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John R. Stanley, Mina Yaar, Pamela Hawley-Nelson, Stephen I. Katz

J Clin Invest. 1982;70(2):281-288. <https://doi.org/10.1172/JCI110615>.

Research Article

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JOHN R. STANLEY, MINA YAAR, PAMELA HAWLEY-NELSON, and STEPHEN I. KATZ,
Department of Dermatology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814; National Cancer Institute and National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20205

ABSTRACT Pemphigus is an antibody-mediated autoimmune skin disease in which loss of cell-to-cell contacts in the epidermis results in blister formation. Patients with pemphigus develop antibodies that bind to the keratinocyte cell surface, the site of primary pathology. The purpose of this study was to characterize the antigen(s) to which pemphigus antibodies bind. Because we could detect pemphigus antigen by indirect immunofluorescence on the surface of multiply-passaged cells in cultures of both a spontaneously transformed mouse keratinocyte cell line (Pam) and normal human epidermal cells, we used these cells as a source of antigen. In order to demonstrate biosynthesis of antigen and to characterize the antigen(s), we radiolabeled cell cultures with [^{14}C]glucosamine or D-[2- ^3H]mannose and used different pemphigus sera to immunoprecipitate antigen from nonionic detergent extracts of these labeled cells. Specifically precipitated radiolabeled molecules were identified using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and fluorography. Sera from five of seven pemphigus patients specifically precipitated (from extracts of both Pam cells and human epidermal cells) a molecule that, when reduced, was ~ 130 kD, whereas seven normal human sera and two pemphigoid sera did not precipitate this molecule. The findings that (a) these precipitated molecules comigrated on SDS-PAGE and that (b) the 130-kD molecule could no longer be precipitated from cell extracts that had been previously reacted with a pemphigus serum, indicate that reactive pemphigus sera bind the same molecule. The molecule was not detected in the culture medium of these cells. This finding, along with the cell surface

immunofluorescence pattern, suggests that the antigen is bound to the cell surface. Cultured mouse and human fibroblasts do not synthesize the antigen. The antigen contains protein because it was degraded by V8 protease and chymotrypsin, and it could also be labeled with [^{14}C]amino acids. It is probably not a sulfated proteoglycan because it did not label with $^{35}\text{SO}_4$. Taken together, these data indicate that some, but not all, pemphigus sera bind a specific cell surface glycoprotein that is synthesized by keratinocytes.

INTRODUCTION

Pemphigus is an antibody-mediated autoimmune blistering skin disease that is almost always fatal if untreated. The histology of pemphigus blisters demonstrates intraepidermal vesicles that result from acantholysis, a loss of cell-to-cell contacts and rounding up of epidermal cells (1). Direct immunofluorescence of lesional or perilesional skin in pemphigus demonstrates antibody (usually IgG) bound, in vivo, to the epidermis and forming an intercellular staining pattern (2). Indirect immunofluorescence has been used to show that most patients with pemphigus also have circulating antibodies that bind to normal epidermis of most mammals and birds in the same intercellular pattern (2, 3). Although light microscopic tissue immunofluorescence patterns appear to be intercellular, immunofluorescence performed on cell suspensions and immunoelectron microscopy have demonstrated that pemphigus antibodies bind to the keratinocyte cell surface (4-8). Whether pemphigus antibodies also bind to a substance in the intercellular space is not known. Thus, antibodies in patients with pemphigus define an antigen or antigens on the normal keratinocyte cell surface.

Pemphigus antibody is probably pathogenic. Organ culture of skin has been used to demonstrate that pem-

Address reprint requests to Dr. Stanley, Uniformed Services University of the Health Sciences.

Received for publication 8 February 1982 and in revised form 30 March 1982.

phigus IgG, in the absence of complement, can cause acantholysis (9). The binding of pemphigus IgG to the keratinocyte cell surface probably results in the release of proteolytic enzymes that can cause acantholysis (6, 10–12). Thus, the interaction of pemphigus antibody with a cell surface antigen or antigens is probably a mediator of disease. Furthermore, most authors (13–16), but not all (17), have found that pemphigus disease activity correlates roughly with the immunofluorescence titer of circulating antibodies.

