ANTI-CELL SURFACE PEMPHIGUS AUTOANTIBODY STIMULATES PLASMINOGEN ACTIVATOR ACTIVITY OF HUMAN EPIDERMAL CELLS

A Mechanism for the Loss of Epidermal Cohesion and Blister Formation*

By KOJI HASHIMOTO,‡ KERRY M. SHAFRAN, PAMELA S. WEBBER, GERALD S. LAZARUS, AND KAY H. SINGER§

From the Division of Dermatology, Department of Medicine, and the Division of Immunology, Department of Microbiology and Immunology, Duke University Medical Center, Durham, North Carolina 27710

Pemphigus is a severe, blistering disease of the skin and mucous membranes. There is loss of epidermal cohesion manifested clinically by induction of intraepidermal blisters and erosions with mechanical trauma (Nikolski's sign). Biopsies of lesional skin reveal rounding up of epidermal cells with loss of epidermal cell adhesion: this pattern is referred to as acantholysis. Two major types of pemphigus are distinguished clinically and histopathologically. Pemphigus vulgaris exhibits extensive erosions and the vesicles form just above the basal layer of epidermal cells. In contrast, pemphigus foliaceous has very shallow blisters that appear in the more superficial granular layer of the epidermis.

In 1964, Beutner and Jordan (1) demonstrated that serum from pemphigus patients contained autoantibodies that bound to an intercellular substance of skin and mucosa. Skin biopsies revealed in vivo deposition of autoantibodies in the epidermis of pemphigus patients (2). Recently, immunoflorescence techniques have shown that pemphigus antibody binds to the surface of mouse epidermal cells (3–5), human epidermal cells (5, 6), squamous cell carcinoma lines (7, 8), and a cervical carcinoma cell line (K. Singer, unpublished results).

Michel and Ko (9) reported that incubation of pemphigus antibody with organ cultures of normal human skin resulted in histologic changes identical to those seen in biopsies of skin from pemphigus patients. Schiltz and Michel (10) showed that the IgG fraction of serum was responsible for loss of cellular adhesion in a complement-independent manner.

Our laboratory (3) presented evidence that a proteinase was responsible for the

* Publication 130 of the Dermatological Research Laboratories of the Duke University Medical Center; supported by National Institutes of Health grants AM21848 and AM19067 from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases and OH01089 from the National Institute of Occupational Safety and Health.

‡ Present address: Department of Dermatology, Osaka University Hospital, 1-1-50, Fukushima, Osaka, Japan.

§ To whom correspondence should be addressed at Box 2987, Duke University Medical Center, Durham, NC 27710.

J. Exp. Med. © The Rockefeller University Press · 0022-1007/83/01/0259/14 $1.00 259
Volume 157 January 1983 259–272

acantholysis found in pemphigus. Incubation of cultured mouse epidermal cells with pemphigus antibody resulted in detachment of cells from the culture vessel. Detachment was blocked by the addition of the proteinase inhibitors soybean trypsin inhibitor and alpha$_2$-macroglobulin. These data suggested that after binding of pemphigus antibody to the surface of epidermal cells, a proteinase was activated that was involved in loss of cell adhesion. Using skin explant cultures, Morioka et al. (11) confirmed a role for proteinases in acantholysis. They reported that the acantholysis observed after incubation of normal skin with pemphigus IgG was inhibited by soybean trypsin inhibitor and pepstatin A, inhibitors of serine and carboxyl proteinases, respectively.

Plasminogen activators (PA)[1] are serine proteinases that catalyze the conversion of plasminogen to plasmin. Recently, the role of PA in the regulation of localized proteolysis within the microenvironment of cells has received much attention. A wide variety of normal vertebrate adult and embryonic cells produce PA under certain circumstances, and this production is highly regulated (for review see ref. 12–14). Included among these cells are macrophages (15, 16), granulocytes (17), ovarian granulosa cells (18, 19), fibroblasts (20–24), trophoblasts (25), endothelial cells (26), and mouse epidermal cells (27). Reich (12) has suggested that the significance of this highly regulated enzyme production is that PA is of critical importance in tissue remodeling and cell migration. PA levels are elevated in primary malignant tumors (28), in cells transformed by oncogenic viruses (20, 21, 29), and in cells treated with tumor promoters (22, 30–32). PA levels may be modulated by agents such as hormones (17, 25), retinoids (33, 34), and lectins (31).

Recently, Becker et al. (35) reported that IgG that binds to cell surface antigens can modulate PA production in a variety of cell types. Their results suggested the possibility that PA might be the serine proteinase stimulated in epidermal cells after the binding of pemphigus autoantibody. The studies presented here indicate that binding of pemphigus autoantibody to human epidermal cells results in elevated levels of both cell-associated and secreted PA, and that PA is involved in the pathogenesis of pemphigus.

Materials and Methods

Chemicals and Other Materials. Chemicals and supplies were obtained from the following sources: Hanks' balanced salt solution, fetal calf serum (FCS), dog serum, and antibiotic/antimycotic from Gibco Laboratories, Grand Island Biological Co., Grand Island, NY; Dulbecco's modified Eagle's medium (DME) from B & B Research, Baltimore, MD; trypsin, mitomycin C, bovine fibrinogen, dexamethasone, Triton X-100, casein, bovine serum albumin, aprotinin, and lima bean trypsin inhibitor (LBTI) from Sigma Chemical Co., St. Louis, MO; hydrocortisone and cycloheximide from Calbiochem-Behring Corp., La Jolla, CA; cholera toxin and Tris from Schwarz/Mann, Orangeburg, NY; Coomassie Blue, Bromphenyl Blue, and sodium dodecyl sulfate from Bio-Rad Laboratories, Richmond, CA; lysine-Sepharose and protein A-Sepharose from Pharmacia Fine Chemicals, Piscataway, NJ; cluster 6 culture dishes from Costar, Data Packaging, Cambridge, MA; Linbro multiwell plates from Flow Laboratories, McLean, VA; microfuge tubes from Walter Sarstedt, Inc., Princeton, NJ; sodium heparin from Upjohn Co., Kalamazoo, MI; epidermal growth factor from Collaborative Research, Waltham, MA; Millex 0.45-μm filters from Millipore Corp., Bedford, MA; urokinase reference standard from Leo Pharmaceutical Industries, Ballerup, Denmark.

