In Vitro Complement Binding on Cytoplasmic Structures in Normal Human Skin: Immunoelectronmicroscopic Studies

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ABSTRACT We have previously provided evidence that suggests that exposure of cryostat skin sections to normal human serum (NHS) results in the antibody-independent Clq binding to cytoplasmic structures of various cell types, leading to classical complement pathway activation as evidenced by cytoplasmic C3 deposition.

In the present study, we have employed immunoelectronmicroscopic methods to clarify the exact nature of cytoplasmic C3 binding structures. Incubation of cryostat skin sections with NHS followed by peroxidase-labeled rabbit anti-human C3 serum (HRP-R/Hu C3) revealed that intracytoplasmic binding of C3 occurred in suprabasal keratinocytes, melanocytes, fibroblasts, smooth muscle cells, endothelial cells, pericytes, Schwann cells, and nerve axons, but not in basal keratinocytes, Langerhans cells, and other cellular constituents of the skin. C3 binding, as revealed by the deposition of HRP reaction product, was exclusively confined to intermediate-sized filaments (ISF), which can therefore be considered to represent the subcellular site for classical complement pathway activation. Under experimental conditions that do not allow classical complement pathway activation, ISF were not decorated.

Our observation that ISF of ontogenetically different cell types share the capacity of complement fixation is in accordance with the recent finding that different ISF types, despite their biochemical and antigenic heterogeneity, have common alpha-helical domains and may provide a clue to the mechanism and site of interaction between complement components and ISF.

In a previous study (18), we reported that the incubation of cryostat sections of normal human skin with normal human sera (NHS) followed by a fluorescein-isothiocyanate-labeled rabbit-anti human C3 antiserum (FITC-R/Hu C3) results in a bright cytoplasmic staining of certain cell populations within the skin. We have also presented evidence that cytoplasmic deposition of C3 is most likely an antibody-independent phenomenon that is due to binding of Clq to cytoplasmic structures and subsequent activation of the classical complement (C) cascade. Although the immunofluorescence (IF) procedure employed allowed us to conclude that keratinocytes of the upper epidermal layers and smooth muscle cells within the dermis displayed the C-binding cytoplasmic components, we observed a variety of positively stained cells in the dermis and epidermis whose exact nature could not be determined by IF. Another limitation of the IF technique employed was that it did not permit the morphological identification of the structural component relevant to complement binding. In the present study, we report on immunoelectronmicroscopic studies that show that intermediate-sized filaments (ISF) represent the only cytoplasmic substrate upon which C3 deposition occurs. Within the epidermis, HRP-reaction product enveloped ISF of suprabasal keratinocytes and melanocytes whereas ISF of basal keratinocytes and of Langerhans cells did not exhibit C3-binding properties. Within the dermis, C3-binding ISF were identified within fibroblasts, endothelial cells, smooth muscle cells, pericytes, Schwann cells, and nerve axons.

MATERIALS AND METHODS

Biopsies

Specimens of normal human skin, obtained from five individuals undergoing corrective plastic breast surgery under general anaesthesia, were embedded in Tissue-Tek II O.C.T. compound (Lab-Tek Products, Division Miles Laboratories Inc., Elkhart, IN), snap frozen in liquid nitrogen and stored at −70°C until sectioning. 4-μm cryostat sections were either immediately incubated with the various immunoreactants listed below or, in a few experiments, subjected to an

THE JOURNAL OF CELL BIOLOGY · VOLUME 95 NOVEMBER 82 543-551
 · 0021-9525/82/11/0543/09 $1.00

extraction procedure that is known to dissolve cellular constituents leaving behind the insoluble ISF network (5, 7, 20, 27, 36, 37, 40). Briefly, sections pretreated with or without 0.2% Triton X-100 at 4°C were reacted with a series of low/high/low ionic strength buffers with or without the enzyme inhibitors phenylmethylsulfonylfluoride (1 mM, Sigma Chemical Co., St. Louis, MO) and 1-l-tosylamide-2-phenylethyl chloromethyl ketone (2 mM, Sigma Chemical Co.), washed with PBS and incubated for immunoperoxidase studies (36).

Sera

Fresh sera were obtained from 20 healthy individuals and from two patients with hereditary C4 deficiency (43). Serum samples were either used immediately or stored at −70°C until use.

Conjugates

Fluorescein-isothiocyanate (FITC)-labeled rabbit antisera directed against human IgG, IgM, and IgA were purchased from Kent Laboratories Ltd, North Vancouver, B.C., Canada and used at a dilution of 1:10. HRP-labeled rabbit antisera directed against either human C3 or human Ig were obtained from Dako Immunoglobulins, Copenhagen, Denmark and used at a 1:20 dilution.

Immunofluorescence Procedure

For the detection of circulating antibodies against cytoplasmic antigens of skin cells, cryostat sections of normal human skin were first incubated with 1:10 diluted NHS or C4-deficient sera and, after extensive PBS washes, with the respective FITC-labeled rabbit anti-human immunoglobulin sera. Only sera devoid of IF-detectable amounts of antibodies directed against cytoplasmic antigens in any skin cell were used in further immunoperoxidase procedures.

