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Iron–dextran antibody conjugates: General method for simultaneous staining of two components in high-resolution immunoelectron microscopy

(cell ultrastructure/Imposil/ferritin/ultracryotomy)

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ABSTRACT **We report the preparation and properties of an electron-dense antibody conjugate, made by the covalent bonding of an iron–dextran (Imposil) particle to an antibody molecule. Transmission electron microscopic experiments with the Imposil–antibody conjugates demonstrate their suitability as specific immunostains at high resolution, particularly for simultaneous double staining experiments in conjunction with ferritin–antibody conjugates.**

The development of fluorescent-labeled antibodies as specific stains for the light microscopy of biological specimens (1) has been of great importance to cell biology. Particularly valuable has been the technique of double immunofluorescence—the simultaneous application of fluorescent conjugates of *two* different antibodies, made with two spectrally distinct fluophores, such as fluorescein and rhodamine. In order to develop generally useful antibody-staining methods at the much higher resolution available in transmission electron microscopy (immunoelectron microscopy), conjugates of antibodies must be prepared that are highly electron scattering (i.e., have a density of heavy atoms). The first conjugate of this kind was made by the covalent coupling of the iron-rich protein ferritin to antibodies (2). Ferritin–antibody conjugates are now widely used and permit the localization of specific components in biological specimens with a resolution of about 30 nm (3). However, a general technique for carrying out double immunoelectron microscopic experiments at this resolution, by using two distinguishable electron-dense antibody conjugates, has been hampered by the lack of a suitable second electron-dense label that could be used in conjunction with ferritin (see *Discussion*). In this paper we report that this problem has now been met by the preparation of an antibody conjugate that uses iron–dextran as the electron-dense label.

MATERIALS AND METHODS

Horse spleen ferritin was isolated and recrystallized as described (4). An iron–dextran preparation called Imposil (Fisons Ltd., Loughborough, England) is distributed in the United States by Burns-Biotec (Oakland, CA) under the name of Nonemic. The preparation contains 100 mg of Fe per ml in 0.5% phenol. Rabbit antiserum to whole human erythrocytes was obtained from Cappel Laboratories (Downington, PA). Goat antibodies to human spectrin, goat antibodies to rabbit IgG, and rabbit antibodies to goat IgG were prepared by standard procedures and were then affinity purified (5).

Preparation of Imposil–Antibody Conjugates. In order to make a covalent conjugate, the dextran shell of Imposil was partially oxidized with periodate under conditions that did not alter the structure of Imposil. The aldehyde functions that were thus formed were allowed to react with amino functions on the subsequently added antibody molecules to form Schiff bases. These were then reduced to stable secondary amine linkages at the same time that unreacted aldehyde functions were reduced to alcohols by reaction with borohydride. This scheme is similar to that used by Sanderson and Wilson (6). Experiments were carried out to define the optimal conditions of reagent concentrations, pH, temperature, and time to prepare conjugates in high yield and with full retention of antibody activity. These experiments will be presented elsewhere. Here the final procedure only is described.

The Imposil was dialyzed against 0.15 M NaCl to remove the phenol in the commercial preparation and was then freed of uncomplexed dextran by three cycles of centrifugation (6 hr at 60,000 rpm in a Beckmann model LS-75 ultracentrifuge), discarding of the supernatant, and resuspension of the pellet. The final solution contained 80 mg of dextran per ml as determined by the phenol/sulfuric acid method (7) and 40 mg of Fe per ml as determined by a modification of the bathophenanthroline method (8). To 0.5 ml of this solution was added 0.5 ml of 0.1 M sodium periodate in 0.2 M sodium acetate (pH 6.0). Reaction was carried out for 1 hr at 22°C in the dark with stirring. The oxidized Imposil was separated from residual periodate by passage through a Bio-Gel P6 column in 0.15 M NaCl. The solution of oxidized Imposil was then adjusted to pH 8.0 with 0.1 M $NaHCO_3$, and 3 mg of affinity-purified antibody was added. These proportions ensured that there was a large excess of Imposil particles over antibody molecules (unpublished data). The final reaction volume was kept below 5 ml. The conjugation reaction was carried out with stirring for 20 hr at 22°C. After that time, 0.5 ml of a freshly prepared 1% sodium borohydride solution was added and after 15 min the reaction was terminated by dialyzing against 0.1 M sodium phosphate buffer (pH 7.5). Imposil conjugated to antibody was separated from any unconjugated protein and higher molecular weight complexes on a Sepharose 4B column (2.9 × 90 cm) that was equilibrated with 0.1 M sodium phosphate (pH 7.5), with an elution rate of 45 ml/hr. The Imposil in the column fractions was estimated from the absorption of iron at 430 nm; the protein content was measured by using the radioactivity of a small amount of ^{125}I-labeled IgG that had been added with the antibody at the beginning of the reaction. [The protein was iodinated by the chloramine T method (9).] This analytical information allowed us to pool those column fractions that contained predominantly conjugates of one Imposil particle to one antibody molecule (unpublished data). This pool, which contained, along with the conjugate, a substantial amount of unconjugated Imposil, was concentrated by ultracentrifugation for 6 hr at 60,000 rpm. The supernatant was discarded, and the pellet containing the Imposil–antibody conjugate and free Imposil was resuspended to a protein concentration of approximately 1 mg/ml in 0.1 M phosphate buffer (pH 7.5). Stock