[1] *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; LBTI, lima bean trypsin inhibitor; PA, plasminogen activator; PBS, phosphate-buffered saline; Pf, pemphigus foliaceous; Pv, pemphigus vulgaris; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

Epidermal Cell Cultures. Human epidermal cells from newborn foreskins were cultured using a modification of the technique of Rheinwald and Green (36). Foreskins were obtained within 3 h of circumcision and incubated briefly in antibiotic- and antimycotic-containing tissue culture medium. After removal of fatty tissue, the foreskin was placed epidermal side down in 0.25% trypsin in Hanks' balanced salt solution and incubated at 4°C for 18–24 h. The epidermis was then easily removed from the dermis and teased with forceps to obtain a cell suspension. Cellular debris was allowed to settle and the cells remaining in suspension were plated into the wells of Costar cluster 6 tissue culture dishes ($1-2 \times 10^5$ cells/8 cm^2 well), which had been seeded with mitomycin C-treated (15 μg/ml, 2 h, 37°C) mouse 3T3 fibroblasts (CCL 92; American Type Culture Collection, Rockville, MD). Epidermal cells were grown in DME supplemented with FCS to a final concentration of 20%, 20 ng/ml epidermal growth factor, 0.4 μg/ml hydrocortisone, 10^{-10} M cholera toxin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone (antibiotic/antimycotic). Cultures were confluent within 3–5 wk. All experiments were performed on cell cultures that had reached confluence.

Harvest of Conditioned Medium and Cell Lysates. After reaching confluence, the cells were incubated overnight with DME containing 20% FCS and no other additives. The cultures were then incubated with DME (1 ml/well) with or without IgG. 24 h later this conditioned medium was harvested, centrifuged at 6,500 *g* for 5 min, and assayed for extracellular PA activity using the fibrin plate method described below. The cells were then washed three times with phosphate-buffered saline (PBS), pH 7.3, and treated with 0.5% Triton X-100 in 0.01 M Tris-HCl, pH 7.8, for 5 min at 4°C (1 ml/well). The wells were scraped with a rubber policeman and the contents were transferred to a 1.5-ml microfuge tube and centrifuged at 6,500 *g* for 5 min. The supernatant (cell lysate) was assayed to determine cell-associated PA activity. The wells used in a single experiment contained cells originating from the same foreskin. Preliminary experiments documented that, at confluence, the cell number in different wells from a single foreskin varied by <15% (data not shown). Cell numbers were not routinely determined in each well because it was desirable to obtain cell lysates from each well. Determination of cell numbers would have required trypsinization, which would have altered cell viability and introduced trypsin-related artifacts.

IgG Preparations. Preparations of pemphigus vulgaris (Pv) IgG from two patients were studied. The source of Pv IgG used in the majority of experiments was the kind gift of Dr. Joseph McGuire, Department of Dermatology, Yale University Medical Center. The patient was a 49-yr-old female with a 3-yr history of Pv, who at the time of plasma collection had a flare up of her disease. Her serum had an indirect immunofluorescence pemphigus antibody titer of 1:320. IgG preparations from three patients with pemphigus foliaceous (Pf) were also studied. The results presented here were obtained primarily from serum and plasma collected from an 18-yr-old male patient at Duke University Medical Center. Clinical examination and histological examination were consistent with a diagnosis of Pf. These findings were confirmed by immunofluorescent examination of his skin biopsy, which showed deposition of IgG in the intercellular spaces of the epidermis. Indirect immunofluorescence revealed a serum pemphigus antibody titer of 1:80. None of the patients studied had received therapy for their disease at the time of serum or plasma collection. Serum and plasma were also collected from normal donors.

IgG was prepared from patient serum and plasma and from normal serum and plasma by affinity chromatography. After single-step isolation of IgG using the standard technique of affinity purification on Staphylococcal protein A-Sepharose (37), we detected plasminogen as a contaminant in IgG preparations (38). Consequently, IgG was routinely isolated free of plasminogen by sequential column chromatography on lysine-Sepharose, which binds plasminogen (39), followed by protein A-Sepharose. All IgG preparations used in the experiments described were isolated in this manner. Briefly, the serum was diluted twofold in 0.1 M phosphate buffer, pH 7.0, and applied to a column of lysine-Sepharose that had been equilibrated with the same buffer. The lysine-Sepharose column was connected directly to a column of protein A-Sepharose equilibrated with the starting buffer. After the sample had run from the lysine-Sepharose column into the protein A-Sepharose column, the latter was washed extensively with 0.1 M phosphate buffer, pH 7.0, and the IgG was eluted with 0.1 M glycine-HCl in 0.15 M NaCl, pH 3.0. When IgG was prepared from plasma, 5 U/ml sodium heparin was included in all buffers. Fractions containing IgG were pooled and neutralized using 1 M

Tris. Protein concentrations were determined and adjusted to 8–10 mg/ml. The IgG preparations were dialyzed against DME and filter-sterilized using Millex 0.45-μm filters. IgG preparations isolated in this manner were incubated with urokinase and assayed by the ^{125}I-fibrin plate assay described below to confirm that the immunoglobulin was free of plasminogen.

PA Assay. PA was assayed using multiwell culture dishes coated with ^{125}I-fibrin. Bovine fibrinogen, free of plasminogen, was prepared according to the method of Laki (40) and Mosesson (41) and iodinated using chloramine T (42). ^{125}I-fibrin-coated multiwell dishes were prepared according to Strickland and Beers (19). Each well (surface area 2 cm^2) was coated with 25 μg ^{125}I-fibrinogen (~4,000 cpm/μg). Conversion of fibrinogen to fibrin was accomplished by a 2-h incubation at 37°C with DME supplemented with 5% plasminogen-depleted fetal bovine serum. Each well was washed twice with PBS. Immediately before assay, the wells were washed with 0.1 M Tris-HCl, pH 8.1. The reaction mixture contained 50 μl of samples (either cell lysates or conditioned medium) and 1.2 μg of dog plasminogen, purified from dog serum (39) in a total volume of 300 μl of 0.1 M Tris-HCl, pH 8.1. The dishes were incubated for 60 min at 37°C, after which the supernatant was collected and the release of ^{125}I-labeled peptides from the ^{125}I-fibrin was quantitated using an LKB model 1270 RackGamma II gamma counter (LKB Instruments, Inc., Turku, Finland).