Immunoperoxidase Studies

4-μm cryostat sections were air-dried for 20 min, washed in PBS for 10 min, and incubated for 30 min at 37°C with a 1:10 dilution of either (*a*) untreated NHS, (*b*) NHS containing either 10 mM EDTA or EGTA, concentrations known to inhibit classical and/or alternate pathway activation or (*c*) C4-deficient sera; in some experiments, PBS was substituted for sera. After three washes in PBS, sections were incubated with appropriate dilutions of either HRP-R/Hu C3 or HRP-conjugated rabbit anti-human immunoglobulin (HRP-R/Hu Ig) for 30 min at 37°C. After three PBS washes, sections were fixed in half-strength Karnovsky's fixative (23) for 60 min at room temperature and then washed in 0.1 M cacodylate buffer, pH 7.4 at 4°C. For visualization of the bound immunoreactants, specimens were incubated in DAB medium (16) for 20 min at room temperature in the dark and then processed for both light and electron microscopic examination. For light microscopic purposes, sections were mounted with glycerine:PBS (4:1). For electron microscopy, sections that had been extensively washed in 0.1 M cacodylate buffer at 4°C were postfixed in potassium-ferrocyanide osmium tetroxide (24) for 60 min on ice. This postfixation method enhances the contrast of membranous structures, reduces the overall density of the cytoplasmic background and, thus, accentuates electron-dense deposits. Specimens were then rapidly dehydrated in a graded series of alcohols and embedded in Epon 812. After polymerization (24 h, 60°C), the Epon sheet was detached from the slides by immersion in liquid nitrogen. Small blocks were excised and remounted using Beem (Polaron Equipment Ltd., Watford, England) capsules. In preliminary experiments, vertical sectioning had demonstrated the even penetration of the antibody-HRP complex through the entire depth of the section. Subsequently, horizontal serial sections were cut with a Reichert "Ultracut" ultramicrotome (C. Reichert, Optische Werke AG, Vienna, Austria) and stained with lead citrate, and approximately every tenth section was examined with a Philips 400 electron microscope operating at 80 kV. By doing so, we excluded the possibility that lack of penetration might have mimicked negative results in nonreactive areas of the specimen.

RESULTS

Light Microscopic Findings

When cryostat sections of normal human skin were first exposed to NHS, subsequently reacted with a HRP-labeled R/Hu C3 antiserum, and then incubated for the demonstration of HRP activity, a seemingly homogeneous deposition of brownish reaction product was revealed within the cytoplasm of various cell types, but was not observed on any extracellular structure. The staining pattern depicted in Fig. 1 was virtually identical for all NHS and substrates tested. HRP-positive cells within the basal layer of the epidermis were dendritic in shape and—according to their distribution pattern—were considered to be melanocytes. Residual basal keratinocytes, i.e., the vast majority of cells within the basal layer, were devoid of reaction product. The lack of C3-binding capacity of basal keratinocytes contrasted sharply with the consistent detection of C3-binding sites within suprabasal keratinocytes. Although with varying intensity, HRP reaction product was detected within essentially all suprabasal keratinocytes, and was confined to their cytoplasm, regularly sparing nuclei and intercellular spaces. Within the dermis, cytoplasmic C3-binding was discernible within at least three distinct cell systems. These included endothelial cells, smooth muscle cells, and a population of apparently uniform cells with stellate cytoplasmic processes scattered between bundles of connective tissue throughout the entire dermis that most likely represent fibroblasts.

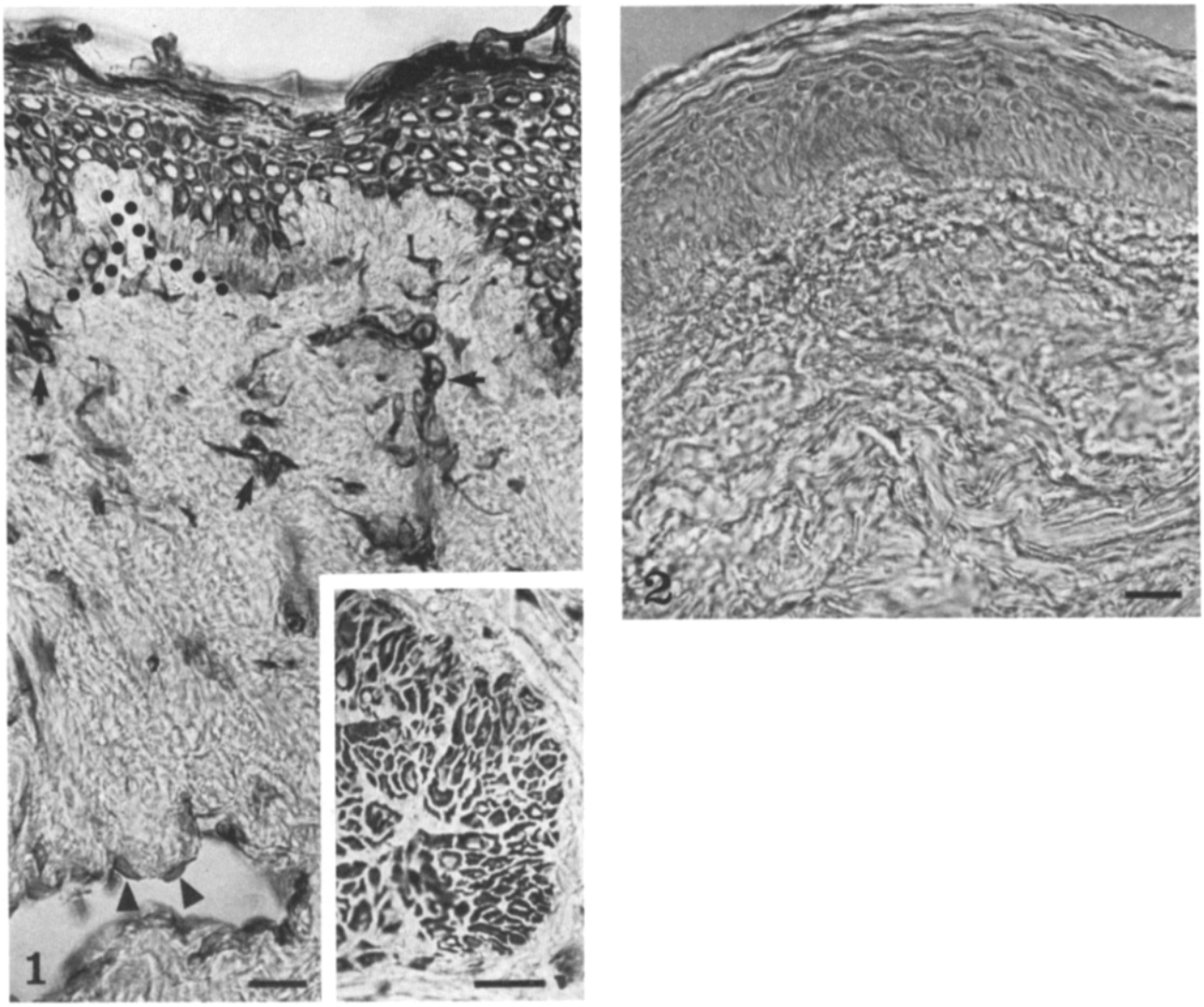
An essentially identical staining pattern was seen when cryostat sections had been previously subjected to extraction procedures described above, but was not observed when NHS was substituted by either C4-deficient sera or PBS, or was used in the presence of 10 mM EGTA and/or EDTA (Fig. 2). Similarly, HRP reaction product was not deposited at any location when HRP-R/Hu C3 was replaced by HRP-R/Hu Ig. These results confirm those obtained in our previous immunofluorescence studies (18).

