin (81 mg/ml)
0.075 ml RIGG (35 mg/ml)
0.38 ml C′
0.26 ml BSS

The two flasks contained equal amounts of iron and approximately equal amounts of gamma globulin. Incubation, fixation and embedding were performed as in the previous experiment.

Results.—The cells in both flasks displayed the typical swelling of the membrane-bounded compartments associated with the action of antibody and complement (1) (Figs. 8 to 10). As in the previous experiment, the experimental cells (flask 1) displayed a much larger amount of ferritin in association with the cell membrane (Fig. 8) than did the control cells. Ferritin within pinocytotic vesicles was evident in both groups. However, cells in both experimental (Fig. 9) and control (Fig. 10) flasks now displayed small amounts of ferritin scattered within the cytoplasmic matrix and nucleoplasm.

The amount of intracellular ferritin found in the experimental cells was greater than in control cells, and a greater proportion of experimental cells contained such intracellular ferritin. Since the control cells were exposed to the same concentrations of ferritin, antibody and complement, the extra amounts of ferritin in the cytoplasm of the experimental cells probably represented ferritin-antibody conjugate. The small numbers of ferritin molecules found within the cytoplasmic matrix did not appear to be in frequent association with organelles, such as mitochondria, endoplasmic reticulum, or cytoplasmic fibrils, but appeared to be distributed at random.

Ascites Cells Incubated with Purified Ferroglobulin in the Presence and Absence of Complement

As the prior experiments were performed with unpurified ferroglobulin solutions containing much uncoupled immune gamma globulin, it could be argued that the described phenomena were secondary to the action of the latter rather than representations of the direct fixation of the conjugated molecule to cellular antigenic sites. Although the absence of ferritin localization in control systems containing free ferritin and RIGG seemed to meet this objection, cells were nevertheless exposed to purified ferroglobulin solutions in the presence and absence of complement.

To 0.3 ml aliquots of cell suspension in 10 ml volumetric flasks, the following additions were made:

Flask 1. Cells incubated with purified rabbit immune ferroglobulin

0.42 ml purified ferroglobulin (ferritin 3.7 mg/ml; gamma globulin 0.44 mg/ml)
0.32 ml BSS

Flask 2. Cells incubated with purified rabbit immune ferroglobulin plus complement

0.42 ml purified ferroglobulin (ferritin 3.7 mg/ml; gamma globulin 0.44 mg/ml)
0.32 ml C′

The quantity of iron in each flask was approximately equal to that added to the flasks in the experiments above (pp. 278 and 279), but the total concentration of gamma globulin (in this instance all of it coupled to ferritin) was only 7 per cent of that used in the earlier experiments. The flasks were incubated for 40 minutes, the contents fixed with equal volumes of 2 per cent OsO_4 and prepared for electron microscopy in the usual manner.

Results.—The degree of agglutination (flask 1) was much less than in previous experiments, presumably owing to the much lower total concentration of antibody in the purified preparations. Correspondingly, the frequency and degree of surface membrane change were also reduced. However, the localization of ferritin was qualitatively similar to that observed in the previous experiments, the ferritin being present along the surface membrane (Fig. 11) and within pinocytotic vesicles.

Only a minority of the cells in flask 2 underwent the osmotic swelling of immune lysis, presumably because of the low antibody concentration. However, in those cells which did swell the ferritin distribution was as observed in an experiment above (p. 279)—on the surface membrane, within pinocytotic vesicles and free within the cytoplasmic matrix.

Electron Microscopic Localization of Immune Ferroglobulin after Incubation with Broken Cells

As the previous experiments did not demonstrate significant attachment of labeled antibody to intracellular organelles, broken cell preparations were employed to test

the affinity of the ferroglobulins for freely accessible intracellular structures. Advantage was taken of the mechanical fragility of the osmotically swollen, antibody-complement treated cells to produce a broken cell preparation. Differential centrifugation of the latter separated a heavy "nuclear fraction" from a light "sphere fraction," the latter consisting principally of spherical profiles derived from mitochondria, elements of smooth and rough surfaced endoplasmic reticulum, and resealed segments of cell surface membranes (17).

A cell suspension was prepared in the usual manner and diluted with BSS to give a cell count of 1.5×10^7 cells/ml. Aliquots of 4.0 ml were placed in each of two 50 ml Erlenmeyer flasks. After gassing, the following additions were made:

Flask 1. Cells incubated with rabbit immune ferroglobulin plus complement

1.5 ml immune ferroglobulin (ferritin 6.6 mg/ml; gamma globulin 5.88 mg/ml)
1.0 ml C′

Flask 2. (Control)—Cells incubated with ferritin plus RIGG plus complement

0.125 ml ferritin (81 mg/ml)
0.26 ml RIGG (35 mg/ml)
1.0 ml C′
1.1 ml BSS

The amounts of gamma globulin and of iron were approximately equal in the two flasks. After 30 minutes incubation at 37°C, the stoppered flasks were removed, shaken sharply to disrupt the cells, and then allowed to stand without agitation for 10 minutes at 37°C. The flask contents were then centrifuged for 10 minutes at 50 *g* at 0°C to sediment the nuclear fraction. These pellets were suspended in BSS to 1 ml, and equal volumes of 2 per cent buffered OsO_4 were added. Fixation was for 15 minutes at 0°C. The supernatants containing the "spheres" were centrifuged at 5,000 *g* for 15 minutes at room temperature. These pellets were fixed in an equal volume of 2 per cent buffered OsO_4 for 7 minutes at 0°C. Dehydration and embedding were performed in the usual manner.

Results.—Sections of nuclear and sphere fractions from the experimental flask contained more associated ferritin than was observed on intracellular structures (p. 279) with whole cell preparations. Nuclear and sphere fractions from flask 1 contained much more ferritin than corresponding fractions from control flask 2. A detailed description follows:

Nuclear fractions: In cells from flask 1, small amounts of ferritin were present in the nucleoplasm, but larger quantities were contained in the adhering fragments of cytoplasm in association with amorphous material and membranous structures derived from endoplasmic reticulum (Fig. 12). Lesser amounts were associated with mitochondria and lipid droplets. However, the distribution of ferritin was variable and in some sections many of the above organelles were free of ferritin. The control sections lacked the characteristic dense accumulations of ferritin in amorphous material and on membranes of the endoplasmic reticulum, but small numbers of ferritin molecules were occasionally identified in apparent association with these structures as well as nucleoplasm, mitochondria, and lipid bodies.

Sphere fractions: The experimental sphere preparations similarly contained much more ferritin than the controls; the largest accumulations were in amorphous material (Fig. 13) and on the outer surfaces of closed profiles formed by smooth membranes (Figs. 14 and 15). A few ferritin molecules were also occasionally present within small spherical profiles (Fig. 15), tentatively identifying the particular profiles as pinocytotic

vesicles, but most of the ferritin was related to the outer surfaces of the smooth membranes. In several instances very dense accumulations of ferritin were present between apposed smooth membranes of spherical profiles (Fig. 16). The majority of the spherical profiles were presumed to arise from smooth surfaced endoplasmic reticulum but the larger profiles were most likely derived from segments of the cell surface (17). Mitochondria and rough surfaced endoplasmic reticulum generally displayed only a few molecules of ferritin on their outer surfaces, and in the latter case the ferritin appeared to be associated with the membrane component rather than with the ribonucleoprotein granules. Some ferritin was also seen on the outer surfaces of lipid droplets. The control cells did not display the characteristic dense accumulations of ferritin in amorphous material or on smooth membranes, but only showed much smaller numbers of ferritin molecules variably distributed between these structures, mitochondria, rough surfaced endoplasmic reticulum, and lipid bodies.