PA activity in conditioned medium and cell lysates was compared with the activity of a standard preparation of human urokinase measured on the same day. In each assay, a standard curve of PA activity was generated over a range of 1.6–25 Ploug milliunits (mU) of urokinase activity. All samples were diluted to fall within the linear range of the standard curve. All assays were performed in duplicate and duplicates varied by ≤10%. The PA activity in each sample was computed from the standard curve using the RackGamma radioimmunoassay data reduction system.

Because the number of cells in confluent wells originating from the same foreskin differed by ≤15%, the PA activity in the samples was expressed as units of activity per well. Extracellular activity was expressed as Ploug milliunits per milliliter conditioned medium collected from one well of a Costar cluster 6 plate (surface area 8 cm^2). Cell-associated PA activity is expressed as Ploug milliunits per milliliter cell lysate extracted from one well.

Skin Explant Cultures. Skin explants were cultured by the method of Morioka et al. (11). Skin was obtained aseptically after face and neck lift surgery, reduction mammoplasty, or laparotomy. After removal of fatty tissue, the skin was cut into pieces ~3 × 3 mm. The pieces of skin were placed dermis-side down on paraffin-rimmed lens paper rafts and floated on 1 ml of tissue culture medium with or without IgG and/or plasminogen and proteinase inhibitors. After appropriate incubation, the skin was processed for tissue sectioning, stained with hematoxylin and eosin and observed microscopically using a Zeiss universal microscope (Carl Zeiss, Inc., New York).

Electrophoretic Analysis of PA. The electrophoretic analysis of epidermal cell PA was performed by the method of Vetterlein et al. (43). Conditioned medium was collected from cultured epidermal cells incubated with or without pemphigus IgG. Samples collected from cells treated with IgG were preabsorbed with protein A Sepharose (0.9 ml sample plus 0.1 ml protein A Sepharose, 4°C, 2 h) to remove the IgG. The samples were then dialyzed to a buffer of 0.06 M Tris, pH 6.8 containing 2.3% sodium dodecyl sulfate (SDS), 10% glycerol, and 0.005% Bromphenyl Blue. Electrophoresis was carried out in slab gels using 0.1% SDS, a 12.5% polyacrylamide running gel, a 5% polyacrylamide stacking gel, and the Laemmli buffer system (44, 45). The gel was removed from the glass plates, agitated in 2.5% Triton X-100 in 0.1 M Tris-HCl, pH 8.1, at 22°C for 1 h, and each lane was cut into 2-mm slices. Each gel slice was assayed for PA activity in the ^{125}I-fibrin plate assay described above.

Assay for Cathepsin D Using [^{3}H]Hemoglobin as Substrate. [^{3}H]Hemoglobin was prepared according to Levine et al. (46). 5 μl of sodium acetate buffer (1.2 M, pH 7.2), 5 μl of water, and 30 μl of sample were incubated at 37°C with 20 μl of [^{3}H]hemoglobin (~300,000 cpm) in 0.4-ml polypropylene microfuge tubes for 2 h. After incubation, 40 μl of a 50 mg/ml solution of bovine serum albumin was added to each tube as carrier protein. 100 μl of a solution of 6% trichloroacetic acid (TCA) was added to each tube and the resulting precipitate was pelleted at 6,500 *g* in a microfuge. 100 μl of the supernatant was removed from each tube and counted in

an Intertechnique liquid scintillation counter (Intertechnique, Zurich, Switzerland). In this assay, 1 μg/ml pepsin caused the release of 5,000 cpm of ^{3}H-labeled TCA-soluble peptides.

Results

Pemphigus IgG Stimulates Extracellular and Cell-associated PA. Confluent cultures of human epidermal cells were incubated for 24 h with various concentrations of Pf IgG, Pv IgG, or normal human IgG. The conditioned medium and cell lysates were then assayed for PA as described above (Fig. 1). These data are pooled from several different experiments and are expressed as percent of control. Production of PA was IgG dose dependent. Incubation of epidermal cells with normal human IgG resulted in a slight increase (1.7-fold) in extracellular PA at all concentrations tested and no increase in cell-associated PA. Incubation of epidermal cells with Pf IgG resulted in a 10-fold increase in extracellular PA and a 5-fold increase in cell-associated PA. The stimulation of both extracellular and cell-associated PA reached a maximum at 2 mg/ml. At higher concentrations of IgG, less stimulation was observed. Incubation of epidermal cells with Pv IgG resulted in a sevenfold increase in extracellular PA and a sixfold increase in cell-associated PA. The stimulation of both extracellular and cell-associated PA by Pv IgG reached a maximum at 0.5 mg/ml IgG. In other experiments, the stimulation of PA by 0.25 mg/ml of Pv IgG was not as great as that seen with 0.5 mg/ml (data not shown). By adding IgG to samples of conditioned medium collected from cells incubated with stimulatory concentrations of IgG, we observed that the higher concentrations of IgG did not inhibit the fibrin plate assay (data not shown). We also tested one additional Pv IgG preparation and two additional Pf IgG preparations. All three stimulated PA in epidermal cells in a dose-dependent manner (data not shown).

Fig. 2 shows the kinetics of PA stimulation by an optimal concentration of Pf IgG

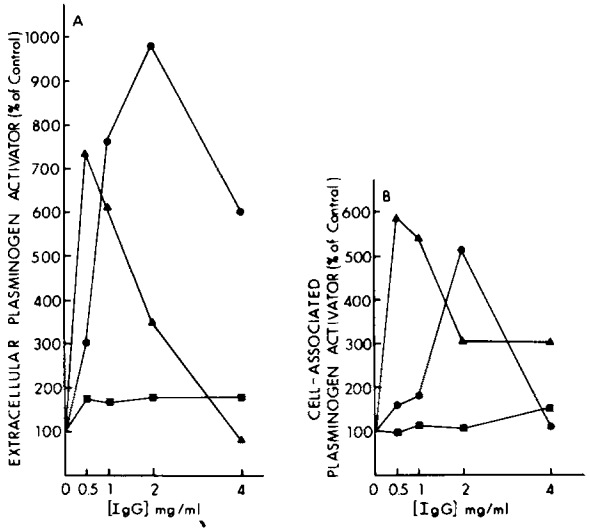

FIG. 1. Stimulation of PA production by pemphigus IgG. Confluent cultures of human epidermal cells were incubated with various concentrations of normal human IgG (■), Pv IgG (▲), or Pf IgG (●) for 24 h at 37°C. Conditioned medium was collected and extracellular PA was quantitated using the ^{125}I-fibrin plate assay as described in Materials and Methods (panel A). Cell lysates were prepared using Triton X-100 and cell-associated PA was quantitated (panel B).

