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Invited Review

Antigen detection on resin sections and methods for improving the immunogold labeling by manipulating the resin

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Summary. Considering the importance of immunolocalization of cellular substances combined with good ultrastructure and ease of use, this review is focused on the use of resin and the possibilities of manipulating the resin before and after embedding in order to improve the immunolabeling of resin sections for electron microscopy. The qualities of acrylic resins and conventional epoxy resin for immunoelectron microscopy are discussed. Acrylic sections are usually more suited for immunoelectron microscopy than conventional epoxy sections. Different etching procedures (sodium ethoxide or sodium metaperiodate) may be applied to conventional epoxy sections to enhance the yield of immunolabeling. Lately, a method which does not involve any kind of etching has been developed for enhancing the immunogold labeling of epoxy sections up to about 8 times. This method involves increased concentration of accelerator in the epoxy resin mixture when processing the tissue. The ultrastructural preservation of the tissue is important in immunoelectron microscopical procedures, and not only the intensity of the immunolabeling; in this respect no resin may compete with the widely used epoxy resins.

Key words: Acrylic resin, LR-White, Epoxy resin, immunogold, Electron microscopy

I. Introduction

IA. History

The most important progresses concerning embedding for electron microscopy occurred in the fifties and sixties. In the very late forties pure resin embedding was introduced by the use of butyl methacrylate (Newman et al., 1949). The methacrylate resins became popular because of their good cutting qualities, but sections of methacrylate blocks were unstable in the electron beam and damage due to polymerisation were often present. In the sixties, epoxy resins became popular because of good preservation of the ultrastructure and because of excellent stability in the electron beam. On the other hand, it was difficult to perform immunoelectron microscopy on epoxy sections. During the last 15-20 years methacrylate resins with crosslinking qualities have been available, and these resins are well suited for immunoelectron microscopy and the stability of the sections in the electron beam is somewhat better than for the original methacrylates (Newman and Hobot, 1987). Examples of such acrylic resins are Lowicryls and LR-White. Even if they have better qualities than older methacrylates for ultrastructural purposes, they do not match the epoxy resins in this respect. Immunolabeling of cryosections has also been popular during the last decades, and by using that technique no resin surrounds the tissue during immunolabeling (Roos and Morgan, 1990).

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IB. Qualities of different resins

Epoxy resins are in several ways superior to other resins as embedding medium. The polymerization occurs without significant shrinkage. Epoxy resin introduces intermolecular crosslinks, and these may act as a "fixative" on proteins and nucleic acids. Epoxy sections are stable when exposed to the electron beam, which allows long observation times in the electron microscope (Hayat, 1989).

The true epoxy monomers in the resin mixture have a chemical structure where the reactive epoxy groups may be concisely described as cyclic ethers with two carbon atoms and one oxygen atom in the ring. Tertiary amines, often DMP-30 (Tris[dimethyl-aminomethyl] phenol), are used as accelerators to start the polymerization process (Mark et al., 1986). When enough epoxy monomers are present, secondary hydroxyl groups produced will add to the epoxies, and the polymerization process starts. To harden the plastic, two kinds of cyclic anhydrides of carboxyl acids are used:

NMA (Nadic Methyl Anhydride); and DDSA (Dodecenyl-Succinic Anhydride). These anhydrides will compete with the secondary hydroxyl groups mentioned above and add to the epoxy groups (Mark et al., 1986). The polyvalent character of the epoxy monomer, makes polymerized epoxy resin highly crosslinked. The reactive epoxy groups have a great tendency to react with hydroxyl and amino groups, which are chemical side groups present in biological macromolecules such as proteins and nucleic acids. Therefore the biomolecules will be parts of the polymer network when embedded in epoxy resin (Causton, 1984; Kellenberger et al., 1987; Hayat, 1989).

