Appearance of "Pemphigus Acantholysis Factor" in Human Skin Cultured with Pemphigus Antibody

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These studies deal with the mechanism of pemphigus IgG-induced epidermal acantholysis. When normal human skin was cultured with defined medium containing IgG from pemphigus serum, extensive epidermal acantholysis developed and heat-labile proteolytic enzyme(s) were recovered in the culture medium. The enzyme(s) displayed maximal activity at pH 6.5 when a \( ^{3} \)-amino acid-labeled, insoluble epidermal cell material was used as substrate. The enzyme activity increased during the first 3 days of culture and the appearance of maximal activity coincided with the time of onset of acantholysis. Acantholysis did not occur in control cultures incubated with normal IgG and the enzyme did not appear in the medium or in aqueous extracts of cultured tissues. The enzyme(s) is probably not of lysosomal origin because low pH-active proteases characteristic of these organelles remained within the cells. The effects of puromycin on appearance of enzyme activity, acantholysis, and cell viability was studied. At cytotoxic concentrations, the appearance of the enzyme(s) and acantholysis were prevented, whereas at less toxic concentrations enzyme activity and acantholysis were not prevented. Because inhibition of protein synthetic rates by puromycin could not be dissociated from the cytotoxic effects, it is uncertain whether enzyme appearance and acantholysis were dependent upon living tissue or on specific protein synthesis. After pemphigus IgG was removed from the conditioned medium by DEAE cellulose and affinity column chromatography, the remaining material contained enzyme activity and caused acantholysis in fresh skin explants. Similar activities were not present in normal IgG-containing conditioned medium or unfractionated epidermal extracts from normal skin.

These data indicate that when the pemphigus IgG autoantibody interacts with epidermal cell surface antigens, the cell responds by synthesis or activation of a non-IgG "pemphigus acantholysis factor" (PAF) which may be a nonlysosomal proteolytic enzyme. It is suggested that PAF causes loss of adhesion between keratinocytes and ultimately produces the characteristic acantholytic cells of pemphigus.

Experimental evidence has demonstrated that antiepithelial antibodies from pemphigus patients are capable of producing acantholysis in stratified squamous epithelia [1-8]. When normal human skin [1-4] or monkey skin [5,6] is cultured in vitro with whole pemphigus serum, characteristic suprabasal epidermal acantholytic "lesions" develop. Schiltz and Michel [7] showed that it is the IgG fraction from the serum which induces the acantholysis and that complement is not required to produce the damage in this in vitro system. Ultrastructural studies have shown that the acantholysis produced in cultured human skin [8] or monkey skin [6] is identical to that seen in the patient's lesions. Initially, suprabasal epidermal cells lose their attachments in nondesmosomal areas, and later the desmosomes disappear and the tonofilaments become clumped in a perinuclear position. Hemidesmosomes remain unaffected and the basal cells remain attached to the basal lamina.

In a recent study, Schiltz, Michel, and Papay [9] showed that when suspensions of primary human epidermal cells were incubated with pemphigus IgG, protein synthesis was inhibited, a rapid solubilization of insoluble cellular material occurred and the cells were eventually killed. These effects of pemphigus antibody were time- and antibody concentration-dependent. It was suggested that the solubilization was caused by a nonlysosomal proteolytic enzyme(s), maximally active at pH 6.5 in our assay system, which was synthesized, released or activated by the epidermal cell. It was proposed that this enzyme (or group of enzymes) causes acantholysis. For the present study we tested the enzyme hypothesis for pemphigus acantholysis using whole skin explants cultured with pemphigus IgG. Our results demonstrate that a non-IgG factor(s) (pemphigus acantholysis factor or "PAF") is synthesized by or activated in human epidermal cells following their interaction with the pemphigus antibody. This factor causes epidermal acantholysis in fresh skin explants, and may be the pH 6.5 active epidermal proteolytic enzyme.

