PRODUCTION OF EPIDERMAL ACANTHOLYSIS IN NORMAL HUMAN SKIN IN VITRO BY THE IgG FRACTION FROM PEMPHIGUS SERUM

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Normal human skin was maintained in organ cultures for several days in Ham's F-10 medium with good preservation of the epidermal cells. When the partially purified IgG fraction from the pooled sera of patients with pemphigus vulgaris or pemphigus foliaceus was added to this culture system, after 24 hr some evidence of epidermal acantholysis was seen. By 72 hr, extensive suprabasilar epidermal acantholysis had occurred in which the acantholytic cells were indistinguishable histologically from the acantholytic cells in biopsies from skin lesions of patients with pemphigus vulgaris. In the control cultures (i.e., F-10 medium or F-10 medium + normal human serum IgG), none of these changes was seen. Direct immunofluorescent staining of these explants using fluorescein-labeled goat antihuman IgG showed that by 6 hr binding of the pemphigus IgG had occurred in the intercellular cement substance of the epidermis. The staining intensity was maximal by 18 to 20 hr.

When the pemphigus serum was fractionated by DEAE–cellulose column chromatography, three major IgG-containing peaks (presumably IgG subclasses) were eluted which bound to the epidermal intercellular substance and caused acantholysis in culture. The complement system did not play a role in the antibody-induced acantholysis since complement was not included in this system and heating the reconstituted F-10 + pemphigus IgG for 1 hr at 58 °C did not destroy the acantholytic activity. Autoradiographic experiments showed that after about 2 days in culture the rates of incorporation of RNA and protein precursors in the suprabasilar cells in the presence of pemphigus IgG were reduced to less than 10% of the normal IgG controls, whereas these synthetic activities of the basal cells were only slightly affected.

These observations lead to the proposal that it is the interaction of the pemphigus autoantibody(s) with the suprabasilar epidermal cell which initiates and possibly sustains the process(es) of acantholysis.

Pemphigus is characterized by the formation of intraepidermal clefts or bullae which form above the basal cell layer in pemphigus vulgaris and vegetans or through the stratum granulosum or upper stratum malpighi in pemphigus foliaceus and erythematous. The most distinguishing histologic feature of pemphigus is the presence of acantholytic epidermal cells which have small pyknotic nuclei surrounded by a fine “halo,” an eosinophilic cytoplasm, and rounded borders. In the early electron-microscopic (EM) studies of pemphigus [1,2] acantholysis was attributed to damage of the tonofilament–desmosome complex, whereas in later EM studies Hashimoto and Lever [3] reported that acantholysis was primarily an extracellular event with dissolution of the intercellular cement substance first in the nondesmosomal areas.

In 1964, Beutner et al [4] demonstrated the presence of tissue-fixed immunoglobulins in the epidermal intercellular cement substance (ICS) of pemphigus patients. Furthermore, most of these patients had a circulating autoantibody in their serum which was directed against antigen(s) in the intercellular space [5]. The role of these autoantibodies in the production of acantholysis became the subject of some controversy. Sams and Jordon transfused the plasma from patients with pemphigus into rhesus monkeys and demonstrated that the IgG antibody became bound to the monkey
epidermis, although no clinical or histologic lesions were obtained [6]. These observations tended to cast doubt on the role of the pemphigus antibodies in pemphigus acantholysis. Wood et al [7], by repeated intradermal injections of pemphigus sera into monkey buccal mucosa, were able to produce histologic and immunofluorescent findings of pemphigus and these workers argued in favor of the pathogenic significance of pemphigus antibody. Using a somewhat different approach, Grob and Inderbitzin prepared antiepithelial autoantibodies in rabbits by immunization with antigens derived from rabbit esophagus epithelium [8]. Although these autoantibodies could be shown to be present in the serum and to bind to the ICS of stratified squamous epithelium in vitro, they did not bind in vivo and acantholysis did not occur. In subsequent work [9], they were able to circumvent the diffusion barrier by treatment of the skin of these immunized rabbits with a combination of sodium dodecyl sulfonate and dry ice. Under these conditions the autoantibodies did bind to the epidermis and in some cases acantholysis resulted.

