THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 68:265–271, 1977

Vol. 68, No. 5
Printed in U.S.A.

ORGAN CULTURE STUDIES OF PEMPHIGUS ANTIBODIES

II. Ultrastructural Comparison Between Acantholytic Changes in Vitro and Human Pemphigus Lesions

MICHAEL L. BARNETT, D.D.S., ERNST H. BEUTNER, PH.D., AND TADEUSZ P. CHORZELSKI, M.D.

Department of Periodontology, School of Dentistry (MLB), Department of Microbiology, Schools of Medicine and Dentistry (EHB), State University of New York at Buffalo, Buffalo, New York, U. S. A., and Department of Dermatology, Warsaw School of Medicine (TPC), Warsaw, Poland

The ultrastructural and light microscopic features of acantholysis produced in organ culture were compared with those of human pemphigus lesions. In both, an intraepidermal split was seen and typical suprabasal acantholytic cells were present. These cells contained small bundles of tonofilaments, usually located away from the cell periphery. Desmosomal plaques with inserted tonofilaments frequently remained along the periphery of acantholytic cells and along the upper portion of the periphery of basal cells. The ultrastructural similarity between in vitro and in vivo lesions provides additional evidence to suggest that organ cultures may provide a valid model for studying the dynamics of pemphigus lesion formation.

It has been well established that intercellular antibodies are found in association with pemphigus lesions [1,2]. Moreover, correlations between the serum titer of these pemphigus antibodies and the severity and/or activity of the disease have suggested a direct role of pemphigus antibodies in the pathogenesis of the disease [2]. As indicated previously [3], further experimental support for such a role has come from passive transfer studies employing repeated injections of pemphigus antibodies into monkey skin [4] and lip [5].

In order to study the mechanism of lesion formation, an organ culture system was developed [3,6] to allow for a variety of experimental manipulations as well as frequent sampling of tissue. This paper describes the light and electron microscopic features of Epon-embedded tissue from acantholytic "lesions" produced in vitro in the presence of intercellular antibodies, and compares these features with those of biopsies of human lesions.

MATERIALS AND METHODS

Split-thickness explants from normal abdominal skin of rhesus monkeys were incubated with pemphigus sera (17 explants) or normal human control sera (17 explants) at 31°C for periods up to 72 hr as previously described [3]. Three pemphigus sera with intercellular antibodies capable of binding to the explants and 4 control sera were used. At the time of culturing and at 24, 48, and 72 hr, explants were fixed in a modified Karnovsky fixative [7] for 2 hr, postfixed in osmium tetroxide for 1 hr, and routinely processed for electron microscopy [8]. Biopsies of 3 human pemphigus vulgaris lesions were similarly processed. These included 2 skin lesions from a 59-year-old male and a 71-year-old female, respectively, and 1 gingival lesion from a 64-year-old female. One-micron-thick sections were stained with toluidine blue for surveying of the tissue,

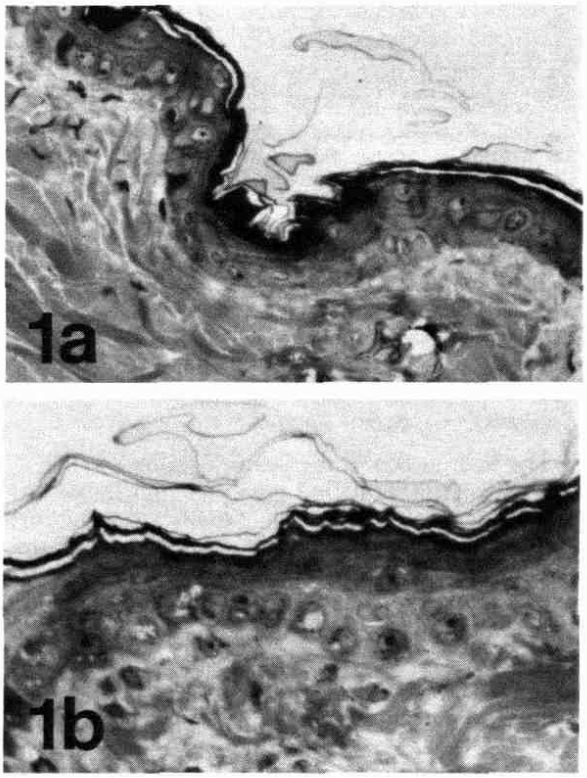


FIG. 1. *a*: Monkey skin at the time of explantation. *b*: Control explant at 72 hr. Features characteristic of normal monkey skin in vivo have been retained (*a* and *b*: Epon embedded, × 800).

Manuscript received October 19, 1976: accepted for publication December 30, 1976.

