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Immunoelectron Microscopy of Skin Basement Membrane Zone Antigens: A Pre-embedding Method Using 1-nm Immunogold with Silver Enhancement

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There is no single immunoelectron microscopical method for invariably effective localization of both intracellular and extracellular antigens. We describe a simple and practicable immunogold technique that can be used to localize various skin basement membrane zone antigens at the ultrastructural level. Small pieces of skin were incubated with primary antibodies recognizing epitopes on a range of basement membrane zone-related antigens (two different lamina lucida-associated antigens, laminin, type VII collagen, fibrillin and keratin 14). This was followed by incubation with 1-nm colloidal gold-conjugated secondary antibody and subsequent silver intensification. The specimens were then processed for transmission electron microscopy. Precise immunolocalization with good ultrastructural preservation was achieved for all basement membrane zone antibodies tested. The results of basal cell keratin immunostaining showed that this microscopic approach could also be applied to some extent in the characterization of intracellular antigens. This immunoelectron microscopy technique provides a useful approach to the study of macromolecules at the basement membrane zone. ***Key words: dermal-epidermal junction; immunogold electron microscopy; lamina lucida; lamina densa; fibro-reticular network.***

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Basement membranes are specialized extracellular matrices associated with various epithelia and other types of tissue. In skin, the basement membrane zone (BMZ) at the dermal-epidermal junction consists of intracellular and transmembrane regions, lamina lucida, lamina densa and sub-lamina densa zone along with hemidesmosomes, anchoring filaments, sub-basal dense plates, anchoring fibrils and elastic microfibril bundles (1). Several macromolecules, predominantly synthesized by keratinocytes, are located in this region and contribute to the function of the BMZ in maintaining epidermal-dermal adherence, also acting as a selectively permeable barrier.

Altered expression of certain BMZ antigens has been implicated in the pathogenesis of various mechanobullous disorders (2, 3) and has also been noted in some forms of acquired blistering disease (1, 3, 4). Recently, a number of new BMZ antigens have been described, e.g. 19-DEJ-1 antigen and nicein (5), and their precise ultrastructural localization has been a matter of interest in understanding the function of this "interface" zone.

Previous attempts to immunolocalize macromolecular antigens or antigenic epitopes by pre-embedding immunoelectron microscopy have been performed using peroxidase as a marker (6, 7). However, the peroxidase reaction products are known to diffuse from the site of production and can be absorbed by adjacent structures in a non-specific manner (8). This may lead to misinterpretation of the antibody-binding sites. In addition, the diffuse peroxidase reaction may obscure underlying structures, making precise ultrastructural immunolocalization difficult to determine.

Alternative pre-embedding labelling techniques, such as the use of 5-nm immunogold, have provided good results with more precise immunolocalization for some extracellular antigens, particularly within the fibro-reticular network (9–11). The method is, however, less effective for localizing antigens in the lamina lucida or on the basal cell surface, probably because the lamina densa prevents transfer of the immunoreagents from the dermal side. We have therefore assessed more sensitive methods using 1-nm gold to examine a wider range of BMZ antigens and have succeeded in immunolabelling lamina lucida antigens (12) as well as type VII collagen on altered or poorly formed anchoring fibrils in various forms of dystrophic epidermolysis bullosa (13).

Table I. *Details of the primary antibodies*

Antibody	Ig type	Antigen	Dilution	Source/reference
19-DEJ-1	Murine IgG	Not characterized	1:3	[6]
GB3	Murine IgG	Nicein/BM600	1:3	[12]
Anti-laminin	Murine IgG	B1 chain of human laminin	1:5	Calbiochem Novobiochem, Nottingham, U.K.
LH 7:2	Murine IgG	NC-1 (amino) terminus of type VII collagen	1:2	[22]
Fibrillin	Murine IgG	350kD glycoprotein fibrillin	1:20	[10]
LLOO1	Murine IgG2a	C-terminus of keratin 14	1:6	[23]

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Table II. *Immunolabelling procedure*

PBS = phosphate buffered saline, pH 7.4; NGS = normal goat serum; BSA = bovine serum albumin; 1/2 K = half-strength Karnovsky fixative.

