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Desmosomal Antigens Are Not Recognized by the Majority of Pemphigus Autoimmune Sera

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Sera from 7 patients with pemphigus vulgaris and both mouse and rabbit antisera against bovine epidermal desmosomes contained antibodies that bound to cell surface components of the spinous layer of bovine epidermis. The antidesmosomal sera showed significant binding to purified desmosomal proteins in an enzyme-linked immunosorbent assay (ELISA). Two of 7 pemphigus sera bound to desmosomal protein-coated microtiter plates at low dilution titers. Two of 6 normal human sera also bound to desmosomal protein-coated microtiter plates

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Abbreviations:
BSA: bovine serum albumin
ELISA: enzyme-linked immunosorbent assay
PBS: phosphate-buffered saline
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

at titers comparable to those of the pemphigus sera. Indirect immunofluorescent labeling of frozen sections of monkey esophagus revealed striking differences in the distribution of pemphigus antigens and desmosomal constituents. Pemphigus antisera produced rather uniform fluorescence around the borders of spinous cells of the esophageal epithelium, while anti-desmosomal antibodies bound in a punctate pattern. Anti-desmosomal antibodies labeled cells of the basal layer in a strongly punctate pattern. Only 1 pemphigus serum appreciably labeled basal cells. Two of 3 anti-desmosomal antisera bound avidly in the upper differentiating layers of the epithelium. Pemphigus antibodies did not. Pemphigus sera that reacted with desmosomal proteins in ELISA were absorbed by affinity chromatography on immobilized desmosomal proteins. This treatment did not alter the immunofluorescent labeling patterns produced by these sera. From these results we conclude that the pemphigus autoantibodies studied here bind to epithelial cell surface antigens which are distinguishable from the structural components of desmosomes.

Pemphigus vulgaris is a human autoimmune disease characterized by the presence of circulating antibodies that react with the intercellular regions of the living cell layers of stratified squamous epithelia [1,2]. These antibodies induce cell separation in epithelial tissues, resulting in blistering lesions of the skin and oral cavity. Antibodies from sera of pemphigus patients can induce acantholysis in vitro when applied to explants of normal human [3] or monkey [4–6] skin (reviewed in [7]).

Depending upon tissue and species source and method of isolation, several chemically diverse antigens have been reported as possible targets of pemphigus autoantibodies [8–14] (reviewed in [15]). Though some early investigators suggested that defective desmosomal contacts might contribute to the pathogenesis of pemphigus [16], later workers have generally concluded that the primary lesion occurs in the "intercellular cement substance" of nondesmosomal cell surfaces, desmosomal contacts being broken only secondarily [17–19]. In contrast, immunoelectron microscopic labeling of acantholytic lesions from pemphigus patients [20] and of dispersed guinea pig epidermal cells that were treated with pemphigus antibodies [21] has detected pemphigus antigen on both desmosomal and nondesmosomal regions of the cell surface. Moreover, pemphigus vulgaris autoantibody reacts most strongly with the spinous layer of the epidermis, the layer in which desmosomes are most abundant. These observations raise the question of whether pemphigus antigens are components of epidermal desmosomes.

We have recently described procedures for the purification of both "whole" desmosomes and desmosomal "cores" from bovine muzzle epidermis [22]. Ultrastructural characterization of these fractions shows that whole desmosomes contain both intercellular and cytoplasmic components of desmosomes, while cores consist primarily of intercellular elements. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the whole desmosome fraction reveals about 12 major protein bands, some of which are glycosylated. The glycoproteins are highly and specifically enriched in gel profiles of the desmosomal core fraction.

We have prepared antisera against whole desmosomes and cores, and have compared their sites of binding in the epidermis and esophagus with those of antibodies from the sera of 7 patients afflicted with pemphigus vulgaris and from 6 normal human sera. Two of 7 pemphigus sera and 2 of 6 normal human sera contain low titers of antibody that react with components of the desmosomal core preparation. None of these 4 sera label tissue in a pattern similar to that produced by the anti-desmosomal antibodies. The nature of the immunoreactive material is not known, but depletion of antibody that binds to it by affinity chromatography on immobilized desmosomal protein does not alter the immunofluorescent labeling pattern of the pemphigus sera. These results suggest that pemphigus autoantibodies are directed primarily against target sites that are not structural elements of the desmosome.