Electron Microscopic Findings

EPIDERMIS:—Light microscopic studies had already shown that, within the basal layer of the epidermis, the only HRP-reactive cells were dendritic in shape and displayed a distribution pattern suggestive for melanocytes. At the ultrastructural level, these C3-binding cells were located directly above the basal lamina, frequently protruded in a teardroplike fashion towards the underlying dermis (Fig. 3 *a*), contained singly dispersed melanosomes at all stages of development within their cytoplasm and, thus, were identified as melanocytes (3). Within these cells, HRP reaction product was deposited continuously along virtually all cytoplasmic ISF, but was not associated with any other cellular structure nor with the cell membrane. These HRP-reactive nonbranching filaments were either located mainly in the perikaryon region (not shown) or were seen in the dendrites (Fig. 4), as is characteristic for ISF within melanocytes (22). The major portion of cells constituting the basal layer was completely devoid of reaction product. These HRP-negative cells contained melanosome-aggregates and loosely packed tonofilaments, were attached to the underlying basal lamina via hemidesmosomes and therefore represented basal keratinocytes (Figs. 3 *a* and 4, reference 3).

In contrast to HRP-negative basal keratinocytes, the reaction product was associated with tonofilament bundles of suprabasal keratinocytes (Fig. 3 *a* and *b*). Although tonofilaments within the cells of the spinous layer are tightly packed and appear as electron-dense structures, HRP deposition was easily recognized in the majority of these cells in which it enveloped the individual tonofibrils. However, the intensity of staining varied in individual cells and in some cells HRP deposits were not unequivocally demonstrable, i.e., we were not able to decide whether dark-appearing tonofibrils were covered with only minute amounts of the tracer or appeared electron-dense due to dense packing.

The epidermis harbors a third distinctive cell population, the Langerhans cells (42). These cells are highly dendritic in shape and are located at a suprabasal position. Langerhans cells have a convoluted nucleus (Fig. 5 *a*) and contain unique trilaminar



FIGURES 1 and 2 Light microscopic visualization of cytoplasmic C3-binding sites in normal human skin: cryostat sections were incubated with (Fig. 1) NHS or (Fig. 2) C4-deficient serum and then with HRP-R/Hu C3. Fig. 1: Suprabasal keratinocytes, fibroblasts (arrows), endothelial cells (arrowheads) and smooth muscle cells of Mm. arrectores pilorum (inset) exhibit strong cytoplasmic staining. Within the basal layer of the epidermis, cytoplasmic HRP deposition is only seen within dendritic cells (melanocytes). Cell nuclei and extracellular structures are devoid of HRP reactivity. The dermal-epidermal junction is marked by a dotted line. Bars, 10 μm. Main field × 800, inset × 1,000. Fig. 2: HRP deposition is not seen under conditions that do not allow classical pathway activation. Bar, 10 μm. × 800.

cytoplasmic structures termed Langerhans cell granules (Fig. 5 *b*). 10-nm filaments within these cells did not display complement-binding properties as was evidenced by a complete HRP-negativity (Fig. 5 *b*).

DERMIS:—Deposition of HRP reaction product within the dermis was confined to cellular elements and never seen in association with components of the extracellular space. One group of cells possessing C-binding ISF was intermingled between collagen bundles, characterized by long and thin cytoplasmic processes (Fig. 3 *a*) and was therefore identified as either active (round nuclei, well-developed rough endoplasmic reticulum, stellate shape) or resting (flattened nuclei, scanty cytoplasm, spindle shape) fibroblasts. HRP precipitates enveloped each ISF in a continuous fashion (Fig. 3 *c*).

Careful sampling of vascular structures revealed that endothelial cells and pericytes comprising the capillary wall also contained C-binding ISF (Fig. 7). Again, this was a regular finding with all NHS and substrates tested. In cutaneous nerves, HRP reaction product was deposited along 10-nm filaments within nerve axons, i.e., neurofilaments (Fig. 8 *a*) and along ISF of Schwann cells (not shown). Myelin sheaths did not exhibit C-binding capacity, which is in accordance with a recent report from Vanguri et al. (44) indicating that central nervous system, but not peripheral nerve myelin leads to the activation of the classical complement pathway in the presence of fresh serum.

Another cell population exhibiting C-binding cytoplasmic structures is the smooth muscle cells of Mm. arrectores pilorum. Within these cells, HRP reaction product was, at a varying intensity, only seen in association with filamentous structures, but spared the perinuclear portion that characteristically lacks cytoskeletal elements. Smooth muscle fibers contain three different classes of myofilaments, i.e., actin (~7.5 nm in diameter), myosin (~15 nm in diameter), and ISF (~10 nm in diameter), which form an interconnected filamentous network linking together cytoplasmic dense bodies and membrane-bound dense plaques (5). Even in routine electron microscopic specimens it is difficult to identify the ISF scattered in between other

