USE OF HYBRID ANTIBODY WITH ANTI-γG AND ANTI-FERRITIN SPECIFICITIES IN LOCATING CELL SURFACE ANTIGENS BY ELECTRON MICROSCOPY*

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The structure of cell surface membranes has been intensively studied by electron microscopy. Characteristically the cell is bounded by a double membrane. Although this "unit" membrane appears morphologically homogeneous, the complex biological functions of the cell imply that it has specialized parts, which presumably may constitute an integral part of the membrane or may be additions to it.

The antigens that have been recognized on cell surfaces offer a lead to studying this implicit diversity of surface structure. On the thymocytes of the mouse, for example, five systems of alloantigens are distinguishable (1), providing unambiguous markers for components of the cell surface. These we have shown to be arranged on the cell surface in a characteristic pattern (by a technique based on the principle that antibodies to closely adjacent antigens compete with one another for attachment) (2).

For visualizing the location of these components, the obvious choice is electron microscopy with ferritin-tagged antibody. Ferritin can be coupled to antibody with bivalent chemical agents, such as metaxylylene diisocyanate (3), toluene 2,4-diisocyanate (4) or *p,p'*-difluoro-*m,m'*dinitrodiphenyl sulfone (5), and many others (6, 7). Unfortunately these compounds can react at many sites on protein molecules, and so cause linkage of two, or more than two, molecules without discriminating between ferritin and immunoglobulin. The product is therefore highly heterogeneous and requires extensive purification. In addition, chemical manipulation of antibody, particularly of mouse antibody, often results in considerable loss of activity.

In our studies of the topography of thymocyte surface antigens, we have largely overcome these limitations by developing a method of labeling that depends not on covalent linkage of ferritin to antibody globulin, but on the specific combination of ferritin with anti-ferritin antibody. It was suggested by Nisonoff's finding that anti-

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body molecules of dual specificity can be obtained by combining univalent fragments of pepsin-treated antibodies of different specificities (8).

Incubation of γG antibody with pepsin causes cleavage and degradation of the Fc fragment, the combining activity of the antibody being preserved (9, 10). Pepsin-treated antibody is a dimer [$F(ab')_2$] of two identical units, the Fab' fragments, each with one combining site, linked by at least one disulfide bond. Loss of the Fc fragment renders this bond highly susceptible to reduction with mercaptans. The bond can then be selectively split because its oxidation potential is smaller than that of the disulfide bonds linking the light chain to the remainder of the heavy chain in each Fab' fragment. Thus in low concentrations of cysteamine the bivalent $F(ab')_2$ fragment dissociates into two Fab' fragments (9). This is reversible by mild oxidation, which reconstitutes the bivalent $F(ab')_2$ (11). When reconstitution takes place in a mixture of Fab' pieces from antibodies of different specificities, some of the $F(ab')_2$ molecules so formed are "hybrids" with double specificity (8). Recombination of Fab' fragments is thought to occur at random, i.e., without preferential union of Fab' pairs with the same serological specificity.

A hybrid antibody with one specificity for a cell surface antigen and the other for ferritin would provide an ideal reagent for visual labeling. Alternatively, hybrids of anti-ferritin and anti-γG may be made, providing a single reagent that will locate *any* cell surface antigen to which antibody is attached. We report here the preparation of hybrids of the latter type, and their use in locating known antigens on the cell surface.

Materials and Methods

A list of abbreviations appears in Table I.

Antisera.—

Rabbit anti-ferritin: Adult female New Zealand rabbits were immunized with 6 × crystallized horse ferritin (6) incorporated in complete Freund's adjuvant, administered into the footpads and subcutaneously at several sites. Subsequent injections of ferritin in incomplete adjuvant were administered subcutaneously at intervals of 4–6 wk.