MATERIALS AND METHODS

Preparation of Sera

Pemphigus serum A was a gift of Dr. Jean-Claude Bystryn (New York University Medical Center, New York, New York). Pemphigus sera B–G were a gift of Dr. Luis Diaz (Department of Dermatology, Johns Hopkins University Medical School, Baltimore, Maryland). Normal human sera were obtained at the McCosh Infirmary, Princeton University. The isolation of whole desmosomes and desmosomal cores is described elsewhere [22]. Desmosome fractions were solubilized in SDS and used to elicit antibodies in mice through i.p. followed by i.v. injections. Blood was collected from the mice by cardiac puncture. A rabbit received multiple subcutaneous injections. Blood was obtained by bleeding from the ear vein. Blood was allowed to clot, was centrifuged briefly, and the serum was removed.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA of desmosomal proteins is described elsewhere [23]. Briefly, proteins solubilized in SDS were adsorbed to microtiter plates. After washing in buffer, the desmosomal protein-coated plates were incubated with the human, mouse, or rabbit antisera, washed, incubated with peroxidase-conjugated antihuman, antimouse, or antirabbit IgG, washed again to remove unbound antibody, and the peroxidase reaction was developed.

Immunofluorescence

Fresh calf muzzles were obtained from a local slaughterhouse. Pieces of epidermis with some attached dermis were sectioned at 5 μm on a cryostat. The sections were collected on slides, fixed for 3 min in 95% ethanol, and air dried. Monkey esophagus sections were obtained commercially (BioDx, Denville, New Jersey). Whole desmosome fractions were centrifuged for 45 min at 200,000 *g* (maximum acceleration) in hemihyperboloid BEEM capsules fitted into Epon adaptors [24] in the SW 41 rotor of a Beckman L5-75 centrifuge. The pellet was then frozen and sectioned at 10 μm.

All antibody labeling steps were carried out at room temperature and all labeling solutions were centrifuged at 15,000 *g* for 2 min in a Fisher microcentrifuge (Fisher Scientific, Springfield, New Jersey) just prior to use, to remove precipitated material. Tissue sections were washed in phosphate-buffered saline (PBS), pH 7.4, for 30 min and treated with either normal rabbit serum or normal goat serum, as appropriate, before incubation with the immune sera. Pemphigus sera B–G and control human serum were diluted 1:25, pemphigus serum A and control serum were diluted 1:50, mouse and rabbit sera were diluted 1:100 in PBS containing 2% bovine serum albumin (BSA) and 0.3% Triton X-100. Diluted sera were applied to tissue sections for 30 min. Slides were rinsed and washed in 2 changes of PBS containing 1.0% Triton X-100 for 15 min each. Normal rabbit or goat serum was then reapplied for 15 min, followed by fluorescein-conjugated rabbit antihuman IgG (Miles Laboratories, Elkhart, Indiana) or antimouse IgG (Cappel Laboratories, Cochranville, Pennsylvania), or goat antirabbit IgG (Tago Inc. Burlingame, California) diluted 1:50 in PBS containing 2% BSA and 0.3% Triton X-100. Just before use, 1 ml of the diluted antihuman and antimouse conjugates were preabsorbed for 1 h against 250 mg of acetone powder from chicken gizzard. The absorbed conjugates were centrifuged for 5 min in the microcentrifuge, the supernatants were collected, recentrifuged, and applied to the sections for 30 min. The preabsorption step was omitted for the goat antirabbit conjugate, which was supplied in an affinity purified form. After incubation with the fluorescent conjugate the slides were rinsed and washed twice for 15 min each with PBS containing 1% Triton X-100. Coverslips were mounted and the slides were examined on a Zeiss Universal microscope equipped with epifluorescence accessories.

