

## PEMPHIGUS SERUM-INDUCED LOSS OF MICROVILLI FROM HUMAN EPIDERMAL CELLS

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**Abstract.** Scanning electron microscopic examination of human epidermal cells prepared from suction blister roofs by treatment with trypsin and dithioerythritol and incubated (37°C, 18 hours) with pemphigus sera revealed a smooth or wrinkled surface texture in 83 to 96% and surface microvilli in only 17 to 4%. The disappearance of microvilli was not affected by the presence of complement. In the control specimen 54% of the cells retained their microvilli. The difference between cells incubated with pemphigus and those incubated with control sera was highly significant ( $p < 0.001$ ). Epidermal cell suspensions were also incubated with pemphigus vulgaris sera plus a protease inhibitor (ovomucoid). The loss of microvilli from human epidermal cells induced by pemphigus vulgaris serum was highly significantly inhibited by ovomucoid ( $p < 0.001$ ). The results suggest that the loss of microvilli induced by pemphigus sera, irrespective of the presence of complement, may be an important factor in acantholysis of pemphigus. Proteolytic enzymes may be responsible for the loss of microvilli and smoothing of the cell surface of human epidermal cells in vitro.

**Key words:** Acantholysis; Microvilli; Complement; Scanning electron microscopy; Ovomucoid; Protease inhibition

A study using scanning electron microscopy (SEM) has revealed that normal human epidermal cells separated by treatment with trypsin and dithioerythritol have numerous microvilli on their surface (7). On the other hand, it has been suggested by Lever (15) that with the progress of acantholysis and desmosomal loss the epithelial cells in pemphigus develop numerous cytoplasmic processes. Transmission electron microscopic studies of the lesions in pemphigus and in Hailey-Hailey disease have demonstrated numerous villous projections on the cells of the acantholytic areas (2, 3, 9, 10, 16, 17).

In an SEM study of acantholytic cells in oral pemphigus, Hietanen (11) found that the cells displayed either smooth, wrinkled or microvillous surfaces.

The histological changes typical of pemphigus

can be induced in vitro by pemphigus sera, as reported by many authors (1, 12, 18, 19, 21-25). These in vitro changes are not dependent on the presence of active complement (4, 12, 15, 18, 19, 21-26), but proteolytic enzymes seem to be involved in the acantholytic process (4, 19, 21, 25, 26).

The present study was performed to examine the effect of pemphigus sera in vitro on the surface texture of human epidermal cells separated with trypsin and to examine the effect of ovomucoid, an enzyme inhibitor of proteolysis, on the pemphigus serum-induced surface changes in isolated epidermal cells.

### MATERIALS AND METHODS

#### *Source of skin samples and separation of epidermis*

Suction blisters were produced on the abdominal skin of two clinically healthy volunteers according to the method described by Kiistala and Mustakallio (13). Suction time was usually 1½ hours and the pressure varied from 160 to 210 mmHg.

#### *Preparation of epidermal cell suspension*

The epidermal cell suspension was prepared *ad modum* Gommans et al. (7) with slight modifications: the blister roofs were removed with scissors and tweezers and transferred to test tubes containing a solution of trypsin (0.25 mg/ml Difco trypsin, 1:250, Difco Laboratories, Detroit, Michigan, USA) and dithioerythritol (3 mg/ml DL-dithioerythritol, Sigma® Chemical Company, St. Louis, USA) in 10 ml of phosphate-buffered saline (PBS Ca & Mg free, Orion Diagnostica, Helsinki, Finland).

Blister roofs were incubated in this solution at 37°C for 30 min. After incubation the intact blister roofs were transferred with a loop to test tubes containing 0.4 ml of fresh serum from a clinically healthy volunteer (blood group AB) and 2 ml of MEM (Eagle) Minimum essential medium (Orion Diagnostica, Helsinki). MEM was supplemented each time with 100 I.U./ml penicillin and 50 µg/ml streptomycin. In two test tubes the complement of the serum was inactivated at 56°C for 30 min. The test tubes with intact blister roofs were agitated gently ("Vortex"

mixer) for 1 min. This caused separation of the epidermal cells. After all the visible clumps of cells and horny layers had been removed with a loop the cells were washed twice with MEM (10 min at 2000 r.p.m.).