This study was supported by USPHS Grants DE 04162 and DE 01766, and by a grant from the Summerhill Foundation.

Reprint requests to: Dr. M. L. Barnett, SUNY/AB School of Dentistry, Department of Periodontology, Buffalo, New York 14214.

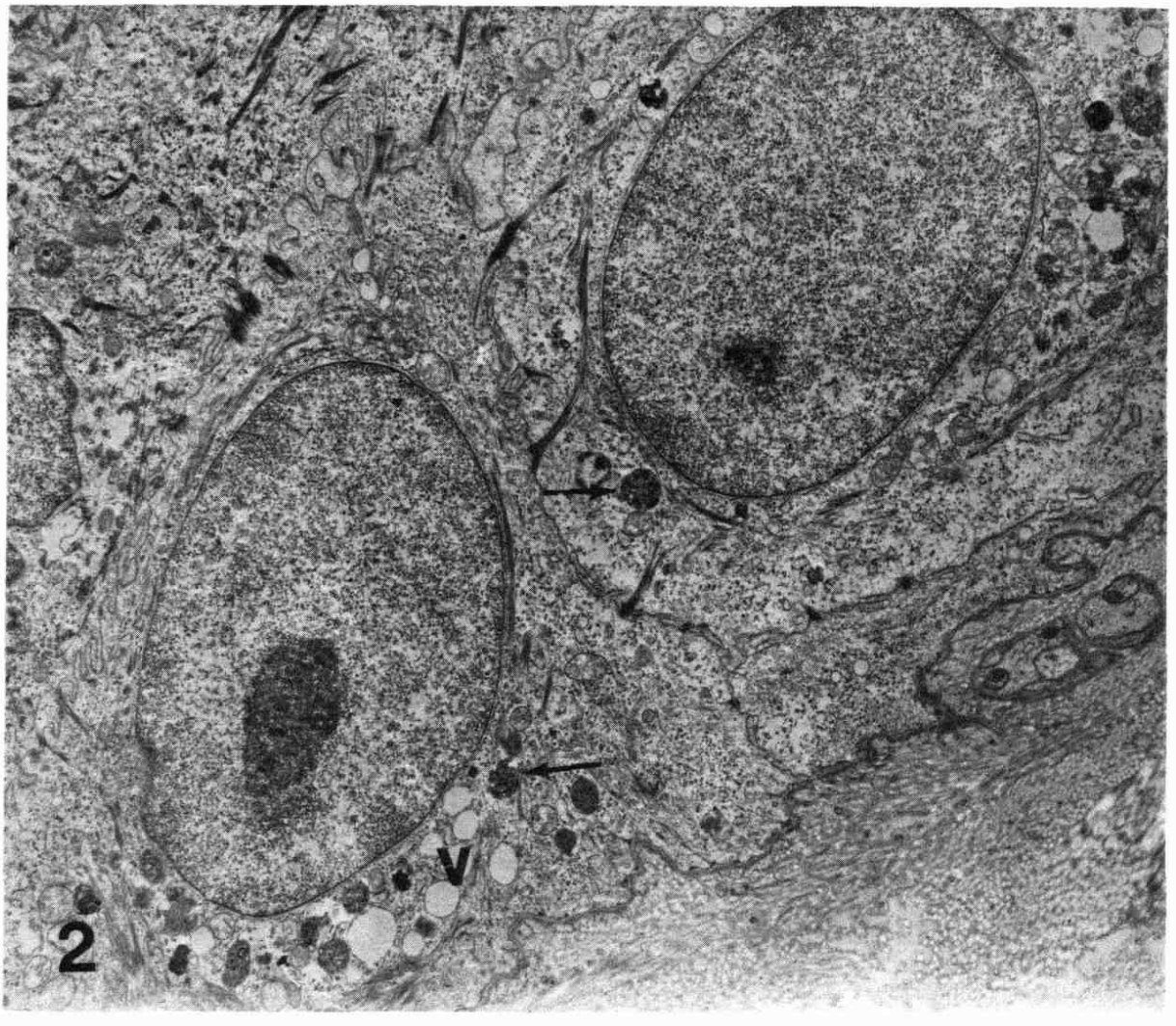


FIG. 2. Control explant at 48 hr. The ultrastructural features of these basal cells and dermal–epidermal junction are similar to those of cells in vivo, with the exception of small cytoplasmic vacuoles (*v*) and organelles resembling secondary lysosomes (*arrows*) (× 12,000).

and thin sections were stained with uranyl acetate and lead citrate [9]. The thin sections were photographed with either an AEI EM6B, Zeiss EM9S-2, or Hitachi HS-8 electron microscope.

