Further Analysis of Pemphigus Autoantibodies and Their Use in Studies on the Heterogeneity, Structure, and Function of Desmosomes

Jonathan C. R. Jones, Karen M. Yokoo, and Robert D. Goldman

Department of Cell Biology and Anatomy, Northwestern University Medical School, Chicago, Illinois 60611

Abstract. Pemphigus is an autoimmune disease that causes blistering of human epidermis. We have recently shown that autoantibodies in the serum of three pemphigus patients bind to desmosomes (Jones, J. C. R., J. Arnn, L. A. Staehelin, and R. D. Goldman, 1984, Proc. Natl. Acad. Sci. USA., 81:2781-2785), and we suggested that pemphigus blisters form, at least in part, from a specific antibody-induced disruption of desmosomes in the epidermis. In this paper, experiments are described that extend our initial observations. 13 pemphigus serum samples, which include four known pemphigus vulgaris (Pv) and four known pemphigus foliaceus (Pf) serum samples, have been analyzed by both immunofluorescence and by immunoblotting using cell-free desmosome preparations. Tissue sections of mouse skin processed for double indirect immunofluorescence using each of the pemphigus serum samples and a rabbit antiserum directed against a component of the desmosomal plaque (desmoplakin) show similar punctate cell surface staining patterns. This suggests that all 13 pemphigus serum samples contain autoantibodies that recognize desmosomes. These autoantibodies appear specific for stratified squamous epithelial cell desmosomes and do not recognize desmosomes of other tissues (e.g., mouse heart and mouse intestine). Cultured mouse keratinocytes, which possess well-defined desmosomes, were processed for indirect immunofluorescence using the pemphigus serum samples. Eight of the 13 sera (including the four known Pv samples but not the known Pf sera) stain desmosomes in these preparations. By double indirect immunofluorescence the desmoplakin antiserum stains a double fluorescent line along the contacting edges of cultured keratinocytes, whereas the positive pemphigus serum samples stain a single fluorescent line along this same border. We believe that these pemphigus autoantibodies recognize extracellular antigens located somewhere within the region between the two apposing membranes that comprise the desmosome. The pemphigus sera exhibit positive immunoblotting reactions with desmosome-enriched fractions obtained from bovine tongue epithelium. Three serum samples (including two of the four known Pf serum samples) react with 160- and 165-kD desmosome-associated polypeptides (Koulu, L., A. Kusimi, M. S. Steinberg, V. Klaus-Kovtun, and J. R. Stanley, 1984, J. Exp. Med., 160:1509-1518). Another eight serum samples (including the four known Pv sera) recognize a 140-kD desmosome-associated polypeptide. We propose that the antigens recognized by these human autoantibodies may play important roles in the adhesion of cells within the epidermis. Furthermore we discuss the heterogeneity of desmosome structure in light of our immunofluorescence and immunoblotting results.

Pemphigus is a devastating autoimmune disease of the skin whose clinical features are characterized by the formation of numerous intraepidermal blisters that cover varying portions of the body (4, 13, 24). There is variation in the disease, in that some patients have extensive blistering in most skin regions, while others, for example, exhibit only mouth blisters (4, 13, 24). Histological examination of biopsies of blistering regions demonstrate that dysadhesion of keratinocytes (acantholysis) produces blistering of the epidermis (4, 13, 24). According to most workers in the field, pemphigus has a number of variants, classified mainly by the clinical symptoms of patients and the histopathology of skin lesions (4, 13, 24). The two main forms of the disease are termed pemphigus vulgaris (Pv) and pemphigus foliaceus (Pf). In the former case, blisters result from dysadhesion of keratinocytes in the epidermis just above the basal cell layer (suprabasally), and in the latter case blisters form because of a loss of cell–cell contacts higher within the stratum spinosum (4, 13, 24). These variations of pemphigus appear to affect only stratified squamous epithelial tissues (4, 24).