It is important in understanding the pathophysiology of this acantholytic process to know whether there is a particular cell surface antigen that can trigger acantholysis or whether the appropriate antibodies to any of a number of cell surface antigens can cause this reaction. Specifically, considering pemphigus, do different patients have antibodies to the same or different keratinocyte cell surface antigens? The present study was designed to characterize the cell surface antigen or antigens that bind antibodies in pemphigus sera.

METHODS

Cell cultures. A spontaneously transformed BALB/c mouse keratinocyte cell line called Pam 212, which we have previously characterized (18), was used at passages 10–12. Normal human epidermal cells (HEC)¹ derived from neonatal foreskins were cultured on collagen substrates as previously reported (19), and used at passage 3. Fibroblasts were derived from the dermis of BALB/c mice (20) and used in the first passage, or from the dermis of human foreskins (19), and used at passage 11.

Radiolabeling and extraction of cell cultures. Labeling medium consisted of basal medium Eagle (M. A. Bioproducts, Walkersville, MD), 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B plus one of the following: D-[1-¹⁴C]glucosamine hydrochloride (sp act 55 mCi/mmol, Amersham Corp., Arlington Heights, IL) at 12.5 µCi/ml, a mixture of ¹⁴C-amino acids (sp act 55 mCi/matom carbon, New England Nuclear, Boston, MA) at 12.5 µCi/ml or D-[2-³H]mannose (sp act 10 Ci/mmol, New England Nuclear) at 125 µCi/ml. Labeling with Na₂³⁵SO₄ (Amersham Corp.) at 30–50 µCi/ml was done in sulfate-free Dulbecco's modified Eagle medium (National Institutes of Health media unit). Cells were labeled at confluence for 42–48 h with [¹⁴C]glucosamine, and for 24 h with ¹⁴C-amino acids, [³H]mannose or ³⁵SO₄. The medium was then removed, centrifuged at 700 g for 15 min, and then dialyzed against 0.01 M Tris-HCl-buffered 0.9% saline, pH 7.2 (TBS). The cell layer, on a 150-mm tissue-culture dish, was scraped into a small volume (1–1.5 ml) of 0.5% Nonidet P-40 in TBS with 1 mM phenylmethylsulfonyl fluoride, vortexed, then centrifuged at 100,000 g for 1 h. The supernatant was dialyzed against 0.3% Nonidet P-40 in TBS. The extraction and subsequent immunoprecipitations were carried out at 4°C.

Sera. Sera from seven patients with pemphigus were used. Several sera were kindly provided from the serum

banks of Dr. E. Beutner and Dr. K. Judd. We did not know the clinical type of pemphigus (e.g., foliaceus vs. vulgaris) for most of these patients but, by indirect immunofluorescence, the sera bound cryostat-sectioned skin in a typical epidermal cell surface pattern at titers of 80 to 320. Two bullous pemphigoid sera, previously described (21), and seven normal human sera were used as controls.

Indirect immunofluorescence of cultured cells. To detect pemphigus antigen in cultured cells we used a modification of a previously described technique (22). We found that using living rather than fixed cells in the indirect immunofluorescence procedure markedly improved the sensitivity of the assay. Sodium azide was used to prevent pinocytosis of antibody. Confluent cells on glass cover slips were washed with basal medium Eagle with Hanks' salts, 25 mM Hepes buffer, and 0.2% sodium azide (washing medium). Antibodies were diluted with Dulbecco's phosphate-buffered saline containing 0.2% sodium azide. Pemphigus serum or normal human serum was diluted 1:10 and applied to the cells for 30 min at room temperature. The cells were washed four times with washing medium and then a fluorescein-isothiocyanate-conjugated goat anti-human IgG (N. L. Cappel Laboratories, Cochranville, PA) was applied for 30 min. Cells were washed three times with washing medium, once with phosphate-buffered saline, then air-dried and mounted on slides as previously described (22).