Acrylic resins (Lowicryls, LR-White) are polymerized by another mechanism: free radical chain polymerization. Free radicals react with double bounds of the acrylic monomer, and a new radical, which is one monomer larger, is produced. Monomers will continue to be added in this way and the polymer grows larger until its growth is terminated (Munk, 1989). The free radicals have no affinity for proteins and nucleic acids, and therefore biomolecules are not incorporated into the polymer network (Kellenberger et al., 1987).

This article reviews the use of resin and the possibilities of manipulating the resin in order to improve the immunolabeling of resin sections. To do this properly, it is necessary to consider the mechanism for how antigens are detected when being located in resin sections with special interest being shown to the interaction between resin and antigens. How knowledge of these mechanisms is used to improve the immunolabeling of epoxy sections will be illustrated.

II. Immunoelectron microscopy on thin sections

There are two fundamentally different ways of performing postembedding immunoelectron microscopy of sections on grids. The first one is to perform immunoEM on a section where the embedding media is present. The alternative is to use a immunoEM technique where the embedding media is removed from the section prior to immunolabeling. Intuitively, it seems reasonable that immunoreagents will have better opportunities to penetrate into the sections if there is no embedding media present. An example of the first type is immunolabeling of acrylic sections and epoxy sections, and an example of the second type is immunolabeling of cryosections and deplasticized epoxy sections (Mar and Wight, 1988; Roos and Morgan, 1990). We may also perform types of postembedding immunoelectron microscopy which may be classified between the two groups mentioned above where the resin is chemically manipulated prior to immunolabeling without being removed.

IIA. Immunolabeling of acrylic sections and epoxy sections

Some of the 'new' acrylic resins mentioned in the introduction were not originally constructed for immunoelectron microscopy. For instance, LR-White was fabricated to be a non-toxic alternative to epoxy resins for use in conventional, ultrastructural EM. Other acrylic resins, like Lowicryl K4M, were constructed to be a hydrophilic resin. The primary reasoning is that when the water is removed from the tissue, hydrophilic components in the monomer mixture will interact with the macromolecules, and thereby the network of hydrogen bounds is maintained. In this way the biological structures will be kept as natural as possible (Carlemalm et al., 1982).

The acrylic resins showed generally better qualities for immunoEM than epoxy resins, and it was suggested that the reason was that aqueous immunoreagents penetrated easily into the 'hydrophilic' sections. It was claimed by Newman and Hobot (1987) that antibodies and secondary peroxidase reagents can penetrate about 200 nm into LR-White sections with an incubation time of 30 minutes for the primary antibody. Similar results were claimed by Ellinger and Pavelka (1985). The theory of penetration of antibodies into LR-White sections was rejected by Brorson et al. (1994), and the claimed 'penetration' was unveiled as an artefact. Other scientists examined the ability of antibodies and antibody gold conjugates to penetrate into fixed and thawed cryosections (Stierhof et al., 1986; Stierhof and Schwarz, 1989) by reembedding immunolabeled cryosections in epoxy resin. No general penetration of antibodies or their conjugates was observed. Immunomarkers were only seen inside the reembedded cryosections in areas where the sections were interrupted. Examination of the capability of antibodies to penetrate deplasticized epoxy sections did not show any general antibody penetration into these sections (Brorson and Skjørten, 1995). 'Penetration' of antibodies was only observed in the periphery of compact structures such as hormone vesicles.

Another theory to explain why acrylic sections are generally better suited for immunoelectron microscopy than epoxy section was proposed by Kellenberger et al. (1987): "Epoxies are able to form covalent bonds with biological material, particularly with proteins. Co-polymerization of epoxies with embedded tissues occurs, while polymerized acrylic resins permeate embedded tissues without binding to them. Accordingly, during cleavage the behaviour of embedments with epoxies and acrylics is different. Without co-polymerization (acrylics), the surface of cleavage tends to follow the areas of least resistance, e.g., the interfaces between resin and proteins. In epoxy-embedded material, however, the resistance in these interfaces is not very much less than in the proteins, and when cutting epoxy-embedded tissue, the surface of cleavage has greater tendency to divide the proteins. Therefore antigens will protrude more easily from acrylic sections than from epoxy sections, and acrylic sections will achieve larger amplitudes on their surface". This means that penetration of immunoreagents into the resin section is not necessary

to explain the amount of immunogold labeling observed (see also Eneström and Kniola, 1995).