RESULTS

Explants in Control Sera

The skin at the time of explantation consisted of a keratinizing stratified squamous epithelium and a portion of the subjacent dermis (Fig. 1*a*). The control explants retained this basic architectural pattern for periods as long as 72 hr, i.e., the longest in vitro period studied (Fig. 1*b*).

The ultrastructural features of the 48-hr control explants were very similar to those of normal skin at the time of explantation, and consistent with those previously reported for normal skin [10]. The basal cells were cuboidal to columnar in shape. Complex interdigitations of adjacent cell processes with periodic desmosomes were present, and the intercellular space appeared to be of normal width (Fig. 2). These features were seen in the stratum germinativum and the stratum spinosum, as well as in the more superficial layers. The epidermal–dermal interface was intact, with hemidesmosomes, basal lamina, and anchoring fibrils present.

With respect to the ultrastructure of individual cells (Fig. 2), the nuclei were relatively homogeneous with only small amounts of heterochromatin present. Nucleoli frequently were prominent. The cytoplasm contained numerous ribosomes, for the most part in the form of polysomes although occasional profiles of rough endoplasmic reticulum could be found. Mitochondria were present, as were bundles of tonofilaments, some of which inserted into desmosomal attachment plaques. The ultrastructural features of the 48-hr explants which distinguished them from normal, in situ skin were the presence of small, clear vacuoles in some of the cells and the presence of round, membrane-bounded organelles containing a heteroge-

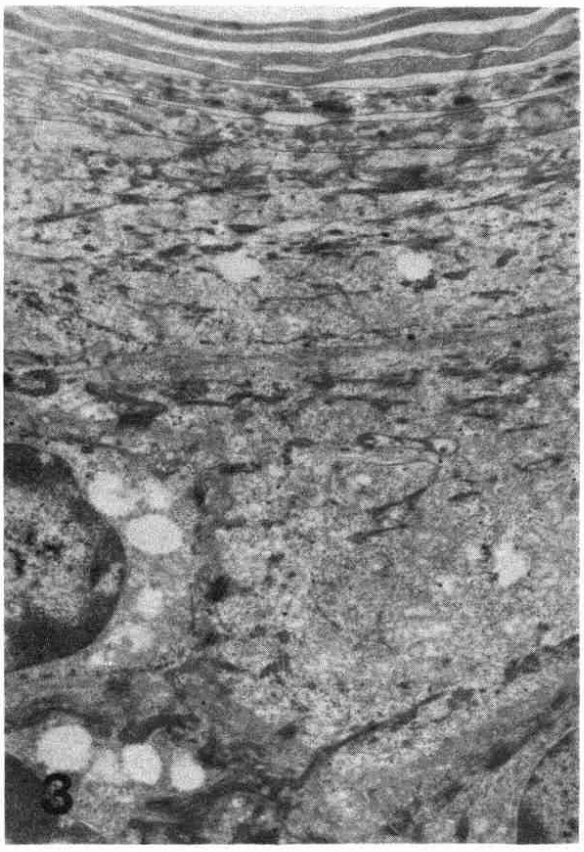


FIG. 3. Control explant at 72 hr. Cells of the stratum spinosum remain closely apposed. Keratinization continues in culture. Desmosomes are present and intercellular spaces are of normal width (× 8,000).

FIG. 4. Control explant at 72 hr. A portion of 3 cells of the stratum spinosum. The desmosomes and the intercellular space have normal morphology, though cell peripheries are relatively straight (*arrows*) (× 24,500).

neous, electron-dense material (Fig. 2). The latter were thought to be secondary lysosomes.

The ultrastructural changes in the epithelium of the 72-hr explants were somewhat more profound. The cytoplasm contained larger and more numerous clear vacuoles. In occasional cells, the cytoplasm was unusually electron lucent, having lost most of its organelles. Significantly, however, the overall tissue architecture remained intact and keratinization continued (Fig. 3). In addition, desmosomes were present and the intercellular spaces remained narrow. Because there were fewer interdigitations of cell processes, cell peripheries appeared straighter than in earlier specimens (Fig. 4).

Explants in Pemphigus Sera and Biopsies of Human Pemphigus Lesions

The appearance of all the explants incubated with 2 of the 3 pemphigus sera was very different from that of the controls by 72 hr. The following description applies to the explants incubated with the pemphigus sera which induced acantholytic changes. Immunofluorescence revealed that all 3 sera contained antibodies capable of binding to epithelium. The 2 sera that yielded demonstrable changes in explants as seen with standard histologic methods and as revealed by the present ultrastructural study also gave the strongest reactions in the indirect immunofluorescent tests described in a previous paper [3].