Immunoprecipitation. To characterize the pemphigus antigen, a double antibody immunoprecipitation technique was used. For each immunoprecipitation, ~100 µl of radiolabeled cell extract containing ~1 × 10⁶ cpm (1 × 10⁵ cpm in the case of ³⁵SO₄-labeled cell extract) was adjusted to 0.3% Nonidet P-40, 0.3% sodium deoxycholate, and 0.1% bovine serum albumin. In order to reduce nonspecific background precipitation a preabsorption step was carried out, before all immunoprecipitations, with ~5–10 µl of normal human serum. After incubation for 2 h, 80–160 µl of goat anti-human IgG, heavy-chain specific, was added. (Goat anti-human IgG was obtained from Cappel Laboratories and had the following characteristics: antibody protein 5 mg/ml, total protein 16.5 mg/ml, a single arc precipitated when tested by immunoelectrophoresis against normal human serum or human IgG). After incubation for 4 h, the sample was centrifuged at 1,500 g and the supernatant was used for the actual immunoprecipitation. The supernatant was incubated overnight with 4 µl of one of several pemphigus sera or, as controls, one of several normal human sera (different from the serum used in the preabsorption) or pemphigoid sera. To precipitate the immune complexes and IgG, 64 µl of goat anti-human IgG was added and incubated for 4 h. The precipitate was washed six times with TBS containing 0.3% Nonidet P-40 and 0.3% sodium deoxycholate. The immune complexes were dissociated by heating at 100°C for 2 min in 100 µl of gel sample buffer containing 2% SDS with or without 0.1 M dithiothreitol. For these experiments the optimal amount of goat anti-human IgG was determined by titrating varying volumes of normal human serum against a constant amount of goat anti-human IgG and determining the point of maximal precipitation by dissolving the precipitates in 0.1 N NaOH and measuring the optical density at 280 nm. In some experiments, protein A-bearing staphylococci were used to precipitate immune complexes, as previously described (21). Results using either method were identical.

SDS-polyacrylamide gel electrophoresis (PAGE) and fluorography. The immunoprecipitated radiolabeled antigen was identified using SDS-PAGE followed by fluorography (21, 23–25). ¹⁴C-labeled molecular weight standards were purchased from New England Nuclear.

¹ Abbreviations used in this paper: HEC, human epidermal cells; kD, kilodalton; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

Proteolytic digestion of immunoprecipitates. To demonstrate that the immunoprecipitated pemphigus antigen contained protein, we used techniques of proteolysis in SDS developed by Cleveland et al. (26). Immunoprecipitates were dissolved at 100°C for 2 min in 0.125 M Tris-HCl, pH 6.8 with 0.5% SDS, 0.01% bromophenol blue, 10% glycerol (protease gel buffer). *Staphylococcus aureus* V8 protease (Miles Laboratories, Elkhart, IN) 100 µg/ml or chymotrypsin (46 U/mg, Worthington Biochemical Corp., Freehold, NJ) 200 µg/ml were added to some samples, and all samples were incubated at 37°C for 15 min. After adjustment to 2% SDS and 0.1 M dithiothreitol, samples were heated at 100°C for 2 min and electrophoresed.

RESULTS

Immunofluorescence of pemphigus antigen in cultured cells. Previously we have shown that pemphigus antigen can be detected by indirect immunofluorescence on the cell surface of the large plate-like cells located superficially in cell colonies of methanol and acetone fixed cultures of HEC (22) and Pam cells (18). Using an indirect immunofluorescence procedure on unfixed, living Pam cells we found a greater sensitivity and could detect pemphigus antigen on the cell surfaces of most cells, both the large superficial cells and the smaller cells lower in the colonies and on the substrate (Fig. 1). The fact that pemphigus antigen could be detected in these cultures of multiply-passaged cells suggested that they were appropriate to use for biosynthetic labeling, isolation, and characterization of the antigen.