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solutions prepared in this manner were used in the immunostaining experiments.

Ferritin-conjugated antibodies were prepared as described by Kishida *et al.* (10).

Electron Microscopy Experiments. Three types of experiments were performed to test the Imposil–antibody conjugates either by themselves or together with ferritin–antibody conjugates. (*i*) *External surface antigens on human erythrocytes.* Freshly drawn blood was used for the preparation of erythrocytes and ghosts. The ghosts were incubated with rabbit antiserum directed against whole human erythrocytes, and then with Imposil or ferritin conjugates of affinity-purified goat antibodies to rabbit IgG or mixtures of the two conjugates. After appropriate washing, the treated ghosts were fixed in 2% glutaraldehyde for 1 hr at 4°C and without further staining were mounted on carbon-coated formvar grids.

(*ii*) *Spectrin in human erythrocyte ghosts.* This preparation was intended to be closely similar to that used in previous ferritin–antibody experiments (11). After treatment of intact erythrocytes with 1 mM $ZnCl_2$ (11) for 15 min at room temperature, the cells were gently homogenized, the ghosts were pelleted, and the pellets were frozen and thawed twice to render them permeable to the antibody reagent. After addition of the goat antispectrin antibodies followed by Imposil-conjugated rabbit antibodies to goat IgG and appropriate washing, the labeled ghosts were then fixed with 2% glutaraldehyde and 2% osmium tetroxide for 2 hr each and then embedded in Spurr's medium (12).

(*iii*) *Spectrin in ultrathin frozen sections of intact human erythrocytes.* Erythrocytes, washed in isotonic phosphate buffer (pH 7.4), were fixed by first treating them with 0.2 M ethylacetimidate/1% formaldehyde for 1 hr at 5°C, followed by 1% glutaraldehyde/1% formaldehyde for 1 hr at 5°C (13). The fixed erythrocytes were washed in the phosphate buffer and stored overnight at 5°C. They were then infused with 0.6 M sucrose, frozen, and ultrathin-frozen sectioned at −70°C. After thawing and treatment with goat antispectrin antibodies, the sections were then mixed with Imposil or ferritin conjugates of affinity-purified rabbit antibodies to goat IgG or with mixtures of the two conjugates. After the antibody staining, the sections were post-stained with uranyl acetate (14).

All specimens were examined in a Philips EM-300 electron microscope at 60 kV.

RESULTS

Preparation and Properties of Imposil–Antibody Conjugates. The conjugation reaction was carried out with an excess of Imposil particles over IgG molecules such that no free IgG remained, as determined by gel chromatography of the reaction product mixture. Fractions from this chromatographic separation were obtained and pooled that contained predominantly a 1:1 conjugate of Imposil and antibody, along with free Imposil, for use in the immunoelectron microscopy experiments. Details of the gel chromatographic separation and the calculation of the stoichiometry of the conjugates, together with