It has also been proposed that the hydrophobic character of the conventional epoxy sections has reduced their ability to be used for immunolabeling. But it was demonstrated by Dürrenberger et al. (1991) that there is now significant difference in immunolabeling due to whether hydrophobic or hydrophilic sections are used, assuming that unspecific labeling is prevented. This was proved by showing that the hydrophobic Lowicryl resin HM20 is equally well suited for immunoelectron microscopy as the hydrophilic Lowicryl resin K4M.

IIB. Etching and deplasticizing of epoxy sections

Etching agents such as $NaIO_4$ and H_2O_2 have been used prior to the immunoprocedure to enhance the yield of immunolabeling (Herrera et al., 1993). If the tissue has been fixed by osmium tetroxide before embedding, these oxidation agents will reoxidize the osmium tetroxide and remove it from the section. This may contribute to facilitate immunolabeling (Baskin et al., 1979). But the immunolabeling is increased after such etching even if osmium tetroxide is not initially used as fixative (Eneström and Kniola, 1995). But the immunolabeling is normally not enhanced so much with these etching agents as with sodium ethoxide, even if interesting results are achieved by using boiling solutions of sodium metaperiodate (Stirling and Graffs, 1995). The oxidation of the section will introduce hydrophilic chemical groups on the epoxy resin (Causton, 1984). It has been claimed that this increased hydrophilicity facilitates immunodetection. From the results of Dürrenberger et al. (1991) mentioned in chapter IIa, this explanation seems dubious. A more reliable theory proposes that the oxidation breaks epoxy resin bonds and thereby increases the accessibility of the epitopes on the surface of the section. This theory has also been suggested to explain the observation that oxidized epoxy sections are easier to stain than untreated sections (Pfeiffer, 1982).

Heating the thin sections by microwaves or by other methods has been used for antigen retrieval by releasing some of the fixations bounds produced by formaldehyde or glutaraldehyde (Wilson et al., 1996). It is possible that such treatment also releases some of the bounds between epoxy resin and the fixed tissue.

The epoxy resin may be totally removed from the sections by treating them with strong sodium ethoxide solution, which will enhance the immunolabeling (Mar and Wight, 1988; Baigent and Müller, 1990; Brorson and Skjørten, 1995, 1996b), but the ultrastructural preservation may suffer. It was confirmed by Brorson and Skjørten (1995) that general penetration into the deplasticized sections is not the mechanism for the improved yield of immunogold labeling. Antibody 'penetration' was only detected in the periphery of compact structures such as hormone vesicles. It was also demonstrated that the labeling intensity for deplasticized epoxy sections was not crucially different from the corresponding labeling of LR-White sections. By a mathematical approach, Brorson and Skjørten (1996b) concluded that proteins embedded in acrylic resin gain immunolabeling by having less tendency to get the outermost calotte cut off during the cutting procedure (Kellenberger et al., 1987), while they lose by having some parts hidden by resin. Deplasticized proteins, originally embedded in epoxy resin, win by having no part hidden by resin, while they lose by having a great tendency to get the outermost calotte cut off during the cutting procedure. Often, high concentrations of sodium ethoxide do not themselves destroy epitopes in such a way that the intensity of the immunogold labeling suffers (Brorson, 1997).