Rabbit anti-mouse γ-globulin: Serum from C57BL/6 mice was chromatographed on Sephadex G200 followed by chromatography of the 7S components on DEAE-Sephadex A50 in 0.08 M Tris [tris(hydroxymethyl)aminomethane] HCl, pH 8.0. The front of the peak consisted predominantly of γ2. Rabbits were immunized with this material as described for anti-ferritin (above).

Histocompatibility-2 alloantisera: The positive serum was strain A ($H\text{-}2^a$) anti-C57BL/6 ($H\text{-}2^b$); and the reciprocal immune serum ($H\text{-}2^b$ anti-$H\text{-}2^a$) was used as a negative control.

Isolation of Anti-ferritin Globulin.—Rabbit anti-ferritin serum was fractionated on DEAE-Sephadex A50 in 0.08 Tris HCl buffer, pH 8.0, at 5°C, and the antibody precipitated with a minimal amount of ferritin. The immune precipitate was washed twice with phosphate-buffered saline (PBS) and then homogenized with 0.3 M glycine buffer, pH 2.5, in the cold. The released ferritin was removed by centrifugation at 150,000 *g* in the cold for 30 min. The supernatant was brought to pH 7 and dialyzed against PBS; it is referred to in the text as "purified anti-ferritin." In later experiments, the separation of anti-ferritin from other γG was carried out *after* preparation of $F(ab')_2$ fragments.

Preparation and Hybridization of $F(ab')_2$ Fragments of Antibody.—(Method of Nisonoff [8]). Rabbit γG, anti-mouse γG, or anti-ferritin (before or after isolation from other γG, see above), at a concentration of 10 mg/ml in sodium acetate buffer at pH 4.5, was incubated

with 2% of its weight of pepsin for 16 hr at 37°C. The mixture was neutralized with 1 N NaOH and chromatographed on Sephadex G150. The resultant $F(ab')_2$ preparation was concentrated by vacuum dialysis in collodion bags. 8 mg anti-ferritin $F(ab')_2$ was mixed with 2 mg anti-mouse γG $F(ab')_2$ in 1 ml sodium acetate buffer, pH 5.0. 2-Mercaptoethylamine/HCl (K and K Laboratories, Plainview, N. Y.) was added to a final concentration of 0.015 M and the mixture incubated under N_2 at 37°C for 60 min. The reducing agent was then removed by passage through a column (10 × 120 mm) of a cation exchange resin (AG 50W-X4, Calbiochem, Los Angeles, Calif.) at pH 5.0, and the mixture neutralized with NaOH and reoxidized gently by stirring in an atmosphere of O_2 for 2 hr. Fab' fragments that had failed to dimerize

TABLE I

Table of Abbreviations

Fragment	Rabbit γG	Abbreviation
Fab'	Anti-ferritin	aF
	Anti-mouse γG	aγG
	Nonspecific*	Nγ
$F(ab')_2$	Anti-ferritin	$(aF)_2$
	Anti-mouse γG	$(a\gamma G)_2$
	Nonspecific	$(N\gamma)_2$
$F(ab')_2$ hybrid recombinant	Anti-mouse γG / Anti-ferritin	aγG/aF
	Anti-mouse γG / Nonspecific	aγG/Nγ
	Anti-ferritin / Nonspecific	aF/Nγ
$F(ab')_2$ nonhybrid recombinant	Anti-mouse γG	aγG/aγG
	Anti-ferritin	aF/aF
	Nonspecific	Nγ/Nγ

* Nonspecific rabbit γG: all rabbit γG present other than anti-ferritin or anti-mouse γG.

were removed by chromatography on Sephadex G100 (Fig. 1). Further relevant purification (exclusion of aγG/aγG and aγG/Nγ, see Table I) was achieved by precipitation with a slight excess of ferritin, followed by dissociation of the washed antigen-antibody complex with glycine buffer, pH 2.5, as above. (Precipitation is brought about by reformed bivalent aF/aF molecules, the hybrid aγG/aF being coprecipitated.)

