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Anti-epidermal-cell-surface pemphigus antibody detaches viable epidermal cells from culture plates by activation of proteinase*

(epithelial cell/concanavalin A/acantholysis)

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ABSTRACT **Immunoglobulin from pemphigus patients binds to the surface of mouse epidermal cells in culture. Cells incubated with the pemphigus antibody are easily detached from culture plates whereas cells incubated with serum from normal patients remain on the plate. Pemphigus antibody-mediated cell detachment is blocked by the addition of the proteinase inhibitors soybean trypsin inhibitor and α_2-macroglobulin to the culture media. Detachable cells are viable, and activation of the complement cascade is not necessary for cell detachment. The anti-cell-surface antibody of pemphigus appears to disrupt adhesion between viable epidermal cells by activation of proteinase.**

Pemphigus is a severe blistering disease of the skin that is characterized by the production of circulating autoantibodies directed against the epidermal intercellular cement substance (1, 2). Pemphigus provides a unique opportunity to study the effect of a specific anti-cell-surface antibody upon cell function. Skin biopsies from pemphigus patients reveal deposition of immunoglobulin between epidermal cells and rounding up and loss of adhesion between cells (3); this latter change is referred to as acantholysis (3). Michel and coworkers (4, 5) and others (6, 7) have shown that explants of whole human skin grown in the presence of immunoglobulin from pemphigus patients develop acantholysis of the basal epidermal cells. The mechanism by which binding of pemphigus immunoglobulin to epidermal cells induces loss of adhesion between cells is unknown. The studies reported here, utilizing an epidermal cell culture system, suggest that pemphigus antibody produces loss of adhesion of cells by activation of a proteinase and that the biological effect of pemphigus antibody *in vitro* does not induce cell death or require complement.

METHODS

Sera. Pemphigus sera were obtained from Ernst Beutner (Buffalo, NY), Jean Claude Bystryn (New York, NY), Robert Jordon (Milwaukee, WI), Steven Katz (Bethesda, MD), Thomas Provost (Buffalo, NY), and Ronald Reisner (Los Angeles, CA). These pemphigus sera were titered for anti-epidermal-cell antibody by standard immunofluorescent techniques (3); the sera had titers ranging from 1:160 to 1:640. Bullous pemphigoid sera were titered against monkey esophagus and the sera had titers of 1:1240 of anti-basement-membrane antibody (3). Normal control sera were obtained from healthy adult donors. All sera were stored at −20° and they were heated at 56° for 30 min prior to use.

Preparation of Immunoglobulin. Immunoglobulin-enriched material was prepared from serum by fractionation with ammonium sulfate (50% saturation, 25°) followed by exhaustive dialysis against borate-buffered saline [0.1 M H_3BO_3/0.025 M $Na_2B_4O_7$/0.075 M NaCl plus 0.145 M NaCl, 5:95 (vol/vol), pH 8] for 1 week in the cold.

Preparation of α_2-Macroglobulin. Partially purified (50%) human α_2-macroglobulin (α_2M) was obtained from Y. L. Hao (American National Red Cross, Blood Research Laboratory, Bethesda, MD) and further purified by chromatography on Bio-Gel A 1.5 (1.5 × 90 cm, 4°, 7 ml/hr) with phosphate-buffered saline (P_i/NaCl; 0.14 M NaCl/2.7 mM KCl/1.5 mM KH_2PO_4/8.4 mM Na_2HPO_4, pH 7.4) as eluent (8). The α_2M fraction was quantitated by radial immunodiffusion (M-partigen, α_2M radial immunodiffusion plates, Behring Diagnostics, NJ). The purified α_2M was characterized by immunoelectrophoretic techniques (9) with anti-whole human serum (goat anti-whole human serum, 4.8 mg of Ab/ml, Miles Research Laboratories, Elkhart, IN) and anti-human α_2M (goat anti-human α_2M serum, 2.1 mg of Ab/ml, Miles Research Laboratories, Elkhart, IN) antiserum. The purified α_2M demonstrated a single arc in immunoelectrophoresis plates against anti-whole human serum. This arc corresponded to that seen when the protein was allowed to diffuse against monospecific α_2M antibody.