MATERIALS AND METHODS

Culture Techniques

Clinically normal human skin obtained from surgical mastectomy specimens was used in these experiments. The skin was prepared as previously described [7] and cultured as explants which were floated on lens-paper rafts. The medium was Ham's F-10 which contained twice the normal concentrations of amino acids and sodium pyruvate + 10% heat-denatured fetal calf serum + a mixture of antibiotics/antimycotics (Grand Island Biological Co) + purified IgG fractions from pooled normal or pemphigus serum [7]. The pemphigus sera were all from patients with pemphigus vulgaris. The antiepithelial antibody titer of the reconstituted pemphigus IgG was adjusted to 160 as determined by reaction with monkey esophagus substrate and the IgG protein content varied between 20 and 40 mg/ml medium. Control normal IgG was prepared from sera of persons with AB blood type, and was adjusted to the same protein concentration as the pemphigus IgG. The explants were cultured at 37°C in a humid incubator containing 95% air/5% CO\(_2\). In the experiments where enzyme activity from medium was assayed, complete equilibration between the explants and the medium was assured by gentle mixing with a Pasteur pipette prior to removal of the sample. In some experiments in which "pemphigus acantholysis factor" was obtained, the fetal calf serum was omitted from the medium. For these experiments, approximately 50 explants (3 mm x 3 mm x 0.3 mm thick) were placed on lens paper rafts and floated on 3 ml medium containing control or pemphigus IgG. After 5 days the explants were immersed, briefly equilibrated in the medium...
and the IgG removed using ion exchange and affinity columns as described below. The pH profiles of intracellular enzymes from intact epidermis cultured with antibodies (Fig 2) was determined as follows. Human skin (8 pieces, each 1 cm \( \times \) 0.3 mm thick) was placed into a trypsin solution (0.4% in Simms’s Ca\(^++\) - Mg\(^++\) -free saline) for 2 hr and the epidermis removed from the dermis by microdissection. The viable, intact epidermis was washed several times in phosphate-buffered saline (PBS) and cultured in F-10 medium (without fetal calf serum) containing either pemphigus IgG (anti-epithelial antibody titer 160, antibody protein 24 mg/ml) or normal IgG (24 mg/ml). After 3 days the tissues were rinsed in PBS, suspended in distilled water, and the soluble intracellular enzymes extracted by a freeze/thaw technique [9].

**Enzyme Assay**

Epidermal proteolytic enzymes were assayed by a modification of the technique previously reported [9]. Insoluble \(^3\)H-amino acid labeled substrates were prepared as described previously and precipitated with trichloroacetic acid (TCA, 5% final concentration), resuspended and dialyzed extensively against distilled water. This substrate contained 10,000–20,000 DPM and 75–100 \( \mu \)g protein per 50 \( \mu \)l. The incubation mixture contained 50 \( \mu \)l of this substrate + 50 \( \mu \)l of the enzyme source + 50 \( \mu \)l of 0.45 M phosphate buffer at the specified pH. After 20 hr incubation in 0.4 ml polypropylene tubes, the mixture was precipitated by addition of an equal volume of 10% TCA and centrifuged for 4 min in a Beckman 152 Microfuge. The supernatant was collected, diluted with distilled water to 0.5 ml, mixed with a Triton X-100-based aqueous scintillation fluid and the radioactivity determined in a Beckman LS 230 liquid scintillation counter.

**Puromycin Studies**

For the experiment shown in Fig 4, 5-mm discs of skin (0.5-mm thick) were prepared from surgical specimen using a circular punch biopsy instrument and cultured 48 hr on rafts in the presence of puromycin·2HCl (0–30 \( \mu \)M, Sigma Chemical Co., P-7255). Then, to deplete puromycin and amino acids from the tissue, the explants were washed for 45 min in 3 changes of F-10 medium followed by 30 min in PBS. To assess the extent of inhibition of protein synthesis, triplicate explants which had not been cultured with puromycin were immersed in 1 ml F-10 labeling medium which contained 0.01 times the normal concentrations of amino acids + 2 \( \mu \)Ci \(^3\)H-amino acid mixture (New England Nuclear, NET-250) + various concentrations of puromycin. To assess epidermal cytotoxicity, explants which had been cultured 48 hr with different puromycin concentrations were incubated with the labeling mixture in the absence of puromycin. The percent recovery of protein synthetic rate was considered to be inversely proportional to the protein per 24 mg/ml) or normal IgG (24 mg/ml). After 3 days the tissues were rinsed in PBS, suspended in distilled water, and the soluble intracellular enzymes extracted by a freeze/thaw technique [9].