In 1974 Michel and Ko [10] cultured normal human skin in whole pemphigus sera at 31°C and were able to produce suprabasilar acantholysis in vitro, and immunofluorescent staining showed that the antibody became fixed to the ICS prior to the onset of acantholysis. The work to be presented here demonstrates that it is the IgG fraction from the pemphigus serum that is responsible for the observed acantholysis. Based on autoradiographic data on the rates of incorporation of RNA and protein precursors by antibody “target” cells and on the lack of complement dependence, we propose that the autoantibody interacts with the surface or ICS of the upper epidermal cell and that this interaction initiates the events which culminate in the production of pemphigus acantholysis.

**IgG Preparation**

The IgG fractions from pooled normal or pemphigus sera were prepared by a multiple ethanol precipitation procedure [13], and the final IgG precipitates were dissolved in and dialyzed extensively against F-10 medium. These reconstituted normal and pemphigus antibody-containing media are referred to as F-10 + N<sub>igG</sub> and F-10 + P<sub>igG</sub>, respectively. The preparations were evaluated by polyacrylamide disc gel electrophoresis in sodium dodecyl sulfate (SDS) and by immunoelectrophoresis.

Pemphigus serum was fractionated on a 2.5 cm x 20 cm column of diethyl aminoethyl (DEAE)-cellulose (Whatman DE-52). Five milliliters of serum was dialyzed into 10 mM potassium phosphate buffer, pH 8.0, and pumped onto the column. After the sample was layered, a continuous and linear gradient elution was performed. The gradient consisted of a decreasing pH (pH 8.0-6.6) and an increasing NaCl concentration (0-0.3 M) in 10 mM potassium phosphate (total volume = 1 liter; flow rate = 1 ml/min).

**Autoradiography**

For the autoradiographic experiments, skin explants were removed from culture and immersed for 2 hr periods in control F-10 medium (without antibodies) containing 1 μCi/ml of either <sup>3</sup>H-lysine (New England Nuclear, NET-250) or <sup>3</sup>H-thymidine (New England Nuclear, NET-028). The explants were fixed in buffered formalin, embedded in paraffin, cut into 5-μ sections, mounted on glass slides, taken to distilled water, and air dried. The slides were then dipped in Kodak NTB-3 emulsion and allowed to expose for 3 weeks. The silver grains were developed with Kodak D-19, fixed with Kodak Rapid Fixer, and stained by standard H & E procedures. In order to quantitate grain density, 4 complete fields (1,000×) of cells were counted on 3 separate sections, and the results were expressed as grains per cell. A distinction was made between grains in the basal cell layer and cells in the stratum malpighi and granular layer in the same fields.

**RESULTS**

**Production of Epidermal Acantholysis In Vitro**

The IgG fractions prepared from the pooled sera of pemphigus or from normal sera (*Materials and Methods*) were considered to be greater than 90% pure based on molecular weight determinations on SDS-polyacrylamide disc gels and immunoelectrophoresis. The major components migrated on SDS gels with molecular weights of approximately 150,000 and when the samples were reduced with 1% β-mercaptoethanol, the major components migrated with the mobility of heavy and light immunoglobulin chains (data not shown). When the samples were subjected to immunoelectrophoresis they were shown to consist almost entirely of IgG (see Fig. 1). No IgA or IgM was evident.

The purified pemphigus IgG fraction was dissolved in Ham’s F-10 medium containing 10% fetal calf serum (FCS) and dialyzed extensively against F-10 medium. The pemphigus titer, as determined by indirect immunofluorescence, was adjusted to 320. Typically, the protein concentration as contributed by the immunoglobulins was between 30

**MATERIALS AND METHODS**

**Culture Techniques**

Normal human breast skin which had been obtained from surgery was used in these experiments. The skin was cut into 1 cm x 5 cm strips and sliced to a thickness of 0.4 mm with a Castroviejo keratome. Squares of about 4 mm<sup>2</sup> were placed dermis down into sterile 2.5 cm<sup>2</sup> lens paper “rafts” as described by Sarkany et al [11]. The rafts were floated on top of the proper medium in 35-mm Falcon plastic tissue culture dishes and cultured in a humid atmosphere containing 5% CO<sub>2</sub> in air at 37°C. The F-10 medium [12] contained 10% fetal calf serum (Grand Island Biological Co.) + 2× concentrations of the amino acids and sodium pyruvate and an antibiotic-antimycotic mixture containing penicillin, streptomycin, and fungizone (GIBCO).