The light and electron microscopic features of the human lesions were consistent with the variety of morphologic changes described in past reports [11–13], although it should be noted that the authors of these papers differed as to the interpretation of the morphologic findings. Since these changes have been well documented previously, they will not be discussed here, although micrographs of the human lesions will be included for comparative purposes.

Light Microscopic Changes

By 72 hr the epidermal architecture of all the explants was markedly altered; in addition, changes were obvious in some of the earlier explants. In all 72-hr explants, suprabasal acantholysis was apparent and small "vesicles" were seen (Fig. 5). The roof of the vesicle varied in thickness in different areas. In many regions it consisted of the stratum corneum and several flattened layers of the upper stratum spinosum. Within the vesicle, acantholytic cells seemed to be floating in a fluid medium (Fig. 5). The individual acantholytic

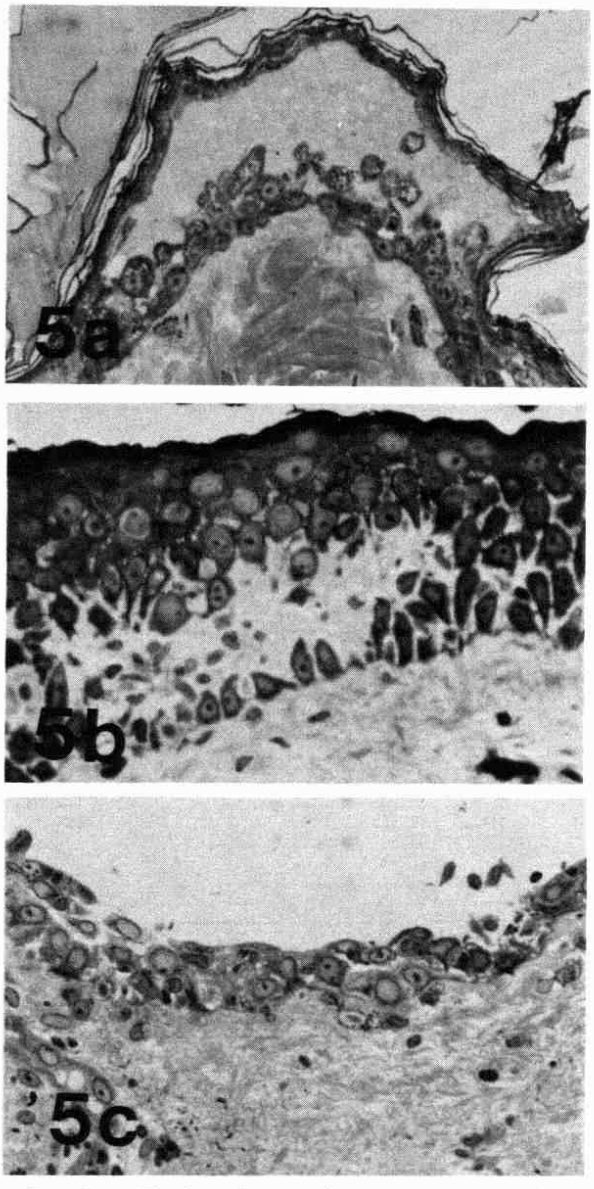


FIG. 5. *a*: Explant in pemphigus serum at 72 hr. A region of "vesicle" formation with acantholytic cells. The basal cells, although rounded, remain adherent to the dermis. *b*: Human skin lesion. Suprabasal acantholysis is present, along with characteristic "tomb-stone" basal cells. *c*: Human skin lesion. Floor of vesicle with several layers of rounded basal and suprabasal cells. In many areas the intercellular spaces appear to have widened (*a*, *b*, and *c*: × 880).

cells were rounded and had degenerative changes. In some cells, the cytoplasmic periphery was intensely stained with toluidine blue, giving these cells an appearance reminiscent of that of Tzank cells.

The appearance of the basal cells varied. In some regions, they retained a relatively normal appearance on a light microscopic level, although their shape was more rounded than usual. In other regions they had nuclear and cytoplasmic changes suggestive of cell degeneration.

When changes were seen in the earlier explants, they were similar to those described above. In regions where obvious vesicle formation had not yet occurred, the suprabasal cells were rounded and often were separated from each other by a widened intercellular space. The so-called "intercellular bridges" normally seen in the light microscope were absent.