Abbreviations used in this paper: PBSa, a solution of 6 mM Na+, K+ phosphate, 171 mM NaCl, 3 mM KCl, pH 7.4; Pf, pemphigus foliaceus; Pv, pemphigus vulgaris; TPBS, 0.05% Tween 20 in PBSa.
The autoantibodies found in pemphigus patients appear to be directed against an intercellular cement substance (4, 13, 24). Pemphigus autoantibodies actually bind to the epidermis in blistering regions of the skin of diseased patients as determined by microscopy of biopsy material (4, 13, 24). Both immunofluorescence (3) and immunojet microscopy (31) observations of these biopsies suggest that the autoantibodies bind to the surface coat or glycocalyx of individual epidermal cells. Indeed it is now considered that these autoantibodies play a pathogenic role in the disease (13, 24) since, for example, incubation of skin explants in the presence of pemphigus IgG induces acantholysis (26).

Recently, we have shown that certain autoantibodies present in the serum of pemphigus patients appear to bind specifically to desmosomes (19), which are intercellular junctions considered to play a vital role in the adherence of the cells within the epidermis (2). Indeed, we have proposed, based on observations of living epidermal cells, that pemphigus blisters form, at least in part, from a specific antibody-induced disruption of desmosomes in the epidermis.

Our finding that certain autoantibodies in pemphigus recognize desmosomes has been supported, at least in part, by a recent publication by Koulu et al. (22). These authors showed that autoantibodies from certain Pf patients recognize polypeptides found in cell-free, so-called "core" (15) preparations of desmosomes. However, these same authors report that Pv autoantibodies do not react with the same desmosome fractions. Gorbsky et al. (16) have also suggested that Pv patients do not possess any circulating anti-desmosome antibodies. The results of these two publications are based primarily on either immunoprecipitation and immunoblotting analyses or ELISA using enriched fractions of bovine muzzle desmosomes as antigenic substrates. Furthermore, in both of these studies (16, 22), the authors either did not or were unable to show by means of immunofluorescence that any of their pemphigus serum samples contained anti-desmosome autoantibodies. This has led Koulu et al. (22) to suggest that our previous finding, which demonstrated the presence of autoantibodies directed against desmosomes, could be explained in light of their data, if all of our serum samples were derived from Pf patients (19, 22).

In this study, in contrast to the aforementioned reports of Koulu et al. (22) and Gorbsky et al. (16), we show that the serum samples of known Pv patients, in addition to certain Pf patients, recognize desmosomal components as determined by both immunofluorescence and immunoblotting criteria using cell-free preparations of desmosomes. We propose that the antigenic determinants of desmosomes recognized by these autoantibodies may play important roles in the adherence of cells within the epidermis. Furthermore, data is presented that suggests that these autoantibodies reveal heterogeneity in desmosome components both within and amongst the cells that comprise different epithelial tissues. These results are discussed in light of the possibility that desmosomes may contain specific cell-cell adhesion molecules and not merely represent spot welds between neighboring cells.

**Materials and Methods**

**Cell Culture**

Primary mouse keratinocytes were prepared and maintained in culture as reported elsewhere (18, 21).
neonatal mouse skin (Fig. 1), using the pemphigus serum samples and the desmoplakin antiserum. The patterns observed with all the pemphigus serum samples are similar to those generated by the desmoplakin antiserum (i.e., in every case a coincident punctate staining at the surface of epidermal cells is revealed [Fig. 1, a–d]).

Figure 1. Cryostat sections of mouse skin were processed for double indirect immunofluorescence using pemphigus serum samples (a and c) and the desmoplakin antiserum (b and d). a shows the punctate intercellular immunofluorescence pattern generated by a Pv serum sample in mouse skin (see Fig. 3, lane G for the immunoreactivity of this serum sample with an enriched fraction of desmosomes). Note the co-localization of this staining pattern with that generated by the desmoplakin antiserum in the same section (b). c shows the punctate intercellular staining pattern produced by a Pf serum sample (see Fig. 3, lane B for the immunoreactivity of this serum sample with a desmosome fraction). This staining pattern co-localizes with that produced by the desmoplakin antiserum in the same section (d). Bar, 10 μm.
Our results to date strongly suggest that pemphigus sera contain autoantibodies that are directed against components of desmosomes. Since desmosomes have been considered to be ubiquitous organelles of epithelial tissues (5), we wondered, therefore, whether the sera we analyzed recognize desmosomal antigens in tissues other than stratified squamous epithelia. Thus cryostat sections of several other desmosome-containing tissues were prepared for indirect immunofluorescence using the pemphigus serum samples and the desmoplakin antiserum as a control. The intercalated discs of mouse heart, which possess desmosome-like structures, and the desmosomes of mouse intestine (5) are readily visualized in tissue sections using the desmoplakin antiserum, whereas these same structures are not stained by any of the pemphigus serum samples (results not shown).