#### *Incubation of epidermal cells*

**Experiment A.** The epidermal cell suspensions were incubated with MEM at 37°C for 18 hours in a 1:20 dilution of the following sera:

1. Serum from a patient with untreated pemphigus vulgaris verified by histology, direct and indirect immunofluorescence tests. Antibody titre 1:160.
2. Serum 1 inactivated 30 min at 56°C.
3. Serum from a patient with pemphigus vulgaris undergoing steroid treatment (70 mg prednisone daily), diagnosis verified as in 1. Antibody titre 1:160.
4. Serum 3 inactivated 30 min at 56°C.
5. Pooled serum from 20 dermatological patients with diseases other than pemphigus.

The inactivated pemphigus sera were also used for incubation of the cell suspensions from which trypsin was inactivated with serum heated previously at 56°C, 30 min.

**Experiment B.** The epidermal cell suspensions were prepared and incubated as in Experiment A with the following sera:

1. (a) Serum 1 of Experiment A.  
(b) Serum 3 of Experiment A.
2. Serum 1 (a) or 1 (b) with ovomucoid (100 µg/ml, Trypsin inhibitor No. T-9253 from egg white, Sigma®, St. Louis, Missouri 63178, USA).
3. Serum 5 of Experiment A.

All operations were conducted under sterile conditions and the sera were filtered through an Acrodisc® single use filter unit (0.45 µm, Gelman Sciences Inc., Ann Arbor, Michigan 48106, USA).

#### *Preparation of cells for SEM*

After incubation for 18 hours at 37°C the cells were washed twice with MEM (10 min at 2000 r.p.m.) resuspended in 0.5 ml of MEM, and 0.2 ml of this cytocentrifuged (Cytospin® Shandon, 10 min at 800 r.p.m.). Agralon® (previous name Melinex®, ICI) polyester film with grid marks (approximate size 8×37×0.075 mm) was used instead of a microscope slide to collect the cells. They were immediately covered with a large drop of 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. During the first 10 min of fixation the Agralon® plastic was laid flat to prevent cell detachment. It was then transferred to a bottle (10 ml) filled with 2% glutaraldehyde. The fixation time was approximately 2½ hours. When the fixation was completed, the samples were rinsed twice with 0.1 M phosphate buffer, pH 7.2, dehydrated in graded alcohols, and critical point dried in an Aminco critical point drier (American Instrument Co.).

The samples were mounted on stubs for SEM examination. Gold coating was performed with a JEOL FINE COAT JFC-1100 sputtering device, using air. The specimens were coated at 1.2 kV and 7 mA for 4 min, yielding a gold coating approximately 30 nm thick. The samples were studied with a JEOL JSM-35C scanning electron mi-

croscope at the Department of Electron Microscopy, University of Helsinki, at 16 kV using a tilt angle of 45°. Photographs were taken using Ilford FP4 (ASA 125) black and white film and prints were made. From each sample approximately 100 basal or spinous cells were studied and the number of microvilli was counted. SEM was performed on coded samples and the investigator (J. H.) did not know the code used.

The  $\chi^2$ -test was used in the statistical analysis of the results.

## RESULTS

In all samples, cells from all layers of the epidermis were encountered. However, the number of superficial epidermal cells was small. In all specimens, clumps of epidermal cells were also found. The basal and spinous epidermal cells were round or slightly ovoid, and three main types were identified on the basis of surface appearance. These were smooth-surfaced (Fig. 1), wrinkled, and microvillous (Fig. 2) basal or spinous epidermal cells. The number of microvilli on the cell surface varied from fewer than 10 to over 100. The thickness and length of the microvilli varied too, the control sample presenting the thinnest and longest microvilli.