Modifications of this staining procedure showed that the pretreatment with normal serum, the use of BSA and Triton X-100 in the labeling and washing buffers, and the absorption of the fluorescent conjugates against chicken gizzard acetone powder did not affect specific labeling. These treatments greatly reduce nonspecific background staining, defined as diffuse labeling observed when nonimmune control sera were applied to the sections.

RESULTS

Antisera raised against desmosomal cores in a mouse and in a rabbit and an antiserum raised against whole desmosomes in

a mouse bind at high dilution titer to desmosomal proteins in ELISA (Table I). Five of 7 pemphigus sera tested reacted very weakly in ELISA, at levels comparable to the normal human serum. Two pemphigus sera reacted at dilution titers of 1/30 and 1/90 with whole desmosome- and desmosomal core-coated microtiter plates. Two of 6 normal human sera also reacted with desmosomal core-coated microtiter plates at dilution titers of 1/30 and 1/90.

We considered the possibility that the lack of reaction of some of the pemphigus antibodies with desmosomal proteins in ELISA might be due to the denaturation of the desmosomal proteins prior to their attachment to the microtiter plate. To test this possibility we assayed the binding of the mouse anti-whole desmosome serum and pemphigus serum A to frozen sections of pellets of whole desmosomes isolated from bovine muzzle, using indirect immunofluorescence. While the anti-whole desmosome serum, as expected, stained the sections very intensely, pemphigus serum A showed virtually no binding (Fig 1).

Immunofluorescent labeling of bovine muzzle skin indicates that the mouse antisera and pemphigus serum A label the cell borders in the living cell layers of the epidermis (Fig 2). In most instances we observed that the anti-desmosomal sera also strongly labeled the corneum layer, while pemphigus serum A did so only weakly. Due to the high incidence of anti-corneum antibodies in the general human population [25], the labeling of stratum corneum by antidesmosomal antibody and to a lesser extent by pemphigus autoantibody is not a reliable indicator by which to distinguish these antibodies.

In contrast with the slight differences in the pattern of binding of anti-desmosomal antibody and pemphigus serum A to bovine epidermis, their patterns of binding to monkey esophagus were strikingly different. All 7 pemphigus sera bound primarily to the cells of the spinous layer, but anti-whole desmosome and anti-core sera reacted with all layers of the esophageal epithelium, including the basal, spinous, and differentiated cell layers (Fig 3). Pemphigus serum B differed from the other pemphigus sera in that it appreciably labeled cells of the basal layer, in addition to the characteristic labeling of the spinous cell layer. Examination at higher magnification revealed that while pemphigus antigens are found more or less continuously over the spinous cell surface, desmosomal constituents are distributed in a punctate pattern (Fig 4). Moreover, in the fully differentiated layers of the esophageal epithelium, where pemphigus antigens are sparse, the mouse anti-desmosomal sera show a secondary pattern of labeling (Fig 5). In this region, desmosomal constituents become concentrated at the circumference of each flattened cell (the cell vertices as they appear in section). The rabbit anti-core serum does not label this secondary pattern. The 2 normal human sera that reacted in ELISA labeled monkey esophagus in a pattern distinctly different from that generated by either the anti-desmosomal antisera or the pemphigus sera. These 2 sera predominantly labeled the surfaces of cells in the most basal layer of the epithelium (Fig 3*i*). There was also a weaker diffuse cytoplasmic labeling in all layers of the esophagus.

In order to assess the contribution of the anti-desmosomal antibodies to the characteristic immunofluorescent labeling pattern of pemphigus, anti-desmosomal antibodies were specifically depleted from pemphigus sera B, D, and E by incubation with desmosomal proteins covalently coupled to CNBr-Sepharose. The quantity of beads used was capable of depleting anti-desmosomal antibody from an equal volume of rabbit anti-core serum containing a higher titer of anti-desmosomal antibody than that found in the pemphigus sera. Depleted sera D and E retained virtually no antibody capable of binding to desmosomal core-coated microtiter plates (not shown). Removal of anti-desmosomal antibody from these sera did not alter their patterns of immunofluorescent labeling on monkey esophagus sections. The immunofluorescent labeling pattern of pemphigus serum D is fairly typical of pemphigus autoantibody (Fig 3*h*).