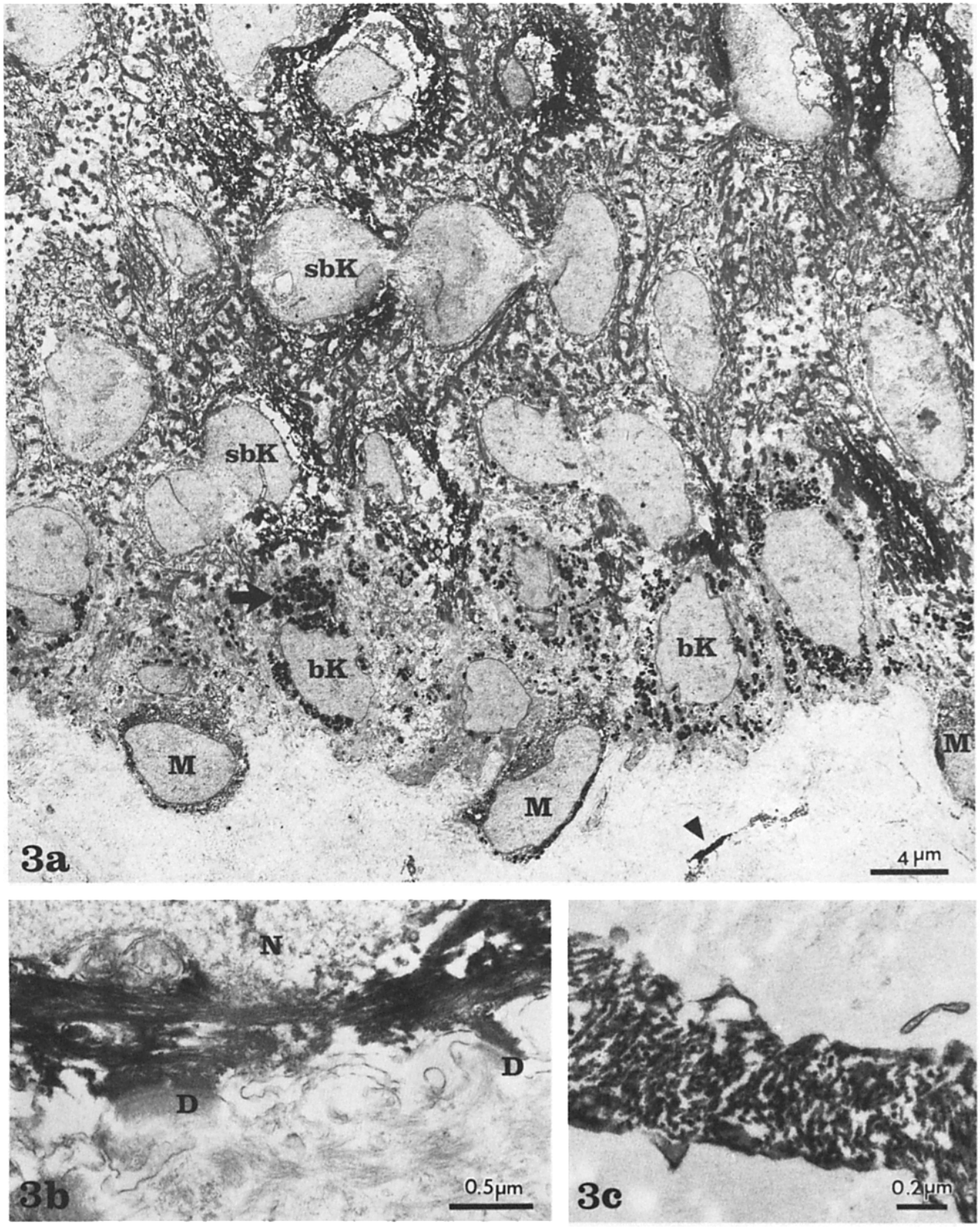


FIGURE 3 Immunoelectronmicroscopic visualization of cytoplasmic C3-binding sites in epidermis and papillary dermis of normal human skin: cryostat sections were incubated with NHS and then with HRP-R/Hu C3. (*a*) Within the epidermis, the HRP reaction product decorates keratin filament bundles within suprabasal keratinocytes (*sbK*) at a varying intensity. In some *sbK* staining is only slight so that it is not readily apparent at this low magnification. By contrast, basal keratinocytes (*bK*), which characteristically contain numerous melanosome aggreates (arrow) always appear unstained. Melanocytes (*M*) which protrude into the dermis in a teardroplike fashion exhibit intracytoplasmic HRP-reactivity as do fibroblast processes within the dermis (arrowhead). Bar, 4.0 μm. × 3,500. (*b*) Close-up of a basal keratinocyte (*bK*) neighboring a suprabasal keratinocyte (*sbK*). HRP deposits decorate the tightly packed keratin filaments of *sbK*, but spare the loosely arranged keratin filaments of *bK*. Note cut-off of filament staining at the desmosomal attachment plaque of the *sbK*. *N*, nucleus of *sbK*, *D*, desmosomes. Bar, 0.5 μm. × 30,000. (*c*) Close-up of the fibroblast process shown in *a* reveals heavily HRP-decorated ISF. Bar, 0.2 μm. × 47,000.