Sensitization of Cells.—10×10^6 washed viable EL4 (C57BL, H-2^b) ascites leukemia cells were incubated at room temperature with excess H-2^a anti-H-2^b serum. Control EL4 cells were incubated in the same concentration of either normal mouse or the reciprocal immune serum H-2^b anti-H-2^a (see above). (Mouse serum lacks effective lytic C′, the cells therefore remaining viable after sensitization with mouse antibody.) The sensitized cells were washed twice in medium 199 and resuspended in the aγG/aF antibody preparation (concentration

adjusted approximately to 0.05 mg/ml with respect to anti-γG). The cells were then spun down and resuspended in ferritin 0.5 mg/ml. After incubation for 30 min, the cells were washed twice with medium 199. At the completion of these procedures >80% of the cells were viable according to their exclusion of trypan blue.

Preparation of Sensitized Cells for Electron Microscopy.—The cell pellets were covered with 4–5 ml of 1% glutaraldehyde (pH 7.2–7.3; osmolality approximately 520–550 milliosmols [12]), for 20 min in ice. After postfixation for 1 hr in cold 1% osmium tetroxide (pH 7.2–7.3; osmolality approximately 290 milliosmols [13]), they were dehydrated in alcohol and em-

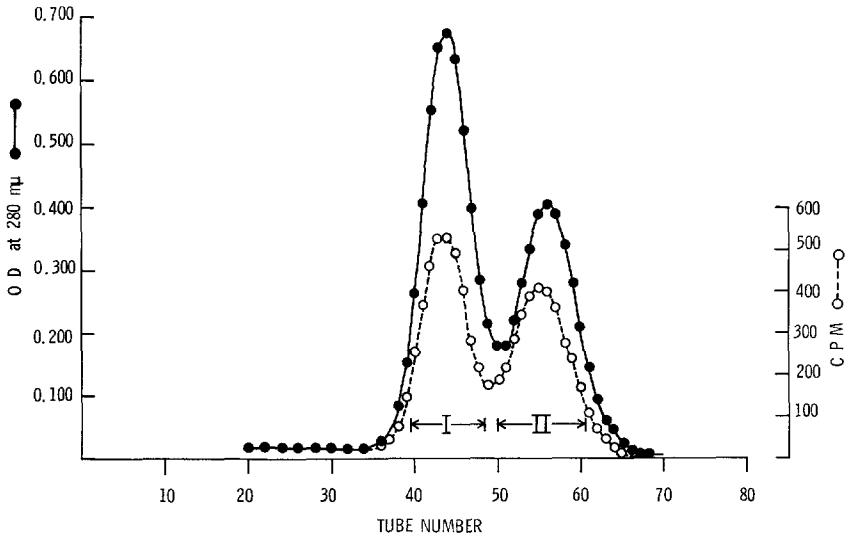

FIG. 1. Sephadex G100 chromatography of reaction mixture after hybridization. Column size, 18 × 1100 mm. 50 mg of protein chromatographed in phosphate-buffered saline. Volume of fractions, 3 ml. Optical density (●—●) monitored at 280 mμ. Radioactivity (○- - - - ○) determined in Bray's solution. Fraction I (tubes 40–49, dimers) was further purified. Fraction II (tubes 50–61, monomers) was again subjected to the hybridization procedure.

bedded in Epon as usual. The blocks were cut on a Sorvall MT-2 ultramicrotome with a diamond knife, and the unstained thin sections were examined with a Siemens Elmiskop 1A electron microscope.