Unlike the other pemphigus sera, serum B labels cells of the basal layer of monkey esophagus in a punctate pattern (Fig. 3*g*). This pattern is qualitatively different from and of lower

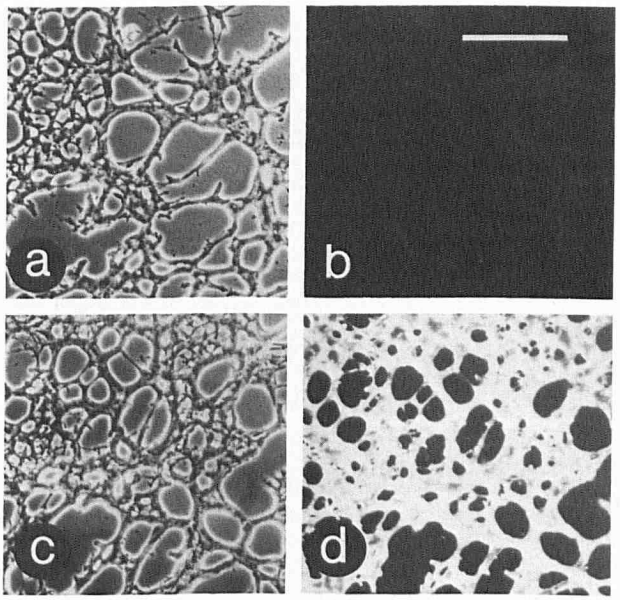

FIG 1. Immunofluorescent staining of frozen sections of a pellet of whole desmosomes. *a*, Phase contrast photograph of a section labeled by indirect immunofluorescence with pemphigus serum A. *b*, Same section as *a* viewed by fluorescence microscopy. *c*, Phase contrast photograph of a section labeled by indirect immunofluorescence with anti-whole desmosome serum. *d*, Same section as *c* viewed by fluorescence microscopy. Pemphigus serum A, which shows little or no binding to denatured desmosomal proteins in ELISA, also does not label undenatured whole desmosomes. Mouse anti-whole desmosome serum labels the section intensely. Vacuolization of the pellet is an artifact of the specimen preparation. *Bar* = 100 μm.

TABLE I. *Screening for anti-desmosomal antibodies by ELISA against proteins from either whole desmosomes or desmosomal cores*

Serum	Titer	
	Core[a]	WD[b]
Mouse anti-whole desmosome		450
Mouse anti-desmosomal core	≥900	
Rabbit anti-desmosomal core	≥810	≥270
Pemphigus serum		
A	100[c]	<50
B	10	10
C	10	10
D	30*	30*
E	90*	90*
F	10	10
G	10	10
Control human serum		
1	100[c]	<50
2	10	—[d]
3	10	—
4	30*	—
5	90*	—
6	10	—

Seven pemphigus sera and 6 normal human control sera were tested for the presence of anti-desmosomal antibodies, each serum was tested in duplicate by serial 3-fold dilution to determine the anti-desmosomal antibody titer. Antisera raised against desmosomal proteins were used for comparison. The human antisera that reacted with desmosomal protein-coated microtiter plates above the background level defined by the other sera are labeled with an asterisk.

[a] Desmosomal core protein coated microtiter plate.

[b] Whole desmosomal protein-coated microtiter plate.

[c] Pemphigus serum A and control human serum 1 were screened in a separate experiment from the other human sera. The background level of labeling was higher in that experiment.

[d] Not tested.