Preparation of Neonatal Epidermal Cell Cultures. Epidermal cells were prepared by the method developed by Yuspa and coworkers (10). Skin was removed from 24- to 48-hr-old BALB/c mice and then floated dermal side down in Hanks' balanced salt solution containing 0.25% trypsin (type III, Sigma, St. Louis, MO) for 18 hr at 4°. The epidermis and dermis were mechanically separated, and the epidermis was minced and agitated in medium 199 (GIBCO, NY) containing 15% heat-inactivated (56°, 30 min) fetal calf serum (GIBCO) for 1 hr at 37° to obtain a cell suspension. The cell suspension was centrifuged in a discontinuous Ficoll (Sigma) density gradient [24% (wt/vol) Ficoll diluted with medium 199 to 16%, 14%, and 12% (vol/vol) final concentration]. The gradient consisted of 5 ml of 16% Ficoll successively overlayed with 10-ml aliquots of 14% Ficoll and 12% Ficoll. The cells were centrifuged through the gradient at 1200 × *g* for 30 min. The pellet at the bottom of the tube, consisting of purified epidermal cells, was resuspended in medium 199 containing 15% fetal calf serum, counted in a Neubauer Levy Ultraplane counting chamber and assayed for viability by eosin Y exclusion (0.1% eosin Y in P_i/NaCl). The viability of the cells prepared for these experiments exceeded 95%. The cells were suspended in medium 199 containing penicillin (112 units/ml), streptomycin (75 μg/ml), amphotericin (0.2 μg/ml), and 15% heat-inactivated fetal calf serum and plated at 6×10^5 cells (in 2 ml) in a 35-mm^2 tissue culture

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Abbreviations: α_2M, α_2-macroglobulin; P_i/NaCl, phosphate-buffered saline.

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plate (Falcon, no. 3001). The cells were then incubated at 34° in the presence of 5% CO_2 in air for 24 hr.

Preparation of Fibroblast Cultures. Cultures of normal human diploid fibroblasts were a gift from Sheldon Pinnell (Duke University Medical Center, Durham, NC). The fibroblasts were maintained on 100-mm^2 tissue culture plates (Falcon, no. 3003) in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin (112 units/ml), streptomycin (75 μg/ml), and amphotericin (0.2 μg/ml) at 37° and 5% CO_2. After the third passage, cells were grown to confluence, trypsinized, and passed (1:3) to 35-mm^2 tissue culture plates (Falcon, no. 3001). Some cells were labeled with [^{3}H]-thymidine and utilized for detachment studies as described for the mouse epidermal cell cultures.

Immunofluorescence Studies. Binding of pemphigus IgG to mouse epidermal cell surface was demonstrated by indirect immunofluorescence techniques utilizing cells grown on glass cover slips. Pemphigus, pemphigoid, and normal sera were diluted with P_i/NaCl containing 4% bovine serum albumin (Sigma) to (vol/vol) 1:25, 1:50, 1:100, and 1:200. The sera were applied to both fixed [acetone/ethyl alcohol, 1:1 (vol/vol), 10 min] and unfixed cells for 1 hr at 25°. The cover slips were then washed four times with P_i/NaCl containing bovine serum albumin. In the second step, rhodamine-conjugated goat anti-human IgG (Cappel Laboratories, Downington, PA), that had been adsorbed with bovine liver powder (Sigma, St. Louis, MO) and diluted 1:64 (vol/vol) with albumin in P_i/NaCl, was applied for an additional hour. The cover slips were then washed four times with albumin in P_i/NaCl, air-dried (10 min, room temperature), and placed onto a drop of glycerol/P_i/NaCl, 1:9 (vol/vol), on a clean glass microscope slide. The specimens were examined at 580 nm (magnification, ×630) with a Zeiss large universal fluorescence microscope equipped with a IIIRS vertical illuminator.

Detachment Studies. The cell cultures used to study immunoglobulin-mediated detachment were incubated with 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol, New England Nuclear, MA) per plate during the initial 24-hr plating period. Epidermal cells from BALB/c mice have been shown (10) to undergo a round of DNA synthesis and cell division at 18–24 hr in culture. The cultures were washed with four changes of P_i/NaCl to remove radioactive label and fetal calf serum.