The larger total amount of ferritin present in the experimental systems attested to the over-all binding specificity of the immune ferroglobulin. However, non-specific entrapment of ferritin in the sticky cell debris of control and experimental cells made comparisons difficult wherever only small and variable amounts of ferritin were observed. For this reason, one could not assume that every ferritin molecule identified represented antibody bound to antigen and the occurrence of specific binding to such structures as nucleoplasm, mitochondria, and rough surfaced endoplasmic reticulum therefore remained in doubt.

A clear qualitative and quantitative difference from the controls was evident, however, in the case of ferritin associated with amorphous material and smooth surfaced membranes. The former could have been derived from soluble proteins in the "cell sap" or have represented products secondarily released from structured organelles. The smooth membranes were derived from elements of the endoplasmic reticulum and segments of the cell surface membrane.

DISCUSSION

The specific reactivity of ferritin-antibody conjugates prepared by Singer's original method (5) was confirmed by our experiments with pure antigens and mammalian cells. Smith *et al.* (18) have used this technique to demonstrate bacterial antigens, and Rifkind and Morgan *et al.* (19, 20), Singer (11) and Lee (21) have identified viral antigens with these conjugates. Singer has since published an improved method employing toluene 2,4-diisocyanate as the coupling agent, achieving a greater yield of the covalently linked ferritin-antibody molecule (11), but the original method clearly yields a satisfactory product which specifically and directly identifies antigenic sites.

The present study demonstrated that antigens in the cell membrane of animal cells were able to react specifically with ferritin-labeled gamma globulins fractionated from sera of animals immunized with whole cells, and supports the concept that the cell membrane is the primary site of antibody fixation. Earlier chemical and ultrastructural studies had indicated a surface action of antibodies on ascites cells, as they were shown to induce a focal zone of cell

membrane folding but no other structural change (1). The presence of antibody on the cell surface is thought to produce sufficient "stickiness" so that, when normal surface membrane movements bring segments of the cell membrane into apposition, the membranes may become fixed, and extension of the process locally may produce a labyrinthine system of folds. Interdigitation of surface projections from adjacent cells is thought to favor agglutination.

The present experiments have shown that the cell membrane is an effective permeability barrier to the direct passage of ferritin-antibody units into the cytoplasmic matrix. Over the short incubation periods employed, the label appeared in the cell interior only within membrane-bounded, closed profiles and presumably entered by the process of pinocytosis. Our observations generally agree with those of Hiramoto, Goldstein, and Pressman (22) who found that fluorescein-tagged antibodies fixed at cell surfaces but did not penetrate the cells unless they had been damaged.

In our experiments the action of antibody and complement permitted the entrance of ferritin-labeled antibodies into the cytoplasmic matrix. Antibody and complement have been shown to kill mammalian cells by production of a permeability disorder (3). They act on the cell membrane, where they produce functional "holes" that allow the loss of low molecular weight constituents from the cells and equilibration of cations between the cells and the medium. The equilibration establishes an osmotic pressure gradient, and water enters all membrane-bounded compartments of the cell (1, 23). As the cell volume increases, macromolecules of the size of ribosomes (100 to 230 A) escape through the stretched but unbroken cell membrane into the medium (17). It is not surprising, therefore, that such cells can admit molecules of the dimensions of ferritin (100 A) and antibody (250 × 40 A).

Antibody localization on the membranes of mitochondria and elements of endoplasmic reticulum would be a logical requirement if the swelling of these organelles depended upon the same osmotic gradients demonstrated for the whole cell. However, it was not possible to demonstrate any high frequency of association of the ferroglobulins with intracellular membranes after complement action permitted their access to the cytoplasmic matrix. As each of our ultrathin sections represented only a very small fraction (approximately 1/700th) of the entire cell volume, and as only relatively few ferroglobulin molecules managed to penetrate the cell membrane under the above conditions, examination of even a great many different sections might not yield a pattern of localization. It is quite likely, furthermore, that only very few molecules of antibody are required to alter the permeability properties of intracellular membranes. Therefore, the data are still not inconsistent with the view that after they have acted on the cell membrane, antibody and complement may act on the membranes of intracellular organelles.

That intracellular structures can react specifically with antibodies in antisera

prepared against whole cells, was shown by mechanically disrupting the cells and exposing the organelles to high concentrations of immune ferroglobulin. Under these conditions, the majority of localizing antibodies reacted with antigens in the smooth membranes of endoplasmic reticulum and in material probably derived from the soluble proteins of the cell. The detection of a significant degree of ferritin labeling of amorphous cellular material indicates a high concentration of antigens therein, but antibodies to these antigens are probably not critical to the events of immune cytolysis. It could be neither established nor ruled out that antigen-antibody union occurred to some degree on mitochondria and rough surfaced endoplasmic reticulum.

It might be presumed that our antisera, prepared against intact ascites cells, should have contained antibodies directed against all intracellular antigens. It has been shown by the agar diffusion studies of Perlmann *et al.* (24) that mitochondria, microsomal ribonucleoprotein, as well as microsomal membranes and "cell sap," contain specific antigenic components as well as cross-reacting antigens. The failure to demonstrate antigens within some of these sites with ferritin-antibody conjugates must be interpreted in terms of the technical limitations of the procedures employed.

Toolan and Wallace have reported (25) that antisera prepared against the microsome fraction of the transplantable human tumor H. Ep. #3 were cytotoxic for whole cells, whereas antisera against nuclear or mitochondrial fractions possessed little cytotoxic potency. Antisera to microsome fractions containing a high ratio of membrane components to ribonucleoprotein granules were more effective than antisera against fractions with a low ratio. As microsome fractions consist principally of elements of endoplasmic reticulum (26), but may contain segments of cell surface membrane as well (17), the above findings are consistent with ours, and support the general concept that the cell membrane and the membranes of the system of endoplasmic reticulum are the principal sources of the antigens which elicit the formation of cytotoxic antibodies.

Other studies of the cytotoxic activities of antibodies prepared against cell fractions have provided somewhat different conclusions (27, 28), perhaps because of the uncertain purity of the fractions. The nature of antibodies to given cell fractions might be tested by labeling the respective antibodies with ferritin and determining their ultrastructural sites of localization. We believe that antibodies with cytotoxic potency will prove to have membrane-localizing capacities.

SUMMARY AND CONCLUSIONS

Immune gamma globulin has been coupled to ferritin by the diisocyanate method of Singer. The final product ("ferroglobulin") had approximately 13 per cent of its gamma globulin coupled to ferritin, and roughly half of its

ferritin coupled to gamma globulin. The uncoupled gamma globulin could be removed by ultracentrifugal sedimentation of the free ferritin and the ferritin-antibody conjugates. The characteristics of the native antibody were retained by the ferritin-antibody conjugates, for they could be precipitated by anti-gamma globulin antisera, and when used as antibodies, they reacted specifically with soluble and cellular antigens.