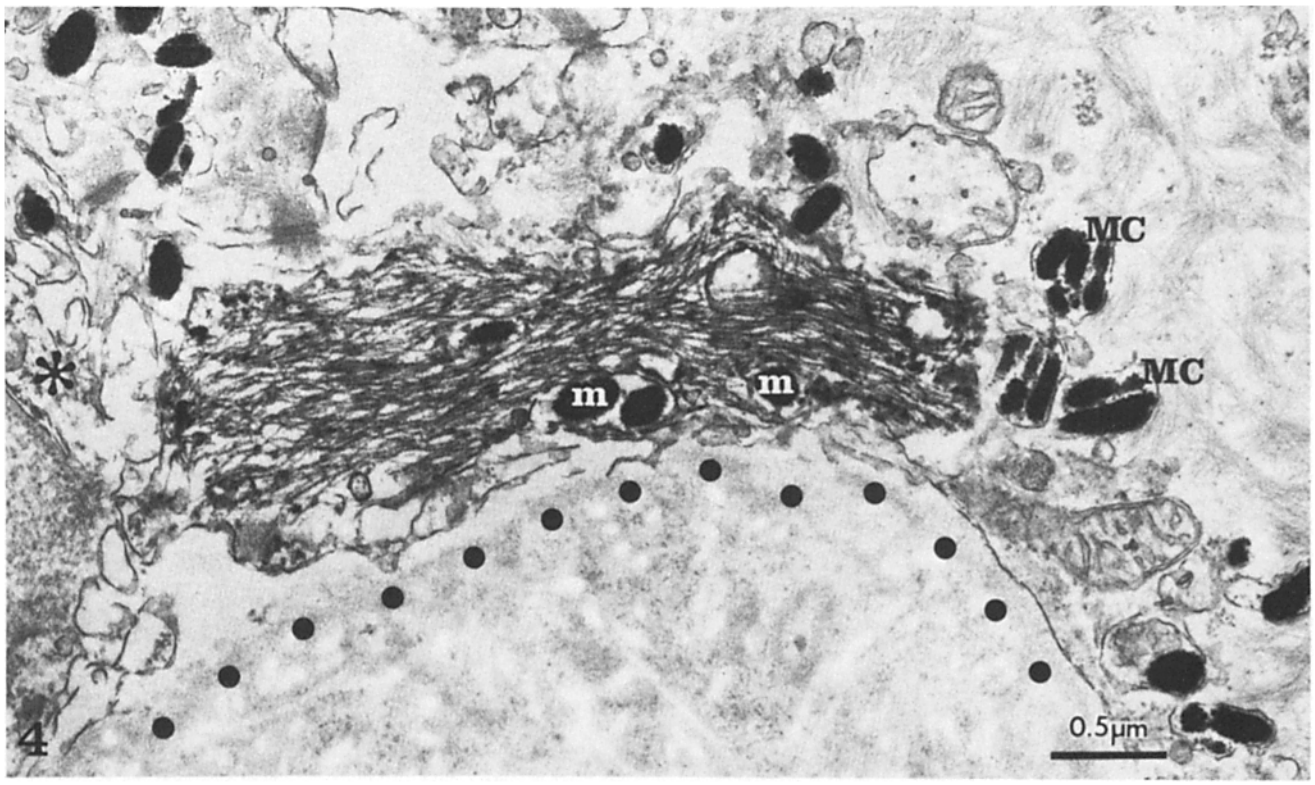


FIGURE 4 Immunoelectronmicroscopic visualization of C3-binding ISF within a melanocyte dendrite. Melanosomes (*m*) are characteristically embedded in the HRP-reactive network of 10-nm filaments; the perinuclear area (*) of this particular melanocyte contains no ISF and thus lacks HRP-reaction product. A dotted line marks the dermal-epidermal junction; *MC*, melanosome complexes within a basal keratinocyte. Bar, 0.5 μm. × 30,000.

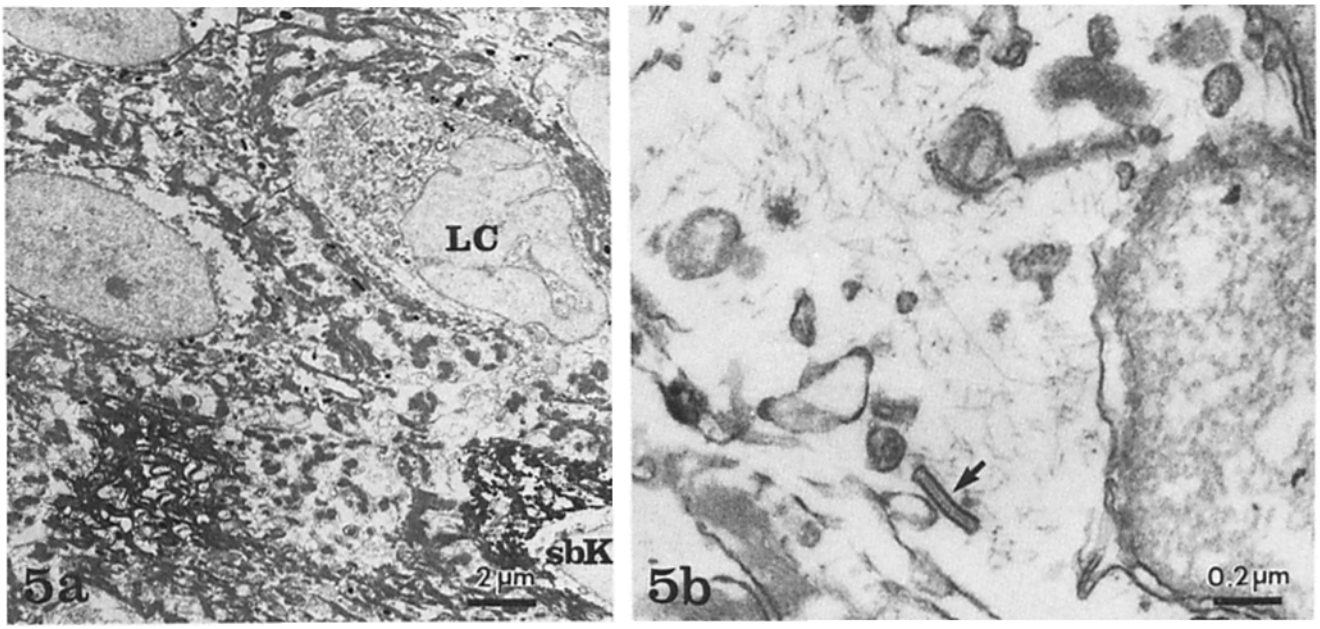


FIGURE 5 (*a*) Cryostat sections of normal human skin reacted with NHS and HRP-R/Hu C3. The cytoplasm of suprabasal keratinocytes (*sbK*) contains ISF-associated HRP-deposits at a varying intensity, whereas the cytoplasm of a Langerhans cell (*LC*) appears unstained. Bar, 2 μm. × 4,500. (*b*) High magnification of the Langerhans cell reveals HRP-negative ISF. Arrow indicates characteristic trilaminar Langerhans cell granule. Bar, 0.2 μm. × 45,000.

filaments. Because the limitations imposed by the requirement for lightly stained sections in peroxidase immunocytochemical procedures prevent the accurate and separate identification of 7.5-nm and 10-nm filaments and, thus, do not allow the unequivocal identification of the structure(s) upon which HRP reaction product is deposited, we employed an extraction procedure with low and high salt buffers that solubilizes actin and myosin filaments, leaving a cytoskeleton composed predominantly of ISF still attached to dense bodies (5, 20, 36). Using this procedure, the staining pattern seen at the light microscopic