Cells were then incubated in 1.25 ml of medium 199 containing heat-inactivated (56°, 30 min) 1:50 dilutions of pemphigus serum, pemphigoid serum, or normal serum. In some detachment studies, medium 199 containing pemphigus serum or normal serum also contained soybean trypsin inhibitor (100 μg/ml, Sigma), concanavalin A (0.5 μg/ml, Sigma), or purified α_2M (0.5 mg/ml). After these additions, all plates were coded and the remainder of the protocol was performed without knowledge of experimental treatment. After 8, 24, and 48 hr of incubation at 35° in 5% CO_2 in air, the cells were gently washed with their overlying medium 15 times by pasteur pipette and suction bulb. The medium was removed and filtered (Whatman filters, 2.4-cm, GL/C). The filters were washed extensively with P_i/NaCl followed by 10% trichloroacetic acid and then assayed for radioactivity in 10 ml of Aquasol II (New England Nuclear) in an Intertechnique SL30 liquid scintillation spectrometer. Trichloroacetic acid-precipitable radioactivity in the medium was taken to represent detachable cells. The tissue culture plates were then scraped with a rubber policeman and the contents were filtered and treated as described for the medium. The trichloroacetic acid-precipitable counts remaining on the plate were taken to represent adherent cells. Cell detachment was expressed as precipitable radioactivity present in the medium and on the culture plate. All studies were done in duplicate and repeated on at least three occasions. Identical results were obtained when cells were preincubated with ^{14}C-labeled amino acids. Cell counts were not used to monitor detachment because epidermal cells were released from the plates in groups; the nature of the experiments precluded incubation of cells with trypsin.

Cellular Viability. Cellular viability was determined by measuring the incorporation of radioactive amino acids into trichloroacetic acid-precipitable protein. At the start of the cell detachment studies, parallel cell cultures without [^{3}H]thymidine prelabeling were incubated with 1 μCi of ^{14}C-labeled mixed amino acids (50 mCi/matom, New England Nuclear). After 8 hr of incubation the cells were scraped from the tissue culture plates and the media were filtered though Whatman filters; the filters were washed with P_i/NaCl and 10% trichloroacetic acid and assayed for radioactivity as above. Similar experiments were performed with the labeled amino acids added to the epidermal cell cultures for 8–24 hr and 24–48 hr of incubation. Results are expressed as cpm of ^{14}C incorporated into trichloroacetic acid-precipitable protein.

Proteinase Determinations. Neutral proteinase was measured by the radioactive caseinolysis assay (11).

Protein Determinations. Protein was measured by the microbiuret method (12).

Fc Receptor. Epidermal Fc receptor determinations were performed according to the procedure of Shevach *et al.* (13). This procedure utilizes ox erythrocytes coated with 7S antibody (7S EA). The percentage of cells rosetting with three or more ox erythrocytes was scored.

RESULTS

Immunofluorescence studies demonstrated that pemphigus immunoglobulin binds to the surface of mouse epidermal cells in culture (Fig. 1 *left*). Cells incubated with normal serum showed no such deposition (Fig. 1 *right*). The binding appeared to be mediated through the Fab fragment of the molecule because no Fc receptor could be demonstrated on our epidermal cells. By contrast, Fc receptor binding was easily demonstrated in a murine macrophage-like tumor cell line, P388D_1, by using our 7S EA reagent. Fc receptor and other macrophage-like characteristics of the P388D_1 cell line have been reported in detail (14, 15).

Epidermal cells incubated in the presence of pemphigus serum detached from culture plates to a significantly greater extent than did cells incubated with normal serum (eight sera, 12 experiments, $P < 0.01$) (Fig. 2). Pemphigus-induced cell detachment was twice the control levels at 8 hr, and detachment continued to increase throughout the 48-hr period of culture. Identical results were seen when the epidermal cells were incubated with preparations of partially purified IgG from pemphigus and normal patients. Examination of the detached cells by both light and phase-contrast microscopy revealed grossly normal cells that excluded eosin Y. High-titer bullous pemphigoid serum, which contains an anti-epidermal-basement-membrane antibody, induced only slightly more cellular detachment than did normal serum controls and significantly less than that induced by pemphigus serum (Fig. 2). Pemphigus immunoglobulin did not cause significant detachment of fibroblasts from culture plates.