**RESULTS**

**Enzyme Proteases in Skin Cultures Incubated with Pemphigus IgG**

Skin was grown in medium containing normal or pemphigus IgG, and at 4 days extensive epidermal acantholysis had occurred only in the culture containing pemphigus IgG (see Fig. 8A as an example of a typical in vitro "lesion"). At this time the media were assayed for enzyme activity at various pH values. These pH profiles (Fig 1) showed the presence of a large peak of activity at pH 6.5 from cultures grown in pemphigus IgG, whereas this activity was absent in the control medium. The activity from conditioned medium containing pemphigus IgG was heat labile since boiling reduced this activity to background levels of conditioned medium which contained normal IgG (Table).

**Heat lability of protease activity**

<table>
<thead>
<tr>
<th>Sample</th>
<th>DPM solubilized ± SD</th>
<th>% Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal IgG</td>
<td>1,062 ± 142</td>
<td>—</td>
</tr>
<tr>
<td>Normal IgG (boiled)</td>
<td>988 ± 60</td>
<td>7.0</td>
</tr>
<tr>
<td>Pemphigus IgG</td>
<td>5,331 ± 253</td>
<td>—</td>
</tr>
<tr>
<td>Pemphigus IgG (boiled)</td>
<td>834 ± 53</td>
<td>84.0</td>
</tr>
</tbody>
</table>

*Triplicate samples (50 \( \mu \)l each) of the medium from the experiment shown in Fig 1 were assayed for enzyme activity at pH 6.5 with or without prior boiling for 15 min.*

**Puromycin Studies**

Normal or pemphigus IgG was removed from the medium in which skin samples had been cultured (i.e., conditioned medium) in the following manner. The medium (6 ml) was first dialyzed against several changes of 0.01 M sodium phosphate buffer, pH 8.0 and then pumped onto a 1 cm \( \times \) 14 cm column of DEAE cellulose (Whatman DE52) which had equilibrated in the same buffer (flow rate 20 ml/hr). After the bulk of the IgG had eluted, the column was stripped with 0.01 M sodium phosphate buffer, pH 6.0 which contained 1 M NaCl. This latter fraction was then dialyzed into 0.15 M sodium phosphate buffer, pH 7.2 and applied to a 1 cm \( \times \) 10 cm column of Sepharose 4B which contained covalently-conjugated goat anti-human IgG (Hyland Labs.). Conjugation of the goat IgG to the cyanogen bromide-activated sepharose was performed using standard techniques [10]. The breakthrough peak from this column (eluted with 0.15 M sodium phosphate buffer, pH 7.2) was dialyzed against distilled water. This fraction was then solubilized by 50 \( \mu \)l of Triton X-100 and the activity determined in the liquid scintillation counter.

**Column Chromatography for Isolation of “Pemphigus Acantholysis Factor”**

Normal or pemphigus IgG was removed from the medium in which skin samples had been cultured (i.e., conditioned medium) in the following manner. The medium (6 ml) was first dialyzed against several changes of 0.01 M sodium phosphate buffer, pH 8.0 and then pumped onto a 1 cm \( \times \) 14 cm column of DEAE cellulose (Whatman DE52) which had equilibrated in the same buffer (flow rate 20 ml/hr). After the bulk of the IgG had eluted, the column was stripped with 0.01 M sodium phosphate buffer, pH 6.0 which contained 1 M NaCl. This latter fraction was then dialyzed into 0.15 M sodium phosphate buffer, pH 7.2 and applied to a 1 cm \( \times \) 10 cm column of Sepharose 4B which contained covalently-conjugated goat anti-human IgG (Hyland Labs.). Conjugation of the goat IgG to the cyanogen bromide-activated sepharose was performed using standard techniques [10]. The breakthrough peak from this column (eluted with 0.15 M sodium phosphate buffer, pH 7.2) was dialyzed against distilled water. This fraction was then solubilized by 50 \( \mu \)l of Triton X-100 and the activity determined in the liquid scintillation counter.

**FIG. 1.** Enzyme activities in the medium of whole skin cultures after 4 days. The medium was F-10 which contained 10% heat-denatured fetal calf serum and 23 mg/ml normal or pemphigus IgG, the latter at an anti-ICS titer of 160. Each culture contained 20 explants in 2 ml medium. The activity is expressed as the average DPM of substrate solubilized by 50 \( \mu \)l (triplicate samples) minus that of unconditioned medium. Pemphigus IgG, \((PIG\)}; Normal IgG, \((NIIG\)}.