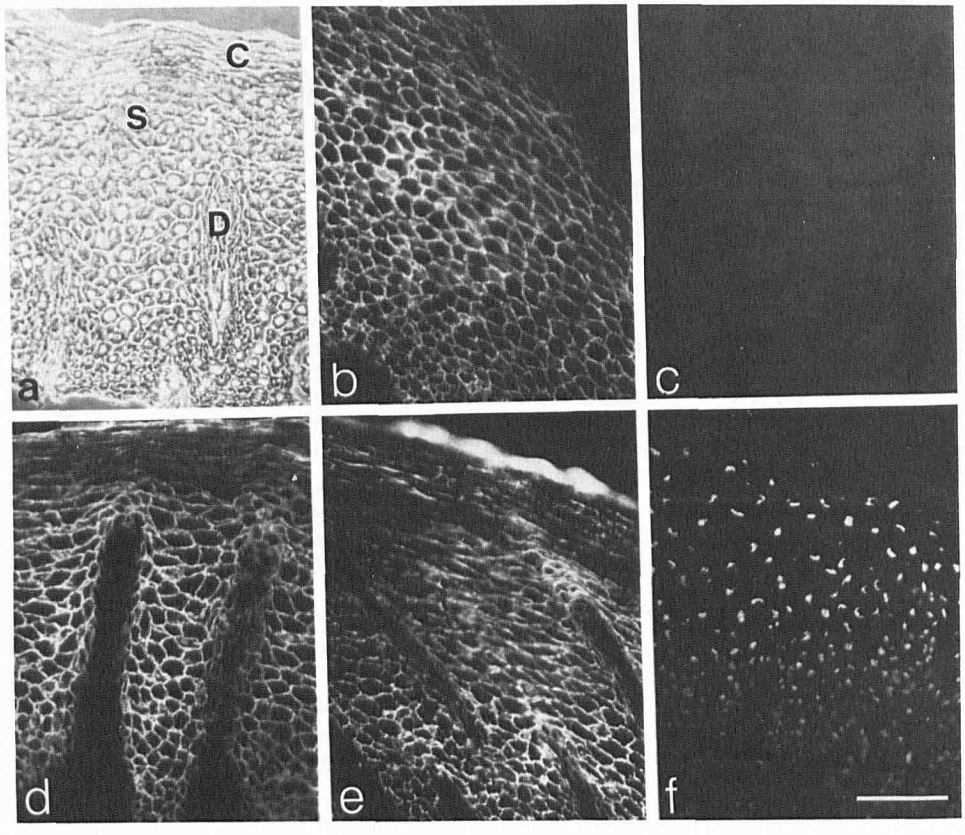

FIG 2. Immunofluorescent labeling of frozen sections of bovine epidermis. *a*, Phase contrast: dermal papillae (*D*), spinous layer (*S*), corneum layer (*C*). *b*, Pemphigus serum A. *c*, Control human serum. *d*, Anti-whole desmosome serum. *e*, Mouse anti-desmosomal core serum. *f*, Control mouse serum showing the presence of antinuclear antibody. Labeling patterns of the pemphigus and anti-desmosomal sera are similar, although the anti-desmosomal sera label more strongly in the corneum layer. *Bar* = 100 μm.

intensity than that generated by the anti-desmosomal antibodies. Serum B does not react with desmosomal proteins in ELISA, and depletion of anti-desmosomal antibody by affinity chromatography does not eliminate labeling of the basal cells. These results suggest that in addition to the pemphigus antigens that are concentrated in the cells of the spinous layer, pemphigus serum B also recognizes an antigen expressed on the surfaces of cells of the basal layer that is not a desmosomal component.

DISCUSSION

Antisera prepared against whole desmosomes and desmosomal cores show a strong and specific binding to both isolated desmosomal components and to desmosomal components in situ in bovine skin and in monkey esophagus. The punctate distribution of desmosomal components on the surface of bovine muzzle keratinocytes may be obscured by the abundance of desmosomes in this tissue (see Fig 1 in [22]). This distribution is, however, readily detected in the epithelium of monkey esophagus, where anti-desmosomal antibodies bind in a discontinuous, punctate pattern around the cell surface. The punctate distribution is most obvious near the basal layer of the epithelium, where desmosomes are less abundant, but it can be detected in all cell layers.