Day 1	[1].	Cut skin samples into small pieces (<2 mm^3) and trim off most of the reticular dermis.
	[2].	Wash in PBS for 1 h at 4°C.
	[3].	Incubate with primary antibody diluted in 1% NGS / 1% BSA / 0.1% gelatin / 0.02% sodium azide / PBS overnight at 4°C.
Day 2	[4].	Wash in PBS for 6 h at 4°C.
	[5].	Incubate with 1 nm immunogold diluted 1:3 in 1% NGS / 1% BSA / 0.1% gelatin / 0.02% sodium azide / 0.9% NaCl / 20 mM Tris-HCl buffer pH 8.2 overnight at 4°C.
Day 3	[6].	Wash in PBS for 2 h at 4°C.
	[7].	Fix in 0.04 M 1 / 2 K for 4 h at room temperature.
	[8].	Wash in PBS for 30 min at 4°C.
	[9].	Rinse in 0.25 M citrate buffer pH 7.4.
	[10].	Silver staining for 6–11 min at room temperature.
	[11].	Stop reaction by immersing specimens in 0.25 M citrate buffer.
	[12].	OsO4 fixation (1.3% for 2 h), alcohol dehydration (35–50%) ...
Days 4–7	[13].	... continue alcohol dehydration (50–100%), uranyl acetate *en bloc* staining, and embedding in resin using standard electron microscopy procedures.
	[14].	Cut superficial semithin sections and then ultrathin sections, staining with uranyl acetate (10 min) and lead citrate (10–20 s).

In this report we describe the practical details and further applications of this method.

MATERIALS AND METHODS

Details of the antibodies used in this study are presented in Table I. Negative control studies were also performed using conditioned medium from the murine myeloma cell line SP2/O-Ag14 or normal goat serum instead of the primary monoclonal antibodies. For immunolabelling, the method of Lah et al. (14) was modified and is described in detail in Table II. One-nanometer immunogold-conjugated antibodies (Amersham U.K., High Wycombe, U.K.) were used. For silver staining, a silver enhancement solution (Amersham U.K.) was diluted with an equal amount of distilled water. Silver enhancement was performed for 6–11 min at room temperature.

RESULTS

Specific immunolabelling for each of the antibodies was obtained (Fig. 1). The silver-enhanced reaction product size varied from approximately 5 nm to 30 nm. 19-DEJ-1 labelling was associated with the anchoring filaments between the sub-basal dense plate and the lamina densa within the mid-lamina lucida (Fig. 1a). GB3 labelling was present on the border between the lamina lucida and the lamina densa, predominantly subjacent to hemidesmosomes (Fig. 1b). In areas of slight widening of the lamina lucida (probably due to the prolonged pre-fixation time), GB3 labelling remained in close association with the upper surface of the lamina densa. Laminin labelling (B1 component) was noted within the mid- and lower lamina lucida and occasionally within the lamina densa (Fig. 1c). Immunoreactivity to the anti-type VII collagen antibody was observed within the lower lamina densa and upper dermis in association with the ends of anchoring fibrils (Fig. 1d). Anti-fibrillin antibody staining was associated with dermal microfibril bundles (Fig. 1e). Keratin 14 labelling was found on keratin filaments, at least on tonofilaments present within the lower one-third of the basal cell cytoplasm (Fig. 1f). The ultrastructure of hemidesmosomes, sub-basal dense plates, anchoring filaments, lamina densa, anchoring fibrils, microfibril bundles and anchoring plaques was generally good, although patchy disruption of the plasma membrane was noted in some sections. Various components of the fibro-reticular network, including anchoring fibrils and elastic microfibrils, were particularly well defined.