Electron Microscopic Changes

The light microscopic changes were reflected on the ultrastructural level. Some of the acantholytic cells retained attachment plaques with small masses of inserted tonofilaments (Fig. 6). Other tonofilaments were arranged in small bundles located away from the cell periphery. Intracytoplasmic clear vacuoles were devoid of a limiting membrane. The nuclei were often indented and nucleoli were not seen. Acantholytic cells were also seen in which no traces of desmosomal attachment plaques remained. These cells were also rounded and contained masses of tonofilaments randomly located within the cytoplasm (Fig. 7). In the acantholytic cells, cytoplasmic constituents, in general, appeared to be disrupted and, on a morphologic basis, the cells did not appear to be functional. Cellular debris frequently was present in the space surrounding the acantholytic cells.

The cells lining the floor of the vesicle usually retained desmosomal attachment plaques at their uppermost surfaces, even though acantholysis had occurred (Fig. 8). Many of these cells appeared to be minimally disrupted although some cytoplasmic changes usually were present. An interesting ultrastructural feature of the pemphigus explants involved the epidermal-dermal interface. It was observed that the basal lamina and anchoring fibrils were intact, but that hemidesmosomes were absent along relatively long portions of the inferior border of the basal cells. The basal cells remained in close proximity to the dermis, however, and the acantholysis still occurred in suprabasal sites.

DISCUSSION

A comparison of the light and electron microscopic features of the explants incubated in pemphigus sera with the features of lesions from pemphigus reveals many similarities between the two and, thus, further supports previous immunologic studies of this organ culture system [3,6,14]. In both explants and human lesions, vesicles were formed in suprabasal locations and typical acantholytic cells were present. Desmosomal components often were seen at the peripheries of the acantholytic cells and remaining basal cells. Small tonofilament bundles were located away from the cell periphery and the cells appeared to be nonfunctional on a morphologic basis. Of interest was the absence of hemidesmosomes at the inferior border of many regions of the basal cells of explants grown in the presence of pemphigus anti-