**The Visualization of Desmosomes in Cultured Cells.** We selected a mouse keratinocyte culture system to determine the presence or absence of desmosome autoantibodies in our 13 pemphigus serum samples. In this culture system keratinocytes grown in low Ca**2+**-containing medium (~0.1 mM) possess no desmosomes (18). Desmosome formation is induced by increasing the Ca**2+** to normal levels (~1.2 mM Ca**2+**) (18). Desmosome-possessing keratinocytes were therefore processed for double indirect immunofluorescence using the pemphigus serum samples and the desmoplakin antiserum. The four Pv serum samples and four of the five unclassified pemphigus serum samples yield a pattern of intermittent single lines along the borders of the contacting keratinocytes (19, Fig. 2b). This pattern was similar in overall distribution to that seen in similar preparations using desmoplakin antiserum. However, this latter pattern usually revealed double lines instead of single lines at the sites of desmosomes (Fig. 2). In double label observations, the nonfluorescent line that separates a double band observed by immunofluorescence using the desmoplakin antiserum appears to be coincident with the single fluorescent line stained by the pemphigus autoantibodies (Fig. 2, a and b).

The known Pf serum samples (see Materials and Methods), one of the unclassified serum samples, and normal human serum did not stain the desmosomes present at the contacting surfaces of keratinocytes maintained in normal levels of Ca**2+** (results not shown).

**Western Immunoblotting Analyses of Pemphigus Autoantibodies**

To determine the target antigen(s) of the pemphigus autoantibodies, we used desmosome-enriched fractions from bovine tongue for analysis by Western immunoblotting (see Materials and Methods).

The pemphigus serum samples react with several polypeptides in the desmosome-enriched preparations, which are not recognized by normal human serum (Fig. 3). There are certain polypeptides that are recognized by subgroups within the 11 pemphigus serum samples shown in Fig. 3. Indeed, in some cases these subgroups can be correlated with known Pv and Pf samples. For example, two of the four known Pf serum samples, in addition to one of the unclassified samples (which fails to stain the cell surface of cultured keratinocytes [see above]) recognize 160 and 165-kD polypeptides (Fig. 3, lanes B, C, and D) (22). Furthermore, the four known Pv serum samples and the remaining four unclassified serum samples all recognize a 140-kD polypeptide in the tongue desmosome preparations (Fig. 3, lanes G–L) (only the immunoblots of two of the known Pv serum samples [Fig. 3, lanes G and H] are shown).

To determine whether autoantibodies directed against the 140-kD desmosome-associated polypeptide contribute to the desmosome staining pattern we observe in cultured keratinocytes (19, see Fig. 2b), absorption of a Pv serum sample...
Figure 3. An enriched preparation of desmosomes isolated from bovine tongue was subjected to SDS PAGE and electrophoretically transferred to nitrocellulose. Lane A shows an Amido black stain of transferred proteins. Note desmoplakins 1 and 2 (D1, D2) (arrows indicate, from top to bottom, 200-, 120-, 92-, 80-, and 50-kD). Lanes B–L are immunoblots using pemphigus serum samples, whereas lane M is an immunoblot using a normal human serum sample. Lanes B, C, E, and F are immunoblots of Pf serum samples. Lanes G and H are immunoblots of Pv serum samples. Note the 160- and 165-kD polypeptides (arrows) which are recognized by serum samples in lanes B, C, and D and the 140-kD polypeptide (arrow) recognized by serum samples in lanes G–L. Polypeptides recognized by both the normal human serum sample, in addition to the pemphigus serum samples, are arrowed in lane M.