Conventional Preparation of Ferritin-Conjugated Rabbit Anti-Mouse γG.—*Six* times crystallized horse ferritin was conjugated to rabbit anti-mouse γG by metaxylylene diisocyanate (3) using the modification of Andres et al. (6), and purified by chromatography on Sepharose 4B.[1]

RESULTS AND DISCUSSION

A number of technical considerations enter into the efficient preparation of a particular hybrid $F(ab')_2$. Specific antibody always occurs together with globu-

[1] Aoki, T., J. Izard, U. Hämmerling, E. de Harven, and L. J. Old. 1968. Ferritin-conjugated antibody for the detection of isoantigens of murine cells. Presented at the 26th Annual Meeting of the Electron Microscopy Society of America.

lin lacking that specificity; the latter, usually comprising the major part, participates equally with antibody in the hybridization process, uniting with specific antibody to form unwanted combinations, and reducing proportionately the yield of the hybrid required. It is therefore desirable to isolate the specific antibody from other γG.

Anti-ferritin γG can be conveniently separated from other γG by acid dissociation of the specific precipitate. Ferritin, having a high sedimentation constant, is then readily removed by ultracentrifugation. By contrast, purification of anti-mouse γG would require large amounts of mouse γG and its conversion into a suitable immunoadsorbent. Fortunately, the availability of pure anti-

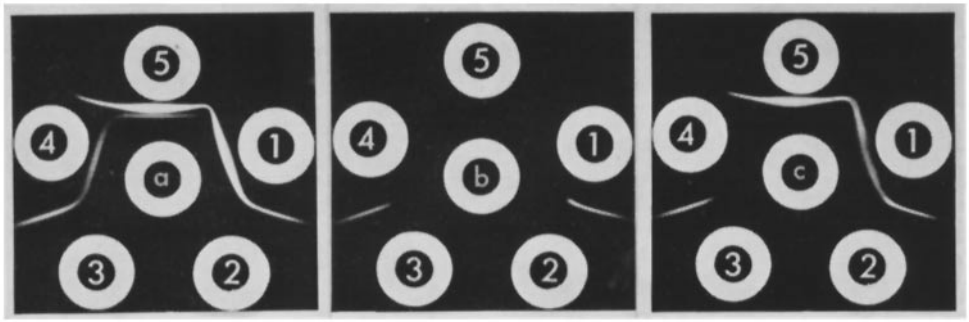

FIG. 2. Immunodiffusion analysis of the hybridization process. Center wells: *a*. Mixture of pepsin-treated purified anti-ferritin $(aF)_2$ and pepsin-treated anti-mouse $\gamma G[(a\gamma G)_2 + (N\gamma)_2]$. *b*. Mixture after reduction by cysteamine (MEA). *c*. Mixture after removal of MEA and reoxidation. Peripheral wells: 1, ferritin; 2, anti-ferritin antiserum; 3, anti-mouse γG-antiserum; 4, mouse γG; 5, mixture of ferritin + mouse γG.

ferritin prepared as above makes the use of purified anti-mouse γG unnecessary. Instead, a considerable excess of $(aF)_2$ can be introduced into the hybridization mixture, thus raising the proportion of $a\gamma$G hybridizing with aF rather than with $a\gamma$G or $N\gamma$.

The events taking place at each step of hybridization are illustrated by Ouchterlony analysis. A mixture of four parts of pepsin-digested purified anti-ferritin $(aF)_2$ with one part of pepsin-digested anti-mouse γG $[(a\gamma G)_2 + (N\gamma)_2]$ precipitates with the corresponding antigens, giving lines of identity with the original antibodies (Fig. 2 *a*). After reduction of the mixture with mercaptoethylamine, precipitation no longer occurs (Fig. 2 *b*). But antigen-binding activity is preserved and can block reaction of antigen with intact antibody; thus in Fig. 2 *b*, univalent antibodies diffusing rapidly from the center well have inhibited the control reactions and produced asymmetry of these precipitin bands. Under the conditions employed, conversion of 5S antibody fragments $[F(ab')_2]$ to 3.5S fragments (Fab') is complete according to analytical ultracentrifugation.

On removal of the reducing agent by passage through a cation exchange resin and exposure to O_2, precipitating [F(ab')$_2$] activity vs. ferritin reappears but precipitating activity vs. mouse γG does not (Fig. 2 *c*), in accord with the expectation of a low yield of unwanted (aγG)$_2$ in comparison with (aF)$_2$ (see below). Asymmetry of the control anti-γG precipitin band can be attributed to blocking by aγG/aF from the center well.

