0022-202X/84/8304-0409$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 83:409–415, 1984

Vol. 83, No. 6
Printed in U.S.A.

Demonstration of Pemphigus Antibodies on the Cell Surface of Murine Epidermal Cell Monolayers and Their Internalization

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The pathogenic effects of pemphigus vulgaris (PV) antibodies on epidermal cells can be demonstrated both in vitro using skin organ culture or primary epidermal cell cultures (PECC) and in vivo by passive transfer of PV antibodies into neonatal BALB/c mice. Although PV antibodies have been localized on the epidermal cell surface by several techniques, little is known about the fate of these autoantibodies subsequent to their surface binding. We have examined this, using murine PECC which express pemphigus antigen on their surface, and followed the fate of the bound antibody molecules. Forty-eight-hour PECC were incubated at 37°C with PV antibodies for 20 min and then with horseradish peroxidase-labelled antihuman IgG. This was considered time 0. The monolayers were fixed with glutaraldehyde after 0, 0.5, 1, 3, 6, 18, and 24 h incubation at 37°C and then processed for electron microscopy. At time 0 hour, PV antibodies is detected bound evenly along the surface of keratinocytes. Within 30 min, the bound PV antibodies becomes clustered, internalized into submembranous vesicles via surface pits, and eventually fused with lysosomes. Widening of the intercellular spaces was also seen in PECC treated with PV antibodies within the first 24 h. PECC treated with normal human IgG in parallel cultures showed no such surface binding, internalization, or cell–cell detachment. Treatment with cytochalasin-D and/or colchicine did not affect the internalization of the PV antibodies, but fusion with lysosomes was not seen in treated cultures.

These findings suggest that PV antibodies binds a surface antigen and the complex is internalized and fused with lysosomes in a process that may have pathophysiologic relevance.

Pemphigus vulgaris (PV) is a chronic bullous disease that presents with blisters and erosions of the skin and mucosal surfaces. Histologic examination of the lesions shows detachment of differentiating keratinocytes from each other (acantholysis) and from the basal cells which typically remain attached to the underlying basal lamina [1–2].

Two features are characteristic of pemphigus: (1) a defect in cell-to-cell adhesion within the epidermis, and (2) an autoimmune antibody-antigen reaction that takes place on the surface of epidermal cells. Immunofluorescence (IF) and immunoelectron microscopy (immuno-EM) have shown intercellular deposits of PV immunoglobulin in the lesional and perilesional epidermis [3–6]. PV antigen, to which PV antibodies bind, has been isolated from the epithelial tissue of several mammalian species and from human saliva, and partially characterized [7–11].

It is clear that PV antibodies are pathogenetically related to the epidermal acantholysis seen in the skin of PV patients. This is shown both by clinical observations and by in vitro and in vivo studies. In vitro studies have shown that IgG fractions from PV sera induce acantholysis in organ skin explants [12, 13] and cell detachment in primary epidermal cell cultures [14–16]. Recently, our laboratory has demonstrated the pathogenic role of PV antibodies in vivo by passive transfer of human IgG into neonatal BALB/c mice [17]. In that report it was shown that mice given i.p. injections of IgG from patients with PV developed cutaneous blisters and erosions with the histologic, ultrastructural, and IF features of PV.

It has been reported previously that neonatal murine epidermal cells in culture [14] and trypsin-isolated guinea pig epidermal cells [18–20] contain cell surface antigens which bind PV antibodies. Presently, however, the fate of surface-bound PV antibody–antigen complex and its importance in the induction of acantholysis is not known. This investigation was carried out to elucidate ultrastructurally the sequence of events that occurs during and following the binding of PV antibodies to the murine epidermal cell surface antigen. We found that PV antibodies bind a cell surface antigen and that the antibody–antigen complex is then internalized and eventually fused with lysosomes. This process may be an important mechanism in the pathophysiology of the disease.

MATERIALS AND METHODS

Pemphigus Vulgaris and Normal Human Sera

Serum was obtained from a single patient with the clinical, histologic, and immunologic features of PV. The titer of PV antibodies was 1:320 when tested against a rat-tongue epithelial substrate by indirect IF. The IgG fraction from this serum has been shown to induce cutaneous disease in mice when injected i.p. (Patient No. 2, described in the paper by Anhalt et al) [17]. Serum obtained from a normal human donor was used as a control. Both sera were sterilized by Millex filtration (Millipore Corp., Bedford, Massachusetts, pore size 0.22 μm) prior to use.

Epidermal Cell Cultures

Epidermal basal cells were isolated as described by Marcelo et al from the skin of neonatal BALB/c mice [21]. Briefly, mouse skin was incubated at 37°C for 1 h with 0.25% trypsin (Sigma, St. Louis, Missouri) in Medium 199 (M-199) containing modified Earle's salt with glutamine (Flow Labs, Rockville, Maryland). The dermal side of the separated epidermis was gently scraped in M-199 containing 50 IU/ml of penicillin and 50 μg/ml of streptomycin (P&S) (Flow Labs)

Manuscript received March 5, 1984; accepted for publication June 11, 1984.