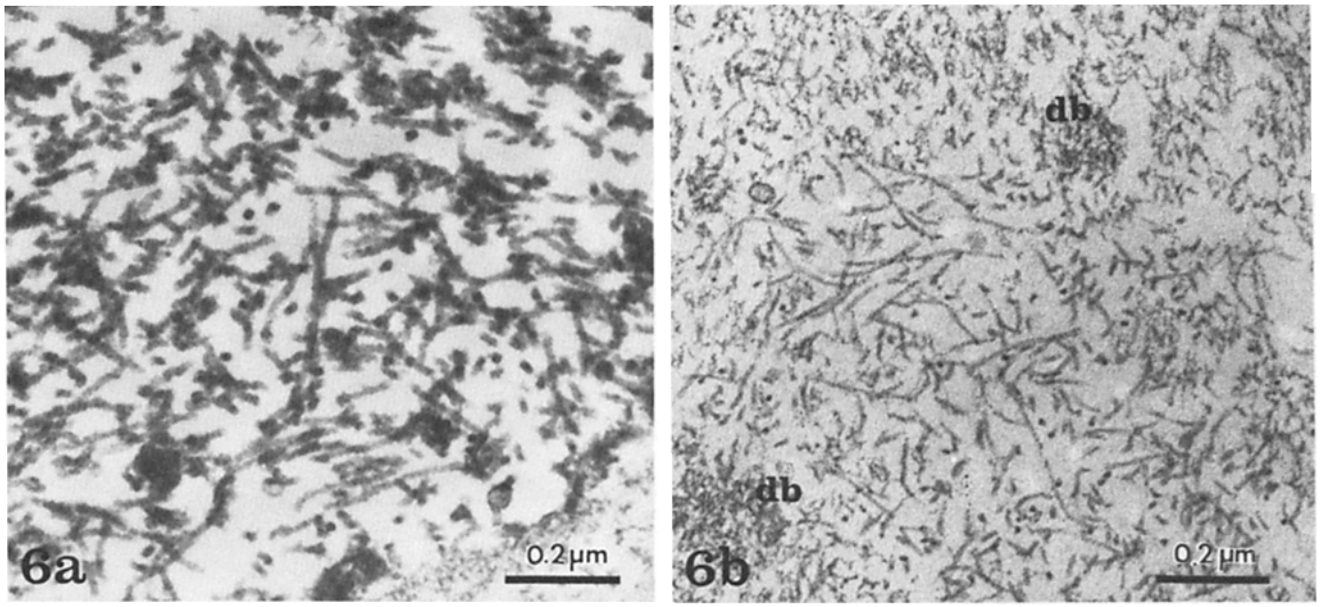


FIGURE 6 Immunoelectronmicroscopic visualization of C3-binding filaments within a smooth muscle cell. Cryostat sections were pretreated with a series of low and high ionic strength buffers and then reacted with either NHS (*a*) or C4-deficient serum (*b*) followed by HRP-R/Hu C3. (*a*) Filaments remaining after the extraction procedure are heavily decorated with HRP-reaction product after the use of NHS. They therefore appear thicker than in the control (*b*). (*b*) Filaments remaining after the extraction procedure are accentuated by conventional OsO_4 postfixation but do not display any HRP reactivity after the use of C4-deficient serum. Filaments have an average diameter of 10 nm and, thus, represent ISF. Bar, 0.2 μm. × 75,000.