Koulu et al. (22) were unable to show reaction of Pv serum samples with any desmosome proteins, either by immunoprecipitation or immunoblotting. Thus their results concerning Pv patients are different from ours. One possible explanation for these differences may lie in the use of desmosome fractions obtained from different tissues. We used enriched fractions of bovine tongue desmosomes, whereas these other workers used bovine muzzle desmosome fractions. We wondered, therefore, whether there were differences in cross-reactivity of pemphigus autoantibodies with bovine tongue and muzzle desmosome preparations. To test this possibility, we isolated and compared desmosomes from bovine muzzle and tongue. These two fractions of desmosomes look identical by ultrastructural analyses (results not shown). However, the SDS PAGE profiles of such preparations differ quite considerably (Fig. 5). Immunoblotting analyses using two of each of the known immunoreactive Pf and Pv serum samples on both bovine tongue and muzzle desmosome preparations are shown in Fig. 6 (see also Table I). The Pf serum samples react with polypeptides in the 160–165-kD range in the tongue desmosome preparation (confirming the results we described above, see also 22) and also in the muzzle desmosome preparation (Fig. 6, lanes B, C, G, and H). In contrast, although the two Pv serum samples react strongly with a 140-kD polypeptide in the bovine tongue desmosome preparation...
Figure 4. A bovine tongue desmosome-enriched preparation was subjected to SDS PAGE and transferred to a nitrocellulose sheet. A thin vertical strip was cut from the sheet and stained with Amido black (lane A). As described in the text, a 140-kD polypeptide (arrow in lane A) was used to absorb out autoantibodies from a Pv serum sample. Lane B shows the immunoreactivity of this Pv serum sample with the desmosome preparation before absorption (see also Fig. 3, lane G). Lane C shows the immunoreactivity of the same serum sample after absorption.

Figure 5. SDS PAGE profiles of a bovine tongue desmosome-enriched fraction (A) and a bovine muzzle desmosome-enriched fraction (B) are shown. Note that although both these preparations are enriched in desmoplakin 1 and 2 (D1 and D2), there are differences between the two profiles.

Our results would thus appear to suggest that desmosomal antigens recognized by the Pf and Pv autoantibodies are different as determined by their different immunoblotting reactions with the bovine tongue desmosome preparation (Fig. 3). Moreover, the 140-kD polypeptide recognized by the Pv autoantibodies is either absent or in greatly reduced amounts in bovine muzzle desmosomes as compared to bovine tongue desmosomes (Fig. 5). These data, in addition to the histology of Pv and Pf blisters (Pv blisters form suprabasally, whereas Pf blisters form higher up within the stratum spinosum) led us to determine whether we could detect differences in the location of Pf and Pv autoantibody binding in cryostat sections of epidermis. Currently all of the Pv and Pf serum samples that we have tested stain most areas of cell contact in neonatal mouse epidermis (Fig. 1). However, one Pf serum sample generates an intercellular staining pattern only in the upper layers of the epidermis of bovine tongue (Fig. 7). This particular serum recognizes the 160/165-kD polypeptides of the desmosome fractions (Fig. 3, lane B). We have found this differential staining pattern only in bovine tongue epidermis and not in neonatal mouse epidermis using this serum sample. These results are probably partly due to the large number of epidermal cell layers in the tongue epithelium, which may afford a better opportunity to resolve such differential staining characteristics.

We previously reported that the sera of three pemphigus patients contained autoantibodies that recognized the desmosomes of keratinocytes maintained in tissue culture (19). We have now extended these observations and have analyzed 13 pemphigus serum samples by immunofluorescence, using both cryostat sections of a variety of tissues and cultured keratinocytes, and by immunoblotting, using enriched preparations of bovine tongue and muzzle desmosomes. The 13 pemphigus serum samples display immunofluorescence patterns on cryostat sections of mouse epidermis that are similar to those observed using the desmoplakin antiserum. Indeed double indirect immunofluorescence using the pemphigus serum samples and a desmoplakin antiserum reveal that the pemphigus autoantibodies localize in the same region as the desmoplakin antibodies, which suggests that they are directed against desmosome components. Eight of the pemphigus serum samples (including the known Pv samples and the three samples we used in our previous study [19]) stain a single fluorescent line at the site of desmosomes of cultured keratinocytes as determined by indirect immunofluorescence. This line appears to co-localize with the material that comprises...
Table 1. Immunofluorescence and Immunoblotting Reactivity* of Pemphigus Autoantibodies