III. Theoretical study of the intensity of immunolabeling of acrylic sections and epoxy sections

As mentioned in chapter II, Kellenberger et al. (1987) proposed that epoxy sections are less suited for immunoelectron microscopy than acrylic sections because of the 'epoxies' tendency to react chemically with the antigens resulting in low amplitudes on the surface of the epoxy sections. Inspired by this theory the ratio of immunogold labeling of LR-White sections and epoxy section (L_{lrw}/L_{ep}) was deduced mathematically by Brorson and Skjørten (1996c), and is given by the formula:

$$L_{lrw}/L_{ep} = \begin{cases} 1 : d \le r_{ep} \\ \left(d/r_{ep}\right)^2 : r_{ep} < d \le r_{lrw} \\ \left(r_{lrw}/r_{ep}\right)^2 : d > r_{lrw} \end{cases}$$

where 'd' is the diameter of the protein carrying the epitopes. The two parameters r_{ep} and r_{lrw} are measures (in nanometers) for how tightly the antigen molecule is bound to the polymer network of epoxy resin and LR-White, respectively (the lower the r-value, the more tightly the antigen is bound to the polymer). The 'r-values' are manifested in different height amplitudes on the surface of the LR-White and epoxy sections (Fig. 1), and are specific for each protein. Typical amplitudes of an epoxy section are 1-3 nm, and corresponding values of an acrylic section are 3-6 nm (Kellenberger et al., 1987). A consequence of the formula for L_{lrw}/L_{ep} is that the advantage of using acrylic resin to epoxy resin is largest when immunolabeling large proteins (Fig. 2).

IV. Improvement of the labeling of epoxy sections without using any etching agents

As mentioned above, the yield of immunolabeling

on epoxy sections may be increased by using strong etching agents to remove the resin partly or totally. But the etching agents may damage the ultrastructural preservation (Mar and Wight, 1988; Baigent and Müller, 1990; Brorson and Skjørten, 1995, 1996b).

Another way of improving the immunolabeling was worked out by Brorson and Skjørten (1996a,c). The method has been produced from the mathematically based theory mentioned in chapter III, which shows that the immunolabeling of epoxy sections would increase if we could make chemical changes in the epoxy resin mixture that made the antigens less tightly linked to the epoxy polymer network. The practical solution was to increase the concentration of accelerator, DMP-30, in the infiltration and embedding steps when processing the tissue. This embedding resin is called high-accelerator epoxy resin, and the sections cut from the resulting blocks are named high-accelerator epoxy sections. When

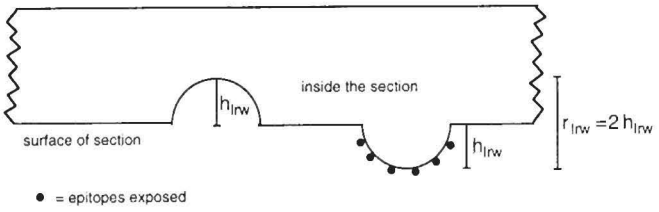

Fig. 1. Illustrates the surface of an LR-White section. The figure shows the maximum height difference r_{lrw} (= $2h_{lrw}$), for the surface when cutting this particular protein (a figure for an epoxy section will be similar).

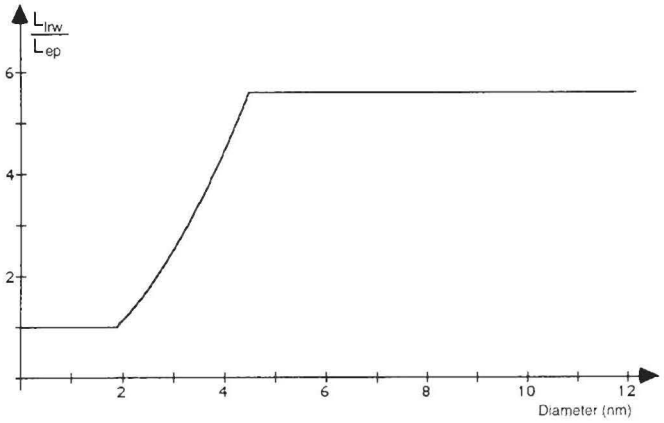

Fig. 2. This graph shows L_{lrw}/L_{ep} as a function of the diameter of the protein when the r_{ep}-value is 1.9 nm and the r_{lrw}-value is 4.5 nm.