Supported in part by U.S. Public Service Grants R01-AM 32081, R01-AM 32599, R23-AM 32079, and R23-AM 32490 from the National Institutes of Health, and a gift from the Estee Lauder Corporation.

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Abbreviations:
ABCPx: avidin-biotin peroxidase complex
DAB: 3,3′-diaminobenzidine
EM: electron microscopy
FCS: fetal calf serum
GBS: glutaraldehyde-borohydride-saponin
ICS: intercellular spaces
IF: immunofluorescence
Immuno-EM: immunoelectron microscopy
M-199: Medium 199
PBS: phosphate-buffered saline
PV: pemphigus vulgaris
P&S: penicillin and streptomycin

supplemented with 13% heat-inactivated fetal calf serum (FCS) (KC Biological, Lenexa, Kansas). The resulting cell suspension was subjected to discontinuous Ficoll density-gradient centrifugation using 12, 15, 18, and 20% Ficoll in M-199 with 13% FCS. The gradients were centrifuged for 30 min at 250 *g* and 4°C. The epidermal basal cells recovered in the 20% Ficoll fractions were washed with M-199 containing P&S and supplemented with 13% FCS. The cells were then resuspended to a known volume in the same medium.

A known number of basal cells (4.0×10^5) in a volume of 1 ml were seeded in 16-mm, 24-well Costar cluster Petri dishes with a 16-mm plastic coverslip (Thermanox, Lux Scientific Corp., Newbury Park, California) covering the bottom of the wells. The cultures were incubated at 37°C in a humid atmosphere with 5% CO_2 and 95% air. Approximately 95% of these cells excluded trypan blue. Following this culture technique, approximately 60% of the epidermal basal cells will attach to the plastic substrate [22], spread, and proliferate. At the end of 48 h, the epidermal cultures attain confluency and show approximately 2–3 layers of stratification [21]. These cultures can be maintained for several weeks if the growth medium is changed every 2 days [21].

Treatment of Epidermal Cell Cultures with Antibodies

The method followed for pemphigus antibody treatment of epidermal cell cultures was similar to the two-antibody technique used by Huet et al [23]. Freshly prepared epidermal basal cells were cultured as described. After 48 h of incubation, the supernatant medium was removed and the cultures were gently rinsed 3 times with M-199. A 1:80 dilution of PV serum in M-199 (0.5 ml) was added to experimental cultures and a 1:80 dilution of normal human serum in M-199 (0.5 ml) was added to control cultures. All cultures were incubated for 20 min at 37°C in a humid atmosphere with 5% CO_2 and 95% air. At this time point, the supernatant media were removed and the cultures were gently rinsed 3 times with M-199 in preparation for immunochemical staining.

Immunochemical Staining of Antibody-Treated Epidermal Cell Cultures

To demonstrate the initial surface binding of PV antibody molecules (arbitrarily labeled at time 0), the cultures were prefixed with freshly prepared 4% paraformaldehyde (Kodak, Rochester, New York) for 30 min before any immunochemical staining [24]. In other experiments, parallel unfixed cultures were treated with a 1:20 dilution of a peroxidase-labeled goat antihuman IgG (Cappel Laboratories, Cochranville, Pennsylvania) in M-199 for 30 min at 37°C in a humid atmosphere with 5% CO_2 and 95% air, followed by 3 rinses with M-199 at room temperature. These cultures were then incubated at 37°C in M-199 for different periods of time: 0.5, 1, 3, 6, and 24 h. Following the second incubation, the cultures were rinsed 3 times with M-199. The cultures, (including those that had been prefixed with paraformaldehyde), were then fixed in the culture plates with 1% glutaraldehyde (Polysciences, Warrington, Pennsylvania) in phosphate-buffered saline (PBS) for 20 min at room temperature, rinsed 3 times with PBS, and reacted with 3,3′-diaminobenzidine (DAB) (Sigma) for 10 min at room temperature.

Alternative Procedure for Immunochemical Staining of Antibody–Epidermal Cell Interaction

To eliminate the possibility that peroxidase-labeled goat antihuman IgG, which is divalent, may have caused cross-linking of the PV antibody–antigen complex, we also used a modification of the glutaraldehyde-borohydride-saponin (GBS) procedure, described by Willingham [25]. This procedure increases the accessibility of some cytoplasmic compartments to antibodies and allowed us to visualize antibody–antigen complexes that had been internalized prior to second antibody treatment. Antibody labeling was done using the avidin–biotin peroxidase complex (ABCPx) method to amplify the degree of immune labeling [26].