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It is likely that this heat-labile medium protease(s) originated from epidermal cells, since similar activity was recovered in medium from intact isolated epidermis incubated for 2 days with pemphigus IgG [9]. The medium pH profile of Fig 1 was also different from that obtained with extracts of intact epidermis which had cultured 2 days with normal or pemphigus IgG. These extracts had low pH optima typically characteristic of lysosomal enzymes (Fig 2).

**Kinetics of Protease Release into the Medium**

The medium from whole skin explant cultures was examined daily for the unique proteolytic enzyme(s). Cumulative enzyme activity is shown in the upper panel of Fig 3. In the normal IgG-treated culture only background activity was present, and this did not change during the experiment. In the pemphigus IgG-treated culture, however, enzyme accumulation increased for the first 3 days and then remained constant. In the lower panel of Fig 3 the rates of appearance of enzyme activity in this same experiment have been plotted to stress the temporal relationship between enzyme activity and acantholysis. To obtain these data, the cumulative activity present the previous day has been subtracted from the activity of a given day. Maximal rate of appearance occurred between 1 and 2 days, and this coincided with the beginning of acantholysis in this experiment.

**Puromycin studies.** The effects of puromycin on epidermal protein synthesis, appearance of protease in the medium, acantholysis and cell viability was studied. Explants were cultured 48 hr in different concentrations of puromycin (without antibodies) and then incubated an additional 24 hr with a 3H-amino acid mixture. To assess inhibition of protein synthesis, puromycin was included in the labeling medium. To determine cytotoxic effects, excess puromycin was washed from the tissue (Materials and Methods) and omitted from the labeling medium. The extent of recovery of synthetic rate was considered to be an index of cell viability. This experiment (Fig 4) clearly demonstrated that it was not possible to dissociate the inhibitory effects of puromycin on protein synthesis from the cytotoxic effects, since inhibition was inversely proportional to recovery.

Several concentrations of puromycin were tested to determine effects on protease appearance and acantholysis during a 5-day culture period. Concentrations of 0.1 µM, 1 µM, and 10 µM, which respectively inhibited protein synthesis 18%, 41% and 82%, did not prevent appearance of the enzyme or acantholysis. The effect of 100 µM puromycin on appearance of lysosomal enzymes (Fig 2) .

**Fig 2.** Activity vs pH profiles of the soluble intracellular fraction from intact epidermis which had incubated 48 h with normal IgG or pemphigus IgG (Materials and Methods).

**Fig 3.** Medium enzyme activities from whole skin during a 5-day culture period with normal or pemphigus IgG. Pieces of human skin (15 per culture) were cultured with medium containing 20.3 mg/ml normal or pemphigus IgG, the latter at an anti-ICS titer of 80. Triplicate, 50 µl samples were removed from the cultures at daily intervals, frozen and later assayed at pH 6.5. The zero point was removed at the start of the culture period. *Upper panel*, cumulative activity; *lower panel*, rate of change of activity (ie, the activity of a given day minus the activity of the previous day).

**Fig 4.** Concentration-dependent inhibition and recovery of epidermal protein synthesis by puromycin. Whole skin explants were cultured 2 days with the indicated concentrations of puromycin, and then incubated 24 hr with 3H-amino acid mixture to assess rates of epidermal protein synthesis (Materials and Methods). The total TCA-insoluble radioactivity of the control (cultured and pulsed without puromycin) was 105,800 ± 9,896 DPM.
Acantholysis was extensive in explants grown in pemphigus IgG alone. Absence of puromycin or pemphigus IgG, but not in cultures with normal IgG (with or without puromycin), enzyme activity did not increase significantly, and acantholysis did not develop in any of these three cultures. In contrast, cultures containing pemphigus IgG alone showed increased enzyme activity and acantholysis occurred.