Ferroglobulin preparations made from rabbit antisera against whole ascites tumor cells were incubated with the cells, and the location of ferritin determined by electron microscopy of thin-sectioned material. It was found that the immune ferroglobulins localized specifically on antigens of the cell membrane. Some of the ferritin label entered the cells by pinocytosis, but the ferritin-antibody units did not appear able to pass directly through the cell membrane into the cytoplasmic matrix.

When cells were incubated with ferritin-labeled antibody and complement, antibody could be located in the cytoplasmic matrix, and it therefore appeared that complement action was required before antibody could pass directly through the cell membrane. This finding was consistent with previous observations that the plasma membrane of an antibody-complement treated cell becomes permeable to large molecules.

In broken cell preparations incubated with ferroglobulin, antibody combined with amorphous material and with structures derived from cell membranes and from smooth membranes of the endoplasmic reticulum. The data favor the concept that antigens contained within the membranous structures are most important in the formation of cytotoxic antibodies.

The reported experiments support the view that cytotoxic antibodies fix primarily to surface antigens of the cell membrane. The subsequent action of complement establishes the permeability defect that induces the osmotic lysis of the cell and permits antibody to pass into the cell where it may act in a similar fashion on intracellular organelles.

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BIBLIOGRAPHY

1. Goldberg, B., and Green, H., The cytotoxic action of immune gamma globulin and complement on Krebs ascites tumor cells. I. Ultrastructural studies, *J. Exp. Med.*, 1959, **109**, 505.
2. Green, H., Fleischer, R. A., Barrow, P., and Goldberg, B., The cytotoxic action of immune gamma globulin and complement on Krebs ascites tumor cells. II. Chemical studies, *J. Exp. Med.*, 1959, **109**, 511.
3. Green, H., Barrow, P., and Goldberg, B., Effect of antibody and complement on permeability control in ascites tumor cells and erythrocytes, *J. Exp. Med.*, 1959, **110**, 699.

4. Green, H., and Goldberg, B., The action of antibody and complement on mammalian cells, *Ann. New York Acad. Sc.*, 1960, **87,** 352.
5. Singer, S. J., Preparation of an electron-dense antibody conjugate, *Nature*, 1959, **183,** 1523.
6. Mayer, M. M., Croft, C. C., and Gray, M. M., Kinetic studies on immune hemolysis. I. A method, *J. Exp. Med.*, 1948, **88,** 427.
7. Saywell, L. G., and Cunningham, B. B., Determination of iron—colorimetric o-phenanthroline method, *Ind. and Eng. Chem., Analytical Edition*, 1937, **9,** 67.
8. Bandemer, S. L., and Schaible, P. J., Determination of iron—a study of the o-phenanthroline method, *Ind. and Eng. Chem., Analytical Edition*, 1944, **16,** 317.
9. Ma, T. S., and Zuazaga, G., Micro-Kjeldahl for determination of nitrogen, *Analyt. Chem.*, 1942, **14,** 280.
10. Eagle, H., Nutrition needs of mammalian cells in tissue culture, *Science*, 1955, **122,** 501.
11. Singer, S. J., and Schick, A. F., The properties of specific stains for electron microscopy prepared by the conjugation of antibody molecules with ferritin, *J. Biophysic. and Biochem. Cytol.*, 1961, **9,** 519.
12. Lewis, W. H., Pinocytosis, *Bull. Johns Hopkins Hosp.*, 1931, **49,** 17.
13. Lewis, W. H., Pinocytosis by malignant cells, *Am. J. Cancer*, 1937, **29,** 666.
14. Palade, G. E., The endoplasmic reticulum, *J. Biophysic. and Biochem. Cytol.*, 1956, **2** (suppl), 85.
15. Holter, H., Pinocytosis, *Internat. Rev. Cytol.*, 1959, **8,** 481.
16. Easton, J. M., Goldberg, B., and Green, H., Demonstration of surface antigens and pinocytosis in mammalian cells with ferritin-antibody conjugates, *J. Biophysic. and Biochem. Cytol.*, 1962, **12,** *in press*.
17. Goldberg, B., and Green, H., Immune cytolysis. I. The release of ribonucleoprotein particles. II. Membrane-bounded structures arising during cell fragmentation, *J. Biophysic. and Biochem. Cytol.*, 1960, **7,** 645.
18. Smith, C. W., Metzger, J. F., Zacks, S. I., and Kase, A., Immune electron microscopy, *Proc. Soc. Exp. Biol. and Med.*, 1960, **104,** 336.
19. Rifkind, R. A., Hsu, K. C., Morgan, C., Seegal, B. C., Knox, A. W., and Rose, H. M., Use of ferritin-conjugated antibody to localize antigen by electron microscopy, *Nature*, 1960, **187,** 1094.
20. Morgan, C., Rifkind, R. A., Hsu, K. C., Holden, M., Seegal, B. C., and Rose, H. M., Electron microscopic localization of intracellular viral antigen by the use of ferritin-conjugated antibody, *Virology*, 1961, **14,** 292.
21. Lee, S., Ferritin-labeled antibody as an electron stain for phage protein, *Exp. Cell Research*, 1960, **21,** 249.
22. Hiramoto, R., Goldstein, M. N., and Pressman, D., Limited fixation of antibody by viable cells, *J. Nat. Cancer Inst.*, 1960, **24,** 255.
23. Green, H., and Silverblatt, F., Effect of antibody and complement on volume control in an ascites tumor cell, *Nature*, 1960, **186,** 646.
24. Perlmann, P., Hultin, T., D'Amelio, V., and Morgan, W. S., Distribution and metabolism of protein antigens in rat liver, *Exp. Cell Research*, 1959, **7,** suppl., 279.

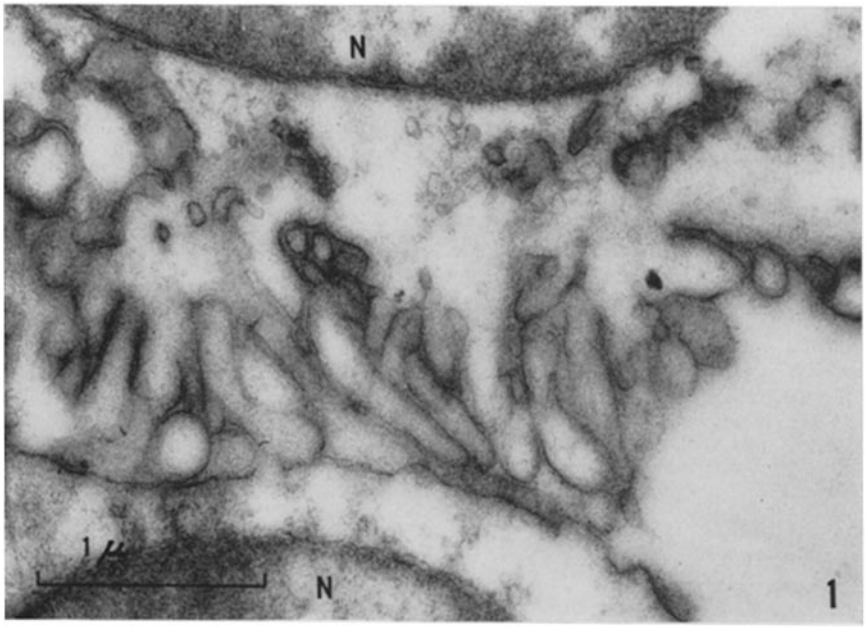
25. Toolan, H. W., and Wallace, R. A., The relative ability of cell-free fractions of the transplantable human tumor, H. Ep. #3, to produce H. Ep. #3 cytotoxins in rats, *Cancer Research*, 1958, **18**, 698.
26. Palade, G. E., and Siekevitz, P., Liver microsomes. An integrated morphological and biochemical study, *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 171.
27. Colter, J. S., Koprowski, H., Bird, H. H., and Pfeister, K., Immunological studies with protein fractions isolated from Ehrlich ascites carcinoma cells, *Nature*, 1956, **177**, 994.
28. Horn, E. C., Ascites tumor development. II. Cytotoxicity of various antisera prepared against Ehrlich ascites tumor cell components, *Cancer Research*, 1956, **16**, 595.