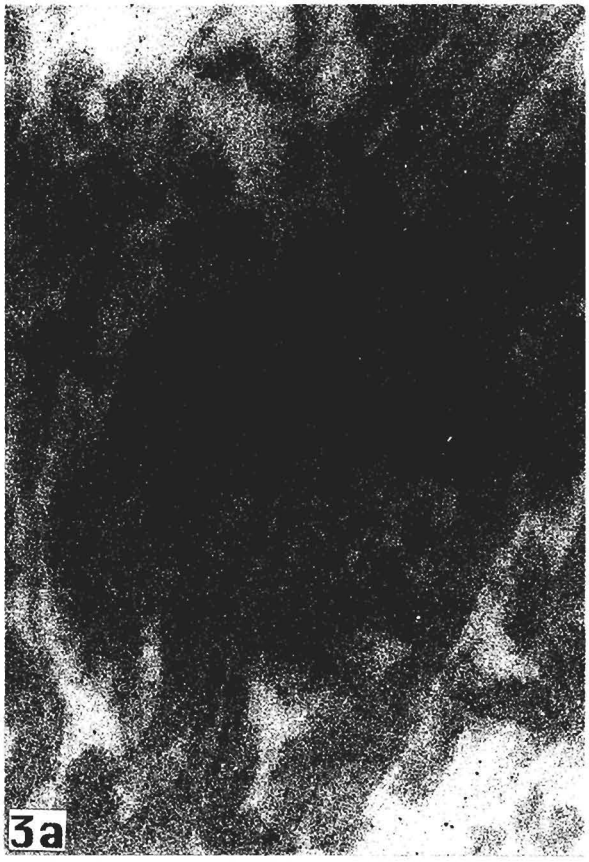

Fig. 3.
a. Illustrates a high-accelerator epoxy section of fibrin (8% accelerator in the first infiltration step, 4% accelerator in the second one, and 6% in the embedding step) immunolabeled with anti-fibrinogen and 5 nm gold particles. There is high density of immunogold labeling, much more intense than for conventional embedding (Fig. 3b).
b. Illustrates a conventional epoxy section of fibrin (normal amount of accelerator) immunolabeled with anti-fibrinogen and 5 nm gold particles. There is a low density of immunogold labeling. x 22,500. Bar: 200 nm.

using high-accelerator sections based on 8% accelerator in the first infiltration step, 4% accelerator in the second one, and 6% in the embedding step, the immunogold labeling increased 7-8 times from the labeling seen in conventional, unetched epoxy sections (Fig. 3a-b). This increase was only seen for *large* proteins like fibrinogen, thyroglobulin and, later on (Brorson et al., 1997), immunoglobulins (IgA, IgG, and IgM), and is an observation which agrees with theoretical considerations (Brorson and Skjørten, 1996c). The immunolabeling on the high-accelerator epoxy sections was only 30-40% less intense than for corresponding LR-White sections. The following theory was proposed to explain the increase in the intensity of immunogold labeling of the high-accelerator epoxy sections: "Increase of the concentration of DMP-30 tends to block the polymer chain synthesis at a low molecular weight (Hayat, 1970; Mark et al., 1986). The epoxy components react with the side groups of proteins (co-polymerization) when *normal* amount of accelerator is used (Kellenberger et al., 1987). The blocked polymer chains which are obtained when using high-accelerator epoxy resin, are not able to react with proteins. The co-polymerization is therefore significantly reduced. On the basis of the theory of Kellenberger et al. (1987), it is reasonable to believe that it will be more favourable for the surface of cleavage to follow the interface between resin and proteins during the cutting process than if the proteins were embedded in a conventional, more co-polymerized epoxy network."