Pemphigus-induced epithelial cell detachment was blocked by the addition of the proteinase inhibitors soybean trypsin inhibitor and α_2M to the culture medium (Fig. 3). Cells pretreated with soybean trypsin inhibitor (100 μg/ml, 24 hr, 35°)

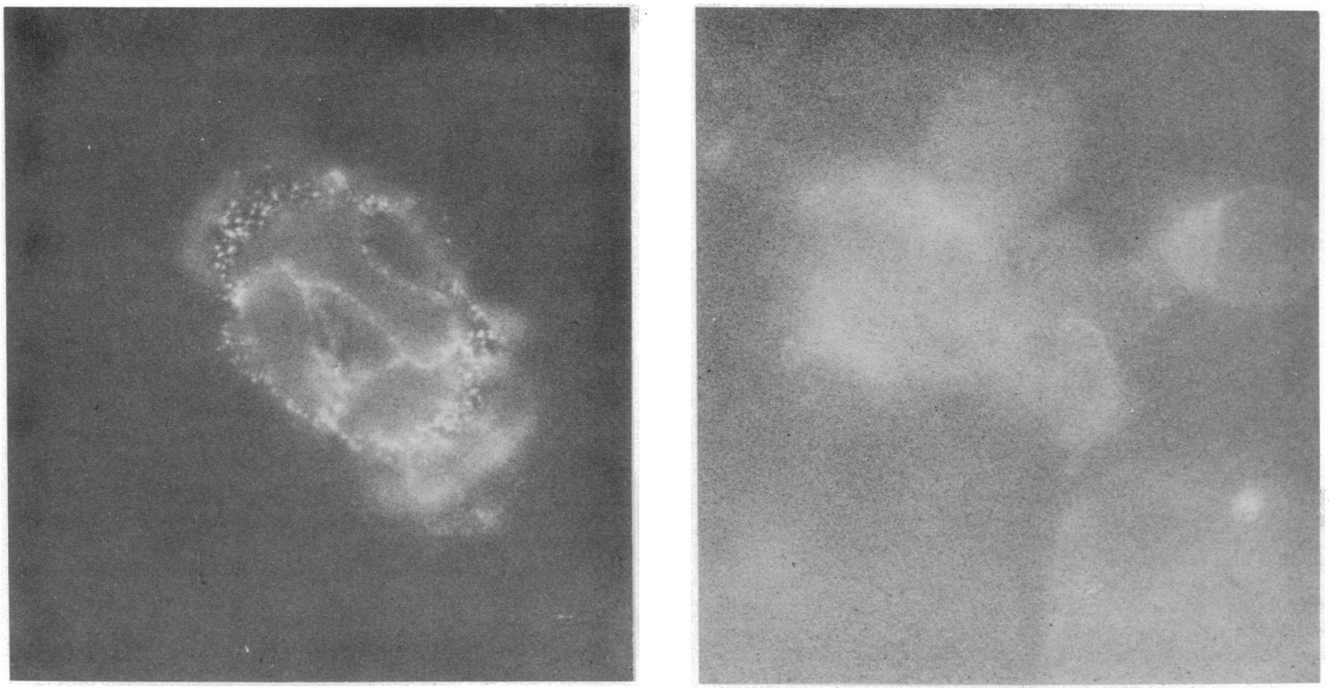

FIG. 1. Binding of pemphigus antibody to the periphery of mouse epidermal cells in culture. (*Left*) Cells incubated with pemphigus serum (1:50) followed by rhodamine-conjugated goat anti-human IgG. (*Right*) Same batch of epidermal cells incubated with normal human serum (1:50) followed by goat anti-human IgG. (×460.)

followed by the addition of pemphigus serum or cells incubated in the presence of soybean trypsin inhibitor throughout the culture period with pemphigus antibody exhibited rates of detachment essentially identical to those of controls. The limiting dilution of pemphigus serum for immunofluorescent staining was 1:200 (final) for cells treated with soybean trypsin inhibitor and cells incubated with P_i/NaCl alone; this suggests that soybean trypsin inhibitor did not prevent binding of pemphigus IgG to the cells. In addition, α_2M (0.5 mg/ml) was as effective in inhibiting pemphigus-induced cell detachment as was soybean trypsin inhibitor. The plant lectin concanavalin A, at concentrations as low as 0.5 μg/ml, induced epithelial cell detachment (Fig. 4). Concanavalin A-induced detachment was not blocked by the addition of soybean trypsin inhibitor.