EXPLANATION OF PLATES

PLATE 24

FIG. 1. Two cells incubated with specific antibody demonstrate focal zones of evagination and invagination of their surface membranes. The labyrinthine complex in the center of the photograph represents interdigitation of the surface projections from each cell; such interdigitation is thought to favor agglutination. The nuclei (*N*) of the individual cells are seen at the top and bottom of the photograph. × 30,000.

FIG. 2. *Cell incubated with rabbit immune ferroglobulin.* The ferritin label (dense 55A granules) is arrayed along the plasma membrane in a zone of antibody-induced surface folding. Micellar structure of ferritin is not resolved at these magnifications. × 68,000.



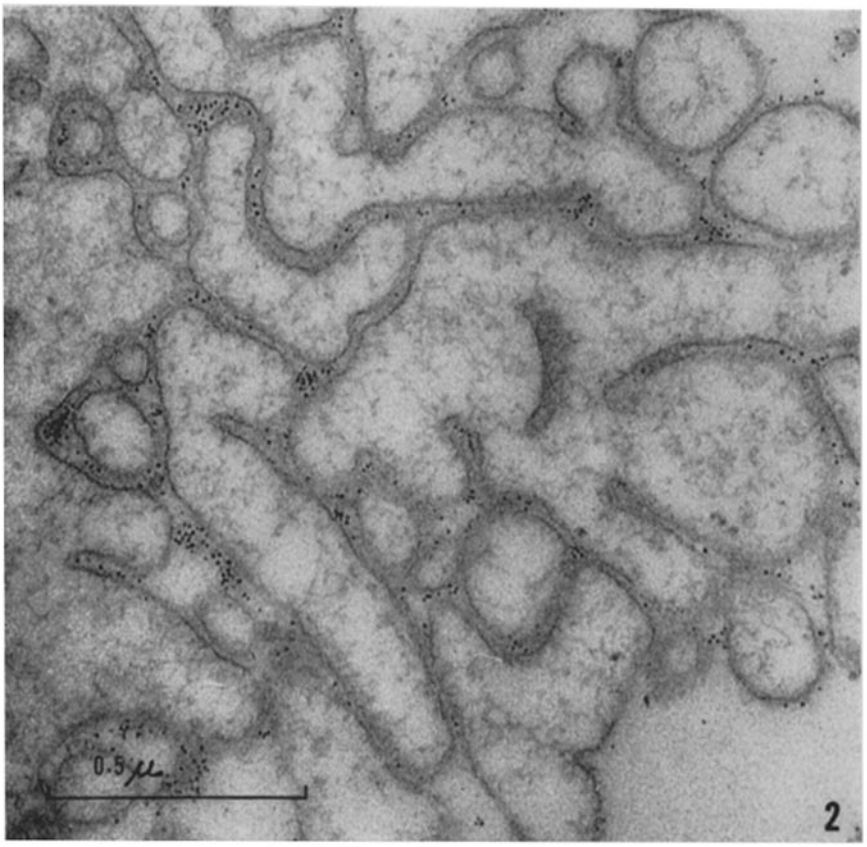


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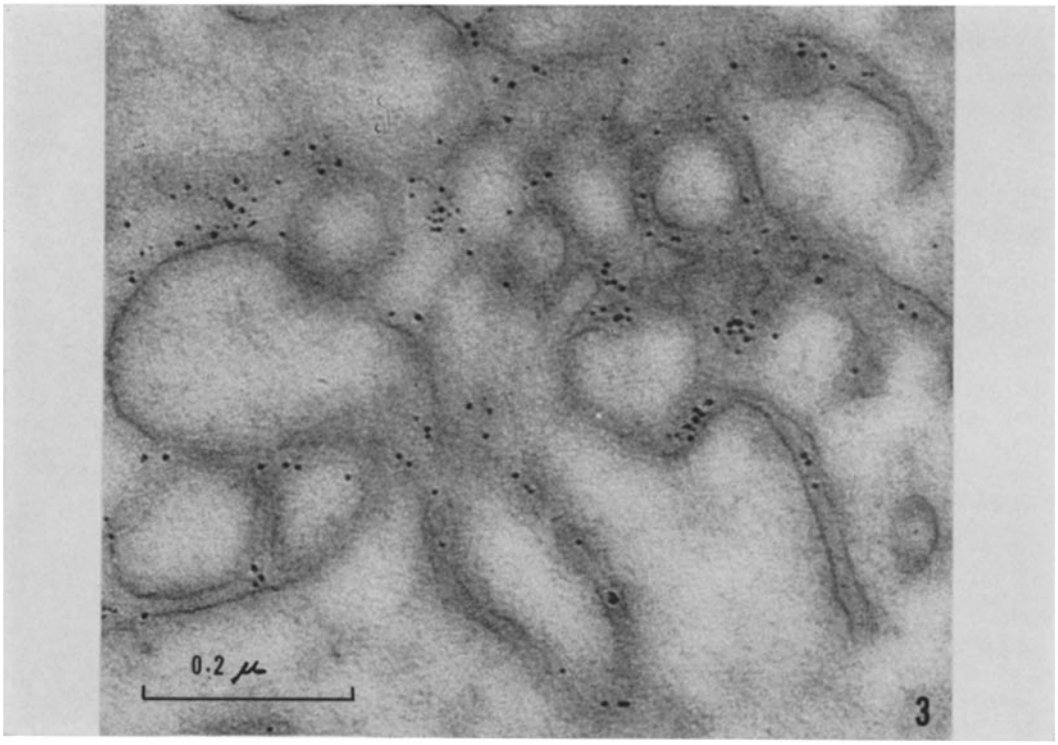
PLATE 25

FIG. 3. *Cell incubated with rabbit immune ferroglobulin.* Zone of surface folding. Antigenic sites in the cell membrane are marked by the ferritin label. × 140,000.

FIG. 4. *Cells incubated with rabbit immune ferroglobulin.* Cell membranes of two adjacent cells indicating pattern of ferritin labeling along folded and unfolded segments of plasma membrane. (*N*) indicates nucleus of one cell. × 68,000.