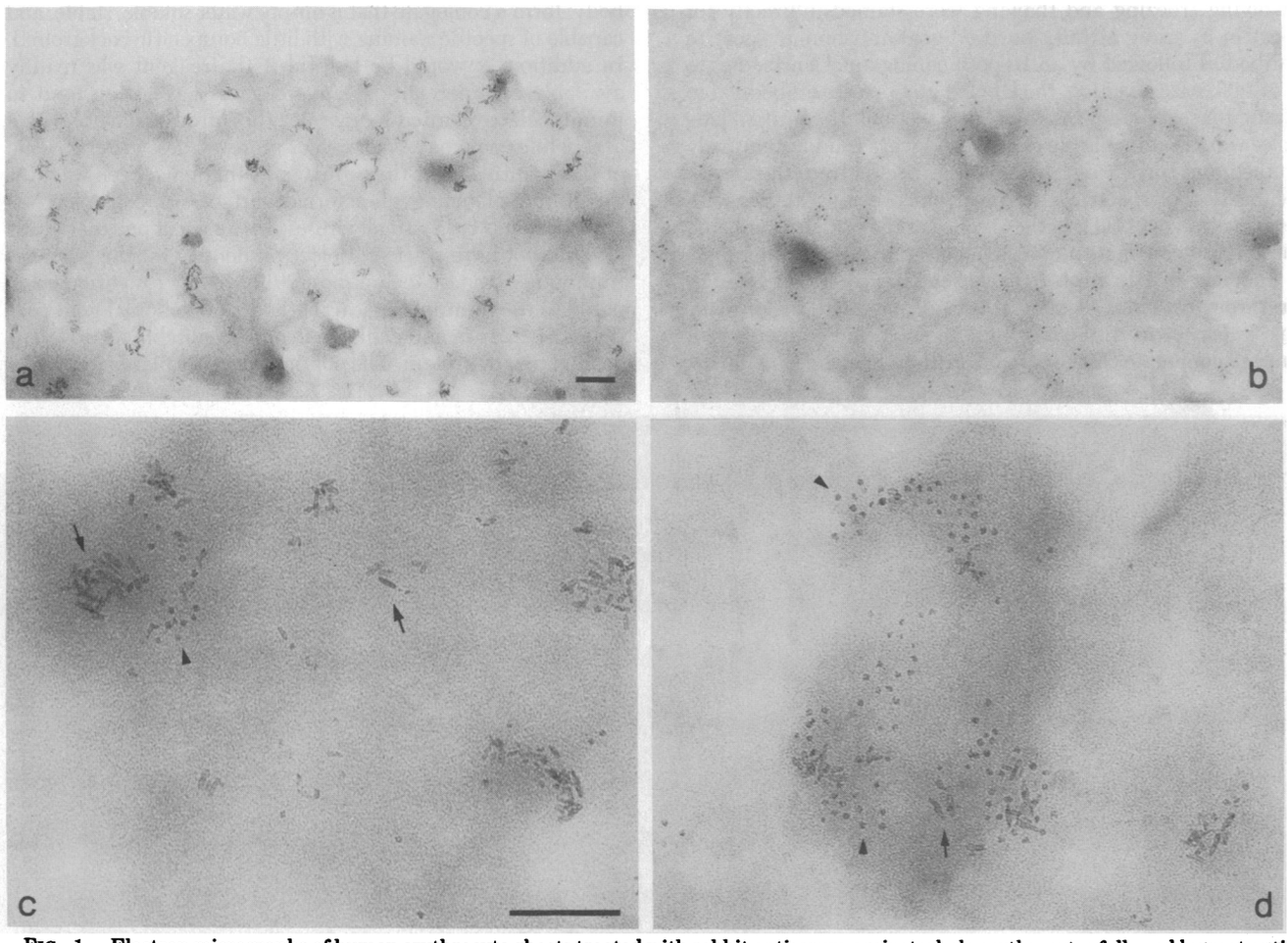

FIG. 1. Electron micrographs of human erythrocyte ghosts treated with rabbit antiserum against whole erythrocytes followed by goat anti-rabbit IgG antibodies conjugated with Imposil (*a*) or ferritin (*b*). (*c* and *d*) A mixture of Imposil-conjugated and ferritin-conjugated goat anti-rabbit antibodies was applied to the ghosts after treatment with the primary antibody. (*c*) An excess of the Imposil-conjugated goat anti-rabbit antibodies over the ferritin conjugate was used. (*d*) Ferritin conjugate was used in excess over the Imposil conjugate. The arrows and arrowhead indicate Imposil and ferritin particles, respectively. Bars represent 0.1 μm.

evidence by Ouchterlony experiments that the conjugates retained the binding activity of the antibody, will be presented elsewhere. After 6 months of storage of the Imposil–antibody conjugates at 4°C in the presence of 0.02% NaN_3, there was no detectable change in their properties.