#### *Experiment A*

The effect on the epidermal cells of incubation with various sera is presented in Table I. Between 83 and 96% of the epidermal cells incubated with pemphigus sera had a smooth or wrinkled surface texture and only 17 to 4% had microvilli on their surfaces. This disappearance of the microvilli occurred irrespective of the presence of active or inactive complement.

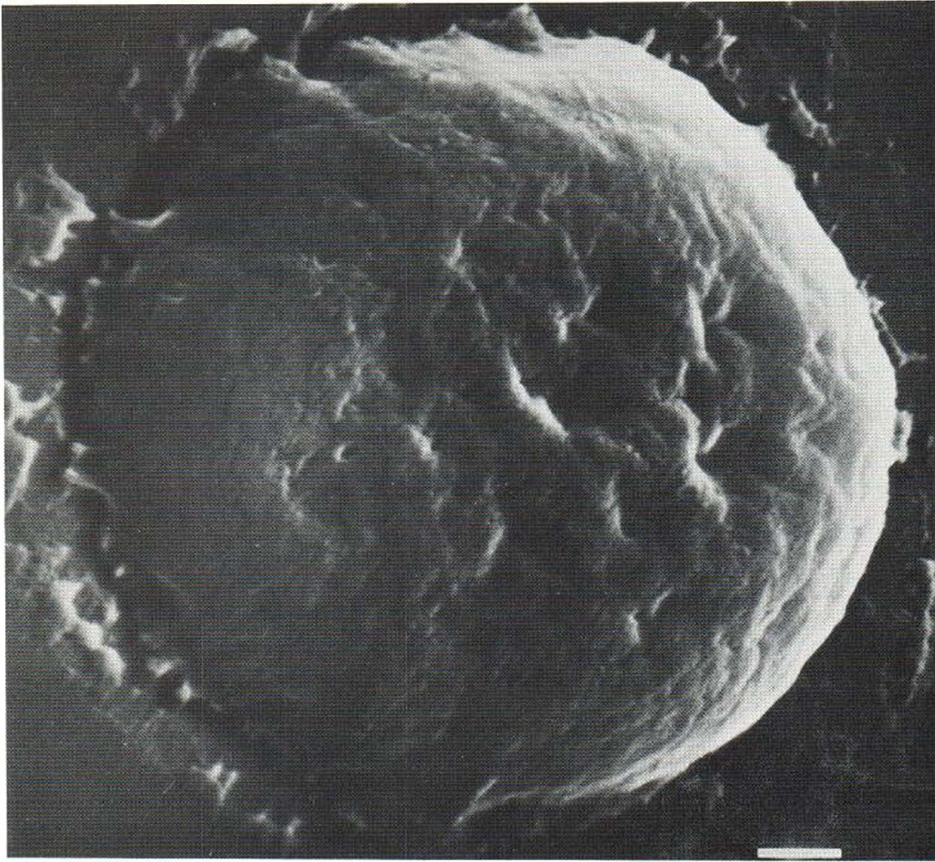
In the control specimen the microvillous cells accounted for 54 out of 100 cells studied. Statistical comparison of samples 1–6 separately with control sample 7 gave a highly significant difference ( $p < 0.001$ ).

#### *Experiment B*

Because the same results were obtained in Experiment A irrespective of the presence of complement, it was not inactivated in Experiment B.

The effect of incubation with various sera on the surface texture of epidermal cells is presented in Table II. Because identical results were obtained in three experiments, two with serum from the untreated pemphigus patient, and one from the treated pemphigus patient, the results are pooled in Table II. Of the cells incubated with pemphigus sera