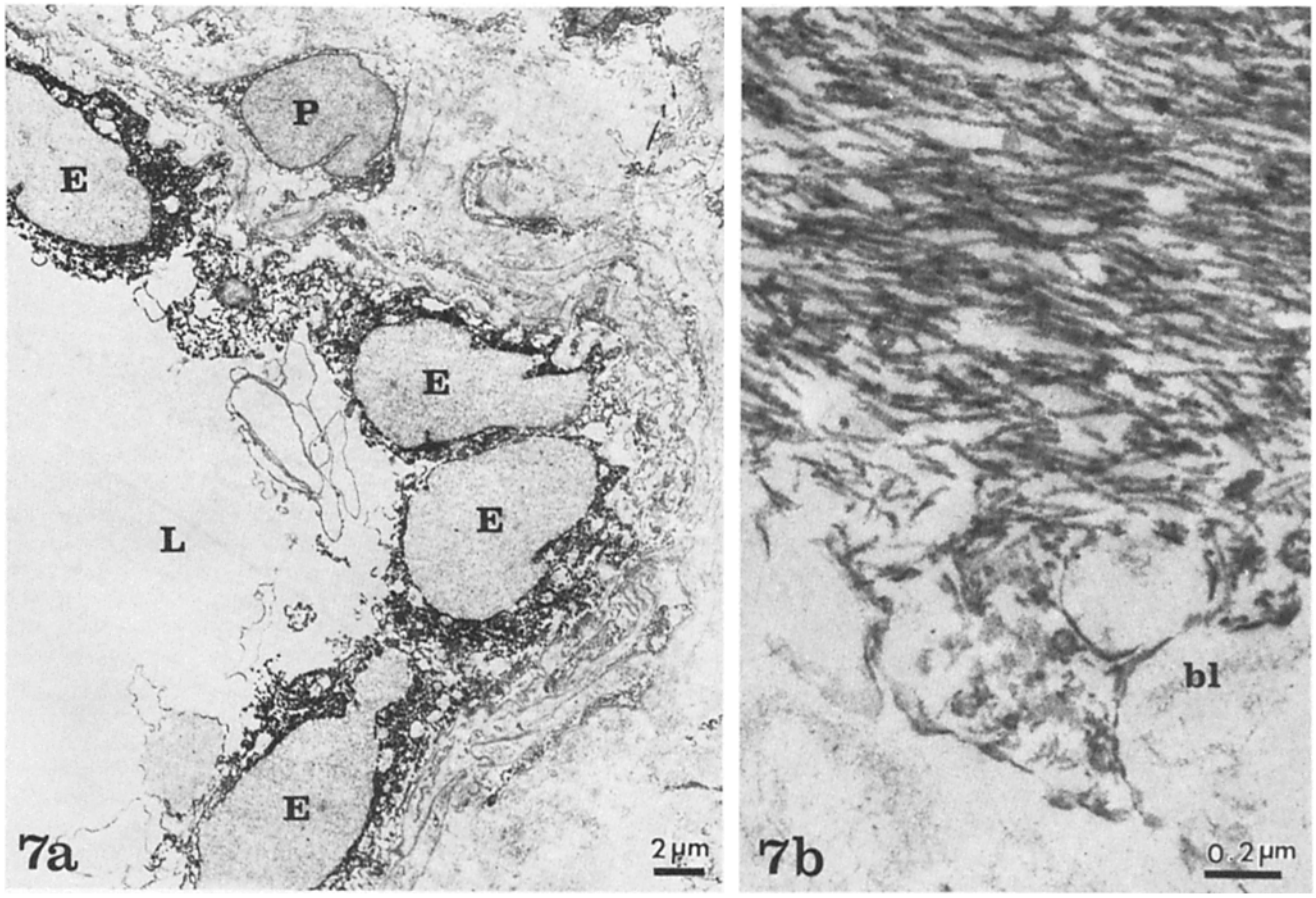


FIGURE 7 Immunoelectronmicroscopic visualization of C3-binding sites within capillary endothelium. Cryostat sections of normal human skin were incubated with NHS and then with HRP-R/Hu C3. (*a*) HRP reaction product is deposited within the cytoplasm of endothelial cells (*E*) and a pericyte (*P*). Bar, 2 μm. × 3,200. (*b*) Higher magnification of the endothelial cell reveals ISF enveloped by the electron-dense marker. *L*, vascular lumen; *bl*, basal lamina. Bar, 0.2 μm. × 48,000.

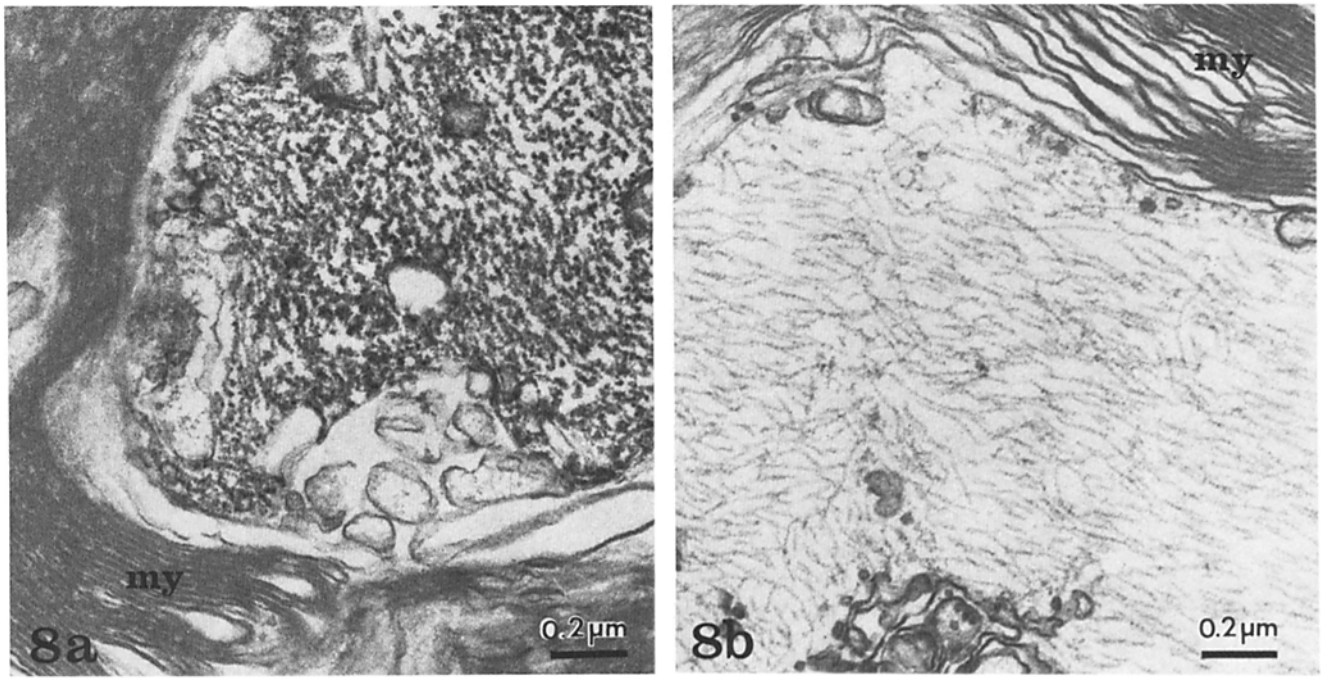


FIGURE 8 Immunoelectronmicroscopic visualization of C3-binding ISF within a cutaneous nerve. Cryostat sections of normal human skin were incubated with either NHS (*a*) or C4-deficient serum (*b*) and then with HRP-R/Hu C3. (*a*) ISF within a myelinated axon appear decorated with HRP-reaction product. Bar, 0.2 μm. × 48,000. (*b*) No reaction product is discernible on ISF. *my*, myelin sheath. Bar, 0.2 μm. × 48,000.

level was indistinguishable from that seen in untreated sections; ultrastructurally, HRP reaction product enveloped all the remaining filamentous structures (Fig. 6 *a*). Using conventionally postfixed (3% aqueous OsO_4 followed by 5% veronal-acetate-buffered uranyl acetate) control sections, filaments remaining after the extraction procedure appeared uniform, had a diameter of ~10 nm and, thus, represented ISF (Fig. 6 *b*).

Similarly, after application of the extraction procedures listed above, an intact insoluble ISF network was left behind in all other cell types tested and HRP-reactivity was identical to that seen in untreated sections.

In addition to the cell types listed, normal dermis also contains a small number of other cellular constituents such as mast cells, lymphocytes, and histiocytes. In the specimens investigated, the number of these cells encountered was extremely small and their preservation not adequate enough to draw valid conclusions with regard to their expression of C-binding cytoskeletal structures.

CONTROLS:—In all control specimens, i.e., after substitution of NHS by PBS, or under conditions that do not allow classical and/or alternate pathway activation, HRP deposition was not observed (Fig. 2, 6 *b*, and 8 *b*). This is also true for specimens in which HRP-R/Hu C3 was replaced by HRP-R/Hu Ig. Thus, no evidence was obtained at the electron microscopic level that autoantibodies participate in the generation of C3 deposition along ISF.