Antibodies from pemphigus sera bind predominantly to the surfaces of cells in the spinous layer of both bovine epidermis and monkey esophagus. Unlike the anti-desmosomal sera, pemphigus sera show little or no labeling of cell surfaces in the basal or corneum cell layers. Within the spinous layer, pemphigus antigens are distributed evenly over the cell surface, in contrast to the punctate distribution of desmosomal antigens. The difference in the 2 labeling patterns is most obvious in monkey esophagus. It has been reported that under certain conditions indirect immunofluorescent labeling with pemphigus antibodies reveals a somewhat punctate distribution of antigen on keratinocytes in tissue culture [26,27]. However Takagawa et al [21] have shown that pemphigus antigens become artificially clustered when living cells are labeled with antibody at 37°C. Moreover, Diaz and Marcelo [26] found that pemphigus antigens become clustered over a period of days on the surface of cultured keratinocytes, first into small patches and eventually into a single, large cap. These results suggest that the reported punctate distribution of pemphigus antigen [26, 27] may be due to antibody-induced patching of cell surface receptors that are mobile in the plasma membrane.

The differences in the labeling pattern of pemphigus and anti-desmosomal sera suggest that the pemphigus sera do not label desmosomal antigens. Within the limits of resolution afforded by immunofluorescence microscopy, we observe a fairly uniform distribution of pemphigus antigen on the cell surface. This observation allows for the possibility that pemphigus antigens might be found both in desmosomes and in nonjunctional regions of the plasma membrane. If pemphigus antibodies label only antigens not found in desmosomes it would be difficult to detect the absence of label in desmosomes by immunofluorescent labeling, given the small diameter of a desmosome in relation to the thickness of the sections examined. Immunoelectron microscopic localization of pemphigus antigens in acantholytic lesions of pemphigus patients [20] and on trypsinized guinea pig epidermal cells [21] has revealed the presence of pemphigus antigens on both desmosomal and nondesmosomal regions of plasma membrane. The antigen localization reported in these studies must be viewed with some caution, since the cells examined were either dissociated with protease [21] or perhaps subject to endogenous protease activity [20]. The acantholytic lesions found in pemphigus have been reported to be caused by endogenous cellular proteases released in response to autoantibody binding [27–30]. The distribution of cell surface components has been reported to be affected by exposure of the cell surface to protease (see e.g., [31]).

In spite of the rather different distributions of pemphigus antigens and desmosomal constituents observed in the immunofluorescent localization experiments described above, the possibility that pemphigus antibody might be directed against