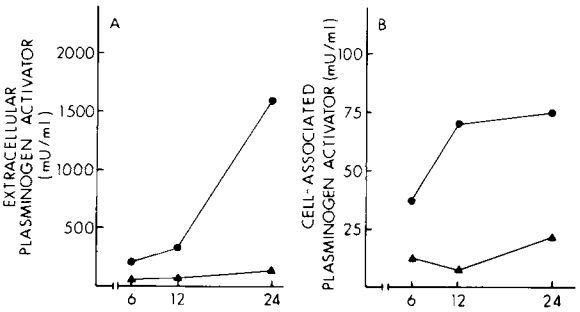

FIG. 2. Time course of pemphigus IgG stimulation of PA production. Confluent cultures of human epidermal cells were incubated with normal human IgG (2 mg/ml) (▲), or Pf IgG (2 mg/ml) (●) for 6–24 h at 37°C. Extracellular (panel A) and cell-associated (panel B) PA was quantitated using the ^{125}I-fibrin plate assay.

(2 mg/ml). By 6 h, extracellular PA in cultures incubated with Pf IgG had risen to 200 mU/ml, or approximately four times that of the cultures incubated with 2 mg/ml normal human IgG. By 12 h, the extracellular PA levels had risen slightly in the Pf cultures but remained the same in the presence of normal IgG. Between 12 and 24 h there was a dramatic increase in the extracellular PA in the presence of Pf IgG to 1,600 mU/ml or ~10 times that in cultures treated with normal IgG for 24 h. Maximum levels of cell-associated PA were reached somewhat earlier. By 6 h about three times more cell-associated PA was detected in Pf IgG-treated cultures as that found in normal IgG-treated cultures. By 12 h, 70 mU/ml cell-associated PA was detected in Pf IgG-treated cultures, more than nine times that detected in normal IgG-treated cultures. The amount of cell-associated PA rose only slightly between 12 and 24 h, the time during which a dramatic increase in extracellular PA was observed.

Antibody Stimulation of PA Is Not Associated with Release of Lysosomal Enzyme Activity. We have documented in previous studies (3, 6) (using trypan blue exclusion, retention of ^{51}Cr, and incorporation of radiolabeled amino acids into protein) that pemphigus antibody in the absence of complement is not cytotoxic for human or mouse epidermal cells, or for a human cervical carcinoma cell line (unpublished results). It was important to determine whether the increased enzyme activity we observed is related to cell damage undetected by the above methods or to regurgitation of lysosomal contents during endocytosis. The conditioned medium was assayed for cathepsin D as a marker for the release of lysosomal enzymes (46). Epidermal cells were incubated for 24 h with IgG preparations at various concentrations up to 8 mg/ml and the medium was then tested for the ability to hydrolyze [^{3}H]hemoglobin (Table I). Medium from cells incubated with 8 mg/ml IgG had slightly higher enzyme activity than that from cells incubated without IgG. Medium collected from cells incubated with 0.5 mg/ml IgG, which is the optimum for PA stimulation, hydrolyzed hemoglobin to the same extent as that collected from cells incubated without IgG. We conclude that the antibody-mediated stimulation of PA activity is not related to cell damage and is not accompanied by a generalized secretion of lysosomal enzymes.

Pemphigus Antibody-induced PA Stimulation Requires Protein Synthesis. Epidermal cells were incubated for 12 h with Pf IgG in the presence of various concentrations of cycloheximide (Fig 3). 20 μg/ml cycloheximide inhibited extracellular and cell-associated PA by 95 and 90%, respectively. This concentration of cycloheximide

TABLE I

Hydrolysis of [^{3}H]Hemoglobin by Conditioned Medium from Human Epidermal Cells Incubated with Pv IgG

IgG	^{3}H peptides released*
mg/ml	*cpm*
8	1,316 ± 78‡
4	1,093 ± 46
2	781 ± 58
1	740 ± 95
0.5	920 ± 26
0	913 ± 68

* 5,000 cpm were released in the presence of 1 μg/ml pepsin.
‡ ± SD.

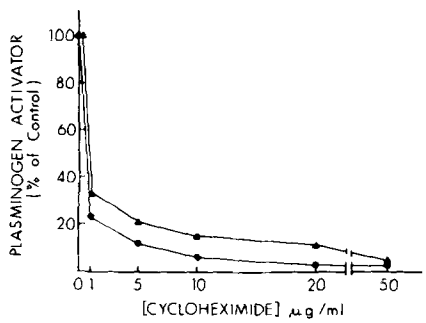

FIG. 3. Inhibition by cycloheximide of pemphigus IgG-stimulated PA production. Confluent cultures of human epidermal cells were incubated for 12 h at 37°C with Pf IgG (2 mg/ml) in the presence of various concentrations of cycloheximide. Extracellular (●) and cell-associated (▲) PA were quantitated using the ^{125}I-fibrin plate assay. Control values in the presence of antibody but absence of cycloheximide were 685 mU/ml in cell lysates and 2,306 mU/ml in conditioned medium.

inhibited incorporation of [^{35}S]methionine into proteins by 82% (data not shown). These data confirm that intact, metabolically active cells are required for the stimulation of PA by pemphigus IgG.

Inhibition by Dexamethasone of Pf IgG Stimulation of PA. Epidermal cells were incubated for 24 h in the presence of Pf IgG and various concentrations of the steroid dexamethasone (Fig. 4). At 10^{-10} M, extracellular PA activity was inhibited by 60%. At 10^{-8} M, 95% inhibition of antibody-induced PA was achieved. Stimulation of PA production by cells treated with lectins (31) and phorbol esters (30, 31), as well as by thioglycollate-stimulated macrophages (15), has been reported to be inhibited by the same concentrations of steroids. The inhibitory concentrations of dexamethasone approximate those used in the clinical treatment of pemphigus.