Immunoprecipitation and characterization of pemphigus antigen. Preliminary studies using var-

ious radiolabeled metabolic precursors (such as ^{14}C -amino acids, [^{35}S]methionine, $^{35}\text{SO}_4$, [^3H]mannose, [^3H]glucosamine, and [^{14}C]glucosamine) demonstrated that pemphigus antigen could best be radiolabeled, immunoprecipitated, and identified from cultures labeled with [^{14}C]glucosamine. Thus, Pam cells and HEC, at confluence, were labeled with [^{14}C]glucosamine, then were extracted with nonionic detergent and the solubilized labeled molecules were immunoprecipitated with different pemphigus sera or, as controls, with normal human sera or pemphigoid sera. The immunoprecipitated radiolabeled molecules were identified by SDS-PAGE and fluorography.

Sera from five of seven pemphigus patients specifically precipitated, from extracts of both Pam cells and HEC, a molecule that when reduced on SDS-PAGE had an apparent molecular mass of 130 kD (Fig. 2). The specificity of this reaction is shown by the finding that seven normal human sera and two pemphigoid sera did not precipitate this molecule (Fig. 2). In order to demonstrate that different pemphigus sera that precipitate this antigen actually precipitate the same molecule, we precipitated the antigen from an extract of Pam cells with one pemphigus serum, then used another to try to immunoprecipitate the antigen from the supernatant. The results of two experiments showed that one pemphigus serum depleted or markedly reduced the antigen with which a second pemphigus serum could react (Fig. 3). In contrast, normal human sera did not deplete the antigen from extracts of these cells. Thus it is likely that the molecules that are pre-

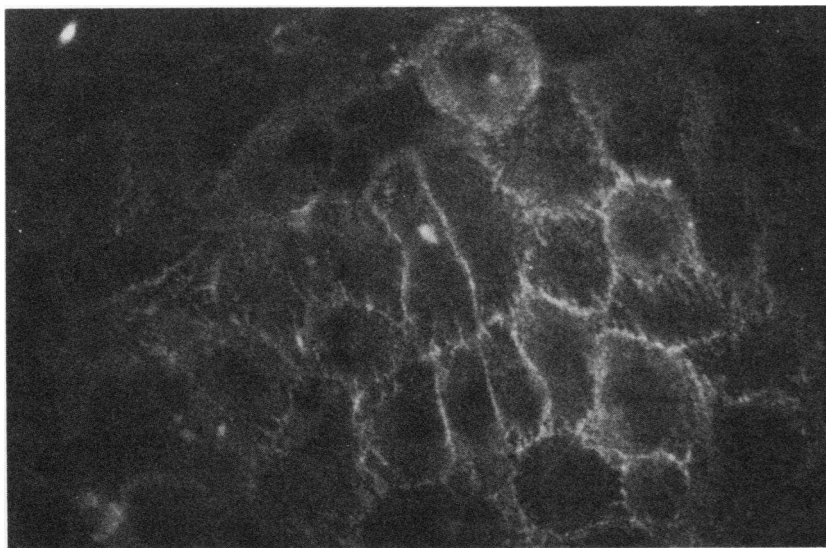


FIGURE 1 Indirect immunofluorescence showing pemphigus antigen in cultured keratinocytes. Living Pam cells at passage 9 were incubated with a pemphigus serum, then, after washing, with a fluorescein-conjugated goat anti-human IgG. The fluorescence pattern indicates that the pemphigus antigen is on the cell surface. Use of normal human serum instead of pemphigus serum did not result in fluorescence. ($\times 430$).