Another way to enhance the immunolabeling of epoxy sections is to use propylene oxide as an additional dehydration/infiltration agent in addition to ethanol or to use 5-10% propylene oxide in the embedding mixture (Brorson, 1996). The mechanism for this kind of enhancing is probably that propylene oxide blocks the polymerization in a similar way as mentioned for the high accelerator procedure. This way of improving the immunolabeling is less potent than the one based on increased concentration of accelerator. The increase in immunolabeling using additional amounts of propylene oxide is largest when the concentration of accelerator is smallest, but the increase cannot compete with the effect of using high-accelerator epoxy resin. Another important consequence of using additions of propylene oxide is that the embedding mixture becomes less viscous. Similar enhancing results have been achieved by mixing water into the partly water miscible epoxy resin Quetol 651 (Abad, 1992), and the mechanism proposed for this enhancing was also reduction of the polymer cross-linking by water blocking the polymerization.

The practical use of high-accelerator epoxy resin has been demonstrated in the diagnosis of renal biopsies (Brorson et al., 1997). Renal biopsies were embedded in high-accelerator epoxy resin, and postembedding immunoelectron microscopy was performed with antibodies directed against IgA, IgG, IgM and C3c to detect immune complex deposits. Parallel samples of renal tissue were subjected to immunofluorescence microscopy (IF). Immunogold labeling of the high-accelerator epoxy resin showed improved sensitivity for detecting small immune complex deposits, especially for IgA (Fig. 4), compared to the IF-method; otherwise there was good correlation between immunoelectron microscopy and IF. The ultrastructural presentation of the glomerular tissue was good on high-accelerator epoxy sections when tannic acid was used to enhance the contrast. Both the stability in the electron beam and ultrastructural preservation was significantly better than for LR-White sections. This shows that immunoelectron microscopy on high-accelerator epoxy sections is well suited for routine use.

V. Fixation and processing of the tissue before embedding

There are more factors affecting the yield of immunolabeling for electron microscopy than the ones mentioned in chapters I-IV. But since this review is focused on the use of resin and the possibilities of manipulating the resin in order to improve the immunolabeling, fixation and processing of the tissue will only be discussed briefly here. Optional fixation for ultrastructural electron microscopy involves perfusion fixation with 2% glutaraldehyde, and postfixation with 1% osmium tetroxide. But such a strong fixation will often be too damaging for sensitive antigens, and a compromise between ultrastructural preservation and immunoreactivity has to be achieved. This is often obtained by using a mixture of 4% paraformaldehyde and 0.1-0.5% glutaraldehyde, osmium tetroxide being avoided. Probably, the fixation network also cooperates with the polymer network to bind the antigens more or less tightly in the block (Kellenberger et al., 1987), resulting in lower or higher amplitudes on the surface of the sections after cutting (Chapter III and Fig. 1).

The dehydration process may introduce disturbance at the cellular and molecular level. To avoid this, cryosectioning may be used (Roos and Morgan, 1990). Another alternative is freeze substitution which combines freezing technique with resin embedding (Hippe-Sanwald, 1993). Freeze substitution dissolves the cellular ice in the frozen specimen by an organic solvent which usually contains chemical fixatives. This procedure is carried out at a very low temperature. The tissue is finally embedded in resin, often the acrylic resin Lowicryl. In this way the molecular disarrangements are held at a very low level facilitating the immunolabeling of sensitive antigens on resin sections.

VI. Concluding remarks

Immunoelectron microscopy of resin sections has been widely used. For research purposes cryosectioning and freeze substitution have become popular. Twenty years ago electron microscopy was expected to have an exploding development in routine pathological diagnostics, but this scenario has failed. Immunocyto-