DISCUSSION

In a previous study (18), we found that exposure of cryostat sections of normal human skin to fresh NHS and FITC-R/Hu C3 resulted in a strong cytoplasmic fluorescence of various skin cells. This finding was in accordance with recent reports by Linder et al. (28–30, 32) who first described cytoplasmic binding to certain mesenchymal cells in tissue sections and culture. For at least two cell types, i.e., suprabasal keratinocytes and smooth muscle cells, we presented evidence that cytoplasmic C3 binding was most likely due to an antibody-independent binding of Clq to cytoplasmic sites followed by activation of the classical C cascade (18). In the case of melanocytes, fibroblasts, and endothelial cells, cytoplasmic C3 binding was also shown to result from classical C activation. However, with regard to these cell types, we could not determine whether this binding had occurred in an antibody-independent fashion or had been initiated by C-binding anticytoplasmic autoantibodies; the occasional occurrence of such antibodies has been described (17, 19, 31). The major limitations of our IF studies were that the exact nature of certain cells with cytoplasmic C3-binding properties could not be determined and, more importantly, that they did not provide a clue to the morphological substrate of C3 deposition. In this report, we unequivocally demonstrate that suprabasal keratinocytes, melanocytes, fibroblasts, smooth muscle cells, endothelial cells, pericytes, Schwann cells, and nerve axons possess cytoplasmic C3-binding structures, whereas basal keratinocytes and epidermal Langerhans cells do not. The visualization of cytoplasmic C3 binding within the cell types listed was a finding consistent for all NHS and substrates tested. This varies slightly from our earlier IF studies in which C3 binding within melanocytes and endothelial cells was only observed in some instances. The reason for this discrepancy may be due to a higher sensitivity of the immunoperoxidase technique as compared with conventional IF (41).

In addition, our results now provide definitive proof that—under the tissue processing techniques used—ISF represent the only cytoplasmic structures upon which C3 deposition can occur, as has been proposed by Linder et al. (30, 32) on the basis of light microscopic studies. However, we cannot exclude the possibility that other cellular constituents, e.g., cytoplasmic matrix and microtubular structures, which are removed and disrupted by freezing, thawing, and washing procedures (45) might also display C-binding properties. This is also true for

actin filaments which, in the case of smooth muscle cells, had to be extracted in order to permit an unequivocal proof for the C3-binding capacity of ISF; in the other cell types studied, actin filaments constitute only a minor portion of the cytoskeleton and, therefore, cannot be easily discerned in the lightly stained sections required for our immunoperoxidase experiments.

Together with microtubules and microfilaments, ISF comprise the cytoskeleton of higher eukaryotic cells (15, 25). Morphologically, ISF are defined as unbranched filaments with an average diameter of 10 nm; on cross-sections, they may exhibit a hollow core (21). Despite their morphological uniformity, ISF display broad differences in size and complexity of their constitutive subunit proteins (2, 4, 6, 8–14, 25, 26, 35, 36).

On both biochemical and immunological grounds, five major classes of ISF can be differentiated, i.e., keratin filaments, desmin filaments, vimentin filaments, neurofilaments, and glial filaments (25). Linder et al. have provided suggestive (light microscopic) evidence for the occurrence of C-binding sites on filaments of the desmin- and vimentin-type (30, 32). Our studies demonstrate that cytoplasmic C3 binding is not confined to these two ISF types known to occur within melanocytes, fibroblasts, smooth muscle cells, endothelial cells, and Schwann cells (25), but can also be detected on keratin filaments and neurofilaments. Thus, at least four of the five ISF types known so far bear C-binding sites. From our studies, we cannot yet draw final conclusions as to the ISF subunit responsible for C3 binding. Although extraction procedures with nonionic detergent and low/high ionic salt buffers excluded the possibility that cytoplasmic structural components loosely attached to ISF were responsible for the C-binding phenomena observed, these experiments did not provide information whether this functional property was inherent to filament core proteins or, alternatively, to consistently and specifically associated proteins. There exists evidence that indicate that alpha-helical segments, although displaying significant amino acid sequence variability, comprise the basic framework of ISF and that the biochemical and immunological differences reported for ISF of different cell types reside within their non-alpha-helical regions (4, 39). The recent characterization of a monoclonal antibody that binds to most and perhaps all ISF subunit proteins that have previously been defined, supports the concept that all ISF share common domains (34). Our finding that ISF in cell types containing different ISF classes have the capacity for C3 binding leads us to the assumption that the C3-binding site is also located within subunit domains common to most, if not all, ISF subunit proteins, i.e., the alpha-helical segment (4, 39). Following this reasoning, the finding that ISF of basal keratinocytes and epidermal Langerhans cells do not bear C3-binding sites may be due to different biochemical or physicochemical properties of ISF alpha-helical regions within these cells or, alternatively, may be due to masking of C-binding filament wall proteins by associated proteins peculiar for these cell types. Recently, it has been shown that a class of cationic structural proteins, termed filaggrin, associated specifically with ISF in a stoichiometric manner that suggests an ionic interaction with the alpha-helical domains of ISF (38). It therefore seems conceivable that differences in C-binding capacity may reflect charge differences between alpha-helical portions of ISF in different cells or in cells at different stages of differentiation. In this context, it is of interest that polyanions can activate the C system via the classical pathway by binding directly to Clq (1, 33). Thus, it does not seem unreasonable to assume that C3 deposition on ISF is initiated by an ionic interaction between Clq and negatively charged alpha-helical portions of ISF. The validity of this concept is currently being investigated on isolated ISF.

We thank Dr. P. Steinert of the National Institutes of Health for helpful criticism and advice. We are grateful to Mrs. Alice Orque, Ursula Stanzl, and Hella Stössl for expert technical assistance, to Mr. Bernhard Sickert for the excellent photographic work, and to Mrs. Janet Gschnitzer for secretarial assistance.

This work was supported in part by grant no. 3228 from the Fonds zur Förderung der wissenschaftliche Forschung, Vienna, Austria.

Received for publication 1 March 1982, and in revised form 2 August 1982.

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