External Surface Antigens on Human Erythrocyte Membranes. With a primary rabbit antibody directed to whole human erythrocytes, indirect staining of erythrocyte membranes with either an Imposil–antibody conjugate (Fig. 1*a*) or a ferritin–antibody conjugate (Fig. 1*b*) directed to rabbit IgG gave very similar results. Clusters of either Imposil or ferritin were of similar size and spatial distribution, and the general density of staining of the membrane surface was similar for the two conjugates. In experiments in which the indirect staining was carried out with mixtures of the Imposil–antibody and ferritin–antibody conjugates, either in an excess of the Imposil conjugate (Fig. 1*c*) or an excess of the ferritin conjugate (Fig. 1*d*), the two types of particles could readily be discriminated even in close proximity to one another. In the absence of the primary rabbit antibody, the Imposil conjugates alone did not bind to the membrane to a significant extent. If, after treatment with the primary rabbit antibody followed by unconjugated goat antibodies to rabbit IgG the membranes were then treated with the Imposil conjugate, no significant staining resulted (not shown). These controls testify to the specificity of the staining observed in Fig. 1.

Spectrin in Human Erythrocyte Ghosts. In these experiments, erythrocyte ghosts rendered permeable by a procedure involving freezing and thawing were stained indirectly for spectrin by using affinity-purified goat anti-human spectrin antibodies followed by an Imposil conjugate of antibodies to goat IgG. After fixation, the labeled ghosts were embedded in plastic for sectioning. In such plastic sections, the Imposil particles were readily visible (Fig. 2) and were found exclusively on the inner surface of the ghost, as expected from the known localization of spectrin (15) and from previous studies with ferritin conjugates (11). In the absence of the primary antibody, the Imposil conjugate alone gave no significant staining.

Spectrin in Ultrathin Frozen Sections of Intact Human Erythrocytes. These sections, after treatment with the primary goat antispectrin antibodies, were then labeled with either an Imposil conjugate (Fig. 3*a*) or a ferritin conjugate (Fig. 3*b*) of rabbit antibodies to goat IgG or with a mixture of the two conjugates (Fig. 3*c*). The patterns and densities of labeling by the two conjugates were very similar (compare Fig. 3 *a* and *b*) and, in mixtures, the Imposil and ferritin particles were easily distinguished. Controls, treated first with goat antispectrin antibodies and then with conjugated rabbit anti-goat antibodies prior to the application of the mixture of Imposil and ferritin conjugates, were negative (Fig. 3*d*).

DISCUSSION

The primary objective of this investigation was to prepare an electron-dense antibody conjugate that, in conjunction with the ferritin–antibody conjugate, could be of general use for the simultaneous staining of two components in the same specimen at high resolution. For many problems in cell biology, such double staining experiments are essential in order to understand the molecular mechanisms involved. The requirements for such a second electron-dense reagent are, however, demanding and difficult to meet. Its molecules or particles must: (*i*) possess a sufficient electron density to be individually visible in transmission electron microscopy, as is the case with ferritin; (*ii*) be characterized by a distinctive and uniform morphology that can be readily distinguished from ferritin; (*iii*) be of the same order of size as ferritin, so as to permit a similar high resolution of staining as ferritin–antibody conjugates (≈30 nm); (*iv*) be capable of covalent conjugation to antibody molecules under conditions sufficiently mild to retain their own morphology as well as the antibody activity; and (*v*) after reaction with antibody, form a conjugate that is amply water soluble, stable, and capable of specific staining with little nonspecific background. In addition, it would be helpful if the reagent was readily available commercially. Many reagents have been used in immunoelectron microscopy since the introduction of ferritin and, while each may meet one or more of these criteria, none of them fulfills all of them. Reagents that have been used for the immunolabeling of external surfaces of cells, such as hemocyanin (16, 17), small viruses (18), or small polymer beads (19), do not have sufficient electron density for the purpose. Immunoperoxidase techniques (20) have low resolution compared to the immunoferritin method, and colloidal gold particles (21, 22) are not available in a form suitable for covalent conjugation to protein. The commercially available iron–dex-