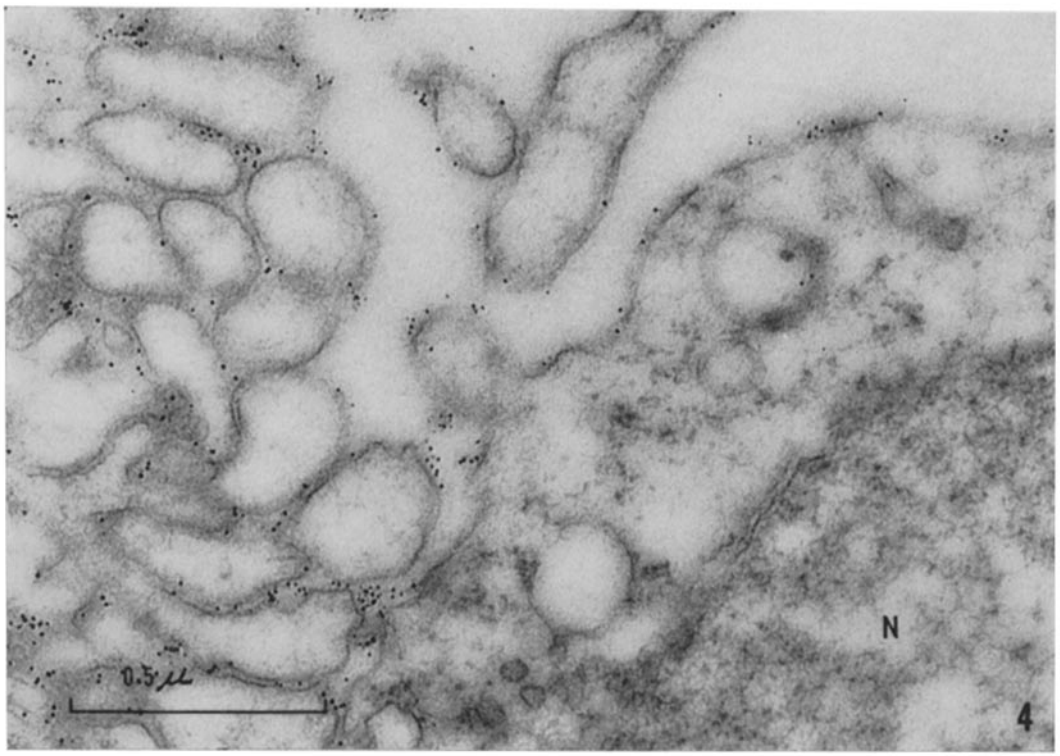


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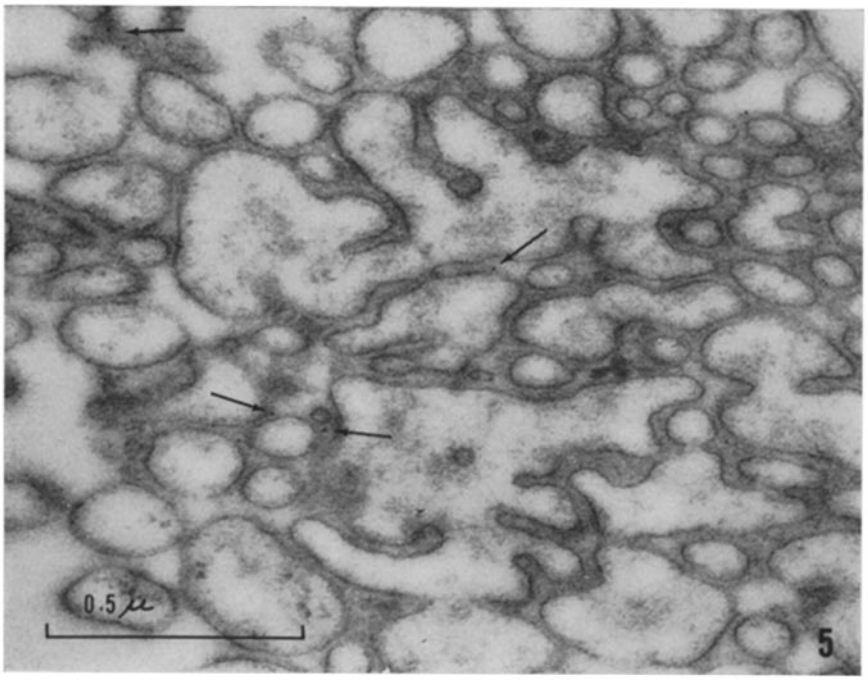
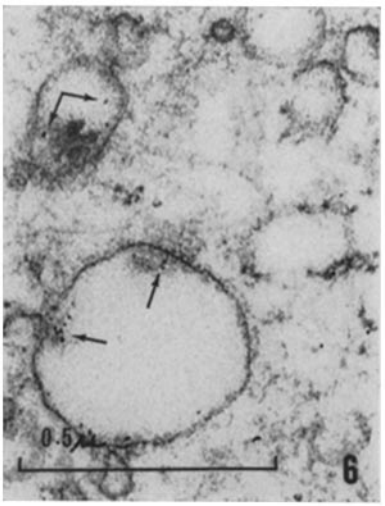
PLATE 26

FIG. 5. *Control cell incubated with ferritin plus rabbit immune gamma globulin.* Note the virtual absence of ferritin in an area of surface folding. Arrows indicate the few ferritin molecules present. Other control cells exposed to free ferritin or to non-immune ferroglobulin also failed to show a significant association of ferritin with the cell membrane. × 68,000.

FIGS. 6 and 7. Pinocytosis of the ferritin label occurred in both experimental and control cells, as indicated by presence of ferritin (arrows) within cavities of the endoplasmic reticulum. Fig. 6, × 68,000. Fig. 7, × 75,000.





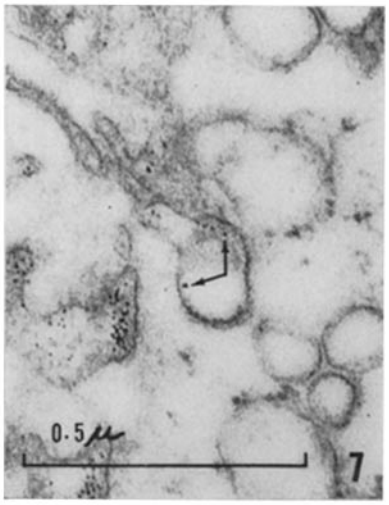


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PLATE 27

FIG. 8. *Cell incubated with rabbit immune ferroglobulin plus complement.* Note swelling of mitochondria (*M*) and rough surfaced endoplasmic reticulum (*ER*). (*N*) indicates nucleus. Ferritin label is seen in characteristic association with the cell surface membrane (brackets). × 63,000.