Forty-eight-hour cultures were treated with a 1:80 dilution of PV serum in M-199 and incubated at 37°C in a humid atmosphere with 5% CO_2 and 95% air for 0.5, 1, and 24 h. At these time points, the supernatant media were removed, and the cultures rinsed 3 times with M-199. The cultures were prefixed with 0.4% glutaraldehyde in 80% PBS for 10–15 min, and incubated for 5 min at room temperature with a fresh solution of 0.05% sodium borohydride in PBS. The cultures were washed in PBS 5 times after each step. The cultures were then treated with a PBS solution containing 0.1% saponin, 4 mg/ml normal human IgG, and 1 mM ethylene glycol tetraacetic acid (EGTA) for 30 min at room temperature and washed 3 times with 0.1% saponin in PBS. Immunochemical staining of the cultures was done at room temperature by incubation with a 1:200 dilution of biotinylated goat antihuman IgG (Vector Labs, Inc., Burlingame, California) in 0.1% saponin and PBS for 30 min, followed by incubation with a 1:200 dilution of horseradish peroxidase avidin D (Vector Labs) in 0.1% saponin and PBS for 20 min. The cultures were washed 3 times with PBS after each step, fixed with 1% glutaraldehyde in PBS, rinsed with PBS, and reacted with DAB for 10 min at room temperature.

Cytochalasin-D and Colchicine Treatment of Epidermal Cell Cultures

Forty-eight-hour cultures were first treated for 0.5 h at 37°C with cytochalasin-D (0.5 μg/ml) in 1% dimethyl sulfoxide, colchicine (40 μg/ml), or a combination of the two, following the technique reported by Knapp et al [27]. The cultures were then exposed to PV antibodies and peroxidase-labeled goat antihuman IgG as described previously, but in the continuous presence of cytochalasin-D and colchicine or a combination of the two.

Processing of Cultures for Electron Microscopy (EM)

The cultures were postfixed in 1% osmium tetroxide (O_8O_4) dehydrated with an ascending series of alcohol and embedded in British araldite (both from Polysciences, Warrington, Pennsylvania). The Thermanox coverslips were peeled off by warming over a hotplate, leaving embedded cells on the block. Ultrathin sections were stained with uranyl acetate alone, or uranyl acetate and Reynold's lead citrate solution [28], and examined with a JEM-100S transmission electron microscope (Joel Ltd., Tokyo).

RESULTS

Demonstration of Binding and Endocytosis of PV Antibody Molecules by Epidermal Cells in Culture

In cultures exposed to PV antibodies and maintained at 37°C, at time 0, the antibody molecules were seen bound diffusely on the cell surface (Fig 1*a*). No cell surface binding was detected on parallel control cultures treated with normal human serum (Fig 1*b*). As early as 0.5 h after exposure to PV antibody, antibody molecules were observed to be clustered on the cell surface (Fig 2*a*), internalized via surface pits (Fig 3*a,b*) into submembranous vesicles (Fig 4*a,b*), and fused with lysosomes (Fig 4*c,d*). A similar distribution of antibody molecules was observed in cultures fixed after 1, 3, 6, and 24 h exposure to PV antibody.

Cultures incubated with PV antibody for 24 h displayed internalization of PV antibody molecules within the cells, and at this point we observed marked widening of intercellular spaces (ICS) of the epidermal cells. Desmosomal attachments between cells remained intact (Fig 5*a*). Cultures incubated with normal human serum in parallel showed no such abnormalities of the ICS (Fig 5*b*).

The results obtained with GBS-ABCPx method showed identical results, i.e., there was initial diffuse binding of PV antibody

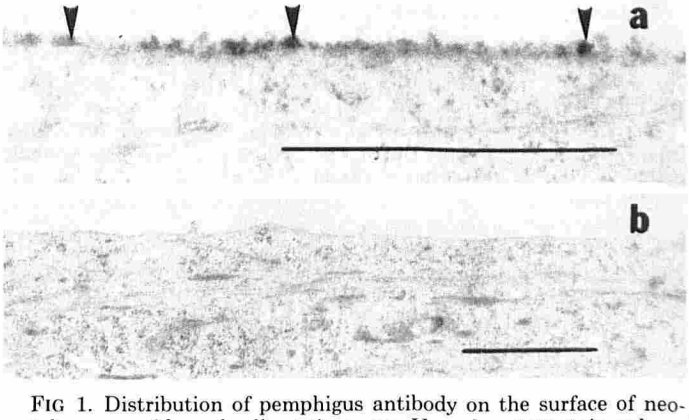

FIG 1. Distribution of pemphigus antibody on the surface of neonatal mouse epidermal cells at time zero. Uranyl acetate stain only. *a*, Pemphigus antibody is seen bound diffusely to cell surface (*closed arrows*). *b*, Normal human serum treated control showing no cell surface binding. *Bars* = 1 μm.

molecules on the epidermal cell surface (Fig 6*a*) followed by internalization of PV antibodies into submembranous vesicles within 1 h (Fig 6*a,b*), and marked widening of ICS of epidermal cells at 24 h (Fig 7*a*).