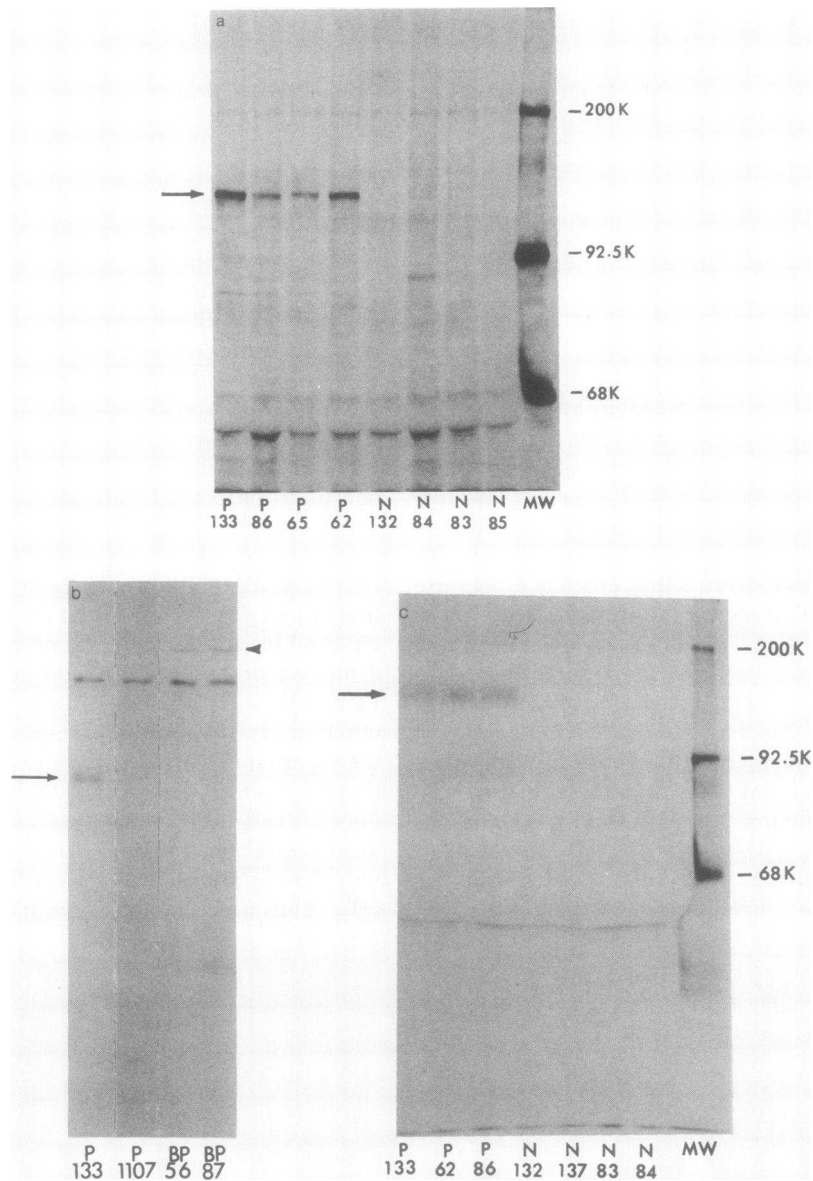


FIGURE 2 Identification of pemphigus antigen synthesized by Pam cells (a, b) and human epidermal cells (HEC) (c). Nonionic detergent extracts of [^{14}C]glucosamine-labeled cultured Pam cells or HEC were immunoprecipitated with several different pemphigus sera (P), normal human sera (N) or pemphigoid sera (BP). (Numbers indicate sera from different patients). Immunoprecipitated radiolabeled molecules were reduced and identified by SDS-PAGE and fluorography. (MW indicates molecular weight standards). (a) Four different pemphigus sera precipitate a molecule, from Pam cell extracts, with apparent molecular mass 130 kD (arrow). Normal sera do not precipitate this molecule. (b) Pemphigus serum 133 precipitates the 130-kD molecule (arrow) from Pam cell extracts but pemphigus serum 1107 does not. Two pemphigoid sera do not precipitate the 130-kD molecule but do precipitate the pemphigoid antigen, molecular mass ~ 220 kD (arrowhead). The 220-kD bands are faint because pemphigoid antigen does not label as well with [^{14}C]glucosamine as it does with [^{35}S]methionine or ^{14}C -amino acids (21). (c) The pemphigus sera precipitate the 130-kD molecule (arrow) from HEC extracts, whereas the normal sera do not. In these gels, and those in subsequent figures, there is a band at 200-kD that is nonspecifically precipitated by all sera.