DISCUSSION

The 1-nm immunogold silver staining method allows improved immunolocalization of several skin BMZ antigens compared with other methods. Comparison with peroxidase methods shows that the localization of the labelling is more precise and more clearly demonstrated by the 1-nm immunogold silver staining method. The use of 1-nm immunogold with silver enhancement also has advantages over the use of 5-nm gold-conjugated probes (9–11). The labelling density in our study was usually greater using the 1-nm immunogold-silver staining method, and labelling could be seen at comparably lower magnification. Indeed, other studies have shown that the labelling density of gold-conjugated antibodies is inversely proportional to particle size (15). One-nm gold particles, therefore, may allow more optimal epitope recognition and are in consequence useful in detecting sparsely distributed antigens. Another advantage of the 1-nm gold particles is that lamina lucida-associated antigens can be accessed more easily. One-nm probes would appear to be able to pass through the lamina densa considerably better than 5-nm or other larger gold-conjugated antibodies. The 1-nm immunogold silver staining method, therefore, is of considerable practical potential in studying structural macromolecules within the lamina lucida or associated with the hemidesmosome-anchoring filament complex.

Similar immunostaining procedures using 1-nm immunogold pre-embedding methods have been used for other tissues (16), cultured cells (14, 16), as well as for a new lamina lucida antigen termed kalinin (17). Recently the similarity between separately described lamina lucida glycoproteins, including nicein, epiligrin and kalinin, has been suggested (12, 17–19), and the immunolabelling of GB3 shown here highlights the probable synonymous nature of many of these BMZ components.

There are other immunoelectron microscopy methods that have their own advantages, including post-embedding methods (20) and cryo-ultramicrotomy (21). However, the presently described pre-embedding method does not require any specialized equipment apart from that which is normally used for transmission electron microscopy. It also has certain advantages over the post-embedding methods we most often use (20), where we have so far failed to achieve labelling with GB3 or 19-DEJ-1 (Shimizu & Eady, unpublished). These antibody binding sites