Effect of Cytochalasin-D and Colchicine on Pemphigus Antibody–Receptor Complex Internalization

In forty-eight-hour cultures incubated with PV antibody in the continuous presence of 0.5 μg/ml cytochalasin-D (Fig 8*a,b*) or 40 μg/ml of colchicine (Fig 8*c,d*) there was no decrease in the observed surface binding of PV antibody or its internalization into submembranous vesicles. Similar results were observed when 0.5 μg/ml cytochalasin-D and 40 μg/ml colchicine were used in combination (Fig 9). The only difference that exposure to these drugs produced was observed in a limited number of cultures treated with cytochalasin-D and/or colchicine, where PV antibodies could not be detected within lysosomes.

DISCUSSION

This study shows that PV antibodies are initially bound to the epidermal cell surface in a uniform and diffuse distribution. If the epidermal cells are incubated with PV antibodies for a short time at 37°C then the antibody molecules become aggregated into clusters on the epidermal cell surface. The results confirm previous observations by Diaz and Marcelo [14] and Takigawa, et al [20]. These earlier studies, however, did not look at the fate of the PV antibody molecule following the initial binding and aggregation that they had detected on the cell surface. In order to follow the fate of PV antibodies, we used the two-antibody technique described by Huet et al [23]. This technique had been used by them to demonstrate antibody-induced clustering and endocytosis of HLA antigens on cultured human fibroblasts. In our study, unfixed epidermal cell cultures were first treated with PV serum for 20 min at 37°C, which allowed the PV antibodies to bind to the PV antigen on the epidermal cell surface without appreciable redistribution of the PV antibody–antigen complexes. This was followed by 3 rinses with M-199 at room temperature to remove any unbound proteins. A second incubation of the cultures was carried out with peroxidase-conjugated antibodies, allowing them to react with the first antibodies for 30 min at 37°C, and then the excess peroxidase-conjugated antibodies were rinsed

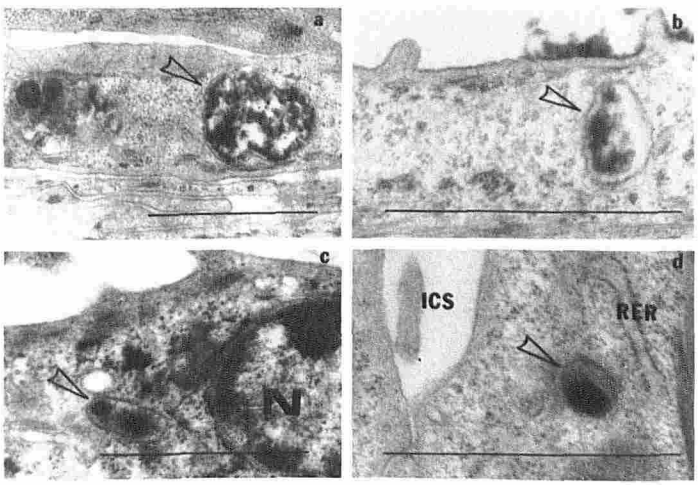

FIG 4. Processing of pemphigus antibody–antigen complexes after binding neonatal mouse epidermal cells. Uranyl acetate and lead citrate stain. *a and b*, Diaminobenzidine deposition seen in intracytoplasmic vacuoles (*open arrows*). *c and d*, Diaminobenzidine deposition seen within secondary lysosome (*open arrows*). N = nucleus; ICS = intercellular space; RER = rough endoplasmic reticulum. *Bars* = 1 μm.

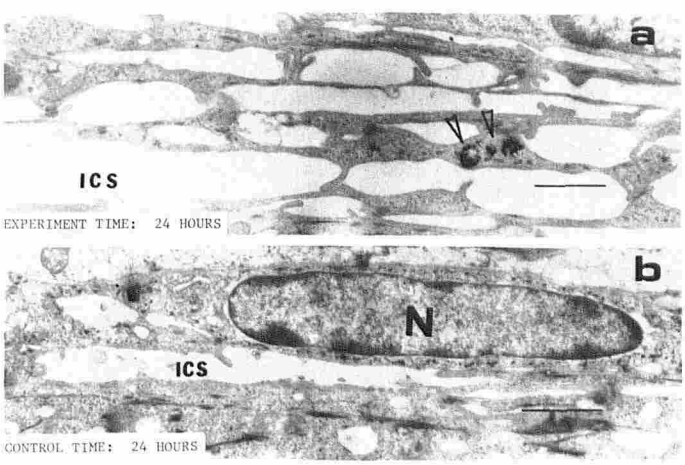

FIG 5. Effect of pemphigus antibody–antigen complex internalization on neonatal mouse epidermal cells at time 24 h. Uranyl acetate and lead citrate stain. *a*, Experimental cultures show widening of intercellular spaces (*ICS*). *Open arrows* point to internalized antibodies. *b*, Control cultures showing *no* widening of intercellular spaces (*ICS*). N = Nucleus. *Bars* = 1 μm.