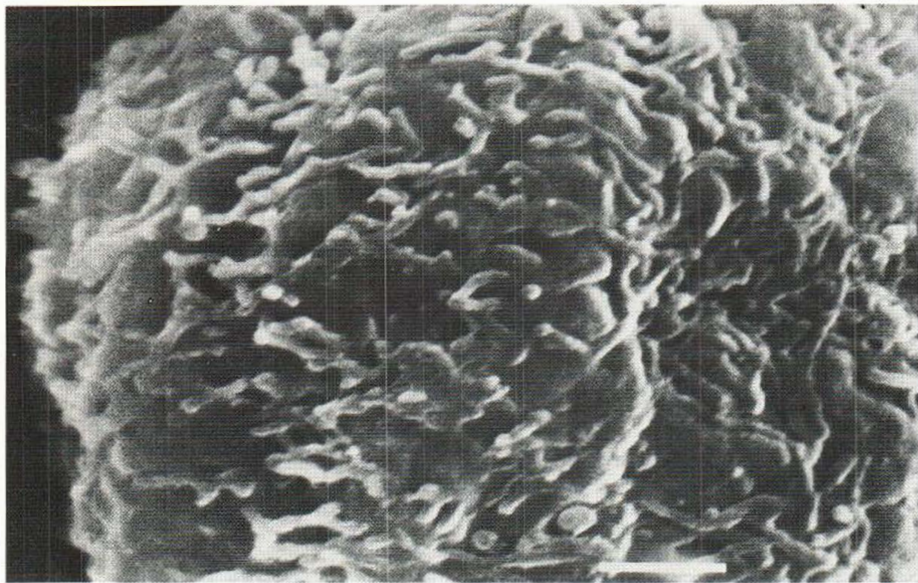
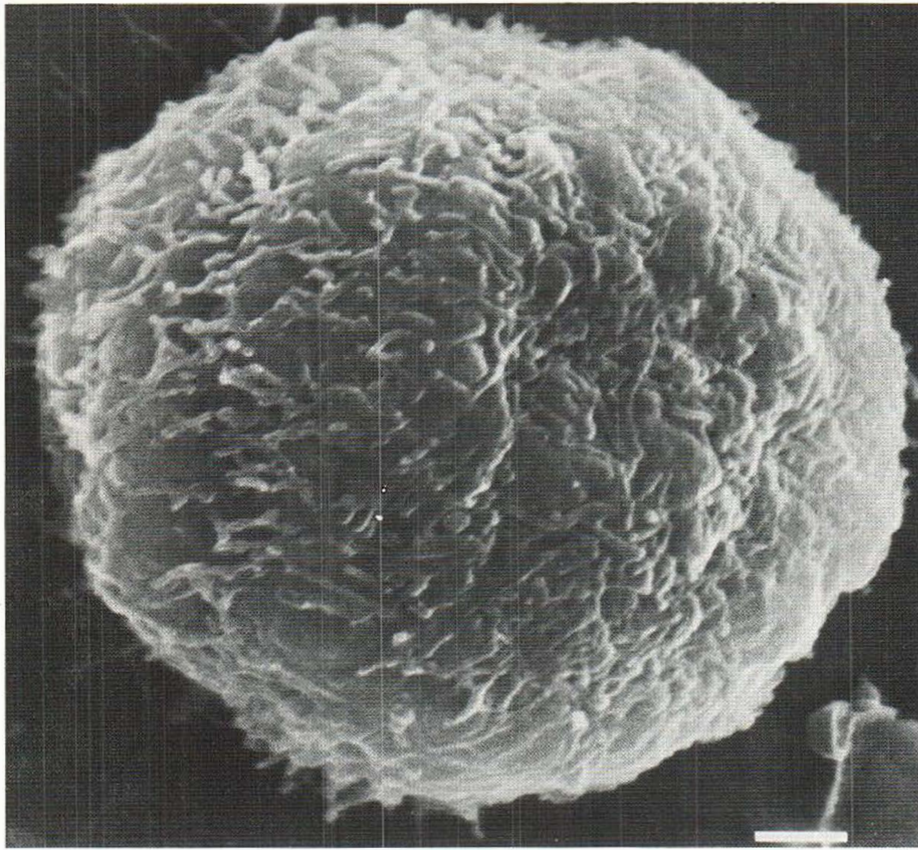




*Fig. 1 (a).* Scanning electron micrograph of a rounded, smooth-surfaced epidermal cell incubated with pemphigus serum in the presence of active complement,  $\times 11\,000$ , bar

$1\ \mu\text{m}$ . *(b)* Higher magnification of the cell in *(a)*, showing smooth surface and variable-sized indentations,  $\times 24\,000$ , bar  $1\ \mu\text{m}$ .