Skin samples were examined by standard hematoxylin and eosin (H & E) staining procedures. Pemphigus autoantibody binding to the epidermis of cultured skin was determined by direct immunofluorescence using fluorescein-conjugated goat-antihuman IgG (Hyland Labs).
and 40 mg/ml of reconstituted medium. Two control media were also used in these experiments, F-10 medium and F-10 + N IgG. In the latter case, normal IgG was prepared in parallel to pemphigus IgG and adjusted to the same protein concentration. Multiple samples of normal human skin were placed on lens paper rafts in the presence of either of these Millipore-sterilized media and samples were removed at specified times for 3 days and examined for epidermal-bound antibody and general histology. Figure 2 is a composite photomicrograph of H & E-stained tissue samples which were collected at 6, 24, 40, and 72 hr. It is seen in the controls A through H (F-10 and F-10 + N IgG) that the histology was similar to that reported by other organ culture methods [11,14,15]. After 3 days, parakeratosis and some edema was evident and slight disorientation of epidermal cells could be seen. Epidermal-derived epiboly was also present in most of the explants, including those grown in the presence of the pemphigus antibody (not shown). In the skin samples grown in the pemphigus IgG (I through L), after 24 hr (J) considerable intercellular edema was seen and a separation had begun above the basal cells. By 40 hr (K) acantholysis was apparent and by 72 hr (L) extensive suprabasilar acantholysis had occurred. When samples were examined by direct immunofluorescent staining, antibody fixation to the IES could be seen in the 6-hr samples and the staining intensity was maximal by 19 hr (not shown).

It should be pointed out that the F-10 medium in these experiments contained 10% FCS. In similar experiments, when the FCS was omitted from the reconstituted media, acantholysis did readily occur in the presence of the pemphigus IgG fraction, but the histology of the control cultures was not well preserved. There was no acantholysis in these controls, but there was much more intracel-

![Fig. 1. Immunoelectrophoretic patterns of the IgG preparations used for making the F-10 + N IgG and F-10 + P IgG media. Upper panel, normal human IgG (NIgG) lower panel, pemphigus IgG (PIgG). In each slide a sample of whole human serum (WHS) was run in the opposing agar wells. Following electrophoresis, rabbit antiserum against whole human serum was applied to the center troughs and the precipitated serum components visualized after 16 hr.](image)

![Fig. 2. Photomicrographs of normal human skin which had been cultured in F-10 medium (A = 6 hr, B = 24 hr, C = 40 hr, D = 72 hr) or F-10 + N IgG (E = 6 hr, F = 24 hr, G = 40 hr, H = 72 hr) or F-10 + P IgG (I = 6 hr, J = 24 hr, K = 40 hr, L = 72 hr). All sections stained with H & E (x 180).](image)
lular and intercellular edema and large necrotic areas.

In order to evaluate a possible role for complement in this in vitro system, the three media were heated at 58°C for 1 hr to inactivate any possible residual activity from the FCS. After 86-hr incubation, the control samples grown in F-10 + N\textsubscript{lgG}, either heated or unheated, were normal and heating did not destroy the acantholytic activity of the F-10 + P\textsubscript{lgG} medium (Fig 3). We conclude that only the IgG fraction from the pemphigus serum was necessary to produce acantholysis, and that complement was not involved.