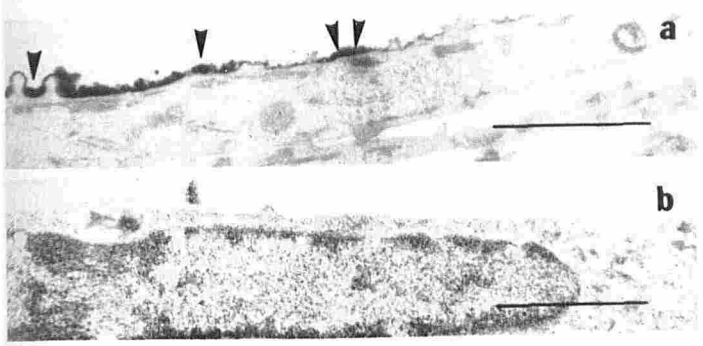

FIG 2. Distribution of pemphigus antibody on the surface of neonatal mouse epidermal cells at time 30 min. Uranyl acetate stain only. *a*, Pemphigus antibody is seen clustering on the cell surface (*closed arrows*). *b*, Normal human serum treated control showing no clustering. *Bars* = 1 μm.

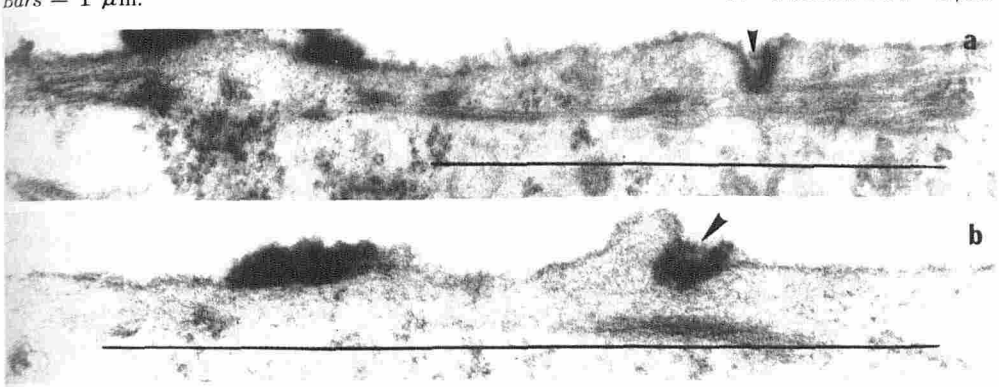

FIG 3. Distribution of pemphigus antibody on the surface of neonatal mouse epidermal cells at time 30 min. Uranyl acetate and lead citrate stain. *a and b*, Higher magnification showing pemphigus antibody–antigen complexes within pits (*closed arrows*). *Bars* = 1 μm.

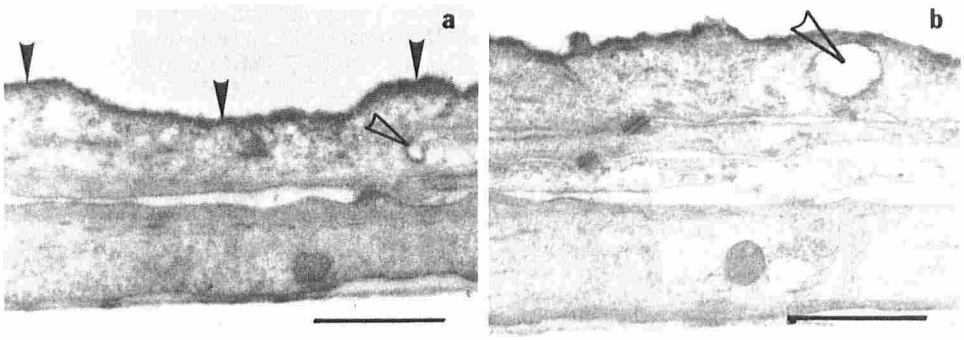

FIG 6. Demonstration of pemphigus antibody binding and internalization on neonatal mouse epidermal cells at time 1 h using GBS and ABCPx method. Uranyl acetate stain only. *a*, Pemphigus antibody is seen bound diffusely to the cell surface (*closed arrow*) and diaminobenzidine deposition seen in intracytoplasmic vacuole (*open arrow*). *b*, Diaminobenzidine deposition seen in intracytoplasmic vacuole (*open arrow*). *Bars* = 1 μm.