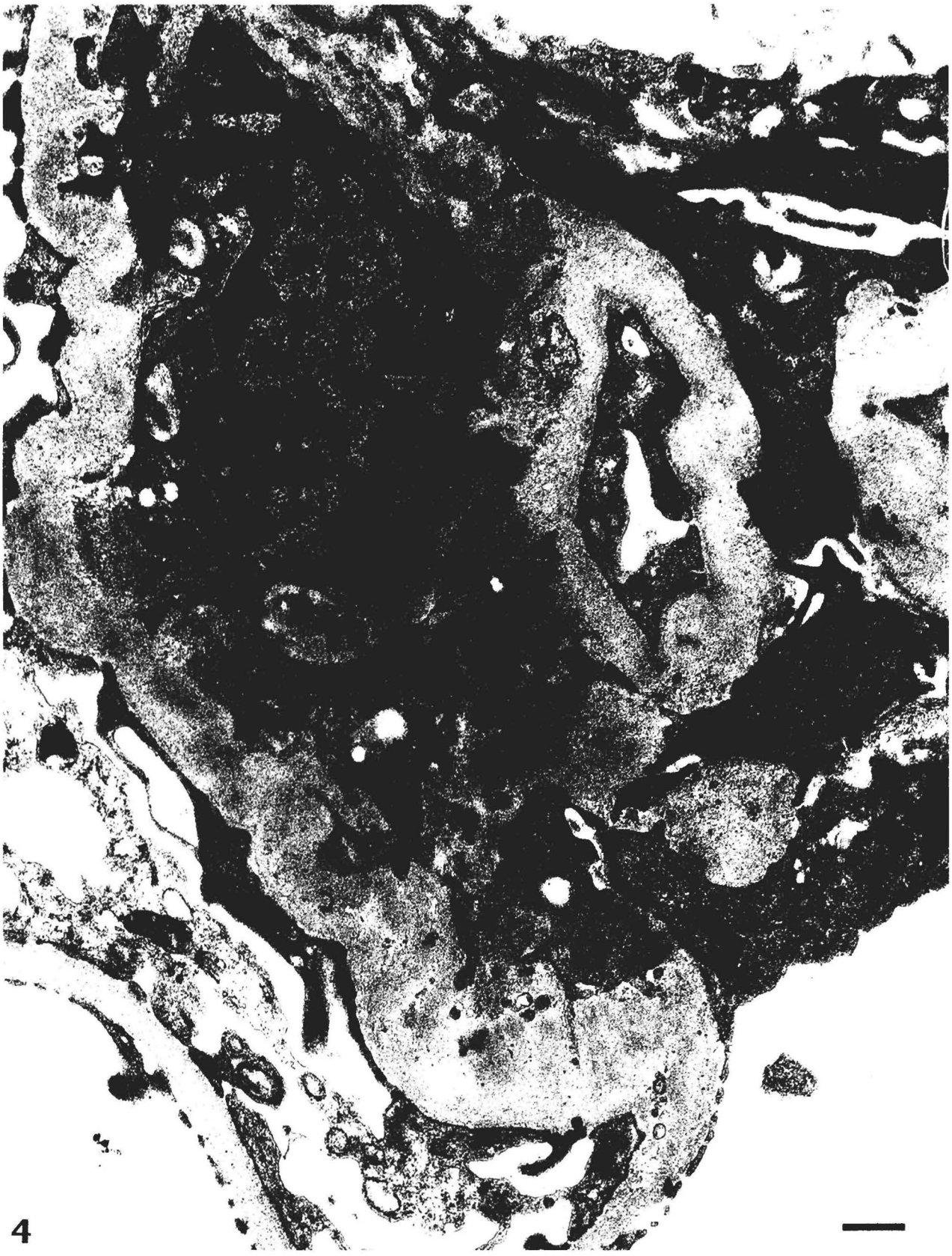

Fig. 4. The ultrastructure and immunolabeling with anti-IgA and 15 nm gold particles of an immune complex deposit in a human glomerulus (Arrowheads show deposits). x 7,500. Bar: 500 nm.

chemistry on frozen sections or paraffin sections for light microscopy has taken over the role that EM was expected to achieve. Immunolabeling of conventional epoxy sections often requires strong etching or deplasticizing procedures to be used. Acrylic sections may be used, but have their shortcomings, like instability in the electron beam, reduced ultrastructural preservation, sensitivity to oxygen during the polymerization process and reduced cutting qualities. The use of high-accelerator epoxy resin for immunoelectron microscopy is well suited for routine use, and may contribute to an increase in the interest for electron microscopy in pathology laboratories.

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