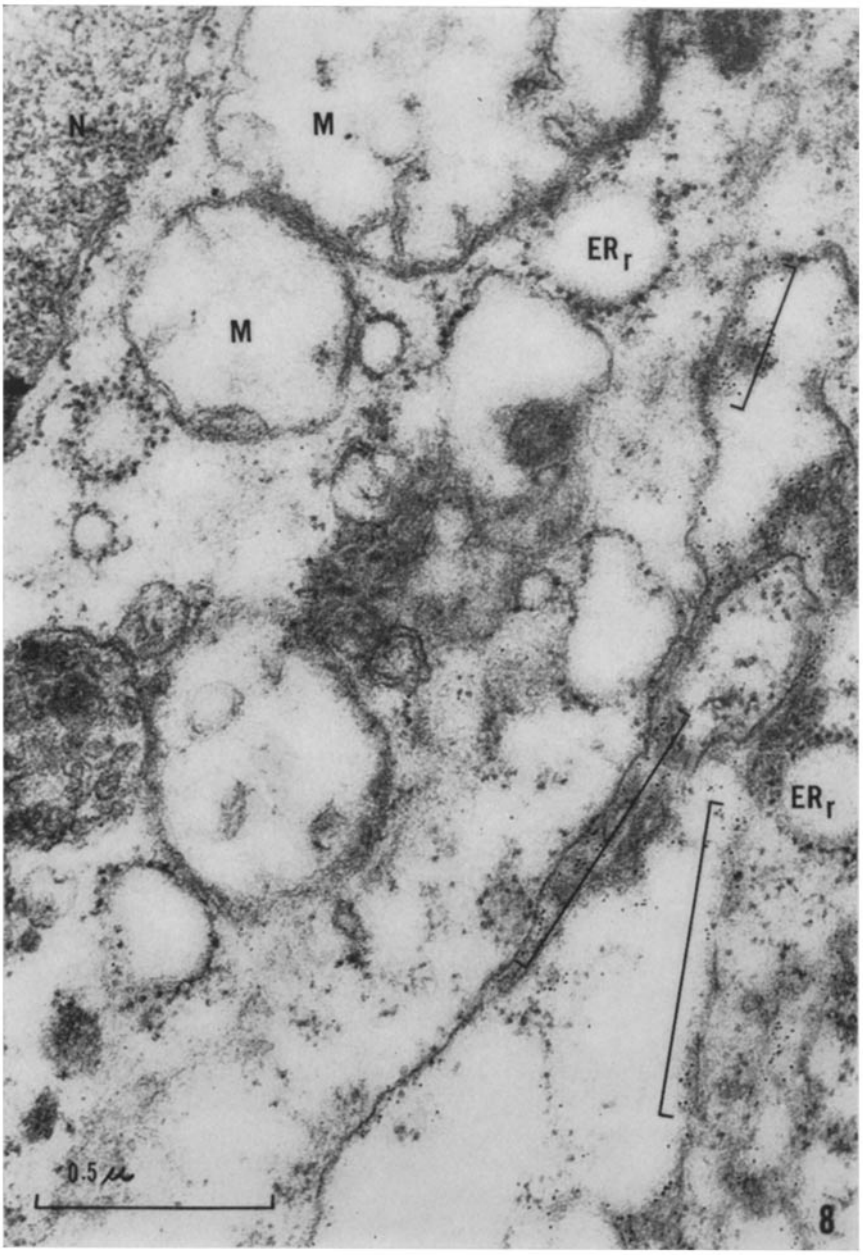

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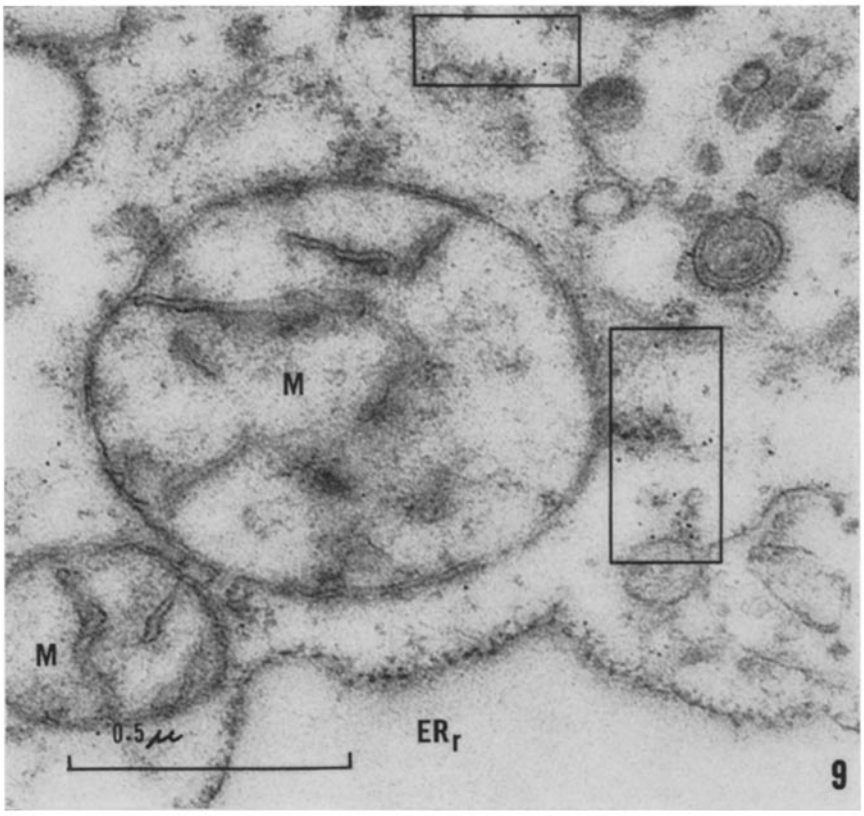
PLATE 28

FIG. 9. *Cell incubated with rabbit immune ferroglobulin plus complement.* Ferritin molecules are scattered throughout the cytoplasmic matrix after antibody-complement action. (*M*), swollen mitochondria; (*ER*), swollen rough surfaced endoplasmic reticulum. × 75,000.

FIG. 10. *Control cell incubated with ferritin plus rabbit immune gamma globulin plus complement.* A few ferritin molecules can be identified in the cytoplasmic matrix. (*M*), swollen mitochondria; (*ER*), swollen rough surfaced endoplasmic reticulum. × 75,000.