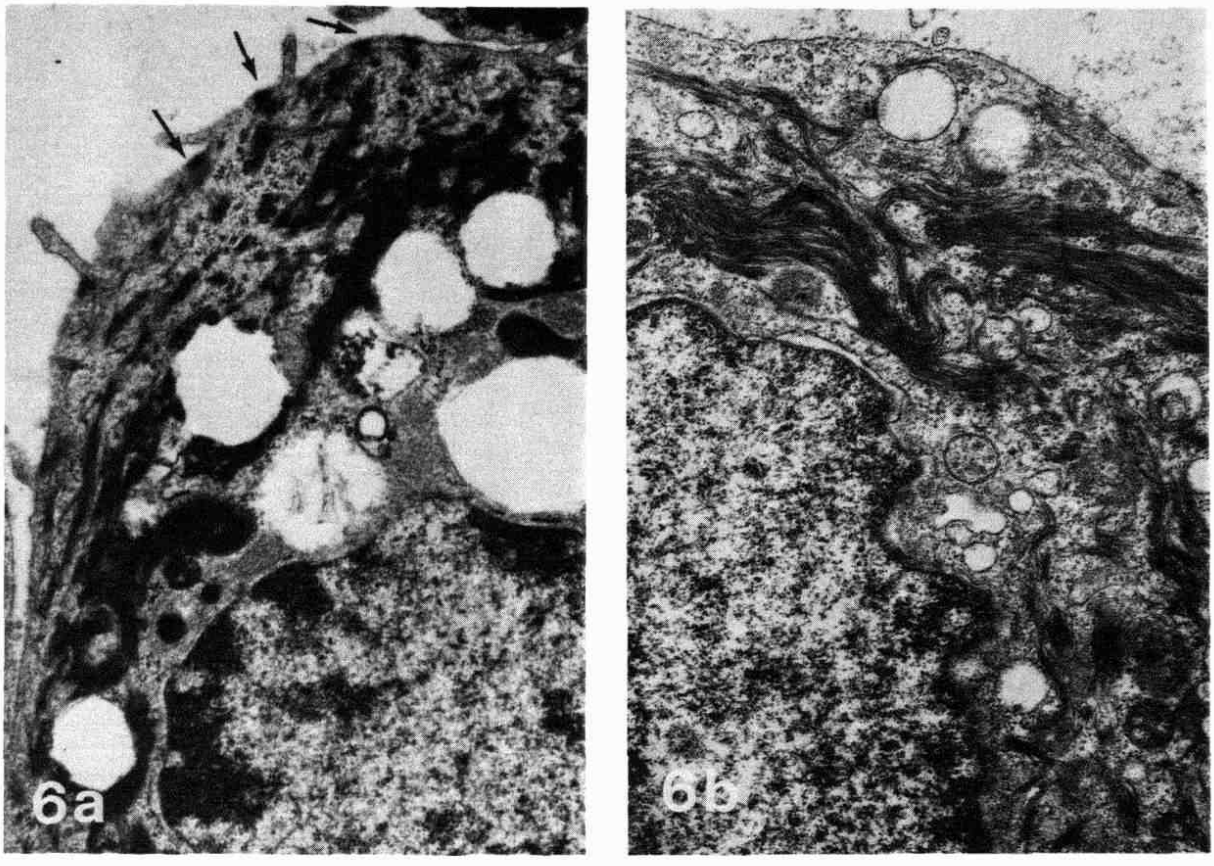


FIG. 6. *a*: Explant in pemphigus serum at 72 hr. A portion of an acantholytic cell showing small bands of tonofilaments and remnants of desmosomal plaques (*arrows*). Clear cytoplasmic vacuoles are evident (× 18,000). *b*: Human skin lesion. A portion of an acantholytic cell having many features similar to the cell in *a* although in the cell shown here the vacuoles are membrane bounded and there are no definitive remains of demosomes present (× 16,000).

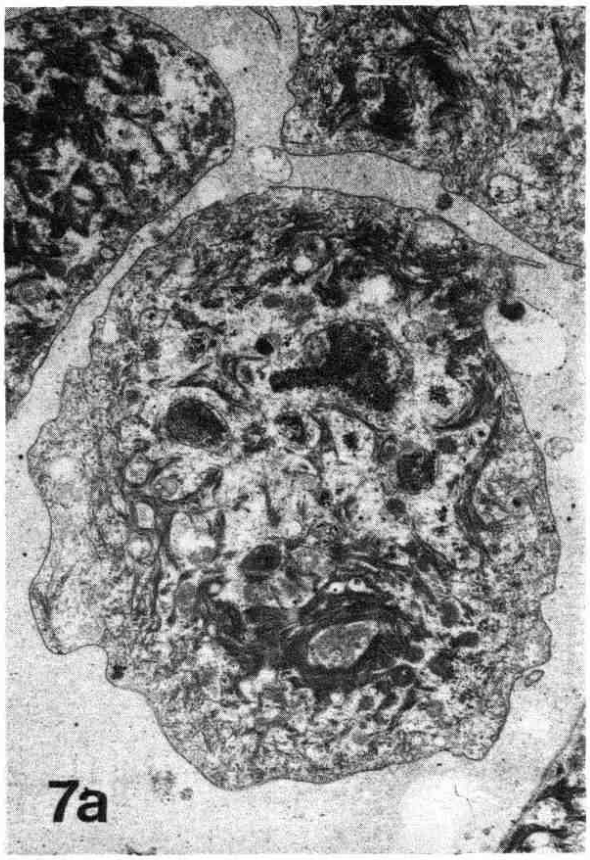
bodies, a feature not seen in the human lesions. Although the reason for this difference is not clear, it is significant that even without hemidesmosomes, the basal cells remained closely apposed to the dermis and an intraepithelial split occurred at a higher level.

That the changes seen in the experimental explants are not a coincidental product of the process of organ culture is evident from past reports [3,6,14], and is borne out by a comparison of the microscopic features of experimental and control explants described in this report. In the control explants, normal tissue architecture was retained at both the light and electron microscopic levels up to 3 days. Intercellular spaces and desmosomes seemed to be unchanged, and keratinization also appeared to continue.

The cellular changes that seem to have resulted from culturing per se include the formation of clear, non-membrane-bounded intracytoplasmic vacuoles, presumably lipid vacuoles, and secondary lysosome formation, as well as a relative straightening of cell peripheries. These changes have been reported previously [15,16] and are important insofar as they must be distinguished from specific alterations produced by pemphigus antibodies.

Previous electron microscopic studies of human pemphigus lesions have given rise to three concepts regarding their pathogenesis. According to one [13], the initial change involves the tonofilament–desmosome relationship. The tonofilaments are thought to separate from desmosomal attachment plaques, as a result of which desmosomes disappear and a loss of cellular adhesion ensues. The second concept [11] is that acantholysis occurs as a result of dissolution of the so-called "intercellular cement." A third group [12] feels that the primary change leading to acantholysis is a loss of the ability of the epithelial cells to complete the development of desmosomal contacts, and that changes in tonofilaments are secondary. The finding in the present study, of attachment plaques with inserted tonofilaments in both acantholytic cells and remaining basal cells, would seem to rule out the first concept, though it certainly does not prove either the second or third.