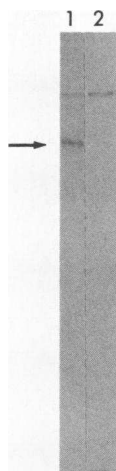


FIGURE 3 Two different pemphigus sera precipitate the same molecule. See Fig. 2 for procedures and abbreviations. Pemphigus antigen was precipitated with P 133 from extracts of Pam cells (lane 1, arrow shows 130 kD molecule). The supernatant of this immunoprecipitate was then incubated with P 62 and the resulting immunoprecipitate (lane 2) did not contain the 130-kD molecule. In contrast, normal human serum did not deplete these extracts of antigen reactive with either P 62 or P 133.

cipitated by the five pemphigus sera and that comigrate on SDS-PAGE are identical. This 130 kD molecule could not be precipitated by pemphigus sera from the labeled medium of these cells. This finding, together with the immunofluorescence data, indicates that the antigen is bound to the cell surface of these cultured cells. In addition, this molecule could not be immunoprecipitated from nonionic detergent extracts of [^{14}C]glucosamine-labeled mouse (Fig. 4) or human fibroblasts, indicating that it is not synthesized by fibroblasts in culture.

Additional studies were done to further characterize this 130-kD molecule. The molecule, when electrophoresed unreduced on SDS-PAGE, could not be detected, presumably because it did not penetrate the gel. This finding suggests that, when unreduced, the molecule may exist as high molecular weight aggregates. In order to demonstrate that the molecule contained protein, we incubated the immunoprecipitates with either *S. aureus* V8 protease or chymotrypsin before electrophoresing them. The specifically precipitated 130-kD molecule was degraded by both proteases (Fig. 5) indicating that it contains protein. The presence of protein in this molecule was also demonstrated by the finding that it could be biosynthetically labeled with a mixture of ^{14}C -amino acids (Fig. 6). However, this molecule could not be radiolabeled in cultures of Pam cells or HEC with $^{35}\text{SO}_4$, suggesting that it is probably not a sulfated proteoglycan. The finding that this molecule labels with [^{14}C]glucosamine,

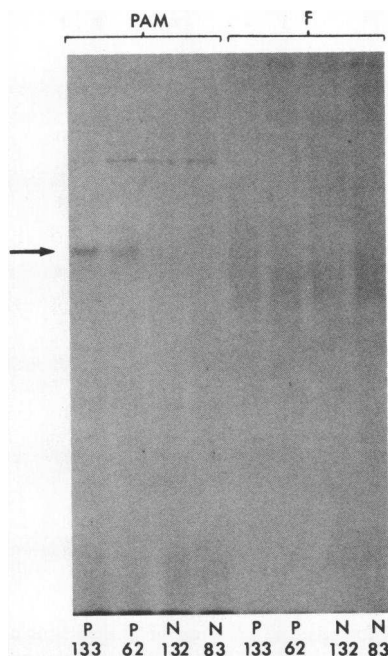


FIGURE 4 Keratinocytes, but not fibroblasts, synthesize the pemphigus antigen. Fig. 2 describes procedures and abbreviations. Pemphigus sera precipitate pemphigus antigen (arrow) from extracts of [^{14}C]glucosamine-labeled Pam cells but not from extracts of [^{14}C]glucosamine-labeled mouse fibroblasts (F). Similar results, not shown here, were obtained with HEC and human fibroblasts.

then, suggests that it is a glycoprotein. This was confirmed by biosynthetically labeling the molecule with D-[2- ^3H]mannose (Fig. 7).