*Fig. 2 (a)*. Scanning electron micrograph of a rounded epidermal cell with many microvilli, incubated with normal control serum,  $\times 12\,000$ , bar  $1\ \mu\text{m}$ . *(b)* Higher magni-

fication of the cell in *(a)*, showing microvillous surface texture and a few indentations,  $\times 17\,000$ , bar  $1\ \mu\text{m}$ .

Table I. Surface texture of normal epidermal cells after 18 hours' incubation at 37°C with various sera

Sample no.	Serum	Serum used for inactivation of trypsin	Surface texture of basal and/or spinous cells. Percentage of cells with						
			No microvilli			Microvilli			
			Smooth	Wrinkled	Total	0-10	10-100	>100	Total
1. Pemph 1	Active	Active	75	10	85	-	8	7	15
2. Pemph 1	Inactive <sup>a</sup>	Active	87	5	92	2	4	2	8
3. Pemph 1	Inactive <sup>a</sup>	Inactive <sup>a</sup>	71	15	86	-	9	5	14
4. Pemph 2	Active	Active	72	11	83	-	9	8	17
5. Pemph 2	Inactive <sup>a</sup>	Active	86	10	96	-	2	2	4
6. Pemph 2	Inactive <sup>a</sup>	Inactive <sup>a</sup>	48	40	88	-	5	7	12
7. Pooled control serum	Active	Active	42	4	46	-	25	29	54

<sup>a</sup> 30 min at 56°C.

81.3% displayed a smooth surface texture, whereas 42.1% and 42.7% of the cells incubated with a combination of pemphigus serum plus ovomucoid or with the pooled control serum, respectively, had a smooth surface texture. Statistically there was a highly significant difference ( $p < 0.001$ ) between the effects of pemphigus serum and the other two incubation media (pemphigus serum plus ovomucoid, pooled control serum). There was no statistically significant difference between the control serum and pemphigus serum plus ovomucoid.

## DISCUSSION

The suction blister method of Kiistala and Mustakallio (13) provides epidermal material which does not contain any dermal contaminating cells. The preparation of an individual cell suspension by the method of Gommans et al. (7) minimizes the detrimental effects of chemical treatment on the plasma membrane, as shown by the viability of the cells. The trypsinization used does not prevent the binding of the pemphigus antibody to the cell surface, thus making acantholytic changes possible in vitro

(23). By combining these two methods it is possible to prepare a suspension containing mostly spinous and basal cells, since the more superficial cells stay attached to the cell clumps as remnants of the blister roof and are removed from the suspension.

Several studies have shown that during in vivo acantholysis in pemphigus and in Hailey-Hailey disease, villous projections are seen on the epidermal cells with transmission electron microscopy (2, 3, 9, 10, 16, 17). On the other hand, it has been shown by Gommans et al. (7) that keratinocytes from normal epidermis display large numbers of microvilli on their surface.

Histological changes typical of pemphigus can be induced in vitro by pemphigus sera (1, 12, 18, 19, 21-25). According to Barnett et al. (1) and Hu et al. (12) the changes are identical under a transmission electron microscope with those occurring in vivo, the cells presenting either villous projections or a smooth surface texture. Most of the acantholytic cells sampled from oral lesions of patients with pemphigus show a smooth surface texture (11), although some cells with a microvillous or wrinkled surface are also present.

Table II. Surface texture of normal epidermal cells after 18 hours' incubation with various sera

Sample no.	Surface texture of basal and/or spinous cells. Number of cells with	
	No microvilli	Microvilli
1. Pemphigus serum	244 (81.3%)	56 (18.7%)
2. Pemphigus serum + ovomucoid	128 (42.1%)	176 (57.9%)
3. Pooled control serum	125 (42.7%)	168 (57.3%)

Tests: 1-2;  $p < 0.001$ . 1-3;  $p < 0.001$ . 2-3; Not significant.