Pemphigus-induced cell detachment could have been a consequence of antibody-mediated cell death. To determine cell viability, amino acid incorporation into trichloroacetic acid-precipitable protein was measured. There was no significant difference in amino acid incorporation into protein between cells incubated in normal serum, pemphigus serum, or pemphigus serum plus soybean trypsin inhibitor during the 48-hr culture period (Fig. 5). Incorporation of labeled amino acids into trichloroacetic acid-precipitable material varied

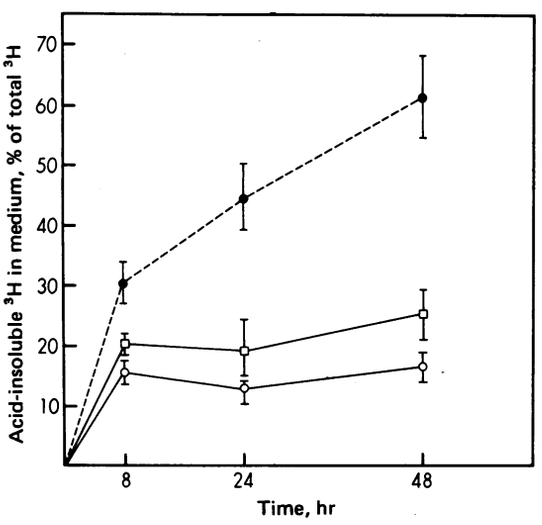

FIG. 2. Detachability of epidermal cells from culture plates incubated with pemphigus serum (1:50) (●), pemphigoid serum (1:50) (□), or normal serum (1:50) (○) as a function of time. Detachment was measured as the amount of trichloroacetic acid-precipitable [^{3}H]thymidine in the medium as a percentage of the total radioactivity in the medium and on the culture plate, shown as mean ±SEM.

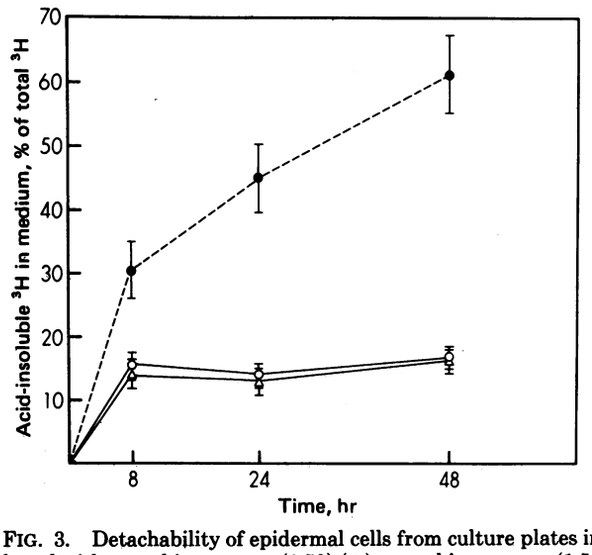

FIG. 3. Detachability of epidermal cells from culture plates incubated with pemphigus serum (1:50) (●), pemphigus serum (1:50) plus soybean trypsin inhibitor at 100 μg/ml (△), and normal serum (1:50) (○) as a function of time. Detachment was measured as the amount of trichloroacetic acid-precipitable [^{3}H]thymidine in the medium as a percentage of the total radioactivity in the medium and on the culture plate, shown as mean ±SEM.

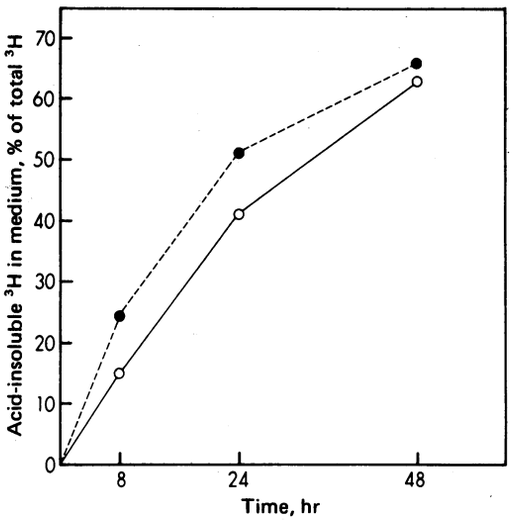

FIG. 4. Detachability of epidermal cells from culture plates incubated with concanavalin A at 0.5 μg/ml (●), or concanavalin A at 0.5 μg/ml plus soybean trypsin inhibitor at 100 μg/ml (○) as a function of time. Detachment was measured as the amount of trichloroacetic acid-precipitable [^{3}H]thymidine in the medium as a percentage of the total radioactivity in the medium and on the culture plate.