Since no valid conclusions regarding a dynamic sequence of events can be drawn on the basis of micrographs taken at a single point in time, stud-



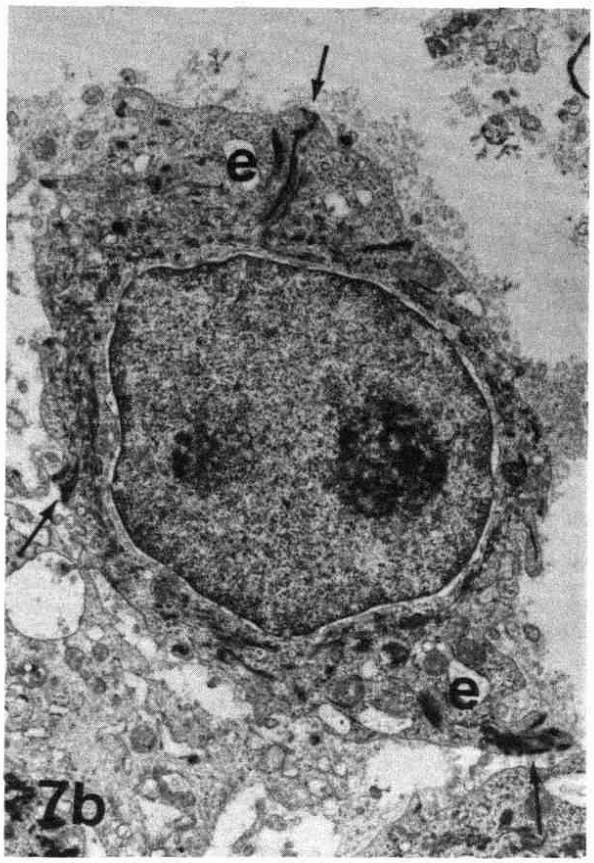


FIG. 7. *a*: Explant in pemphigus serum at 72 hr. An acantholytic cell with bands of tonofilaments located for the most part away from the cell periphery. A well-defined nucleus is no longer present; remnants of desmosomes are also lacking (× 6,100). *b*: Human skin lesion. An acantholytic cell with small bands of tonofilaments distributed within the cytoplasm. Desmosomal plaques with tonofilaments still inserted are present (*arrows*). The dilated profiles of endoplasmic reticulum (*e*) give the cell a vesicular appearance (× 8,100).

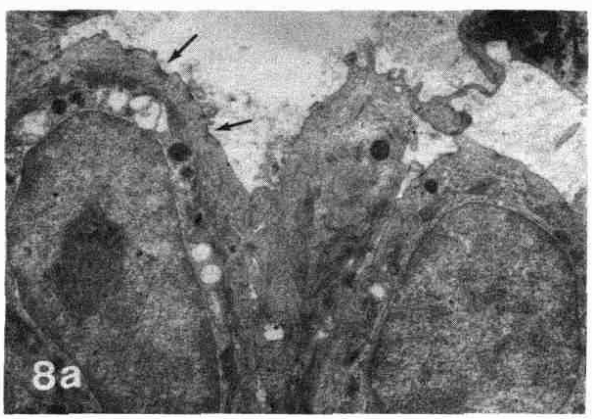
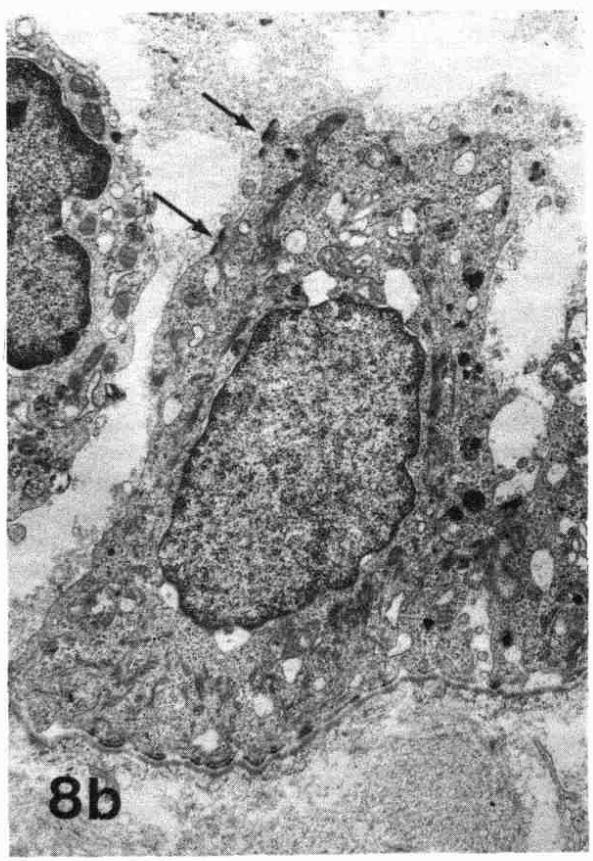