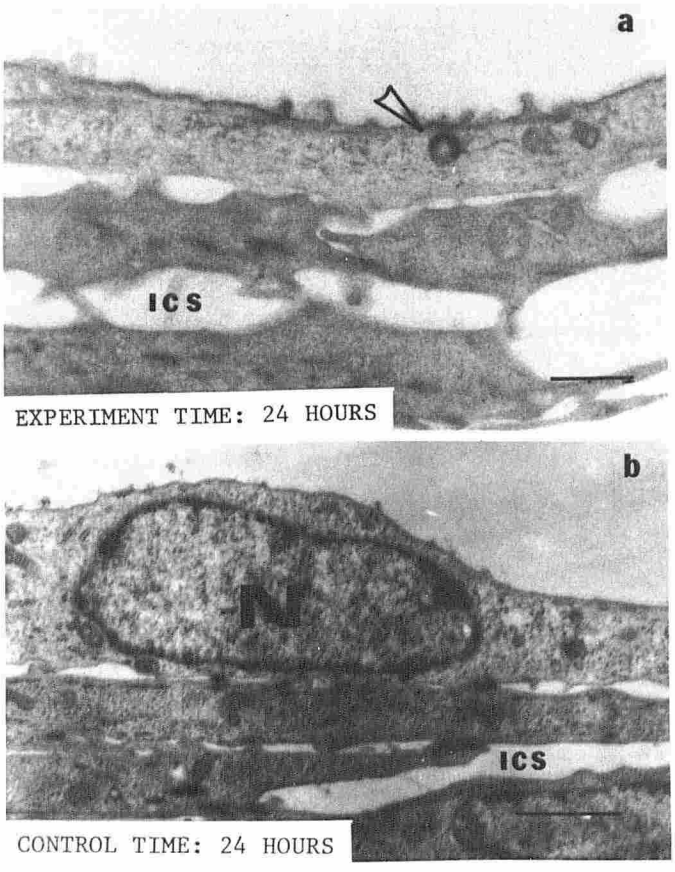

FIG 7. Effect of pemphigus antibody–antigen complex internalization on neonatal mouse epidermal cells at time 24 h using GBS and ABCPx method. Uranyl acetate stain only. *a*, Experimental cultures show widening of intercellular spaces (*ICS*). *Open arrow* points to internalized antibodies. *b*, Control cultures showing *no* widening of intercellular spaces (*ICS*). N = Nucleus. *Bars* = 1 μm.

off. The now-labeled PV antibody was allowed to interact with epidermal cells for different periods of time (0.5–24 h) and its fate at a given time point visualized by EM. Our observations showed that, subsequent to clustering, the PV antibody is internalized into submembranous vesicles via surface pits and eventually fused with lysosomes. Parallel control epidermal cell cultures treated with normal human serum showed no binding or internalization of IgG molecules. This finding strongly indicates that endocytosis of the PV antibody molecules by epidermal cells is a specific phenomenon.

Singer has proposed that clustering of receptor-bound ligands is a prerequisite for endocytosis [29]. It would follow, therefore, that cross-linking and clustering of these epidermal cell surface molecules (the PV antigen) was caused by the PV antibody. It may be argued that in our experiments the use of peroxidase-labeled goat antihuman IgG, which is divalent, may have caused cross-linking of the PV antibody–antigen complex, and then artifactually induce clustering and endocytosis. This possibility has been ruled out, as the internalization of PV antibody–antigen complex was also seen in cultures treated by the GBS-ABCPx method. In this method, unaltered antibody–antigen complexes were visualized after their internalization. This clearly demonstrates endocytosis of PV antibodies in the absence of the cross-linking effect of peroxidase-labeled goat antihuman IgG.

PV antibody-treated cells immediately fixed with paraformaldehyde, a reagent that immobilizes membrane macromolecules [24], showed diffuse surface distribution of PV antibody molecules, indicating that PV antibody–antigen clustering is inhibited by aldehyde fixation. These findings suggest that PV antigen(s) in unfixed cells are mobile in the membrane of epidermal cells as reported by Takigawa et al [20].

Pemphigus antigen may be referred to as a receptor since it specifically recognizes and binds its ligand, the PV antibody. Therefore, binding of PV antibodies to the epidermal cell surface (receptor/ligand interaction) may translate information that subsequently modifies some aspects of cell behavior or metabolism [30]. Interestingly, it is known that the cross-linking of cell surface receptors by autoantibodies is associated with an increased metabolic turnover of these molecules and defective receptor function [31–33]. For example, in autoimmune diseases with autoantibodies against cell surface receptors, such as myasthenia gravia, insulin-resistant diabetes, and Graves' disease, an autoantibody–cell surface receptor cross-linking is a common pathogenetic mechanism [31–38]. It is conceivable that the same cross-linking phenomenon occurs on the epidermal cell surface after binding PV antibodies, which may lead to the cell detachment observed in vivo and in vitro.