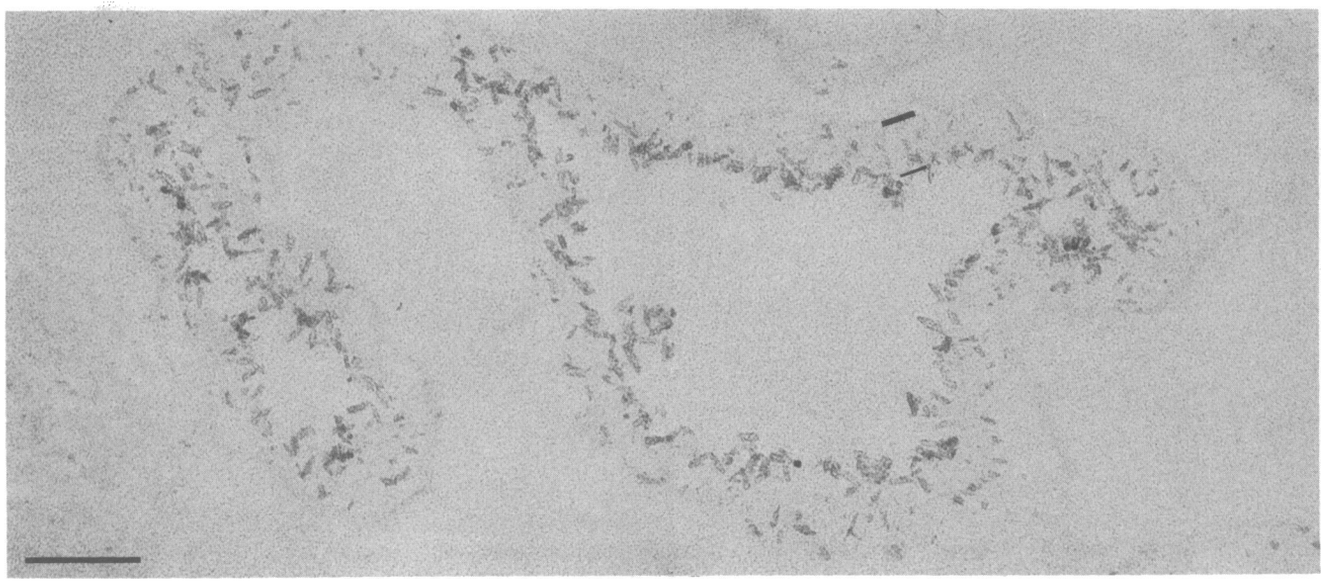

FIG. 2. A thin plastic section of Zn-treated and frozen-thawed erythrocytes labeled with goat antispectrin antibodies followed by Imposil-conjugated rabbit antibodies to goat IgG. Imposil labeling (thin black line) of the spectrin on the inner surface of the erythrocyte ghost may be seen. The thick black line denotes the outer surface of the membrane. Bar represents 100 nm.

tran preparation called Imposil, however, satisfies all the requirements quite well.

Imposil is one of a series of preparations that have been developed for use as hematinics. The particles of Imposil consist of an electron-dense core of a ferric oxyhydroxide micelle complexed to and surrounded by an electron-translucent shell of alkali-modified dextran (23). Electron microscopic studies (23) have shown that the particles of Imposil have an oblong core and a less oblong shell. The particle size distribution is fairly narrow; the average dimensions of the core are 30 × 110 Å and those of the shell, 120 × 210 Å. The oblong shape of the Imposil core is readily distinguished in transmission electron microscopy from the isometric core of the ferritin molecule.

The iron–dextrans have been known for some time, but they have not previously been used in a systematic way for immunoelectron microscopy. An iron–dextran preparation called Imferon, which differs from Imposil in having an isometric core of smaller average size as well as a smaller dextran shell, was used as a noncovalent stain for concanavalin A (for which it has an affinity) by Martin and Spicer (24). We have defined a set of reaction conditions and separation procedures that result in the preparation, in good yield, of an Imposil–antibody conjugate with the following chemical properties (unpublished data). (*i*) The conjugates consist predominantly of one Imposil particle linked by one or more stable covalent bonds to one antibody molecule, together with some unconjugated Imposil that does not interfere with the specific staining. (This free Imposil could be removed from the conjugate, if desired, on some suitable ion-exchange column, but we have not found it necessary to do so.) (*ii*) The reaction conditions are sufficiently mild to have no detectable effect on the structure of Imposil or the activity of the antibody. (*iii*) The conjugates are stable and active for prolonged periods when protected from contamination.