**Recovery of Non-IgG “Pemphigus Acantholysis Factor(s)” from Medium of Skin Cultured with Pemphigus IgG**

The experiments shown in Fig 3 and 5 respectively demonstrated that acantholysis and the appearance of the unique protease enzyme(s) are coincident, and that both can be prevented by puromycin. As an approach to determine whether enzyme activity was responsible for acantholysis, we removed the IgG from the medium of skin cultured for 4 days and tested the remaining material for the 2 activities. The bulk of the IgG was removed by DEAE cellulose column chromatography (Fig 6). As shown previously [7], most of the IgG, but not all the pemphigus antibody, eluted in the void volume of this column. The bound fraction was eluted from this column and applied to an affinity column which contained goat antihuman IgG covalently bound to Sepharose 4B. The breakthrough peak from this column (Fig 7) was collected, reconstituted in F-10 medium and tested for protease activity and ability to cause acantholysis. This material did not contain pemphigus antibody, as shown by indirect immunofluorescence testing, and did not contain IgG, as shown by immunoelectrophoresis. This fraction caused suprabasilar epidermal acantholysis in fresh skin explants after 3 days in culture (Fig 8B), and the histology was similar to that obtained with pemphigus IgG (Fig 8A). Acantholysis did not occur in control medium which contained the affinity column breakthrough peak from normal IgG-containing conditioned medium (Fig 8C). The experiment shown in Fig 6-8 has been repeated 3 times using different batches of pemphigus IgG conditioned medium. Non-IgG acantholysis factor(s) were recovered in 2 of these experiments, and the protease enzyme(s) was present in both. The enzyme activities were small, but demonstrable at the beginning of the secondary culture period, and after 3 days had increased nearly 4-fold.

**Lack of PAF Activity in Epidermal Extracts**

Because our experiments suggested that a non-IgG pemphigus acantholysis factor (PAF) originated from epidermis, we tested epidermal extracts from noncultured skin for ability to cause acantholysis in cultured skin. An epidermal extract was prepared [9], added to F-10 medium (final protein concentration 0.1 mg/ml) and the pH was adjusted to 10 at the appropriate pH, these media were cultured with fresh skin explants. Epidermal acantholysis was not produced in any of the pH-adjusted media.

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**Fig 5. Effects of puromycin on appearance of protease in the culture medium.** Whole skin was cultured with normal or pemphigus IgG (39 mg/ml, anti-ICS titer of pemphigus antibody = 100) in the presence or absence of 100 μM puromycin. Samples of medium were removed and assayed for protease activity as described in Materials and Methods. Acantholysis was extensive in explants grown in pemphigus IgG alone, but did not occur in explants cultured with normal IgG (with or without puromycin) or pemphigus IgG + puromycin.

**Fig 6. DEAE cellulose column elution profile of 6 ml medium from skin explants which had cultured 4 days in F-10 medium containing 10% heatdenatured fetal calf serum + pemphigus IgG (Materials and Methods). The pemphigus antibody titer of the void volume peak was 40 and the stripped peak was 20 (both reconstituted in 12 ml PBS). PAb = pemphigus antibody; “PAF” = pemphigus acantholysis factor; SB = starting buffer.

**Fig 7. Affinity column chromatography of the DEAE cellulose column stripped fraction.** The DEAE cellulose fraction which eluted between 130 and 160 ml (Fig 6) was dialyzed against 0.15 M sodium phosphate buffer, pH 7.2 and applied to a column which contained goat IgG directed against human IgG. The goat IgG was covalently bound to Sepharose 4B (Materials and Methods). The breakthrough peak of 6-8 ml contained a pemphigus antibody titer of zero and the citrate-eluted peak had a titer of 10 (both in 30 ml PBS).
FIG 8. Photomicrographs of normal human skin explants which had cultured 72 hr in F-10 medium containing (A) 39 mg/ml pemphigus IgG, anti-ICS titer 100, (b) "Pemphigus acantholysis factor" (i.e. the breakthrough fraction from Fig 7) or (c) a fraction from normal IgG-containing conditioned medium which was prepared in parallel to the pemphigus acantholysis factor. The medium for (A) contained 10% heat-denatured fetal calf serum, which was omitted in (B) and (C). Hematoxylin and eosin (x 180).
either in the presence or absence of the epidermal extract. Nonspecific epidermal changes did occur with extracts at lower pH values (3.5 to 5.5), and increased intracellular edema was prominent.

**DISCUSSION**

These experiments have demonstrated that when pemphigus antibody-induced epidermal acantholysis occurs in cultured human skin, non-IgG factor(s) appear in the medium which can cause acantholysis in fresh skin explants. This "pemphigus acantholysis factor" (PAF) originates from the skin and does not appear in control cultures incubated with normal IgG.