Electrophoresis of Antibody-stimulated PA. Two major types of human PA are distinguished both biochemically and immunologically. The "urokinase type" has a molecular weight of 52,000–55,000, whereas the "tissue factor type" has a molecular weight of 68,000–74,000 (23, 26, 43). Samples of conditioned medium from cells incubated with or without pemphigus IgG were analyzed for PA activity after electrophoresis on SDS polyacrylamide gels. The electrophoretic profile of enzymatic activity indicated that the major peak of PA activity secreted by unstimulated cultured human epidermal cells is of a molecular weight of ~55,000 (Fig. 5A). A trailing of enzymatic

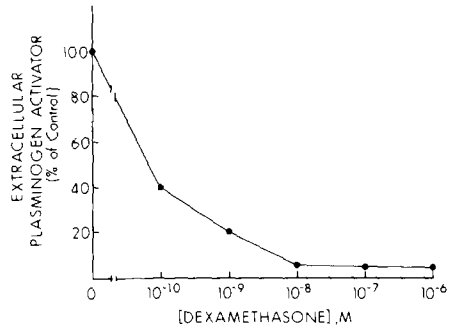

FIG. 4. Inhibition by dexamethasone of pemphigus IgG-stimulated PA production. Confluent cultures of human epidermal cells were incubated for 24 h at 37°C in the presence of Pf IgG (2 mg/ml) in the presence of various concentrations of dexamethasone. Extracellular PA (●) was quantitated using the ^{125}I-fibrin plate assay. Control value in the absence of dexamethasone was 1,576 mU/ml.

activity was seen at higher molecular weights. The small peak of activity at 36,000 mol wt is probably a degradation product of the urokinase type PA, as it also appeared when purified urokinase was electrophoresed as a standard. The electrophoretic profile of conditioned medium from cells treated with Pv IgG (Fig. 5B) or Pf IgG (Fig. 5C) was not different from untreated cells. These data suggest that pemphigus antibody stimulates the production of PA normally produced by cultured human epidermal cells.

Skin Explants. To investigate the role of antibody-induced increases in PA on the loss of epidermal cohesion, skin explant experiments were performed in the presence or absence of pemphigus IgG, human plasminogen, and a variety of proteinase inhibitors. When normal human skin was incubated for 48 h with IgG from Pv patients (4 mg/ml), histologic changes resembling Pv occurred (Fig. 6A). The onset of these changes appeared to be hastened by the inclusion of human plasminogen (200 μg/ml); however, a strict dependence upon exogenous plasminogen was not demonstrated (data not shown). No acantholysis was seen in skin explants incubated with Pf IgG (4 mg/ml) (Fig. 6B); however, when human plasminogen (200 μg/ml) was included along with Pf IgG (4 mg/ml), acantholysis in the upper epidermis was consistently observed (Fig. 6C). No acantholysis was seen in explants incubated with human plasminogen alone (200 μg/ml) (data not shown). When aprotinin, which inhibits plasmin but not PA (47), was included with Pf IgG and plasminogen, production of acantholysis was inhibited (Fig. 6D). Identical results were obtained using LBTI, which also inhibits plasmin but not PA (47). Both aprotinin and LBTI were also able to inhibit acantholysis in explants incubated with Pv IgG and plasminogen (data not shown). In an effort to further implicate plasmin as having a role in acantholysis, skin explants were incubated with plasmin. Acantholytic changes were not observed in explants incubated with 3 Sigma U/ml. At higher concentrations, the plasmin was toxic to the explants. It is possible that activation of plasminogen by antibody-induced PA results in high, localized concentrations of plasmin that were not achieved when explants were incubated with plasmin.

Discussion

Binding of pemphigus autoantibody to epidermal cells stimulated both secreted and cell-associated PA in a time- and dose-dependent manner. The increase in PA

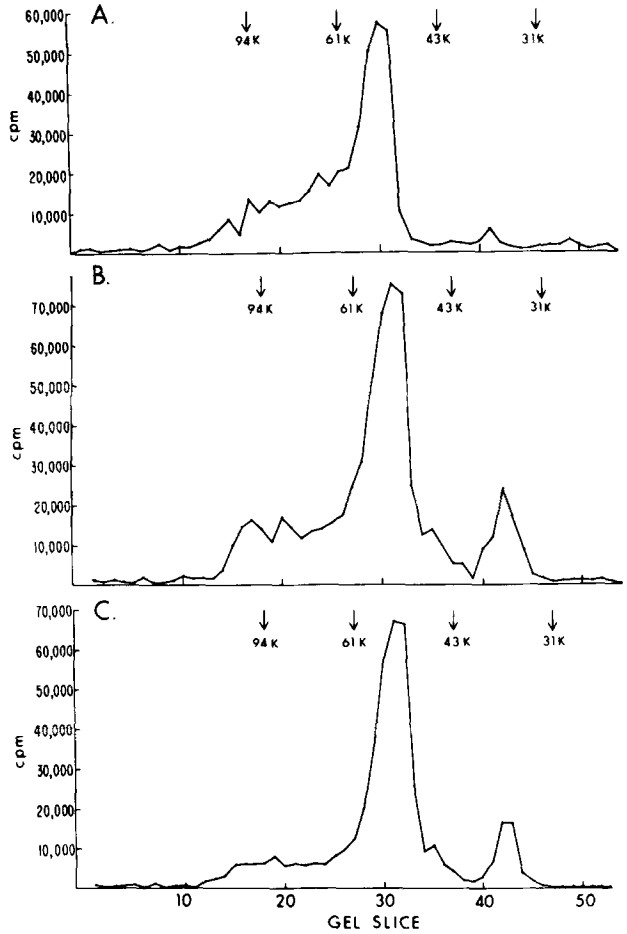

FIG. 5. Electrophoretic profile of PA activity in conditioned medium collected from human epidermal cells incubated at 37°C. (A) without IgG, (B) with Pv IgG (1 mg/ml), (C) with Pf IgG (2 mg/ml). 1.5–2.5 Ploug units/nl of urokinase equivalent activity were applied to each lane of a slab 12.5% polyacrylamide gel and electrophoresed. The gel was agitated in 2.5% Triton X-100 for 1 h at 22°C and each lane was sliced into 2-mm slices. The PA activity in each gel slice was quantitated using the ^{125}I-fibrin plate assay.

was not due to cytotoxicity and was not accomplished by a generalized secretion of lysosomal enzymes. Stimulation of PA after interaction of pemphigus antibody and epidermal cells was dependent upon protein synthesis and was prevented by dexamethasone. The molecular species of PA stimulated by both Pv and Pf IgG appeared to be the same as that found in cultured human epidermal cells not treated with IgG, and the major peak of PA activity co-migrated in SDS polyacrylamide gels with the high-molecular-weight band of urokinase (~55,000).