Appearance of peroxidase-labeled PV antibody molecules within submembranous vesicles and subsequent fusion with lysosomes indicates an endocytic pathway from the epidermal cell surface to lysosomes [39]. However, the final fate of PV antibody–antigen complexes beyond the lysosomes remains unknown. In the majority of systems studied [40], the ligand–cell surface molecule complex that reaches the lysosome is subsequently dissociated, and the cell surface molecule is recycled back to the cell surface to be reutilized. In rat embryo fibroblast, it has been shown that internalized rabbit IgG is partially digested in the lysosomal compartment, with certain Fab′ type fragments remaining undigested [41].

An interesting observation in this investigation was that exposure of these cultures to cytochalasin-D and colchicine, alone or in combination, did not prevent the clustering and internalization of PV antibodies. This would indicate that the cytoskeletal contractile system does not play a role in this process. This finding would be in agreement with results obtained in other cell systems, indicating that internalization of a Ligand–cell surface molecule complex is a process resistant to cytoskeleton-disrupting drugs [42]. In the cell cultures we treated with cytochalasin-D or colchicine, there was persistence of the pemphigus antibody on the cell surface and we were

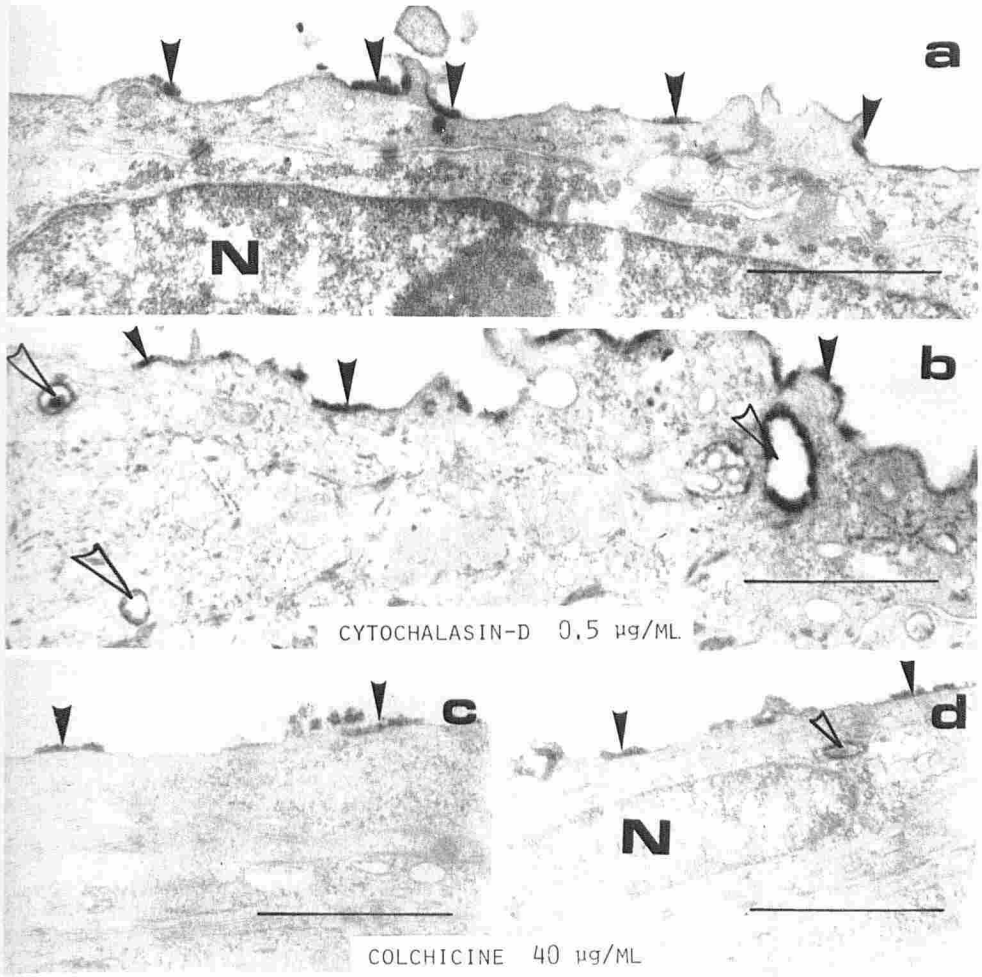

FIG 8. Effect of cytochalasin-D or colchicine on the internalization of pemphigus antibody by neonatal mouse epidermal cells. Uranyl acetate stain only. *a and b*, Pemphigus antibody–antigen complexes are seen bound to the cell surface (*closed arrows*) and within intracytoplasmic vacuoles (*open arrows*) in cultures treated with cytochalasin-D. *c and d*, Pemphigus antibody-antigen complexes are seen bound to the cell surface (*closed arrows*) and within intracytoplasmic vacuoles (*open arrows*) in cultures treated with colchicine. N = Nucleus. *Bars* = 1 µm.