In the present study, incubation of isolated epidermal cells with pemphigus sera caused disappearance of the microvilli. The smooth-surfaced cells were indistinguishable under the scanning electron microscope from the oral acantholytic cells (11).

The disappearance of the microvilli in the present experiment was not affected by the presence of active complement. This agrees with the findings that acantholysis produced by pemphigus serum in organ culture is not dependent on the presence of active complement (4, 12, 15, 18, 19, 21–26).

It seems feasible that one of the functions of the microvilli on epidermal cells is to increase the area available for adhesion. The smoothing of the cell surface induced by pemphigus autoantibody may be an additional factor in the loss of cellular coherence of pemphigus. The dissolution of glycocalyx and desmosomal loss, as seen under the transmission electron microscope, are well established mechanisms in acantholysis of pemphigus (8, 15).

It has been suggested that in vitro pemphigus antibody induces or activates enzyme systems which then cause acantholysis. Schiltz et al. (23) have suggested that hydrolytic enzymes may cause human acantholysis. According to Farb et al. (4) a proteinase might be responsible for acantholysis in vitro in mouse skin. In their study, it was found that the cell detachment from the culture plates could be blocked by the addition of soybean trypsin inhibitor and  $\alpha_2$ -macroglobulin to the culture medium.

In the present study ovomucoid was used as proteinase inhibitor. Ovomucoid is a glycoprotein isolated from the eggs of several avian species (6, 20). It has an inhibitory effect on trypsin, chymotrypsin and possibly other proteinases, as well (5, 6, 14, 20). According to the present results, ovomucoid is capable of inhibiting the smoothing effect of pemphigus sera on epidermal cells. This seems to indicate that the loss of microvilli is caused by a proteolytic process.

The present findings agree with those of Morioka et al. (19), who were the first to demonstrate the inhibition of acantholysis with soybean trypsin inhibitor in human skin in vitro. Schiltz et al. (25) and Schiltz (21) postulated that pemphigus antibody induces the activation of an enzyme (pemphigus acantholysis factor) which degrades glycocalyx and later desmosomes. Our results suggest that the enzyme(s) is probably also responsible for the loss

of microvilli and smoothing of the surface of epidermal cells. As the acantholytic cells found in pemphigus blisters are mostly smooth surfaced (11), it seems possible that the loss of microvilli is also part of the in vivo acantholytic process.

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#### REFERENCES

1. Barnett, M. L., Beutner, E. H. & Chorzelski, T. P.: Organ culture studies of pemphigus antibodies. II. Ultrastructural comparison between acantholytic changes in vitro and human pemphigus lesions. *J Invest Dermatol* 68: 265, 1977.
2. Braun-Falco, O. & Wolff, H. H.: Elektronenmikroskopie von Mundschleimhautläsionen des Pemphigus vulgaris. *Hautarzt* 26: 483, 1975.
3. De Dobbeleer, G. & Achten, G.: Disrupted desmosomes in induced lesions of familial benign chronic pemphigus. *J Cutan Pathol* 6: 418, 1979.
4. Farb, R. M., Dykes, R. & Lazarus, G. S.: Anti-epidermal-cell-surface pemphigus antibody detaches viable epidermal cells from culture plates by activation of proteinase. *Proc Natl Acad Sci USA* 75: 459, 1978.
5. Fossum, K.: Proteolytic enzymes and biological inhibitors. III. Naturally occurring inhibitors in some animal and plant materials and their effect upon proteolytic enzymes of various origin. *Acta Pathol Microbiol Scand (B)* 78: 741, 1970.
6. Francois-Gerard, C., Gerday, C. & Beeley, J. G.: Turtle-dove ovomucoid, a glycoprotein proteinase inhibitor with P<sub>1</sub>-blood-group antigen activity. *Biochem J* 177: 679, 1979.
7. Gommans, J. M., Bergers, M., van Erp, P. E. J., van den Hurk, J. J. M. A., Mier, P. D. & Roelfzema, H.: Studies on the plasma membrane of normal and psoriatic keratinocytes. I. Preparation of material and morphological characterization. *Br J Dermatol* 101: 407, 1979.
8. Graham, J.: Pathology, pp. 398–399. In Ahmed, A. R. (moderator). Pemphigus: current concepts. *Ann Intern Med* 92: 396, 1980.
9. Hashimoto, K.: Electron microscopy and histochemistry of pemphigus and pemphigoid. *Oral Surg* 33: 206, 1972.
10. Hashimoto, K. & Lever, W. F.: An electron microscopic study on pemphigus vulgaris of the mouth and the skin with special reference to the intercellular cement. *J Invest Dermatol* 48: 540, 1967.