To determine the yield of recombination the reaction mixture was chromatographed on a Sephadex G100 column (Fig. 1). It was calculated from the chromatogram that about 60% of the proteins consisted of dimers and 40% of

TABLE II

Expected Ratios of Various F(ab')$_2$ Molecules Formed from a Mixture of Fab' Fragments

These fragments are equivalent to (*a*) 80% separated anti-ferritin γG (see Materials and Methods) and (*b*) 20% γG from anti-mouse γG serum, of which one-third is specific antibody—assuming random reassociation of Fab' fragments.

Recombinants	Relative concentration			Category
I	II	III	IV	V
aγG/aF*	2(0.067 × 0.8)	= 0.107	0.107	Wanted
aγG/aγG	$(0.067)^2$	= 0.004	0.022	Unwanted
aγG/Nγ	2(0.067 × 0.133)	= 0.018		(Interfering)
aF/aF	$(0.8)^2$	= 0.640		
aF/Nγ	2(0.8 × 0.133)	= 0.213	0.871	Irrelevant
Nγ/Nγ	$(0.133)^2$	= 0.018		
Total		1.000		

* For abbreviations see Table I.

monomers. (Nisonoff obtained a similar value by analytical ultracentrifugation [8].) The low molecular weight peak contained univalent antibodies inhibiting the precipitation of ferritin with anti-ferritin and of mouse γG with anti-mouse γG. It is conceivable that failure of these univalent fragments to form dimers was due to blocking of the SH groups, perhaps by reaction with some mercaptan impurities not completely removed by cation exchange chromatography. In accord with this view, when the hybridiziation procedure was repeated with these monomers they were found to be capable of forming dimers in about the same yield and with the same precipitin reactions illustrated in Fig. 2 *c*.

The main peak of the G100 fractionation comprised reconstituted F(ab')$_2$. Assuming random recombination, the yield of each hybrid, theoretically, is proportional to the product of the relative concentrations of the reaction partners. Table II gives the expected ratios of different F(ab')$_2$ molecules produced

by four parts purified anti-ferritin [$(aF)_2$] with one part anti-mouse γG (comprising in our hands approximately one-third specific anti-γG antibody and two-thirds other γG, the ratio assumed in Table II).

In this case the product is expected to contain 10.7% of the desired hybrid $a\gamma G/aF$, with only 2.2% of the $a\gamma G/N\gamma$ and $a\gamma G/a\gamma G$ recombinants capable of interfering with the serological application by occupying antigen sites without binding ferritin. The expected proportion of original $(a\gamma G)_2$ converted to $a\gamma G/aF$ is about 80%.

Formation of hybrid antibody was confirmed in a hybridization experiment with ^3H-labeled γG-fraction of rabbit anti-mouse γG [$=(a\gamma G)_2 + (N\gamma)_2$].[2] Chromatography on Sephadex G100 again showed the product to consist of about 60% dimers and 40% monomers. The tritium/protein ratio was similar in all fractions (Fig. 1) indicating that failure of recombination affected aF, $a\gamma G$, and $N\gamma$ equally. At each step of the hybridization procedure, an aliquot was withdrawn, mixed with an equivalent amount of ferritin, and centrifuged at 150,000 *g* for 30 min. The label appeared neither in the sediment obtained from the unreacted mixture of labeled $(a\gamma G)_2 + (N\gamma)_2$ and unlabeled $(aF)_2$, nor in the sediment from the reduced mixture. But with the final $F(ab')_2$ hybrid preparation, 40% of the label was present in the sediment. The precipitability of radioactivity indicates the presence of hybrid antibody.