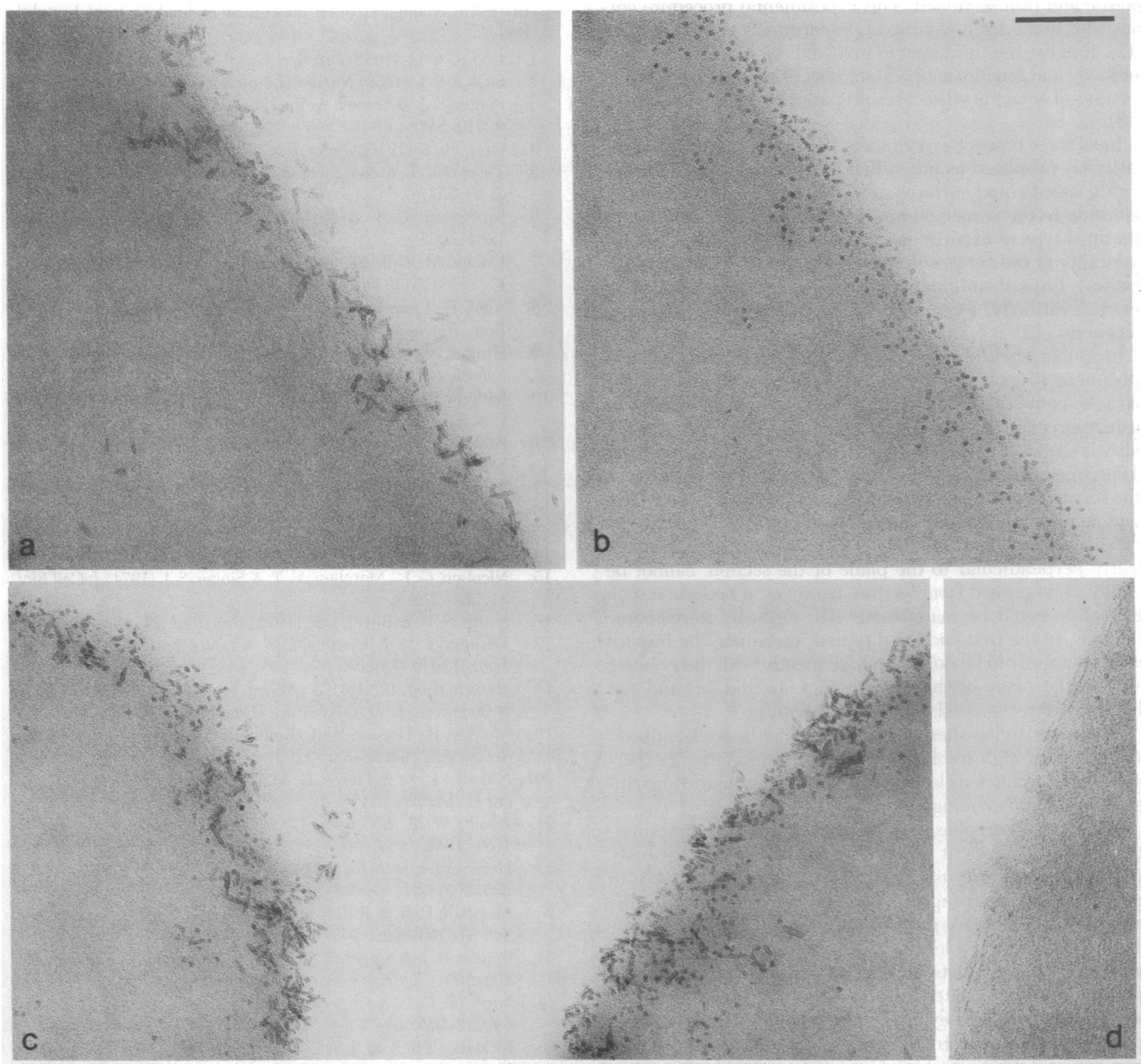

FIG. 3. Frozen sections of erythrocytes labeled with goat antispectrin antibodies followed by Imposil conjugated to rabbit anti-goat antibodies (*a*), goat antispectrin antibodies followed by ferritin conjugated to rabbit anti-goat antibodies (*b*), and goat antispectrin antibodies followed by first the Imposil conjugate and then the ferritin conjugate (*c*). (*d*) A control in which treatment with antispectrin antibodies was followed by incubation with unconjugated rabbit anti-goat antibodies and then a mixture of the Imposil and ferritin conjugates. Bar represents 0.1 μm.