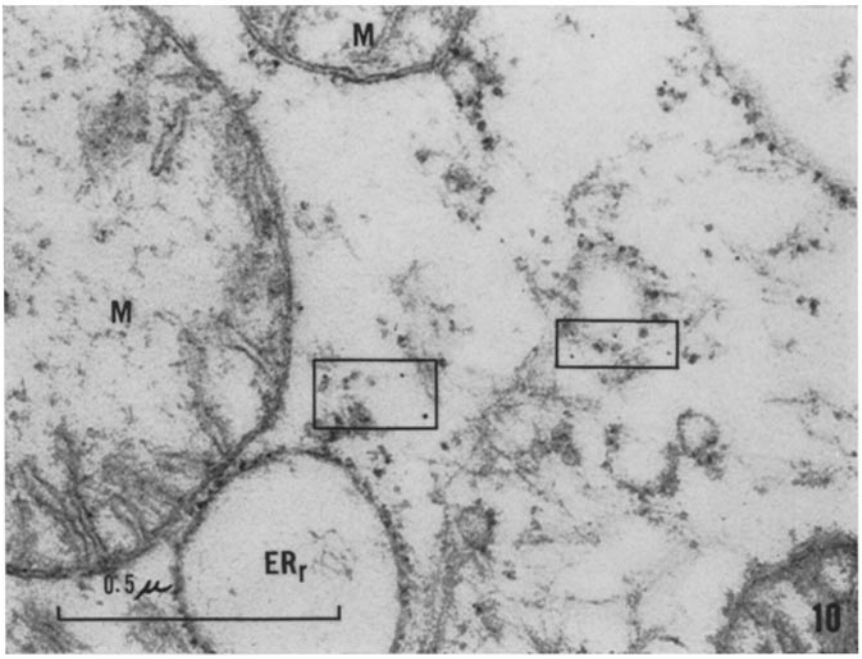


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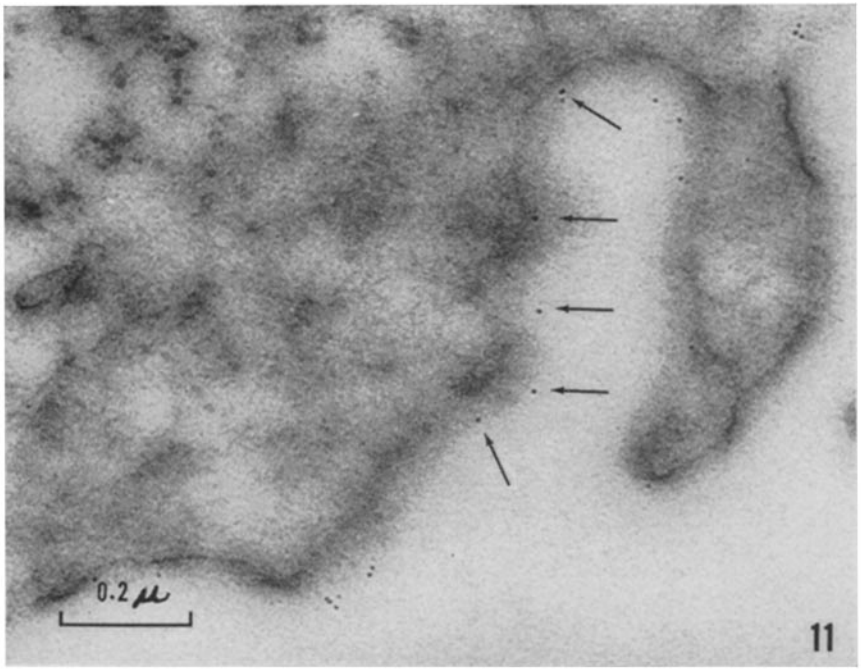
PLATE 29

FIG. 11. *Cell incubated with purified rabbit immune ferroglobulin.* Surface antigenic sites are indicated by the ferritin moiety (arrows) of conjugate. × 91,000.

FIG. 12. *Broken cell preparation-"nuclear fraction."* Some ferritin molecules are seen within the nucleus (*N*), but greater numbers are present on smooth membranes of endoplasmic reticulum (*ER*) and in adjacent amorphous material. × 75,000.



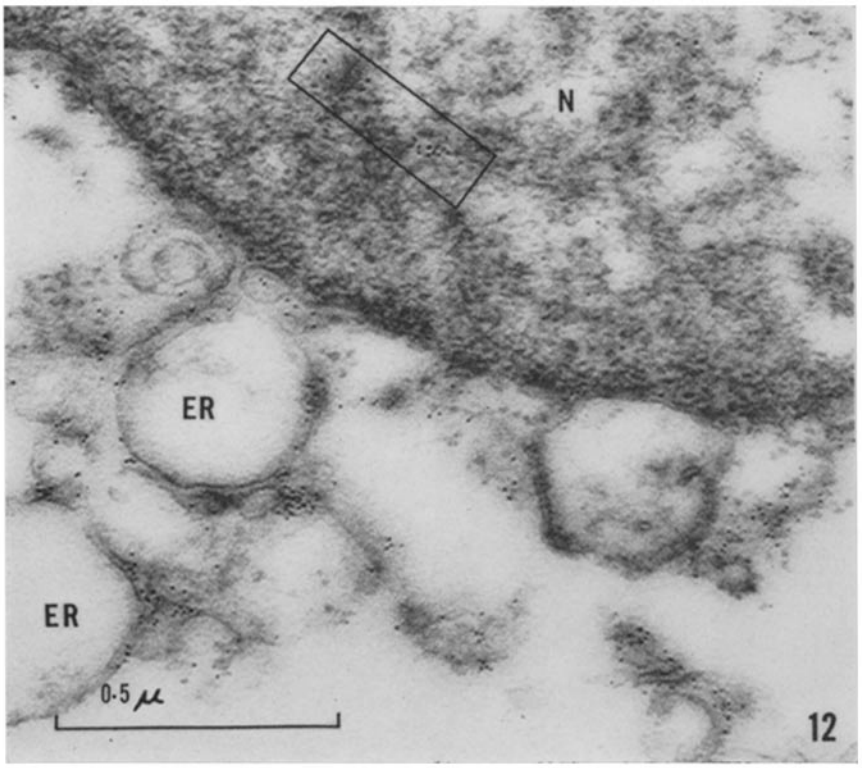


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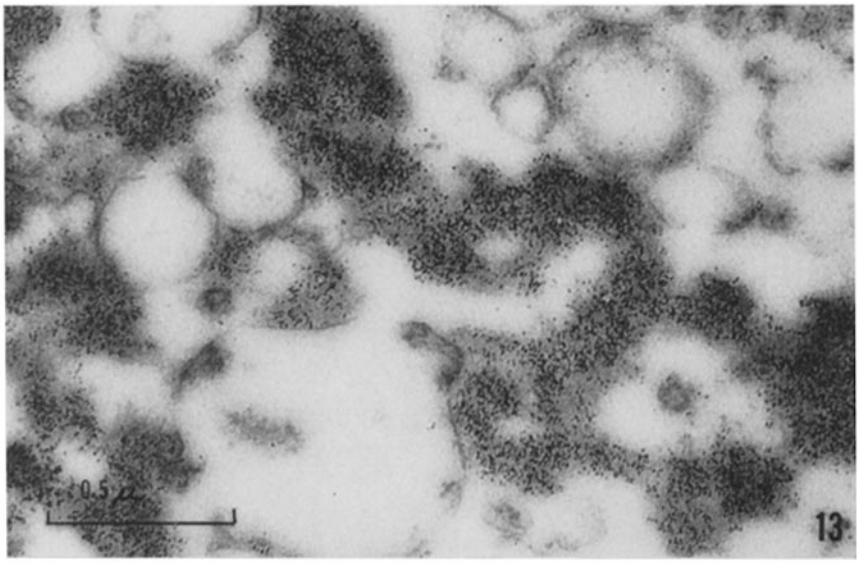
PLATE 30

FIG. 13. *Broken cell preparation-"sphere fraction."* High concentration of ferritin in amorphous material. × 50,000.

FIG. 14. *Broken cell preparation-"sphere fraction."* Association of a large number of ferritin molecules with smooth membrane-bounded profiles. × 75,000.