Sams and Schur have shown that the pemphigus IgG antibody is distributed in all the IgG subclasses [16]. In an attempt to separate these subclasses, we performed a continuous and linear pH and NaCl gradient elution of pemphigus serum from Whatman DE-52 (Materials and Methods). The elution profile is shown in Figure 4. Five peaks of protein were eluted and a sixth was eluted with 1 M NaCl in the starting buffer. Analysis of these peaks by indirect immunofluorescent staining of monkey esophagus showed that the pemphigus autoantibody was equally distributed in fractions II, III, and IV. Although we did not test these fractions for IgG subclass by immunologic methods (due to the lack of specific subclass antibodies), we infer from Sam and Schur’s work [16] and from the known isoelectric points [17] of the various subclasses that they probably represent IgG2, IgG3, and IgG4. Under these conditions IgG1 should have eluted in fraction I [13]; in three separate chromatographic elutions, fraction I had a negative titer against monkey esophagus intercellular cement substance. When the various fractions were

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**Fig. 3.** Photomicrographs of normal human skin which had been cultured 88 hr in (A) F-10 + N\textsubscript{lgG}, (B) F-10 + N\textsubscript{lgG}, which had been heated at 58°C for 1 hr, (C) F-10 + P\textsubscript{lgG}, or (D) F-10 + P\textsubscript{lgG} which had been heated at 58°C for 1 hr. All sections stained with H & E (x 180).
lyophylized and reconstituted in F-10 medium and tested for acantholytic activity on cultured skin, it was shown that whenever the antibody titer against epidermal ICS was at least 80, binding to the epidermis and acantholysis occurred. We tentatively conclude that probably all the pemphigus IgG subclasses which are capable of binding to the ICS are also capable of causing suprabasilar epidermal acantholysis.

**Autoradiographic Studies**

Very little is known regarding the metabolic activities of an acantholytic cell in pemphigus (although such a cell is presumed to be a dead or dying cell) and essentially nothing is known about the effects of pemphigus autoantibody on the synthesis of RNA and protein on the different cell types in the epidermis. In order to answer these questions, we used autoradiography to examine the relative rates of incorporation of precursors into these two classes of macromolecules in the epidermis during the entire sequence of events that occurred in culture from the time of addition of the pemphigus antibody until extensive acantholysis had occurred. Skin explants were prepared and placed into either (i) control F-10 medium, (ii) control F-10 + N_IgG medium, or (iii) F-10 + P_IgG medium. At specified times during the next 4 days samples were removed from these cultures and placed into fresh F-10 medium (no antibodies) containing either [3H]uridine (RNA synthesis) or 3H-amino acid mixture (protein synthesis) and incubated for a 2-hr period. At the end of these short pulses the samples were prepared for autoradiography (Materials and Methods). Average grain counts per cell were determined in both the basal cells and in the remaining epidermal cell population which we refer to simply as “nonbasal” cells. No attempt was made to further break down cell types. Grains were not seen above stratum corneum cells. The average grain counts for uridine and amino acids are shown in Tables I and II, respectively. It can be seen that the relative incorporation of uridine by the controls is fairly constant during the first 67 hr in culture, and this is true for both basal cells and nonbasal cells. By 91 hr, the system had deteriorated. When one examines the effects of the pemphigus antibody on these patterns, however, a striking differential effect on the nonbasal cells is seen by 43 hr. By this time, uridine incorporation by the basal cells was relatively unaffected whereas nonbasal activity had been reduced to less than 10% of the controls. By 67 hr, a time at which acantholysis was extensive, the basal cells were still relatively unaffected, whereas incorporation by the nonbasal cells was completely absent. This differential effect is illustrated in Figure 5. Figures 5A and 5B are photomicrographs of the 67-hr skin cultures grown in F-10 and F-10 + N_IgG, respectively, and Figure 5C is the 67-hr culture grown in F-10 + P_IgG. In the controls, incorporation is nearly equal in both cell layers, whereas grains are absent in the suprabasilar cell layers of the explant grown in the pemphigus IgG. None of the acantholytic cells had grains. In Table II the data for the grain counts derived from the 3H-amino acid pulses are presented, and the patterns are similar to those seen following uridine. By

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43 hr there was a differential decline in incorporation by nonbasal cells, when compared to the basal cells.

