Human autoantibodies against desmosomes: Possible causative factors in pemphigus
(human autoimmune disease/keratinocyte/epidermal cell junctions/cellular adhesion)

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ABSTRACT Pemphigus is a human disease that causes extensive blistering of the skin. This blistering is related to a loss of epidermal cell cohesion and is accompanied by circulating autoantibodies that stain epidermal cell surfaces, as shown by immunofluorescence microscopy. One of the major components involved in epidermal cell cohesion is the desmosome. The pathological changes that accompany pemphigus led us to determine whether the autoantibodies are specific for desmosomes. Incubation of cultured mouse keratinocytes in medium containing pemphigus antiserum leads to cell separation at cell–cell contact sites, which possess desmosomes. Tissue sections of mouse skin processed for indirect immunofluorescence, using pemphigus antiserum or a rabbit antiserum directed against components of desmosomes, show similar punctate cell-surface staining patterns within the epidermis. Cultured mouse keratinocytes possessing well-defined intermediate filament bundles (tonofilaments) and desmosomes were processed for double indirect immunofluorescence, using a monoclonal antibody directed against mouse skin keratin and either pemphigus antiserum or the desmosome antiserum. The keratinocytes exhibit a complex system of keratin-containing tonofilaments. Tonofilaments in contacting cells are separated by thin dark bands at the cell surface, which correspond precisely to desmosomal plaques seen by phase-contrast microscopy. These bands specifically stain with both pemphigus antiserum and the desmosome antiserum. Double indirect immunofluorescence of the cultured mouse keratinocytes, using pemphigus antiserum and the desmosome antiserum, reveals that the pemphigus autoantibodies stain the same areas of cell–cell contact as the desmosome antibodies. Our evidence supports the idea that pemphigus blisters form, at least in part, from a specific antibody-induced disruption of desmosomes in the epidermis.

Pemphigus is a devastating disease of the skin that is frequently fatal if left untreated (1). Its clinical features are characterized by the formation of numerous intraepidermal blisters covering extensive portions of the body. The only evidence regarding the biochemical basis of this disease is related to the fact that pemphigus patients possess autoantibodies within their serum that appear to be directed against a so-called "intercellular cement substance" (2, 3). In addition, autoantibodies (usually IgG) are found within the epidermis of biopsies obtained from pemphigus patients (2, 3). Immunofluorescence and immunoelectron microscopic observations of skin biopsies suggest that pemphigus antibodies bind specifically to the surface coat or glycocalyx of epidermal cells (4). Furthermore, incubation of normal human skin organ cultures with pemphigus antiserum in the absence of complement induces histologic changes similar to those seen in skin biopsies of pemphigus patients (5).

Pathogenic role for pemphigus antibodies has therefore been suggested (6) because of both the clinical symptoms of the disease and the surface-staining properties of antiserum from pemphigus patients.

One of the major components involved in the adhesion of cells in the epidermis is the desmosomal junction (7). We wondered, therefore, whether the loss of epidermal cell adhesion in pemphigus could be related to a specific alteration in desmosome structure and function. To determine more precisely whether pemphigus patients have circulating antidesmosome antibodies, we used a mouse keratinocyte cell culture system in which desmosome formation and maintenance can be controlled by alteration of cell-culture conditions (8, 9). We have demonstrated that desmosome formation can be monitored in live mouse keratinocytes in culture (9). In this system, mouse keratinocytes are grown and maintained for long periods in medium containing low Ca²⁺ (between 0.05 and 0.1 mM). Under these conditions, the cells do not form desmosomes or layers of stratified squamous epithelium (8). The keratinocytes can be induced to produce desmosomes and subsequently to stratify by adjusting the Ca²⁺ concentration in the culture medium to normal levels (=1.2 mM). Under these cell culture conditions, Stanley et al. (10) have been able to immunoprecipitate a Mr 130,000 polypeptide from mouse keratinocytes with some, but not all, pemphigus antiserum. More recently, Stanley and Yuspa (11) have used the mouse keratinocyte culture system to show that there is an induction of pemphigus antigen when keratinocytes are switched from medium containing low Ca²⁺ to medium containing normal Ca²⁺ levels (11).

In this study, we have investigated the properties of antiserum from three patients with pemphigus, and the results demonstrate the presence of antibodies that specifically bind to desmosomes.

MATERIALS AND METHODS

Cell Cultures. Primary mouse epidermal cells (PME) were prepared and maintained in culture as reported (8, 9).