Explants of human skin incubated with plasminogen and Pf IgG underwent acantholytic changes identical to those found in biopsies of Pf patients. Acantholysis was inhibited by aprotinin and LBTI. Skin explants incubated with Pv IgG showed histological changes identical to those seen in patients with Pv. The inclusion of plasminogen hastened the onset of acantholysis in skin explants incubated with Pv

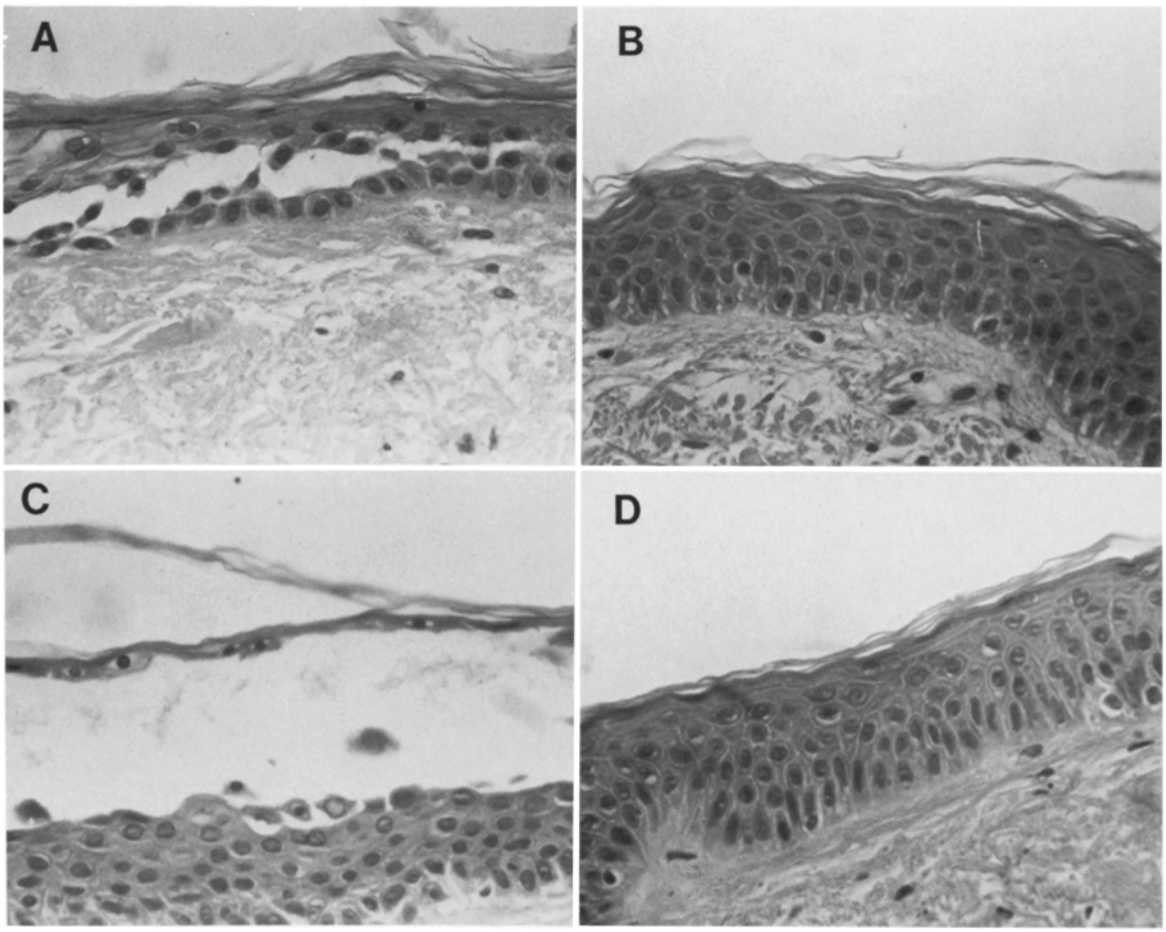

FIG. 6. Explants of normal human skin were incubated for 48 h at 37°C with (A) Pv IgG (4 mg/ml); (B) Pf IgG (4 mg/ml); (C) Pf IgG (4 mg/ml) plus plasminogen (200 μg/ml); (D) Pf IgG (4 mg/ml), plasminogen (200 μg/ml), aprotinin (a total of 60 trypsin inhibitory units was added in doses of 20 trypsin inhibitory units at 0 time, 20 h, and 30 h). × 600.

IgG. This observation is consistent with a recent report by Isseroff and Rifkin (48), who found that plasminogen was preferentially deposited on the basal layer of epidermis. In organ culture experiments using Pv IgG, exogenously added plasminogen might not be necessary if plasminogen is already present on the basal cells of the epidermis.

The results presented here provide evidence for autoantibody-mediated regulation of a specific disease process by activation of proteolytic enzymes. Our data support the following molecular mechanism for the autoimmune disease pemphigus. Binding of pemphigus autoantibody to the surface of human epidermal cells stimulates the production of PA. In the presence of plasminogen, the increased levels of PA result in an increased conversion of plasminogen to plasmin. Plasmin is then responsible for loss of cellular adhesion, probably by degradation of cell surface molecules responsible for cell-cell adhesion within the epidermis. The plasmin-induced dissolution of the intercellular matrix results in acantholysis, which is the hallmark of pemphigus.