Taken together these data indicate that some, but



FIGURE 5 Pemphigus antigen contains protein. Pemphigus antigen immunoprecipitated from extracts of [^{14}C]glucosamine-labeled Pam cells was incubated at 37°C for 15 min without (lane 1) or with the proteolytic enzymes, *S. aureus* V8 protease 100 $\mu\text{g}/\text{ml}$ (lane 2) or chymotrypsin 200 $\mu\text{g}/\text{ml}$ (lane 3). The specifically precipitated 130-kD molecule (arrow) was degraded by both proteolytic enzymes.

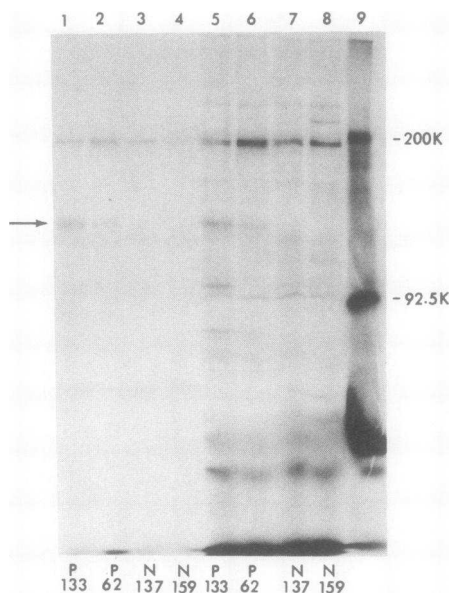


FIGURE 6 Biosynthetic labeling of pemphigus antigen with [^{14}C]glucosamine and ^{14}C -amino acids. Pam cells were labeled with either [^{14}C]glucosamine (lanes 1–4) or a mixture of ^{14}C -amino acids (lanes 5–8). Equal numbers of counts from nonionic detergent extracts of these cells were immunoprecipitated with pemphigus sera (P) or normal human sera (N). See legend Fig. 2 for details. The 130-kD molecule (arrow) is specifically precipitated from both [^{14}C]glucosamine labeled cell extracts (lanes 1, 2) and from ^{14}C -amino acid labeled cell extracts (lanes 5, 6). Normal human sera do not precipitate this band. There is increased background noted on the ^{14}C -amino acid labeled lanes, but only the 130-kD band is specifically precipitated by the pemphigus sera.

not all, pemphigus sera bind a specific cell surface glycoprotein that is synthesized by keratinocytes in culture.

DISCUSSION

This study demonstrates that most, but not all, pemphigus sera bind the same keratinocyte cell surface antigen, a glycoprotein with apparent molecular weight 130 kD, when reduced, as determined by SDS-PAGE. This is in the molecular weight range of the major cell surface glycoproteins of the epidermis (27). Both a spontaneously transformed mouse keratinocyte cell line (Pam) and cultured normal human epidermal cells, but not mouse or human fibroblasts, synthesize this glycoprotein which is bound to the cell surface of these cultured keratinocytes.

Several prior attempts have been made to characterize antigens that react with pemphigus sera. Each of these studies described an antigen of different molecular weight. A 40–50-kD glycoprotein with subunits of 20 and 25 kD was isolated from human saliva and

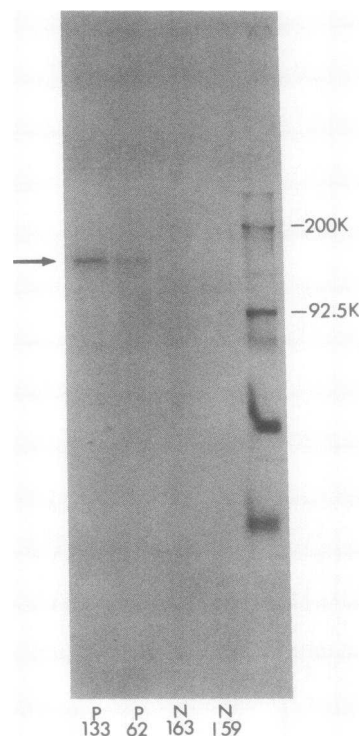


FIGURE 7 Biosynthetic labeling of pemphigus antigen with D-[2- ^3H]mannose. Pam cells were radiolabeled, then extraction and immunoprecipitation were performed as described in the legend of Fig. 2. Labeled pemphigus antigen (arrow) was specifically precipitated.