Indirect evidence suggests that PAF may be a protease enzyme: (i) enzyme activity and acantholysis activity are not generated in control cultures, (ii) during a 6-day culture period maximal protease activity coincided with the time of onset of acantholysis, (iii) puromycin prevented both the appearance of the protease and acantholysis in pemphigus IgG-treated cultures and (iv) both activities were present in conditioned medium following removal of the pemphigus antibody. However, further study is needed to establish a direct cause and effect relationship between the 2 activities. It is possible that protease activity is not the cause of acantholysis but rather results from acantholysis.

It is reasonable to suspect that PAF is a proteolytic enzyme. In previous studies [9], we showed that protease activity was released by human epidermal cells cultured with pemphigus IgG, and that these enzymes caused solubilization of previously insoluble epidermal cellular material. We believe this solubilization to be equivalent to acantholysis. It is also well known that selected proteolytic enzymes are capable of causing epidermal acantholysis, for example trypsin [11], chymotrypsin [12] or papain and elastase [13]. These enzymes, however, do not cause the exact lesion to that produced by pemphigus IgG or PAF. Whereas, the enzymes cause acantholysis throughout the epidermis, pemphigus IgG or PAF-induced acantholysis occurs only in the suprabasilar cells, thus reproducing the pattern of injury seen in spontaneous pemphigus vulgaris lesions.

Farb, Dykes, and Lazarus [14] showed that mouse epidermal cells in monolayer culture became less adherent to culture dishes when pemphigus serum was added to the medium. Because the serine protease inhibitors soybean trypsin inhibitor and α2-macroglobulin prevented this loss of adhesion, they suggested pemphigus acantholysis was caused by the release of a serine proteinase. Since the protease in our studies has not yet been isolated in pure form, we have not tested it for protease class. We have, however, tested soybean trypsin inhibitor for ability to prevent acantholysis in cultured human skin [15]. This compound, at concentrations which completely inhibited trypsin activity, did not prevent the pemphigus antibody from binding to the epidermis and did not prevent acantholysis. This is perhaps predictable, since pemphigus acantholysis readily occurs in human skin explants cultured in fresh, whole pemphigus serum [1-6], which contains large amounts of α2-macroglobulin and α2-antitrypsin. It is possible that the conflicting results may be due to species differences.

We do not know which epidermal cells elaborate PAF or from which intracellular structures it arises. However, the factor likely does not originate from lysosomes because the low-pH-active proteases characteristic of these organelles were not recovered in the medium, but remained within the epidermal cells. PAF may have been synthesized by the epidermal cells in response to the pemphigus antibody because puromycin prevented acantholysis and PAF activity was not present in epidermal extracts. However, since the concentration of puromycin required to prevent acantholysis also appeared to be cytotoxic, it is not known whether acantholysis was dependent upon specific protein synthesis, living epidermis or both. We have determined that pemphigus antibody-induced acantholysis does not occur in skin which had been killed by freezing (Schiltz, unpublished). PAF synthesis in response to the antibody is possible since we know from autoradiographic studies that protein synthesis in cultured explants is not impaired during the initial stages of acantholysis [7]. Furthermore, loss of capacity to synthesize protein coincided with the decline in rates of appearance of the protease enzyme. An alternate explanation for the lack of PAF activity in epidermal extracts may be that in normal epidermis the factor is complexed with inhibitors.

Although pemphigus acantholysis appears to be an immunological phenomenon, in these in vitro studies, the process is unique because it does not require complement activation [5-9]. In this regard, there is a parallel to that which occurs in myasthenia gravis (reviewed by Drachman, reference 16). In vitro studies showed that degradation rates of the cell surface acetylcholine receptor protein of striated muscle cultures doubled in the presence of the IgG autoantibody from these patients, and this occurred without activation of complement [17].

In summary, our studies suggest that pemphigus acantholysis occurs as the result of a dynamic response of the epidermal cell to the pemphigus antibody. We believe the epidermal response consists of synthesis or activation of PAF, the agent which causes the tissue damage. It is intriguing to consider the possibility that PAF generation in epidermis may be a nonspecific response to injury by a variety of external agents, including cantharidin [18,19], staphylococcal toxin exfoliatin [20], mild thermal damage [21] or Herpes simplex virus [22]. All these agents produce acantholysis in epidermis in vitro and in vivo, but probably not by direct action.

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