| Serum sample | Source | Classification | Tongue desmosome fraction | Muzzle desmosome fraction |
|--------------|--------|----------------|---------------------------|---------------------------|
|              |        |                | 160/165 kD | 140 kD | 160/165 kD | 140 kD |
| 1            | A      | P              | +           | -     | +           | -     |
| 2            | B      | Pf             | +           | -     | +           | -     |
| 3            | B      | Pf             | +           | -     | +           | -     |
| 4            | B      | Pf             | +           | -     | +           | -     |
| 5            | B      | Pf             | +           | -     | -           | -     |
| 6            | B      | Pv             | +           | +     | -           | +     |
| 7            | B      | Pv             | +           | +     | -           | +     |
| 8            | B      | Pv             | +           | +     | -           | +     |
| 9            | B      | Pv             | +           | +     | -           | +     |
| 10           | A      | P              | +           | +     | -           | -     |
| 11           | A      | P              | +           | +     | -           | -     |
| 12           | A      | P              | +           | +     | -           | -     |
| 13           | A      | P              | +           | +     | -           | -     |
| 14†          | A      | Normal         | -           | -     | -           | -     |

A, obtained from Northwestern University. B, obtained from Dr. J. Stanley, National Institutes of Health (see Materials and Methods). P, pemphigus. WR, weak reactivity.

* Only reactivity that appears common to groups of patients is tabulated.

† Four normal serum samples were tested.

Figure 6. A bovine tongue desmosome-enriched preparation (lanes A–E) and a bovine muzzle desmosome enriched preparation (lanes F–J) were subjected to SDS PAGE and electrophoretically transferred to nitrocellulose. Lanes A and F show Amido black stains of transferred proteins. Lanes B and G are immunoblots using one Pf serum sample. An immunoblot of this same serum sample is shown in Fig. 3, lane B. Lanes C and H are immunoblots using a second Pf serum sample (see also Fig. 3, lane C). Note that in both tongue and muzzle desmosome fractions these two serum samples recognize 160- and 165-kD polypeptides (arrowed). Lanes D and I are immunoblots using one Pv serum sample (see also Fig. 3, lane G). Lanes E and J are immunoblots using a second Pv serum sample (see also Fig. 3, lane H). Note that these sera recognize a 140-kD polypeptide in the tongue desmosome preparation (lanes D and E). However, these same sera either do not react or react extremely weakly with polypeptides in the 140-kD molecular weight range in the muzzle desmosome preparation (lanes I and J).

the dark line separating the two halves of a desmosome as delineated by use of the desmoplakin antibody preparations (see Fig. 2 and reference 14). These observations suggest that the antigenic determinants recognized by these pemphigus autoantibodies lie somewhat within the region between the two subplasmalemmal desmosomal plaques recognized by the desmoplakin antibodies (14). Furthermore, we feel the pemphigus autoantibodies probably bind to the outer surface of living keratinocytes as indicated by our earlier study that demonstrated that a loss of cell–cell cohesion occurred after exposure of living keratinocytes to pemphigus serum samples only at cell surface sites occupied by desmosomes (19). Based on these data, we further propose that pemphigus antigens are most likely positioned in the extracellular region between...
as determined by immunoblotting using enriched prepara-
the two apposing membranes that comprise the desmosome.

The pemphigus serum samples exhibit a variety of reactions as determined by immunoblotting using enriched preparations of bovine tongue desmosomes. However, even though we have tested a relatively small number of known Pv and Pf serum samples, the immunoblotting data are very provocative. These data suggest that there may be a correlation between the 160- and 165-kD desmosome-associated polypeptides and the autoantibodies found in some but not all Pf patients (see also reference 22) and a 140-kD desmosome-associated protein and the autoantibodies found in the serum of Pf patients. This latter polypeptide may be that described by Stanley et al. (28) as a cell surface glycoprotein recognized by Pf autoantibodies. Furthermore, it is also possible to divide the unclassified pemphigus serum samples into those that react with the 160- and 165-kD desmosome-associated polypeptides (one serum sample) and those that cross-react with the 140-kD desmosome-associated polypeptide (four serum samples). Whether this type of immunoblotting analysis will allow us to differentially diagnose patients with different forms of pemphigus awaits the analysis of a significantly larger number of known Pf and Pv serum samples. However, our results tempt us to suggest that the four unclassified serum samples that recognize the 140-kD polypeptides are from Pf patients, whereas the remaining serum sample is from a Pf patient. Indeed, additional evidence for this supposition is provided by our finding that the four unclassified serum samples stain the desmosomes of cultured keratinocytes similarly to that observed using the known Pf serum samples, whereas the one remaining unclassified serum sample in our collection, like the known Pf serum samples, does not.