From column III of Table II it can be calculated that after hybridization 0.8 of the radioactivity should be sedimented together with ferritin, leaving 0.2 in the supernate. The values actually observed were 0.4 sedimented and 0.6 unsedimented. This discrepancy may indicate that there is a degree of selective recombination between like pairs, and that recombination is not entirely random.

As the ratio of $a\gamma G/aF$ was lower than expected it was considered worthwhile to remove the other $a\gamma G$ recombinants ($a\gamma G/a\gamma G$ and $a\gamma G/N\gamma$) routinely. This was accomplished by precipitation with ferritin followed by acid dissociation of the immunoprecipitate: 70% of the precipitated hybrid antibody was recovered (according to experiments with ^3H-labeled γG).

Direct evidence for the formation of $a\gamma G/aF$ hybrid molecules is furnished by electron microscopy. EL4 cells, first incubated with H-2^b alloantibody, then with a hybrid antibody preparation, and last with ferritin, displayed under the electron microscope typical electron-opaque granules of ferritin (Fig. 3). In numerous control experiments summarized in Table III ferritin was not seen attached to the cell membrane.

The picture produced by labeling with hybrid antibody differs markedly from that produced by labeling with anti-mouse γG coupled chemically with ferritin

[2] Tritium-labeled antibody was obtained by reaction of the γG-fraction with ^3H-alanine-*N*-carboxy anhydride (analogous to [14]). Method to be described elsewhere.

in the conventional manner (Fig. 4). The latter frequently showed ferritin granules piled at several levels, whereas hybrid aγG/aF antibody showed only a single layer, consistent with the expected binding of single ferritin molecules.

SUMMARY

Hybrid antibody [F(ab′)$_2$] with dual specificity for mouse γG and ferritin was prepared from the corresponding rabbit antisera, providing a precise reagent for locating mouse γG on cell surfaces. Viable cells were exposed successively to (*a*) mouse antibody to a cell surface antigen, (*b*) the rabbit hybrid antibody, and (*c*) ferritin, before preparation for electron microscopy. This method of labeling is sensitive and specific and clearly lends itself to the introduction of visual markers other than ferritin. Other advantages are uniformity of labeling, ease of purification of the reagent, and circumvention of the many drawbacks arising from coupling ferritin to antibody chemically.

TABLE III

Control Experiments Showing the Specificity of Hybrid aγG/aF Antibody*

Serum	F(ab′)$_2$ antibody	Labeling of EL4 leukemia cells (C57BL) by ferritin
A anti-C57BL/6	aγG/aF	Positive
C57BL/6 nonimmune	aγG/aF	Negative
C57BL/6 anti-A	aγG/aF	"
None	aγG/aF	"
A anti-C57BL/6	None	"
"	$(aF)_2$	"
"	$(a\gamma G)_2 + (N\gamma)_2$	"
"	$(aF)_2 + (a\gamma G)_2 + (N\gamma)_2$	"

* For abbreviations see Table I.

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FIG. 3. Hybrid-antibody method of ferritin labeling. C57BL ascites leukemia cell (*H-2*^{b}) incubated first with the serum A anti-C57BL (H-2^a vs. H-2^b); second with anti-mouse γG/anti-ferritin hybrid antibody; and third with crystallized ferritin. The plasma membrane has the typical unit-membrane appearance. A discrete segment of the cell surface is labeled with ferritin, arranged mainly in a single layer. The majority of ferritin molecules are separated from the outer layer of the plasma membrane by a distance of approximately 150–160 A (compare with Fig. 4). × 155,000.