11. Hietanen, J.: Acantholytic cells in pemphigus. A scanning and transmission electron microscopic study. *Acta Odontol Scand* 40:257, 1982.
12. Hu, C.-H., Michel, B. & Schiltz, J. R.: Epidermal acantholysis induced in vitro by pemphigus autoantibody. An ultrastructural study. *Am J Pathol* 90: 345, 1978.
13. Kiistala, U. & Mustakallio, K. K.: In-vivo separation of epidermis by production of suction blisters. *Lancet* i: 1444, 1964.
14. Laskowski, M., Jr & Sealock, R. W.: Protein proteinase inhibitors, pp. 465-467. *In* The Enzymes (ed. P. D. Boyer), vol. 3. Hydrolysis: peptide bonds. 3rd ed. Academic Press, New York, 1971.
15. Lever, W. F.: Pemphigus and pemphigoid. A review of the advances made since 1964. *J Am Acad Dermatol* 1: 2, 1979.
16. Lever, W. F. & Hashimoto, K.: The etiology and treatment of pemphigus and pemphigoid. *J Invest Dermatol* 53: 373, 1969.
17. Marsch, W. Ch. & Stüttgen, G.: Generalized Hailey-Hailey disease. *Br J Dermatol* 99: 553, 1978.
18. Michel, B. & Ko, C. S.: An organ culture model for the study of pemphigus acantholysis. *Br J Dermatol* 96: 295, 1977.
19. Morioka, S., Naito, K. & Ogawa, H.: The pathogenic role of pemphigus antibodies and proteinase in epidermal acantholysis. *J Invest Dermatol* 76: 337, 1981.
20. Rhodes, M. B., Bennett, N. & Feeney, R. E.: The trypsin and chymotrypsin inhibitors from avian egg whites. *J Biol Chem* 235: 1686, 1960.
21. Schiltz, J. R.: Pemphigus acantholysis: a unique immunological injury. *J Invest Dermatol* 74: 359, 1980.
22. Schiltz, J. R. & Michel, B.: Production of epidermal acantholysis in normal human skin in vitro by the IgG fraction from pemphigus serum. *J Invest Dermatol* 67: 254, 1976.
23. Schiltz, J. R., Michel, B. & Papay, R.: Pemphigus antibody interaction with human epidermal cells in culture. A proposed mechanism for pemphigus acantholysis. *J Clin Invest* 62: 778, 1978.
24. Schiltz, J. R., Hu, C.-H. & Michel, B.: Corticosteroids, aurothioglucose and soybean trypsin inhibitor do not prevent pemphigus antibody-induced acantholysis in vitro. *Br J Dermatol* 101: 279, 1979.
25. Schiltz, J. R., Michel, B. & Papay, R.: Appearance of "pemphigus acantholysis factor" in human skin cultured with pemphigus antibody. *J Invest Dermatol* 73: 575, 1979.
26. Singer, K. H., Sawka, N. J., Samowitz, H. R. & Lazarus, G. S.: Proteinase activation: a mechanism for cellular dyshesion in pemphigus. *J Invest Dermatol* 74: 363, 1980.

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