Antisera. Antisera from three patients with pemphigus were used in these studies. These were obtained from Robert Marder (Clinical Immunology Laboratory, Northwestern Memorial Hospital, Chicago), who also provided antisera from two pemphigoid patients. Normal human serum was used as a control. A rabbit antiserum directed against the so-called "desmoplakin 2" (Mr, 220,000) component of bovine muzzle desmosomes (12, 13), a rabbit antiserum directed against the 60K K2 (Mr, 60,000) mouse keratin subunit (9), and a rat monoclonal anti-mouse keratin (14) were also used.

Tissues. Neonatal mouse skin was removed and frozen in liquid Freon 22 cooled to liquid nitrogen temperature. Cryostat sections 5–8 μm thick were cut and placed on coverslips. After drying for about 60 min at room temperature, the sections were fixed for 2 min in −20°C acetone and were air

Abbreviation: PME cells, primary mouse epidermal cells.
dried. The pemphigus antiserum, the rabbit desmoplakin antiserum, and the antikeratin antiserum were all diluted 1:20 in phosphate-buffered saline (6 mM Na+, K+ phosphate/171 mM NaCl/3 mM KCl, pH 7.4) prior to use. The antisera were applied to different coverslips containing sections that were subsequently incubated for 30 min at 37°C. After thorough washing in distilled water, the sections were incubated for an additional 30 min at 37°C in fluorescein-conjugated goat anti-human IgG (for pemphigus) or goat anti-rabbit IgG (for the anti-desmoplakin and anti-keratin sera) (Kirkegaard and Perry, Gaithersburg, MD). The coverslips were extensively washed in water and mounted in Gelvatol (Monsanto).

**Cultured Cells.** Single indirect immunofluorescence. After removal of cell culture medium, mouse epidermal cells grown on glass coverslips were fixed for 2 min in -20°C acetone and were air dried. Either pemphigus or desmoplakin antiserum (diluted 1:10 in phosphate-buffered saline) were added to cover the cells. The coverslips were incubated in a moist chamber for 1 hr at 37°C, followed by thorough washing in several changes of distilled water. These coverslips were then incubated for a further 30 min at 37°C with fluorescein-conjugated goat anti-human IgG or fluorescein-conjugated goat anti-rabbit IgG (see above), followed by extensive washing and mounting in Gelvatol.

**Double indirect immunofluorescence.** Mouse epidermal cells were processed with the pemphigus antiserum or desmoplakin antiserum as described above for single indirect immunofluorescence. However, after incubation in either fluorescein-conjugated goat anti-human IgG or fluorescein-conjugated goat anti-rabbit IgG, the coverslips were washed in water and then the coverslips were refixed in 3.7% formaldehyde in phosphate-buffered saline. This step helped to ensure that the fluorescein-conjugated IgGs did not become redistributed during subsequent incubations and contribute to background fluorescence (Hsi-Yuan Yang, personal communication). After another washing in water, the rat monoclonal mouse keratin antibody (hybridoma medium) was overlaid on the cells, and the coverslips were incubated for 1 hr at 37°C. After a wash with distilled water, the coverslips were incubated for an additional 30 min at 37°C with rhodamine-conjugated goat anti-rabbit IgG (Kirkegaard and Perry). Coverslips were washed in distilled water and mounted in Gelvatol. Double indirect immunofluorescence was also performed on mouse epidermal cells using pemphigus sera and the desmoplakin antiserum. The pemphigus serum was used in the first incubation, followed by fluorescein-conjugated goat anti-human IgG. After fixation in 3.7% formaldehyde in phosphate-buffered saline, the coverslips were incubated in desmoplakin antiserum followed by rhodamine-conjugated goat anti-rabbit IgG (Kirkegaard and Perry).

**Light Microscopy.** A Zeiss photomicroscope III equipped with epifluorescence and and phase-contrast optics was used for observations of living, fixed, and stained cells. A Zeiss III RS epi-illumination system was equipped with narrow band filter sets for selective observation of fluorescein and rhodamine. Fluorescein observations were made with a Xenon 75 W dc lamp and rhodamine observations were with a 100 W dc Mercury Arc source. Living cells were prepared for observations as reported (15) and maintained at 37°C with a Sage Instruments (Boston) Air Curtain incubator. Phase-contrast micrographs were taken on Kodak Panatomic-X film. Fluorescence micrographs were taken on Kodak Plus-X film. All films were developed in Diafine (Acufine, Chicago) two stage developer.

**RESULTS**

The Exposure of Living PME Cells to Pemphigus Antiserum. With phase-contrast optics, perinuclear arrays of phase dense fibrils (tonofilaments) can be seen in live PME cells grown in medium containing low Ca2+. No desmosomes are present in such cell preparations (ref. 9; Fig. 1a). When these cells are switched to medium containing normal levels of Ca2+, tonofilaments appear to move rapidly to the cell surface where they associate with phase-dense plaques (ref. 9; Fig. 1b). In a previous study, we showed by electron microscopy and immunofluorescence microscopy that the phase-dense tonofilaments consist of keratin-containing intermediate filament bundles (9) and that the phase-dense plaques correspond to desmosomes (9).