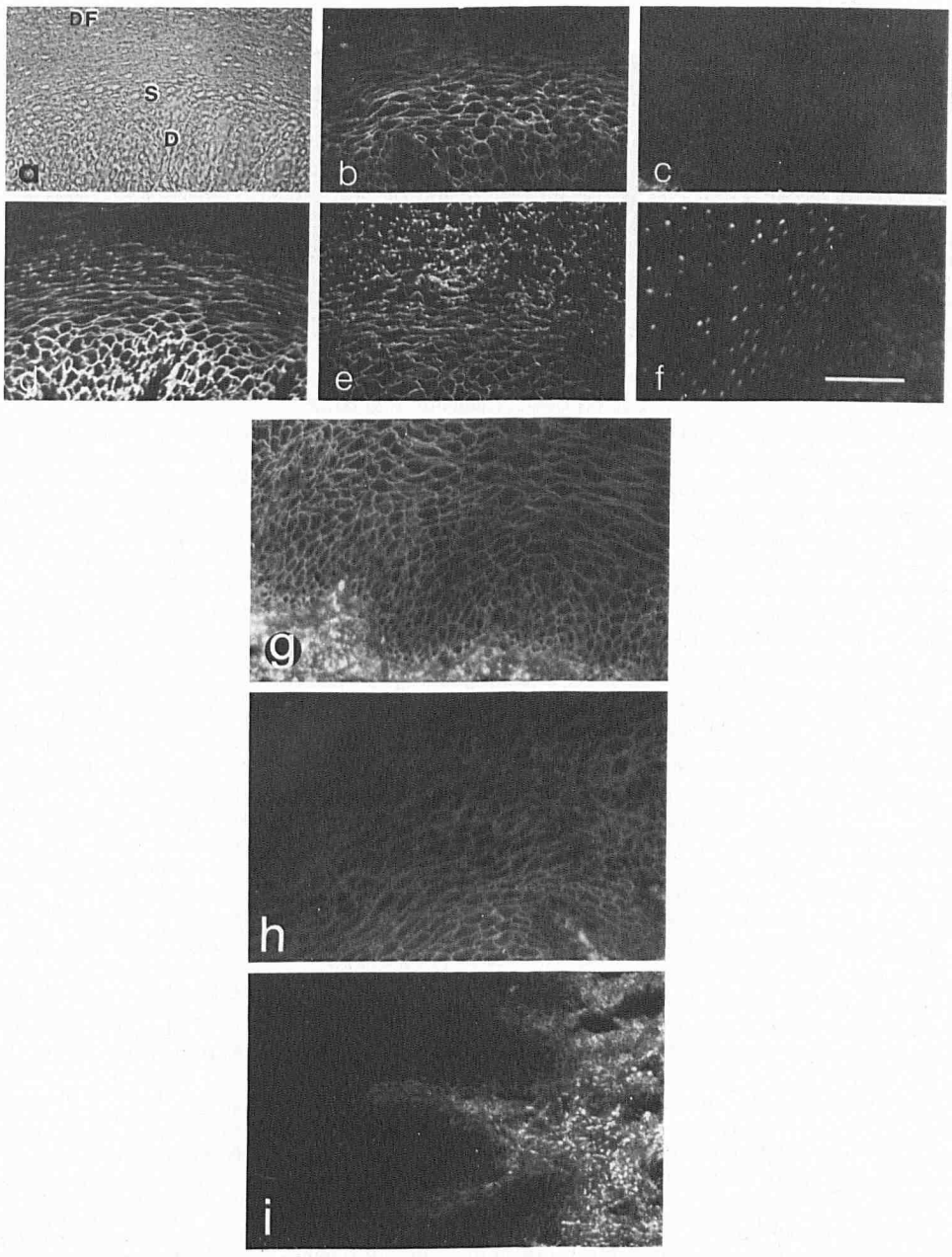

FIG. 3. Immunofluorescent labeling of frozen sections of monkey esophagus. *a*, Phase contrast: dermal papilla (*D*), spinous layer (*S*), differentiated layer (*DF*). *b*, Pemphigus serum A. *c*, Normal human serum 1. *d*, Anti-whole desmosome serum. *e*, Anti-desmosomal core serum. *f*, Control mouse serum. *g*, Pemphigus serum B. *h*, Pemphigus serum D. *i*, Normal human serum 4; the dermis in this illustration is at the right. Pemphigus antibodies bind most strongly to cell borders in the spinous layer. Pemphigus serum B and normal human serum 4 label cells of the basal layers. Anti-desmosomal antibodies label cells of all layers of the epithelium in a distinctive punctate pattern. *Bar* = 100 μm.

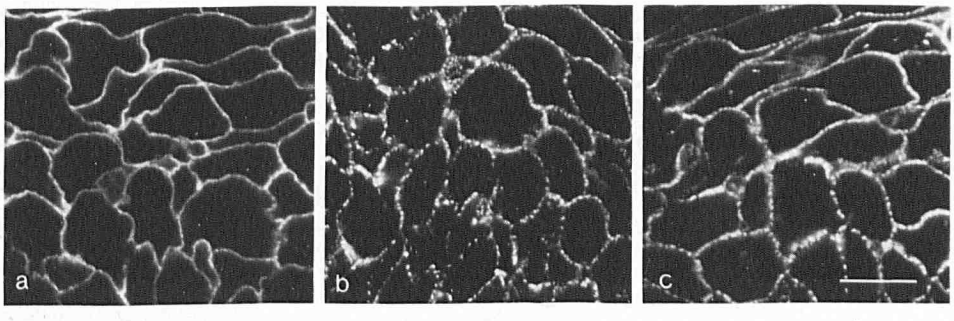

FIG 4. High magnification view of immunofluorescent labeling pattern in the spinous layer of monkey esophagus. *a*, Pemphigus serum A. *b*, Anti-whole desmosome serum. *c*, Anti-desmosomal core serum. Pemphigus antigens are distributed uniformly around the cell borders. Desmosomal antigens are distributed in a punctate pattern. *Bar* = 20 μm.