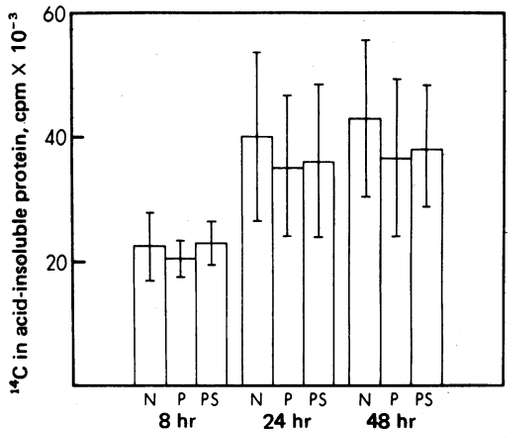

FIG. 5. Incorporation of ^{14}C-labeled amino acids into trichloroacetic acid-precipitable protein in cells incubated with pemphigus serum (1:50) (P), pemphigus serum (1:50) plus soybean trypsin inhibitor at 100 μg/ml (PS), or normal serum (1:50) (N) as a function of time, shown as mean ± SEM.

proportionally with time of incubation of the cells in the presence of labeled amino acid precursors during the first 24 hr of culture. These data suggest that the epithelial cells incubated with pemphigus serum were as viable as the control cells. By contrast, incubation of cells with concanavalin A at concentrations as low as 0.5 μg/ml decreased incorporation of amino acids into acid-precipitable protein by 80%. These observations suggest that concanavalin A-induced cell detachment is the result of cytotoxicity and confirms the evidence that pemphigus-induced cell detachment *in vitro* is not a consequence of cell death.

DISCUSSION

IgG from pemphigus patients specifically binds to mouse epidermal cells in culture. Pemphigus antibody has been shown to bind specifically to epithelial cells in frozen sections of human, guinea pig, and monkey skin (1, 2, 5, 16, 17). These studies indicate that pemphigus antibodies are directed against tissue-specific but not species-specific antigens of the epidermis (3).

The binding of pemphigus antibodies to cultured epidermal cells induces detachment of viable cells from the culture plates. By contrast, incubation of epithelial cells with the anti-basement-membrane antibody found in patients with bullous pemphigoid produced minimal increase in cellular detachment. These data illustrate the specificity of pemphigus antibody.

The possibility that pemphigus antibody induces detachment of epithelial cells by a cytotoxic mechanism was evaluated in a series of amino acid incorporation experiments. Epithelial cells treated with levels of pemphigus antibody sufficient to induce detachment demonstrated no change in protein synthesis ability. In addition, the detachable cells appeared normal morphologically. These data suggest that the release of radioactivity into the medium represents detachment of viable, intact cells and not release of DNA from lysed epithelial cells. All sera and the IgG preparations were heat-inactivated at 56° for 30 min and the cell cultures were washed extensively with serum-free medium and saline before incubation with pemphigus antibody. These data support the hypothesis that acantholysis and loss of attachment do not require activation of the entire complement cascade (4).

Pemphigus antibody-induced cell detachment was blocked by two proteinase inhibitors in the cell culture system. Neither soybean trypsin inhibitor nor α_2M interfered with pemphigus IgG binding to the cell surface as determined by immunofluorescence techniques. Pemphigus serum had no intrinsic assayable proteolytic activity (11). Such data suggest that binding of pemphigus antibody to the cell surface induces secretion or activation of an epithelial proteolytic enzyme which degrades the intercellular cement substance and leads to acantholysis. Further support for this hypothesis is provided by the experiments with concanavalin A. Concanavalin A is cytotoxic to several mammalian cell lines (13). The studies reported here demonstrate that treatment of cell cultures with concanavalin A induces cellular detachment by a cytotoxic mechanism and that release of cells is not blocked by proteinase inhibitors. Whether the proteinase responsible for acantholysis is secreted or activated and whether the enzyme is similar to the enzyme described by our laboratory in human skin (11, 19, 20) and mouse skin (C. A. Thomas, R. Farb, F. J. Yost, and G. S. Lazarus, unpublished data) is not yet known.

Our data may explain the pathogenic role of antibody in pemphigus. Patients with pemphigus have antibody fixed to epidermal cells throughout the skin but lesions occur focally and especially in areas of trauma. It appears that the pemphigus antibody disrupts adhesion between viable epidermal cells by activation of proteinase and that local factors and especially trauma lead to blisters. These observations may also have broad biological interest because they may suggest mechanisms by which membrane-directed antibodies influence cells.

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