Cells on coverslips were maintained in medium containing low Ca2+ and then switched to medium containing normal levels of Ca2+. At 6 hr after the switch, the majority of tonofilaments of neighboring cells are associated at their borders at sites containing desmosomes (Fig. 2a). At this time, the medium was removed and replaced with medium containing a normal level of Ca2+ and 10% antiserum from a pemphigus patient in place of fetal calf serum. Within 20 min of the addition of pemphigus antiserum, contacting cells begin to pull apart at cell contact points that possess desmosomes (Fig. 2b). Within 1 hr the phase-dense plaques are no longer visi-

![Fig. 1. Living mouse keratinocytes viewed with phase optics in medium containing low Ca2+ (a), showing a perinuclear arrangement of intermediate filament bundles (tonofilaments) (arrow). (b) At 3 hr after switching the cells to medium containing normal amounts of Ca2+, the phase-dense tonofilaments can be traced from a perinuclear position to the cell surface where they associate with phase-dense plaques (desmosomes) (arrow). (×2100.)](image-url)
ble and the cells remain connected by fine cytoplasmic strands (Fig. 2c). At this stage, the cells have become more rounded (Fig. 2c). Within 4 to 6 hr, the majority of cells appear moribund, become detached from the coverslip, and float into the medium. In controls incubated with normal human serum, desmosomes remain intact and no alterations in morphology or tonofilament distribution were seen even after 24 hr. It is interesting to note that PME cells maintained in low Ca\(^{2+}\) to inhibit desmosome formation, retain their normal morphology and remain attached to their substrates for periods up to 12 hr after the addition of pemphigus antiserum. Thus, desmosomes are required for the antiserum of diseased patients to have its effect.

Other controls were carried out using antiserum from patients suffering from bullous pemphigoid, another blistering disease of the skin. However, this disease has been associated with an autoimmune response against the epidermal basement (1). In this case, the PME cells remain with their desmosomes and tonofilaments intact even after 24 hr of incubation in medium containing 10% pemphigoid antiserum.

**Immunofluorescence Observations.** Cryostat sections of neonatal mouse skin were processed for indirect immunofluorescence, using pemphigus antiserum, a rabbit anti-desmoplakin antiserum, or a rabbit anti-keratin antiserum. With the pemphigus antiserum and the desmoplakin antiserum there is a punctate intercellular staining pattern of the epidermis of neonatal mouse skin (Fig. 3a and b). This pattern reflects the typical distribution of desmosomes in epidermal tissue (13). With the anti-keratin antiserum, the cytoplasm of all cells within the epidermis is brightly stained (Fig. 3c).

PME cells grown on coverslips in medium containing low Ca\(^{2+}\) for 2 days and then grown in medium containing a normal level of Ca\(^{2+}\) for 1-4 hr were processed for indirect immunofluorescence using pemphigus antiserum. One hour after the Ca\(^{2+}\) switch, apposed surfaces of neighboring cells have established areas of cell–cell contact (Fig. 4c). These points of cell–cell contact are stained with pemphigus antibodies, and this is shown as intermittent fluorescent dots and bands along their surfaces (Fig. 4a). Noncontacting surfaces of PME cells are not stained by pemphigus antiserum (Fig. 4a). Pemphigus antibody does not stain the borders of PME cells grown in low Ca\(^{2+}\) (i.e., cells that do not possess desmosomes), nor does it stain the surfaces of human or mouse fibroblasts (data not shown).

An extensive keratin-containing network of tonofilaments in PME cells is revealed by indirect immunofluorescence microscopy, using the monoclonal antibody preparation directed against mouse skin keratin within 1 hr after the addition of Ca\(^{2+}\). Tonofilaments of neighboring cells appear to approximate each other but are separated by thin nonfluorescent bands (Fig. 4b). By double indirect immunofluorescence using pemphigus antiserum and this anti-keratin, these nonfluorescent bands are seen to react with pemphigus antibodies (Fig. 4a). At 6 hr after the Ca\(^{2+}\) switch, neighboring PME cells are closely apposed and keratin-containing tonofilaments of contiguous cells associate all along the cell–cell borders (Fig. 5b). These fibers are separated by nonfluorescent bands that extend along the borders of contacting cells and that stain with pemphigus antibodies (Fig. 5a and b). These nonfluorescent bands correspond with fidelity to the dense plaques shown by phase-contrast microscopy (Fig. 5c).
Immunofluorescence controls, in which normal human sera or pemphigoid antisera were used in place of pemphigus antisera, do not yield any obvious staining of the surfaces of mouse keratinocytes in culture both in the absence and presence of desmosomes. As further controls, we have also used the anti-keratin monoclonal and a rabbit desmoplakin antiserum in double-indirect immunofluorescence observations of switched PME cells. The desmoplakin antiserum, like the pemphigus antiserum, stains the nonfluorescent band between the keratin-containing tonofilaments of contacting PME cells that have been switched to normal Ca\(^{2+}\) levels for 3 hr (Fig. 6 a and b). A corresponding phase-contrast micrograph in Fig. 6c shows that the desmoplakin antiserum stains the phase-dense plaques present at the sites of cell–cell contact. Double-indirect immunofluorescence observations of PME cells using pemphigus antisera and the desmoplakin antiserum reveals that the pemphigus antibodies stain the same areas of cell–cell contact as the desmoplakin antibodies (Fig. 7 a and b). Furthermore, these areas correspond to the dense plaques shown by phase-contrast microscopy (Fig. 7c).