Our immunoblotting results are, in part, in conflict with two reports in the literature, both of which have suggested that Pf autoantibodies do not react with desmosomal polypeptides by immunoblotting or immunoprecipitation analyses (16, 22). Our results and those from other laboratories appear to differ because of the use of different tissues as sources of desmosomes. The Pf samples show immunoreactivity with desmosome-associated polypeptides obtained from enriched fractions of tongue desmosomes, which appear either to be missing or are available in much lower amounts in similar preparations isolated from muzzle.

These data suggest that desmosomes in different types of stratified squamous epithelia are biochemically distinct. In addition, pemphigus autoantibodies cross-react only with desmosomes of stratified squamous epithelial tissues as determined by immunofluorescence. This raises the distinct possibility that the composition of desmosome components varies both amongst different cells in different epithelial tissues and from cell to cell within a single type of tissue (e.g., the stratified squamous epithelia of tongue and muzzle). Thus it is our belief that desmosomes are more biochemically diverse than previously thought (10, 11). Indeed, this possibility has been suggested previously (6). Moreover, Guidice et al. (17), using monoclonal antibodies directed against desmosome components, have shown diversity of desmosome composition in different tissues.

A striking difference between Pf and Pv serum samples is that the Pf samples stain the desmosomes of cultured keratinocytes, whereas the Pf samples do not. Since all our serum samples appear to contain autoantibodies that co-localize with the desmoplakin antiserum in cryostat sections of mouse skin, we wondered why these same serum samples did not all stain the desmosomes of cultured keratinocytes. We are tempted to speculate that the autoantibodies in Pv serum samples recognize desmosome-associated cell surface antigens responsible for cell adhesion in basal cells, i.e., the types of keratinocytes that grow in cell cultures. On the other hand it may be that the antigens responsible for cell adhesion recognized by Pf autoantibodies (we propose these to be desmosome-associated based on the immunoblotting data and the tissue staining observations) are accessible for antibody binding only in the more differentiated cells present in the upper layers of the epidermis. Indeed, our finding that one Pf serum sample stains primarily the borders of epidermal cells in the superficial layers supports this contention and also confirms reports in the literature (7, 8). In addition, this observation suggests that desmosomal components undergo some form of modification during differentiation of keratinocytes. In support of this notion, it has already been shown that the keratin subunits that comprise intermediate filaments, which are associated with desmosomes (2), undergo modification during keratinocyte differentiation in vivo (29).

Our results indicate that autoantibodies in many pemphigus patients recognize the desmosomes found in stratified squamous epithelia. The desmosomal antigens recognized by certain Pf and Pv autoantibodies are distinct and may be differ-
entially localized within the cells associated with the different layers of the epidermis. Furthermore, some desmosome-associated antigens may be found in varying amounts in different areas of the body within the same tissue type (e.g., stratified squamous epithelia). If these desmosome-specific autoantibodies are pathogenic in the disease, these results may begin to explain why only stratified squamous epithelial tissues are affected by the autoantibodies in pemphigus, and why there is regional blister formation in many pemphigus patients (e.g., in Pv, the oral mucosa may be the only region of the body affected by the disease).

In summary, the autoantibodies in pemphigus may prove to be valuable tools in the determination of the composition of desmosomes of stratified squamous epithelia. These autoantibodies can be used as probes to investigate the structural heterogeneity of desmosomes at both the morphological and biochemical levels. To this end we are currently attempting to further characterize desmosome-specific autoantibodies from pemphigus serum samples by affinity purification of monospecific antibody fractions using desmosome-derived polypeptides. We are also in the process of making chicken polypeptides. We are also in the process of making chicken gifts of the desmoplakin antiserum and also Ms. Laura Davis for her disease.

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