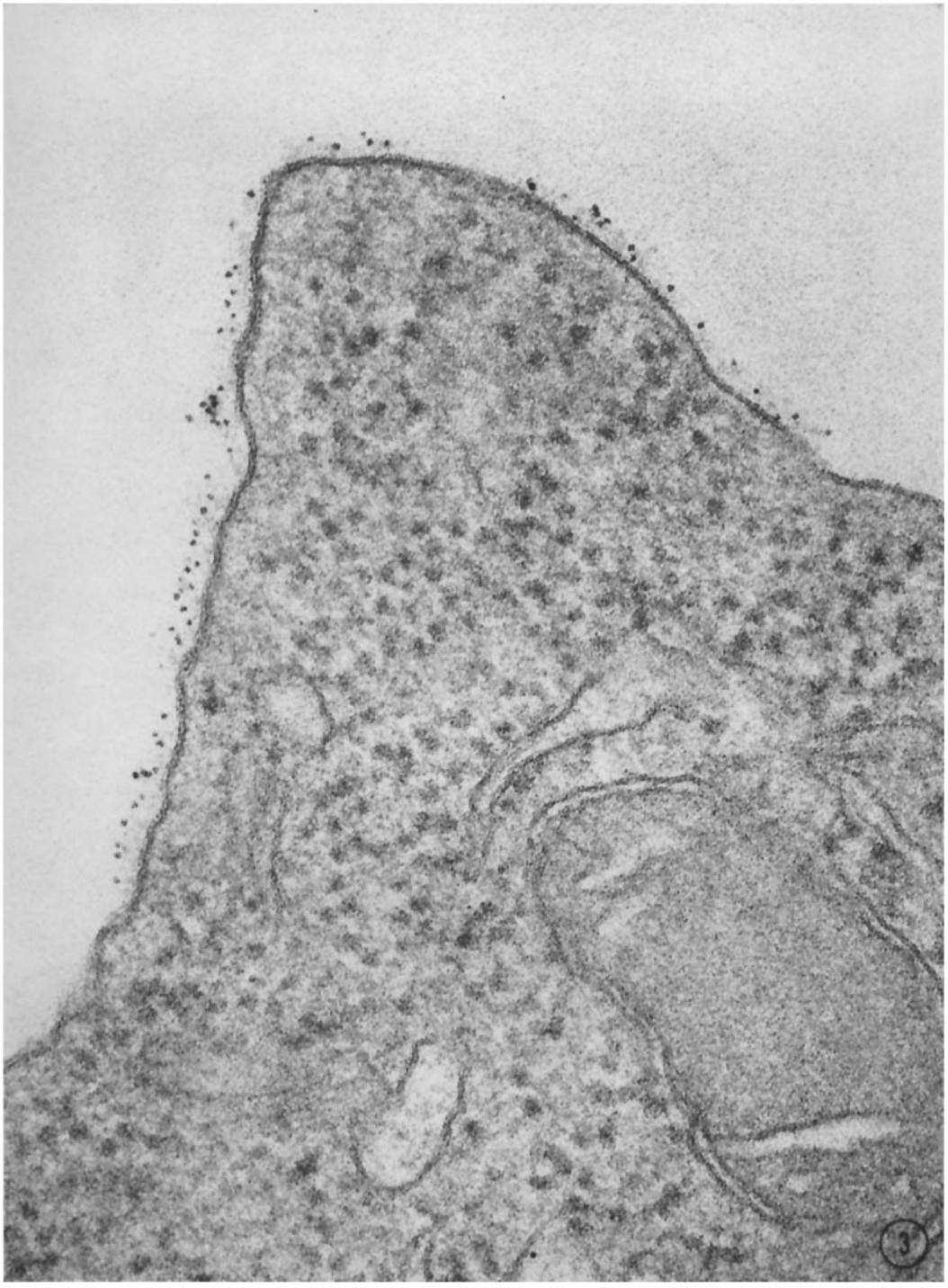
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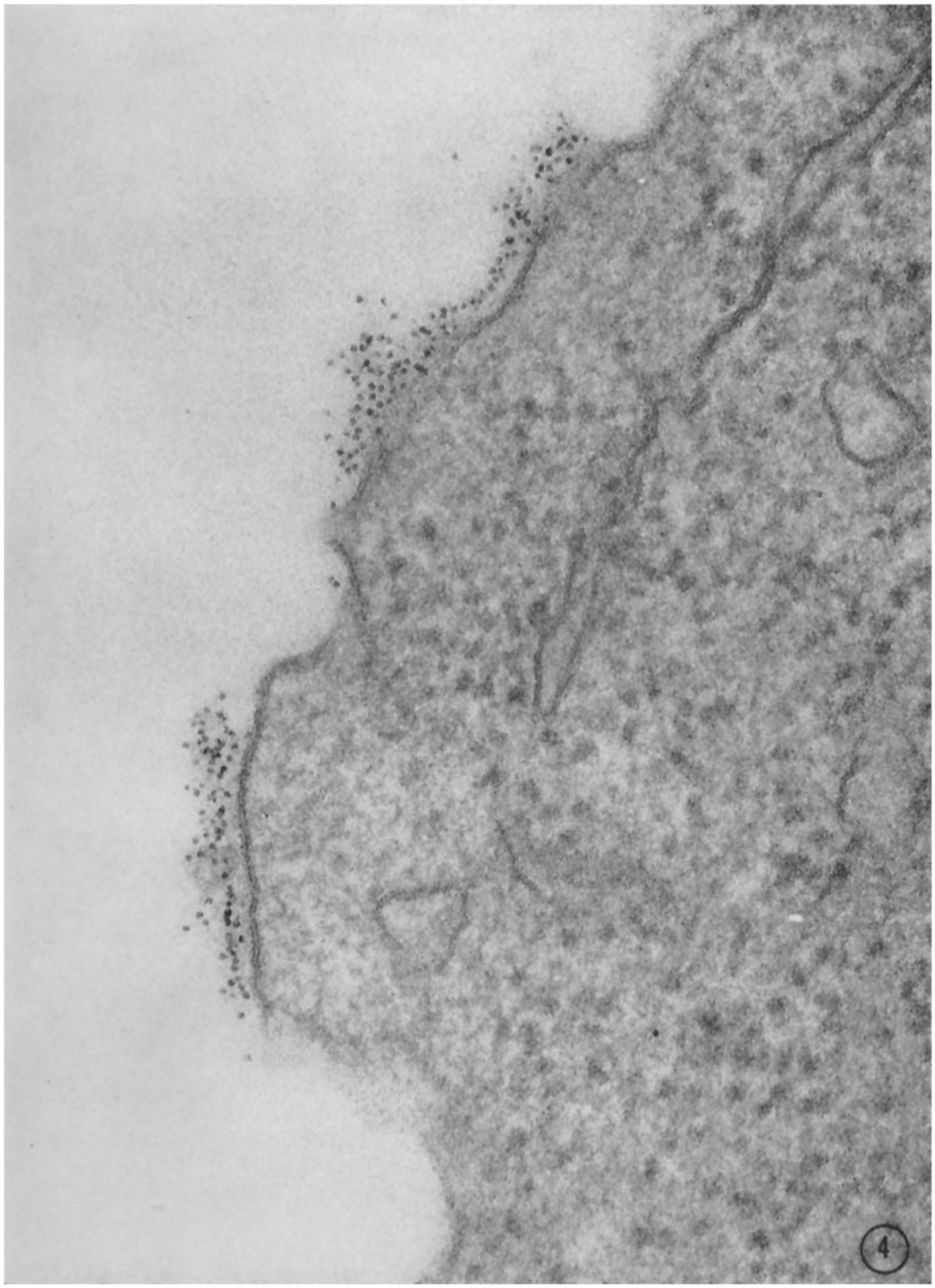
FIG. 4. Conventional method of ferritin labeling. EL4 cell incubated first with A anti-C57BL serum, and second with rabbit anti-mouse γG chemically conjugated with ferritin. Two segments of the cell surface are heavily labeled with ferritin molecules which are situated in dense clusters at various distances from the cell surface. In this case the closest ferritin granules are at a distance of 110–120 A from the cell surface (compare with Fig. 3). × 155,000.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