**DISCUSSION**

Pemphigus is an autoimmune disease of the skin that results in the formation of intraepidermal blisters (1). Epidermal cells in the blisters appear to lose their intercellular adhesions and die. To date, indirect immunofluorescence has shown that pemphigus patients have a circulating antibody thought to be directed against the intercellular epidermal cement (2). Moreover, it is known that the titer of pemphigus antibody reflects the severity of the disease. Therefore, this latter parameter has been used as a diagnostic tool in determining the degree of disease activity and as a guide to therapeutic control (1).

Braun-Falco and Vogell (16) suggested that a loss of cell adhesion in pemphigus may be related to alterations in desmosome structure. However, Wilgram et al. (17) proposed that desmosome disintegration is secondary to changes in the tonofilaments of epidermal cells in diseased patients. Others have refuted both of these proposals and claim that the primary effect of pemphigus is on the intercellular cement, with secondary effects on the desmosome–tonofilament complex (18). Our results once again focus attention on the role of the desmosome in pemphigus.

In this paper, we have shown that antiserum from several patients with pemphigus induces an acantholytic effect; i.e., a loss of cell–cell contact in cultured mouse keratinocytes containing distinct desmosomes. Within 20 min after exposure to a 1:10 dilution of the autoimmune antiserum, areas of cell–cell contact begin to separate only in regions containing desmosomes. The cells begin to round up, and within 4 to 6 hr, the cells detach from their substrate.

In addition to the in vivo effects of pemphigus antiserum, immunofluorescence observations indicate that pemphigus antibodies stain desmosomes in a fashion identical to that seen with an antibody directed against a so-called desmoplakin (M\(_s\), 220,000) component of bovine muzzle desmosomes (12, 13), both in cultured keratinocytes and in whole mouse epidermis. Double-immunofluorescence labeling of mouse keratinocytes, using pemphigus antiserum and the desmo-
plakin antiserum, shows coincident staining of keratinocyte cell surfaces. Based on these observations, we believe that pemphigus antibodies are directed against a component of the desmosomal junction. These results are in direct conflict with a recent report in which Gorbsky et al. (19) concluded that desmosomal antigens are not recognized by pemphigus autoimmune antiserum. These observations were based on ELISA, using bovine muzzle desmosomal proteins and immunofluorescence labeling of frozen sections of bovine epidermis and monkey esophagus. One of the reasons for this discrepancy may be that the autoantibodies in pemphigus recognize only native proteins. This possibility is supported by the finding that pemphigus antiserum causes acantholysis of desmosome-possessing keratinocytes in vivo. Gorbsky et al. (19) used denatured bovine muzzle desmosomal proteins in their assays. It is possible that the denaturing of desmosomal proteins for such an ELISA alters the antigenic determinants for pemphigus autoantibodies.

It is interesting to note that in pemphigus patients the only desmosome-possessing tissues that are affected by the disease are stratified squamous epithelia. We have shown that autoantibodies in pemphigus appear to be specific for desmosomes. However, many other tissues in the body possess desmosomes—e.g., columnar epithelium such as the intestinal mucosa (20)—and yet these tissues appear unaffected by pemphigus autoantibodies. It is thus tempting to speculate that pemphigus autoantibodies specifically recognize a component of stratified squamous epithelial desmosomes that is absent in the desmosomes of nonepidermal tissues.

Desmosomes are considered to be areas of enhanced contact between the epidermal cells of the skin. With their connections to tonofilament bundles, they are considered to be involved in the transduction of stretching and constricting forces within the epidermis (7). Thus, disruption of desmosomes would result in the loss of the integrity of the epidermis. Our results support the idea that the blistering in pemphigus is induced, at least in part, from specific autoantibodies that induce disruption of desmosomes in the epidermis.

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