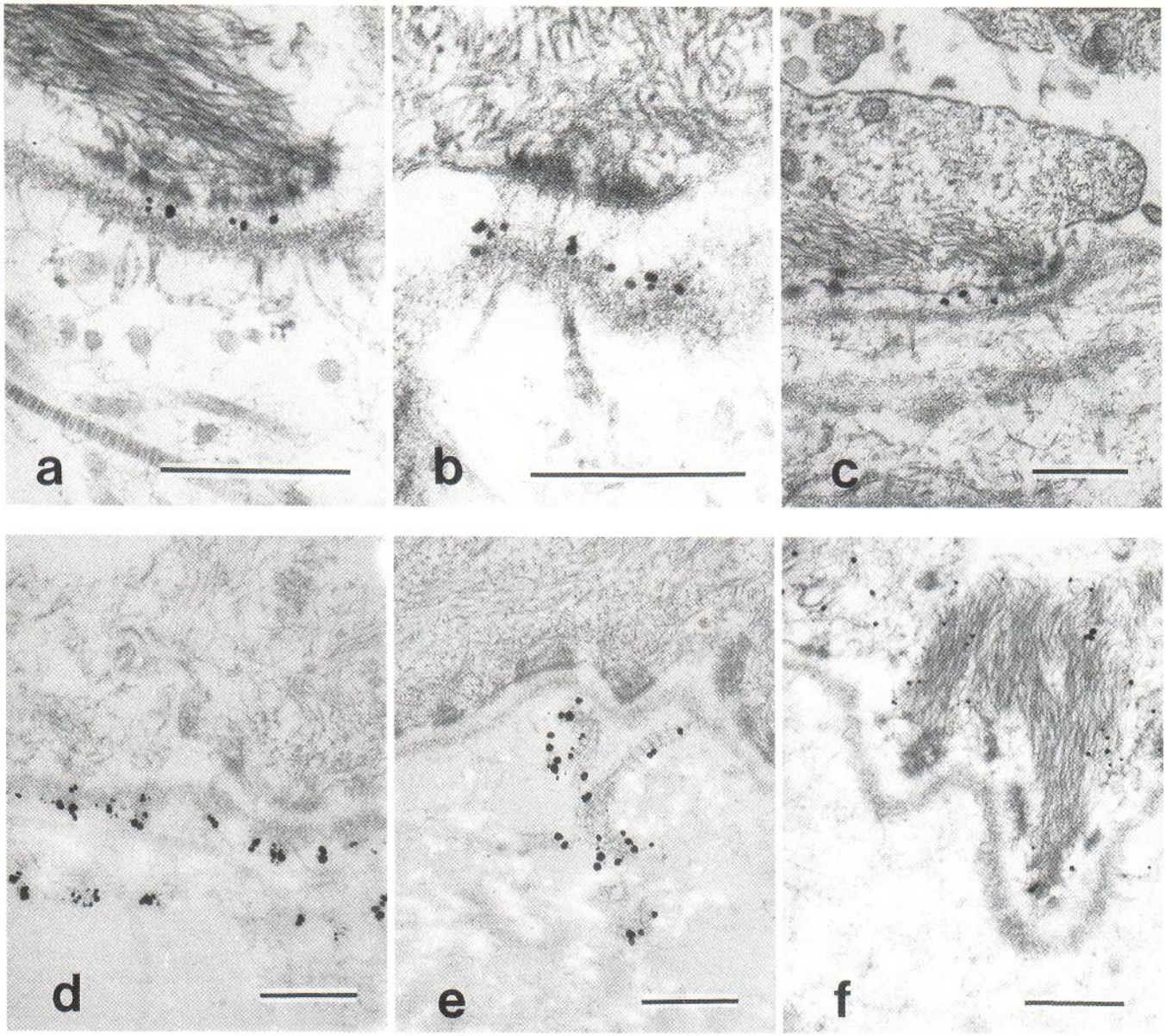

Fig. 1. Immunoelectron microscopy of various skin basement membrane zone-related antigens using a pre-embedding method with 1-nm immunogold silver staining. (*a*) 19-DEJ-1 labelling. Immunoreactivity is seen within the mid-lamina lucida in association with anchoring filaments beneath a hemidesmosome. (*b*) GB3 (nicein antibody) labelling is present on the border between the lamina lucida and the lamina densa. Labelling is maximal subjacent to a hemidesmosome and is often, but not invariably, associated with anchoring filaments. (*c*) Anti-laminin (B1 component) antibody staining. Immunoreaction is seen within mid- and lower lamina lucida. (*d*) LH7:2 (anti type VII collagen antibody) staining. Immunoreactivity is seen within the lower lamina densa and possible anchoring plaques. (*e*) Anti-fibrillin antibody staining is present on net-like dermal microfibril bundles. (*f*) Anti-keratin 14 labelling (LLOO1 antibody). Basal cell keratin filaments are labelled. *Bar* = 0.25 μm.

were clearly demonstrated by our pre-embedding 1-nm immunogold silver staining method.

A disadvantage of this immunoelectron microscopy method is the uneven and variable size of the final reaction products. Although diluting the original silver enhancement reagents with distilled water helped to control this variability, it remains to be seen whether further steps, including strict control of temperature and light (14), and possibly the addition of gum arabic to the silver enhancement solution (16), will help to provide a more uniform reaction product size. The concentration and duration of secondary fixation in osmium tetroxide may also be an important variable in determining the final silver-enhanced particle size (16).

In summary, this technique should provide a useful alternative for the characterization of BMZ antigens and is particularly useful for lamina lucida as well as sub-lamina densa antigen localization. Nevertheless, there will still be a place for additional immunoelectron microscopy techniques such as post-embedding microscopy and cryo-ultramicrotomy, particularly in the assessment of intracellular antigens.

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