FIG. 8. *a*: Explant in pemphigus serum at 48 hr. A portion of several basal cells with remnants of desmosomes (*arrows*) (× 5,800). *b*: Human skin lesion. A "tomb-stone" cell with desmosomal plaques (*arrows*). The dermal-epidermal interface is intact with hemidesmosomes, basal lamina, and anchoring fibrils (× 8,000).



ies up to now have not provided definitive information about the mechanism of pemphigus lesion formation. The results reported herein comparing the ultrastructure of fully developed acantholytic changes in vitro with that of human pemphigus lesions, provide additional support for the validity of the organ culture system as a means for studying the mechanism of lesion formation. Whereas this study concentrated on the "end-point" of the acantholytic process, as seen in explants cultured for 48 to 72 hr, it is clear that in order to consider mechanisms, explants should be selected from time periods at which overt acantholysis has not yet occurred.

A portion of the electron microscopy was carried out at the Electron Microscopy Laboratory, Department of Pathology, Erie County Laboratory, Buffalo, New York.

We would like to extend our appreciation to Miss Evelyn Flynn and Mrs. Sandra Berntheizel for their technical assistance, to Dr. Michael Newman for providing the gingival biopsy, and to Dr. Shipen Sun for maintaining the organ cultures.

REFERENCES

1. Beutner EH, Lever WF, Witebsky E, Jordon RE, Chertock B: Autoantibodies in pemphigus vulgaris. JAMA 192:682–688, 1965
2. Beutner EH, Chorzelski TP, Jordon RE: Autosensitization in Pemphigus and Bullous Pemphigoid. Springfield, Ill, Thomas, 1970, pp 5–194
3. Deng JS, Beutner EH, Shu S: Organ culture studies of pemphigus antibodies. I. Studies of antigen stability in explants of normal monkey skin and kinetics of acantholytic changes. Arch Dermatol (in press)
4. Wood GW, Beutner EH, Chorzelski TP: Studies in immunodermatology. II. Production of pemphigus-like lesions by intradermal injection of monkeys with Brazilian pemphigus foliaceus sera. Int Arch Allergy 42:556–564, 1972
5. Holubar K, Chorzelski TP, Gauto M, Beutner EH: Studies in immunodermatology. III. Induction of intraepithelial lesions in monkeys by intramucosal injections of pemphigus antibodies. Int Arch Allergy 44:631–643, 1973
6. Michel B, Ko CS: Effect of pemphigus or bullous pemphigoid sera and leukocytes on normal human skin in organ cultures. An in vitro model for the study of bullous diseases. J Invest Dermatol 62:541–542, 1974
7. Ito S, Karnovsky M: Formaldehyde–glutaraldehyde fixatives containing trinitro compounds. J Cell Biol 39:168a, 1968
8. Luft JH: Improvements in epoxy resin embedding methods. J Biophys Biochem Cytol 9:404–414, 1961
9. Reynolds ES: The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J Cell Biol 17:208–213, 1963
10. Odland GF, Reed TH: Epidermis, *In* Ultrastructure of Normal and Abnormal Skin. Edited by AS Zelickson. Philadelphia, Lea & Febiger, 1967, pp 54–75
11. Hashimoto K, Lever WF: An electron microscopic study on pemphigus vulgaris of the mouth and the skin with special reference to the intercellular cement. J Invest Dermatol 48:540–552, 1967
12. Braun-Falco O, Vogell W: Elektronen-mikroskopische Untersuchungen zur Dynamik der Acantholyse bei Pemphigus vulgaris. I. Die klinisch normal aussenende hant in der umgebung von blasen mit positivem Nikolski-phenomen. Arch Klin Exp Dermatol 223:328–346, 1965
13. Wilgram GF: Pemphigus and bullous pemphigoid, *In* Ultrastructure of Normal and Abnormal Skin. Edited by AS Zelickson. Philadelphia, Lea & Febiger, 1967, pp 335–346
14. Schiltz JR, Michel B: Production of epidermal acantholysis in normal human skin in vitro by the IgG fraction from pemphigus serum. J Invest Dermatol 67:254–260, 1976
15. Prose PH, Friedman-Kien AE, Neistein S: Ultrastructural studies of organ cultures of adult human skin. *In vitro* growth and keratinization of epidermal cells. Lab Invest 17:693–716, 1967
16. Barnett ML, Szabo G: Effect of vitamin A on epithelial morphogenesis *in vitro*. Fine structural changes in explants of adult mammalian skin. Exp Cell Res 76:118–126, 1973