The significant mortality from pemphigus was greatly reduced when corticosteroid therapy became available (49). Corticosteroids in high doses rapidly and dramatically reverse blister formation in pemphigus. In our experience, high-dose methyl prednisolone pulse therapy resulted in clearing of blisters within 48 h (G. S. Lazarus, unpublished results). Pemphigus antibody titers, however, drop significantly only after 2–4 mo of steroid therapy, and some patients become clinically free of disease while still possessing an antibody titer (50). The data presented in this report are compatible with the hypothesis that corticosteroids act by inhibiting antibody stimulation of proteinase activation.

Summary

Binding of anti-cell surface pemphigus autoantibodies to cultured human epidermal cells stimulates synthesis and secretion of plasminogen activator (PA). Increases in PA activity were detected within 6 h of the addition of IgG and stimulation was dependent upon IgG concentration. Stimulation of PA activity was inhibited by cycloheximide, which indicates that synthesis of protein was necessary. Pharmacological doses of dexamethasone also prevented IgG-induced stimulation of PA. Electrophoretic profiles of PA secreted by cultured human epidermal cells in the presence or absence of pemphigus IgG were similar. The majority of the PA activity comigrated with the higher-molecular-weight species of human urokinase (~55,000). Explants of normal human skin incubated with pemphigus vulgaris IgG displayed loss of epidermal cohesion similar to that observed in patient biopsies. The histologic changes were potentiated by the inclusion of human plasminogen. Loss of epidermal cohesion in normal skin explants incubated with pemphigus foliaceous IgG was dependent upon the addition of plasminogen and was inhibited by aprotinin or lima bean trypsin inhibitor, which indicated that plasmin is the active enzyme in producing acantholysis. These data support the hypothesis that stimulation of PA by the anti-cell surface autoantibodies of pemphigus results in a localized increase in plasmin, which through proteolysis produces the loss of epidermal cohesion characteristic of pemphigus.

We would like to thank Dr. Shinji Morioka, who provided vital help in the performance of the skin explant experiments, Dr. Joseph McGuire for plasma from a pemphigus patient, and Mrs. Marcia Beard for excellent secretarial assistance.

Received for publication 8 July 1982.

References

1. Beutner, E. H., and R. E. Jordan. 1964. Demonstration of skin antibodies in sera of pemphigus vulgaris patients by indirect immunofluorescent staining. *Proc. Soc. Exp. Biol. Med.* **117**:505.
2. Jordan, R. E., C. T. Triftshauser, and A. L. Schroeter. 1971. Direct immunofluorescence studies of pemphigus and bullous pemphigoid. *Arch. Dermatol.* **103**:486.
3. Farb, R. M., R. Dykes, and G. S. Lazarus. 1978. Anti-epithelial cell-surface pemphigus antibody detaches viable epidermal cells from culture plates by activation of proteinase. *Proc. Natl. Acad. Sci. U. S. A.* **75**:459.
4. Diaz, L. A., and C. L. Marcelo. 1978. Pemphigoid and pemphigus antigens in cultured epidermal cells. *Br. J. Dermatol.* **98**:631.
5. Stanley, J. R., P. Hawley-Nelson, M. Poirier, S. I. Katz, and S. H. Yuspa. 1980. Synthesis

of pemphigoid and pemphigus antigen and keratin by cultured human epidermal cells. *Clin. Res.* **28:**582A.

6. Singer, K. H., K. Hashimoto, and G. S. Lazarus. 1981. Antibody-induced proteinase activation: a proposed mechanism for pemphigus. *Springer Sem. Immunopathol.* **4:**17.
7. Carey, T. E., L. A. Diaz, K. A. Kimmel, D. R. Schwartz, D. E. Richter, and C. J. Krause. 1982. Cell surface antigens of human squamous cell carcinoma. *Clin. Res.* **30:**579A.
8. Spina, C. A., R. E. Murahata, and A. R. Ahmed. 1982. Establishment of lymphoid cell lines producing autoantibody from peripheral blood of patients. *Fed. Proc.* **41:**596.
9. Michel, B., and C. S. Ko. 1974. Effect of pemphigus or bullous pemphigoid sera and leukocytes on normal human skin in organ culture: an *in vitro* model for the study of bullous diseases. *J. Invest. Dermatol.* **62:**541.
10. Schiltz, J. R., and B. Michel. 1976. Production of epidermal acantholysis in normal human skin *in vitro* by the IgG fraction from pemphigus serum. *J. Invest. Dermatol.* **67:**254.
11. Morioka, S., K. Naito, and H. Ogawa. 1981. The pathogenic role of pemphigus antibodies and proteinase in epidermal acantholysis. *J. Invest. Dermatol.* **76:**337.
12. Reich, E. 1978. Activation of plasminogen: a general mechanism for producing localized extracellular proteolysis. *In* Molecular Basis of Biological Degradative Processes. R. D. Berlin et al., editors. Academic Press, Inc., New York. 155.
13. Christman, J. K., S. C. Silverstein, and G. Acs. 1977. Plasminogen activators. *In* Proteinases in Mammalian Cells and Tissues. A. J. Barrett, editor. Elsevier/North-Holland, Amsterdam. 91.
14. Quigley, J. P. 1979. Proteolytic enzymes. *In* Surfaces of Normal and Malignant Cells. R. O. Hynes, editor. John Wiley & Sons, Chichester. 247.
15. Unkeless, J. C., S. Gordon, and E. Reich. 1974. Secretion of plasminogen activator by stimulated macrophages. *J. Exp. Med.* **139:**834.
16. Gordon, S. J., C. Unkeless, and A. Z. Cohn. 1974. Induction of macrophage plasminogen activator by endotoxin stimulation and phagocytosis. *J. Exp. Med.* **140:**995.
17. Granelli-Piperno, A., J.-D. Vassalli, and E. Reich. 1977. Secretion of plasminogen activator by human polymorphonuclear leukocytes. Modulation by glucocorticoids and other effectors. *J. Exp. Med.* **146:**1693.
18. Beers, W. H., S. Strickland, and E. Reich. 1975. Ovarian plasminogen activator:relationship to ovulation and hormonal regulation. *Cell.* **6:**387.
19. Strickland, S., and W. H. Beers. 1976. Studies on the role of plasminogen activator on ovulation: *in vitro* response of granulosa cells to gonadotropins, cyclic nucleotides and prostaglandin. *J. Biol. Chem.* **251:**5694.
20. Unkeless, J. C., A. Tobia, L. Ossowski, J. P. Quigley, D. B. Rifkin, and E. Reich. 1973. An enzymatic function associated with transformation of fibroblasts by oncogenic viruses. I. Chick embryo fibroblast cultures transformed by avian RNA tumor viruses. *J. Exp. Med.* **137:**85.
21. Ossowski, L., J. C. Unkeless, A. Tobia, J. P. Quigley, D. B. Rifkin, and E. Reich. 1973. An enzymatic function associated with transformation of fibroblasts by oncogenic viruses. II. Mammalian fibroblast cultures transformed by DNA and RNA tumor viruses. *J. Exp. Med.* **137:**112.
22. Wigler, M., and I. B. Weinstein. 1976. Tumor promoter induces plasminogen activator. *Nature (Lond.).* **259:**232.
23. Wilson, E. L., M. B. Becker, E. G. Hoal, and E. B. Dowdle. 1980. Molecular species of plasminogen activators secreted by normal and neoplastic human cells. *Cancer Res.* **40:**933.
24. Quigley, J. P. 1976. Association of a protease (plasminogen activator) with a specific membrane fraction isolated from transformed cells. *J. Cell Biol.* **71:**472.
25. Strickland, S., E. Reich, and U. I. Sherman. 1976. Plasminogen activator in early embryogenesis:enzyme production by trophoblast and parietal endoderm. *Cell.* **9:**231.