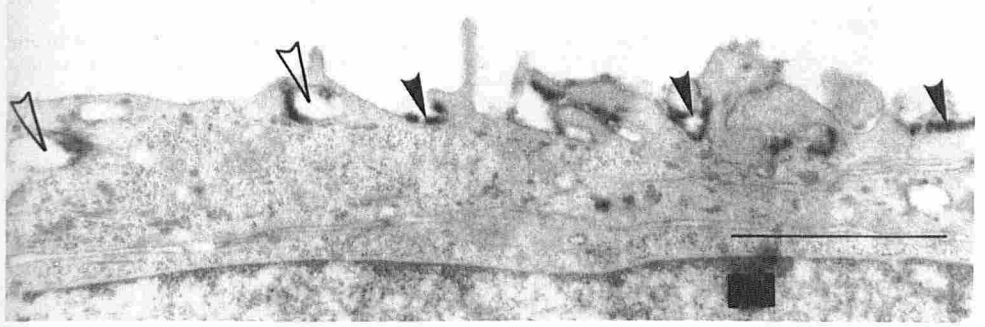

FIG 9. Effect of cytochalasin-D and colchicine in combination on the internalization of pemphigus antibody by neonatal mouse epidermal cells at time 1 h. Uranyl acetate stain only. Pemphigus antibody–antigen complexes are seen bound to the cell surface (*closed arrows*) and within intracytoplasmic vacuoles (*open arrows*). N = Nucleus. *Bar* = 1 µm.

unable to detect any PV antibody within the lysosomes. This suggests that these cytoskeleton-disrupting drugs may partially inhibit endocytosis and block the fusion of endocytosed vesicles with lysosomes, and therefore impair the intracytoplasmic processing of PV antibody molecules. This observation needs future confirmation, for it may be due to sampling error, an inherent problem with EM studies.

An important observation made in cultures incubated with PV serum for 24 h was the appearance of marked widening of ICS and the presence of PV antibody molecules within the epidermal cells. However, the relation of internalization of PV antibody molecules and the dissolution of the ICS producing widening of these spaces remains to be clarified.

Recently, using the neonatal mouse animal model, our laboratory has described ultrastructural changes triggered by the binding of PV antibodies to the mouse epidermis [43]. Briefly, that report describes the time-course of the ultrastructural injury triggered in the epidermis of BALB/c mice by i.p. injections of human PV IgG. These include early widening of the ICS, suprabasal cleft formation, separation of desmosomes into split desmosomes, dissolution and disappearance of attachment plaques, perinuclear tonofilament retraction, and intact basal cell–dermis attachment. These are all consistent with previously reported findings in spontaneous human pemphigus [2,44] and the experimentally induced acantholysis in human skin explants [45]. It is unclear whether the internalization of PV antibody molecules into lysosomes and the dissolution of attachment plaques and retraction of tonofilaments described above are pathogenetically related. It is conceivable that the epidermal acantholysis induced by PV antibodies may go through an early detachment process that may be mediated by proteases, as suggested by several investigators [15,16,46,47].

However, the fully developed acantholytic vesicle may represent the end result of several cytopathologic processes that perhaps are operating within the keratinocyte itself, rather than in the extracellular milieu alone.

It is known that antibodies produced against cell surface molecules (antigens or receptors) may have numerous and profound effects on the cells to which they bind. They may: (a) be cytotoxic to these cells in the presence of complement [48, 49], (b) induce antigenic modulation [50,51], (c) agglutinate cells or cell surface domains, resulting in increased numbers of microvilli [52–54], (d) impair phagocytosis [55,56], (e) impair cell aggregation [57–59], (f) prevent the restoration of the deficient morphology and adhesiveness in transformed cells upon the addition of the deficient cell surface glycoproteins [57,60], (g) alter the normal morphology of cells causing them to become rounded [54,57], (h) increase cell surface receptor turnover and thus impair receptor function [32,33], and (i) activate cell surface proteases [15,16,46,47], including plasminogen activator [61,62]. It is clear from the present studies and those published by others on the pathogenesis of the epidermal injury induced by PV antibodies [15,16,46,47] that the acantholysis in pemphigus is a phenomenon triggered by autoantibodies and that the cell detachment may result from complex intracellular and extracellular mechanisms. The present study shows that PV antibodies bind a cell surface antigen (receptor) and the complex is internalized and fused with lysosomes in a process that may have pathophysiologic relevance.

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