**DISCUSSION**

The work presented here extends the observations of Michel and Ko that pemphigus serum contains a factor(s) capable of producing "pemphigus-like" epidermal acantholysis in normal human skin in culture [10]. We have shown that the IgG fraction from the serum is responsible for the tissue damage, although we have not tested the other major immunoglobulins for acantholytic activity. It is also likely that all the IgG subclasses which bind to the epidermal ICS are capable of producing acantholysis.

Our autoradiographic study showed that during the acantholytic process(es) in vitro, there was a differential decline in the incorporation of uridine and amino acids in the suprabasilar cells relative to the basal cells, and the definitive acantholytic cells did not incorporate either of these precursors. Thus, even though we do not know the primary mechanism by which the pemphigus autoantibody produces acantholysis in human epidermis, from these data it is clear that the antibody "target cells" respond to the antibody by reductions in the net incorporation of precursors to RNA and protein. We do not propose that the primary action of the antibody is to inhibit RNA and protein synthesis in these target cells; instead, it is more likely that these events are secondary to other more basic changes.

The role of complement in pemphigus acantholysis is controversial. Jordon et al [18] have demonstrated reaction products from complement activation in blister fluids from pemphigus lesions, and C3, C1q, C3PA, and properdin are occasionally found to be deposited in acantholytic areas of skin and oral mucosa [19]. These workers have concluded that complement is probably involved in the production of the lesion. On the other hand, it is known from indirect immunofluorescence studies that the antigen–antibody complex which forms when pemphigus serum is incubated with frozen sections of human skin will not fix complement [20]. Sams and Schur [16] separated the pemphigus IgG immunoglobulins into subclasses and showed that they did not fix complement. Our demonstration that heated F-10 + P IgG medium still contained acantholytic activity ruled out complement involvement in our system. The possibility must be considered that complement may be activated in pemphigus only in response to initial damage produced by the autoantibody. This could result from an injury-induced leakage at the site of the lesion of the naturally occurring autoantibodies which are present in all human sera, for example the "stratum corneum" autoantibody [21] or the "upper epidermal" autoantibodies which are present in human serum [22]. These latter antibodies are cytotoxic to epidermal cells in vitro as shown by trypan blue exclusion tests, but they do not normally bind in vivo. Both of these antibodies are capable of fixing complement.

The ultimate mechanism for producing epidermal damage in pemphigus is not known, but the concept of an enzymatic mechanism has been advanced for several model studies. Various proteolytic enzymes, including trypsin [23], chymotrypsin [24], papain, or elastase [25] are capable of producing epidermal acantholysis when injected into excised skin, and Stoughton [26] isolated a factor (presumably an enzyme) from the feces of patients with ulcerative colitis which caused acantholysis on thin, formalin-fixed sections. The concept that the source of hydrolytic enzymes comes from the skin has been supported by the observation of Stoughton and Novak [27] that mild heating of excised skin specimens produces acantholysis as well as dermal–epidermal separation 24 hr later and this is prevented by the presence of sulfhydryl inhibitors. It has also been shown that the acantholysis induced by cantharadin is the result of the action of this compound of enzymes [27]. It should be stressed here that our model for pemphigus acantholysis differs from these other models in that the stimulus for acantholysis is provided by the pemphigus autoantibody.

As an alternative to an enzyme-induced acan-
tholysis, one can readily visualize how the formation of immune complexes with epidermal antigens could in a rather nonspecific way interfere with the normal homeostasis of the epidermis. Since the types and amounts of macromolecules synthesized by a cell are very much affected by the environment of that cell (and this should be particularly true of the heterogeneous population of differentiating cells in the epidermis), it is conceivable that immune complex formation could upset delicate balances between the normal synthesis and turnover of components of the epidermal cell surface. Indeed, the autoradiographic experiments presented here lend some support to this hypothesis. Other probable results would be interference with normal (i) maturation of molecules and (ii) extracellular association of components of the ICS. Such a scheme would predict that almost any agent which binds one or more components of the ICS would result in some type of epidermal damage, and if the agent is the pemphigus autoantibody, this damage is manifested as acantholysis.

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