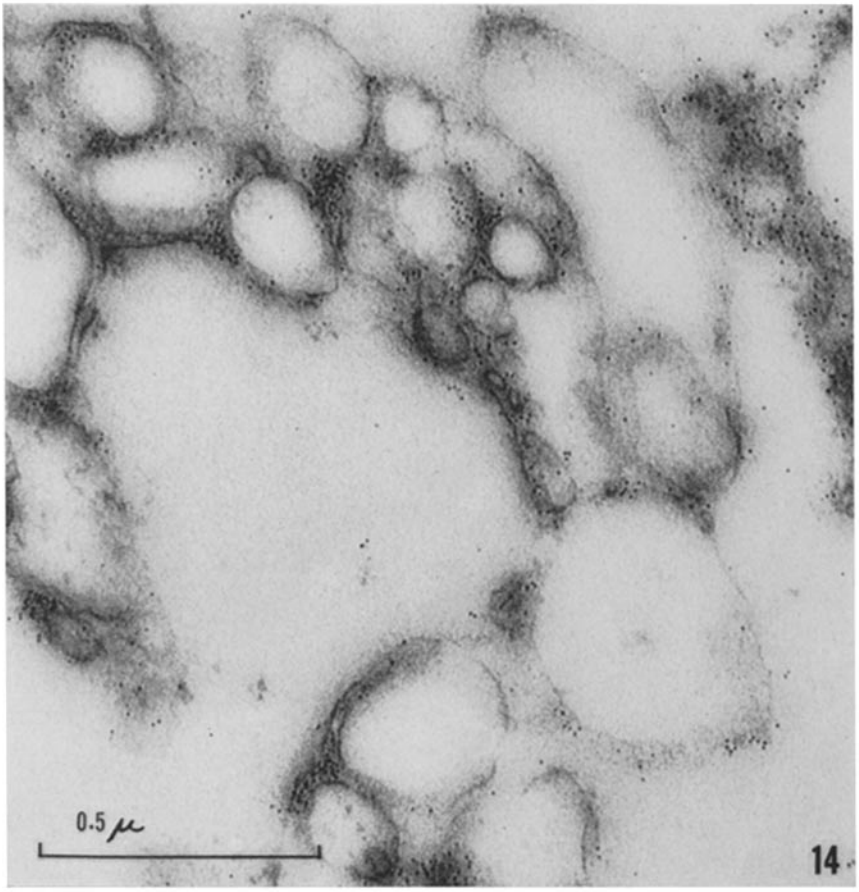


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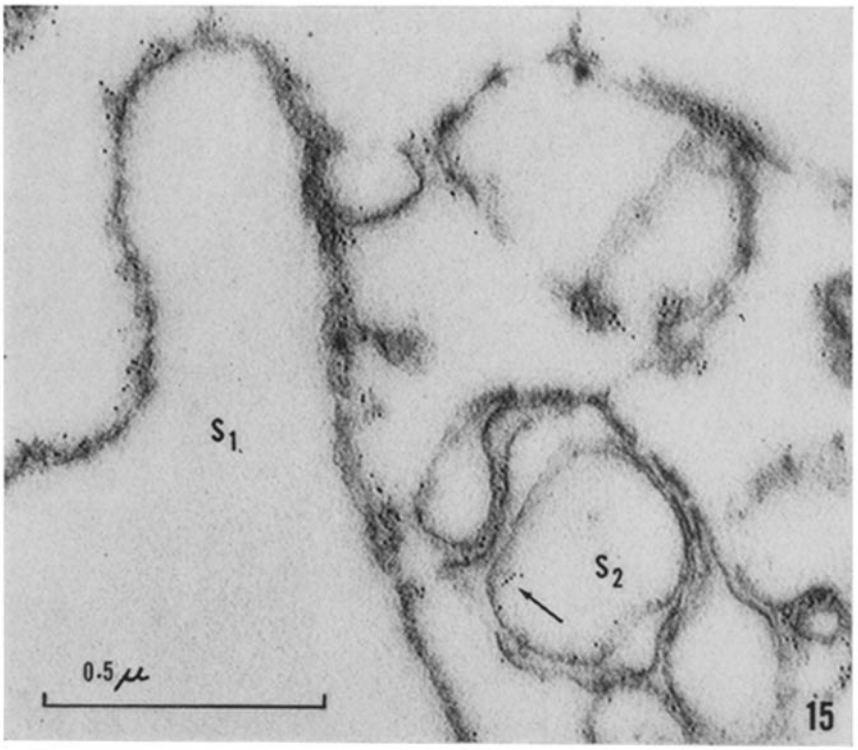
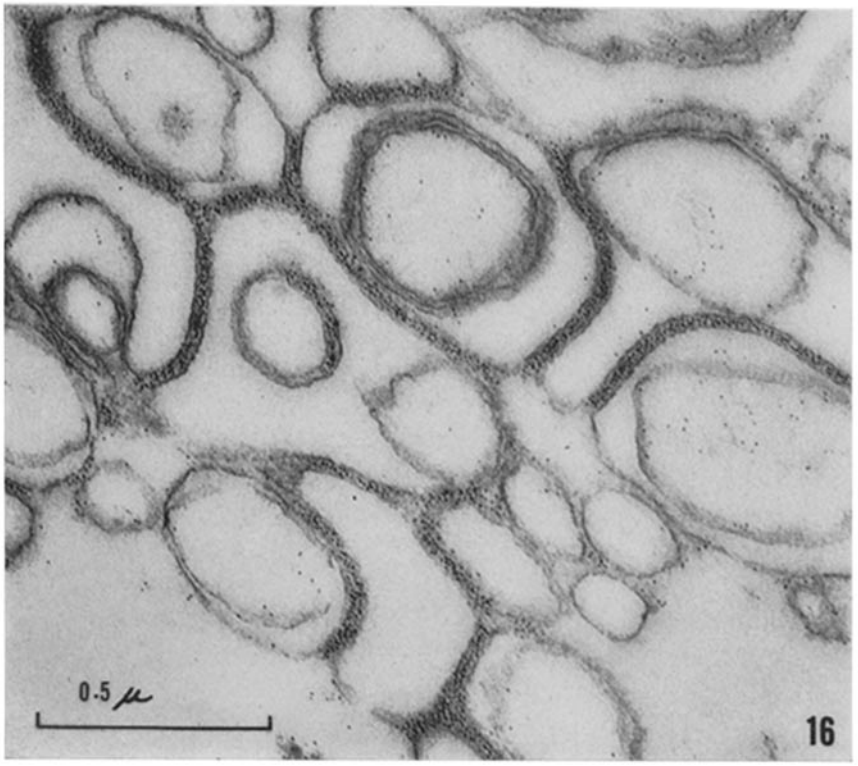
PLATE 31

FIG. 15. *Broken cell preparation-"sphere fraction."* Association of ferritin with surfaces of smooth membranes is illustrated. Very large profile (S_1) is probably derived from a segment of cell surface membrane and smaller spherical profile (S_2) from endoplasmic reticulum. Arrow indicates ferritin molecules within the smaller profile. × 75,000.

FIG. 16. *Broken cell preparation-"sphere fraction."* Very dense accumulations of ferritin between apposed smooth membranes. × 62,000.





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