26. Loskutoff, D. J., and T. S. Edgington. 1977. Synthesis of a fibrinolytic activator and inhibitor by endothelial cells. *Proc. Natl. Acad. Sci. U. S. A.* **75:**3903.
27. Brown, J. M., and D. A. Chambers. 1982. Molecular dynamics of plasminogen activator in epidermal cell cultures. *Clin. Res.* **30:**578A.
28. Nagy, B., J. Ban, and B. Brdar. 1977. Fibrinolysis associated with human neoplasia: production of plasminogen activator by human tumors. *Int. J. Cancer.* **19:**614.
29. Dano, K., and E. Reich. 1978. Serine enzymes released by cultured neoplastic cells. *J. Exp. Med.* **147:**745.
30. Goldfarb, R. H., and J. P. Quigley. 1978. Synergistic effect of tumor virus transformation and tumor promoter treatment on the production of plasminogen activator by chick embryo fibroblasts. *Cancer Res.* **38:**4601.
31. Vassalli, J.-D., J. Hamilton, and E. Reich. 1977. Macrophage plasminogen activator: induction by concanavalin A and phorbol myristate acetate. *Cell.* **11:**695.
32. Quigley, J. P. 1979. Phorbol ester-induced morphological changes in transformed chick fibroblasts: evidence for direct catalytic involvement of plasminogen activator. *Cell.* **17:**131.
33. Miskin, R., T. Easton, and E. Reich. 1978. Plasminogen activator in chick embryo muscle cells: induction of enzyme by RSV, PMA and retinoic acid. *Cell.* **15:**1301.
34. Strickland, S., and V. Mahdavi. 1978. The induction of differentiation in teratocarcinoma stem cells by retinoic acid. *Cell.* **15:**393.
35. Becker, D., L. Ossowski, and E. Reich. 1981. Induction of plasminogen activator synthesis by antibodies. *J. Exp. Med.* **154:**385.
36. Rheinwald, J. G., and H. Green. 1975. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell.* **6:**331.
37. Kronvall, G., U. S. Seal, S. Finstad, and R. C. Williams, Jr. 1970. Phylogenetic insight into evolution of mammalian fragment of G globulin using staphylococcal protein A. *J. Immunol.* **104:**140.
38. Singer, K. H., K. Hashimoto, and G. S. Lazarus. 1982. Plasminogen contaminates IgG preparations isolated by affinity chromatography on staphylococcal protein A-Sepharose. *Clin. Res.* **30:**609A.
39. Deutsch, D. G., and E. T. Mertz. 1970. Plasminogen: purification from human plasma by affinity chromatography. *Science (Wash. D. C.).* **170:**1095.
40. Laki, K. 1951. The polymerization of proteins: the action of thrombin on fibrinogen. *Arch. Biochem. Biophys.* **32:**317.
41. Mosesson, M. W. 1962. The preparation of human fibrinogen free of plasminogen. *Biochim. Biophys. Acta.* **57:**204.
42. Moza, A. K., M. Kumar, and R. P. Sapra. 1976. Satisfactory radioiodination of rabbit fibrinogen using chloramine T. *J. Lab. Clin. Med.* **87:**169.
43. Vetterlein, D., P. L. Young, T. E. Bell, and R. Robbins. 1979. Immunological characterization of multiple molecular weight forms of human cell plasminogen activators. *J. Biol. Chem.* **254:**575.
44. Laemli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* **227:**680.
45. Anderson, C. W., P. R. Baum, and R. F. Gesteland. 1973. Processing of adenovirus 2-induced proteins. *J. Virol.* **12:**24.
46. Levine, N., V. B. Hatcher, and G. S. Lazarus. 1976. Proteinases of human epidermis, a possible mechanism for polymorphonuclear chemotaxis. *Biochim. Biophys. Acta.* **452:**458.
47. Dano, K., and E. Reich. 1979. Plasminogen activator from cells transformed by an oncogenic virus. Inhibitors of the activation reaction. *Biochim. Biophys. Acta.* **566:**138.
48. Isseroff, R. R., and D. B. Rifkin. 1982. Plasminogen is present in the basal layer of the epidermis. *Clin. Res.* **30:**605A.

49. Lever, W. F., and H. White. 1963. Treatment of pemphigus with corticosteroids. Results obtained in 46 patients over a period of 11 years. *Arch. Dermatol. Syphilol.* **87:**12.
50. O'Loughlin, S., G. C. Goldman, and T. T. Provost. 1978. Fate of pemphigus antibody following successful therapy. Preliminary evaluation of pemphigus antibody determinations to regulate therapy. *Arch. Dermatol.* **114:**1769.