blocked the binding of one pemphigus serum to the epidermis as determined by indirect immunofluorescence (28). Affinity chromatography, with a serum from a patient with pemphigus vulgaris, was used to detect a protein of molecular weight 18 kD from an extract of guinea pig epidermis, but this protein also contained RNA and was said to be impure (29). A group of proteins in the 68-kD range partially purified from extracts of human esophagus was able to decrease the immunofluorescence titer of two pemphigus vulgaris sera (30). For several reasons it is difficult to compare the antigens described in these studies to the 130-kD glycoprotein that we have characterized. First, in these prior studies the source of the antigen varied but did not include human or mouse keratinocytes that we used. Second, two of these studies (29, 30) deal with rather impure and heterogeneous substances and did not use protease inhibitors in extraction procedures. Finally, these prior studies rely on immunofluorescence blocking assays using only one or two sera. In the present study, rather than relying on immunofluorescence blocking, which is an indirect method of detecting antigen, we directly characterized the an-

tigen that bound the IgG of several different pemphigus sera.

The present study clearly demonstrates that not all pemphigus sera bind the same molecule. This finding is not simply due to differences in antibody titer among different pemphigus sera because the indirect immunofluorescence titers of the two sera that did not precipitate the 130-kD molecule were 160 and 320, whereas the titers of those sera that did precipitate the molecule were equivalent, 80-320. Whether this difference in specificity can be related to the clinical type of pemphigus (e.g., foliaceus, in which the blister occurs in the superficial epidermis vs. vulgaris, in which the blister is found deeper, just above the basal layer) cannot be determined from this study because most of the sera used were obtained from immunofluorescence laboratory serum banks. Thus, although the diagnosis of pemphigus was confirmed by indirect immunofluorescence, the clinical type of pemphigus could not be accurately determined without histopathology, which was unavailable in most cases. Immunofluorescence blocking studies have suggested that antibodies from patients with Brazilian pemphigus foliaceus may have different specificities than antibodies of patients with pemphigus vulgaris (31), however inability of one serum to block the binding of another serum does not prove that these sera bind to different molecules. The presence of several cell surface pemphigus antigens has also been suggested by the observation that different pemphigus sera tested on the same skin specimen may result in different immunofluorescence patterns (32, 33). For example, some patients with pemphigus foliaceus have antibodies that only bind the superficial layers of normal epidermis (32). However, most patients with pemphigus foliaceus have antibodies that bind all layers of normal epidermis and it has been suggested that a cell surface antigen, normally present throughout the epidermis, is missing in the lower part of the epidermis in pemphigus foliaceus lesions (33). Thus, the superficial or deep location of the blister in pemphigus foliaceus and pemphigus vulgaris, respectively, may be due either to distinct antigenic specificity of antibodies or to a different distribution, in the epidermis, of a single reactive antigen. The present study demonstrates that some pemphigus sera do not react with the specific molecule to which many sera do bind. Further studies are necessary to determine if antibody specificity in pemphigus patients is related to the clinical type of disease.

It is clear, however, from this study that many pemphigus sera react with a specific keratinocyte cell surface glycoprotein. It is likely that the interaction of pemphigus antibody with this glycoprotein is important in triggering acantholysis, the pathologic process

in pemphigus. The understanding of this interaction should yield important insights into the molecular basis of pemphigus.

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. Victor Ginsburg and Dr. Manfred Brockhaus for valuable discussions, Ms. Vera Klaus-Kovtun for technical assistance, and Mr. Harry Schaefer for his photographic expertise.

This work was supported in part by Defense Department grant R08400.

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