desmosomal as well as nondesmosomal components of the cell surface was tested by screening pemphigus sera for antibody capable of binding to desmosomal proteins adsorbed to microtiter plates. Five of 7 pemphigus sera did not contain detectable anti-desmosomal antibody. Two pemphigus sera had low titers of antibody that bound to desmosomal protein. Two of 6 normal human sera also contained antibody that bound to desmosomal protein-coated microtiter plates with titers similar to those of the pemphigus sera. The 2 normal human sera labeled sections of monkey esophagus, but the pattern of antigen localization was distinctly different from that of either the pemphigus sera or the anti-desmosome sera. The nature of the antigen recog-

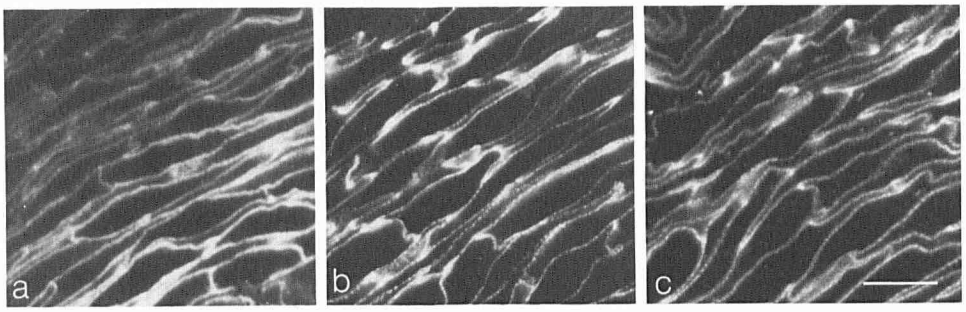

FIG 5. High magnification view of immunofluorescent labeling patterns in the region of transition from the spinous to the differentiated layers of the monkey esophageal epithelium. *a*, Pemphigus serum A. *b*, Anti-whole desmosome serum. *c*, Mouse anti-desmosomal core serum. Pemphigus antigens decrease in amount in the differentiated layers and show only a slight tendency to concentrate at the cell vertices. Desmosomal antigens retain their punctate distribution around the cell border, but also become very concentrated at the vertices of the flattened cells as they transit from the spinous to the differentiated layers. *Bar* = 20 μm.

nized by these sera is not known. The detection of nondesmosomal immunoreactive material in ELISA may indicate the presence of contaminating impurities in the desmosomal core preparation. The antigen recognized in ELISA by the pemphigus sera is also unknown. If this antigen was desmosomal, then the titer of the anti-desmosomal component of the pemphigus sera was presumably below the level of detection in immunofluorescent labeling. When the "anti-desmosomal" activity was specifically depleted from these sera, no change was observed in their patterns of immunofluorescent labeling of monkey esophagus sections.

Although cases of pemphigus can be found in which antibodies to desmosomal constituents may occur, autoimmune recognition of desmosomes is not a requisite feature of the disease. Only 2 of 7 sera tested contained detectable levels of "anti-desmosomal" antibody. Indeed, 2 of 6 normal human sera showed similar levels of "anti-desmosomal" antibody. Although there are differences in detail, all of the pemphigus sera examined share a common general pattern of immunofluorescent labeling in the epithelial tissues. The differences that we have detected among the sera suggest some heterogeneity in the composition of autoimmune antibody generated in different cases of pemphigus. A second indication of such heterogeneity lies in the observation that, unlike the other pemphigus sera, serum B labels cells of the basal layer of monkey esophageal epithelium, appreciably and in a punctate pattern. The antigen recognized on basal cells by this serum is unrelated to desmosomes since serum B does not recognize desmosomal protein in ELISA and depletion of anti-desmosomal antibody by affinity chromatography does not eliminate the labeling of the basal cells. Our comparison of pemphigus autoantibodies and anti-desmosomal antibodies suggests that the target sites of the autoimmune antibodies are not structural elements of desmosomes.

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