Three different types of immunoelectron microscopic experiments were carried out to test the specificity of staining by the Imposil–antibody conjugates and their suitability for double staining experiments with ferritin–antibody conjugates. The three types of experiments represent three different kinds of specimen preparation that have been used in immunoelectron microscopy previously. For such testing, the systems used were chosen for convenience and availability and not with the object of obtaining new biological information. In the first system described, the Imposil–antibody and ferritin–antibody conjugates were used as indirect stains for the same antigenic components on the exterior surfaces of erythrocyte ghosts. The ghosts were then mounted intact and flat on Formvar-coated grids for electron microscopic examination. This preparation is similar to that used by Nicolson and Singer (25) in ferritin–antibody experiments. In the second system, the spectrin of erythrocyte membranes was indirectly stained with an Imposil–antibody conjugate; the specimen was embedded in plastic and thin-sectioned. This experimental procedure corresponds to the ferritin–antibody experiments of Painter *et al.* (11). Finally, the third system involved the indirect Imposil–antibody and ferritin–antibody staining of spectrin on ultrathin sections of intact erythrocytes prepared by frozen-sectioning (13).

In all three types of experiments, the Imposil–antibody could easily be visualized as individual particles. The staining was highly specific and corresponded closely in intensity and distribution to the corresponding ferritin–antibody staining. In the third type of experiment, the results indicate that the accessibility of the antigen in the ultrathin frozen section to the indirect Imposil–antibody stain was comparable to that of ferritin–antibody, a very important consideration in such experiments (13).

In double labeling experiments on the first and third type of specimens (Figs. 1 *c* and *d* and 3*c*) the Imposil and ferritin particles could easily be distinguished from one another, even in an appreciable excess of one or the other. This distinguishability is apparently less efficient in plastic-embedded sections of specimens that have been immunostained before embedding (the second type of experiment, Fig. 2) because of the nearly random orientation of the Imposil particles with respect to the plane of the section; the oblong core of Imposil, when oriented nearly perpendicular to the plane of the section, cannot be readily distinguished from ferritin. However, if necessary, such distinction could be made more efficiently by stereoscopic methods. In the first and third type of specimen, the Imposil particles appear to lie down most frequently with their oblong cores in the plane of the section (Figs. 1*a* and 3*a*) and can therefore be recognized with little ambiguity.

All of these studies attest to the suitability of Imposil–antibody conjugates for high-resolution double immunostaining experiments in transmission electron microscopy in conjunction with ferritin–antibody conjugates.

Although our emphasis has been on such double immunostaining experiments, it is evident that the Imposil–antibody conjugates should be useful as single stains alternatively to ferritin–antibody conjugates. Because the average outer dimension of the Imposil particle is somewhat larger than that of the ferritin molecule, the resolution attainable with Imposil–antibody conjugates might be slightly less than with ferritin–antibody conjugates, but this difference should not ordinarily be significant. They should also be appropriate as specific stains for scanning electron microscopy since the Imposil particles have a distinctive external morphology. In addition, there are other iron–dextran preparations with average particle sizes both smaller (26) and larger (23) than Imposil that could be covalently conjugated to antibodies by the same procedures used in this investigation. This would provide a battery of distinguishable electron-dense antibody conjugates for multiple simultaneous staining of several components in the same specimen. Finally, the conjugation of Imposil to proteins other than antibodies, as has been carried out in many instances with ferritin, should be feasible by the methods described.*

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* The iron–dextrans bind noncovalently to a number of lectins, including concanavalin A, wheat germ agglutinin, and ricin. If Imposil conjugates of lectins are to be prepared or if double immunostaining experiments are to be carried out with Imposil–antibody and ferritin–lectin conjugates, such nonconvalent binding must be inhibited.

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