Abstract. Treatment of bovine tongue mucosa with 1 M KCl induced a split in the lamina densa of the basement membrane zone (BMZ). The epithelium was then separated from the underlying connective tissue. Electron microscopic analysis of the stripped epithelium revealed that hemidesmosomes and their associated intermediate filaments (IF) remain along the basal surface of the epithelium. This surface was solubilized in an SDS/urea-containing buffer. Characterization of components of this protein mixture was undertaken using human autoantibodies from bullous pemphigoid (BP) patients that have been shown to recognize hemidesmosomal plaque elements (Mutasim, D. F., Y. Takahashi, R. S. Labib, G. J. Anhalt, H. P. Patel, and L. A. Diaz. 1985. J. Invest. Dermatol. 84:47-53) and by production of mAbs. Affinity-purified autoantibodies directed against 180- and 240-kD polypeptides present in the protein preparation generated strong immunofluorescence staining patterns along the BMZ of bovine tongue mucosa. Furthermore, immunogold localization revealed that these two polypeptides are associated with the hemidesmosomal plaque. A mAb preparation directed against a 125-kD polypeptide present in this same protein mixture lamina lucida side of the hemidesmosome. Autoantibodies in BP serum samples, affinity-purified 180-kD autoantibodies and the mAb preparation generated a punctate stain along the substratum attached surface of epithelial cells maintained on glass substrata for ~1 wk. The spots appeared to be associated with bundles of IF in cultured mouse keratinocytes. These monospecific antibody probes should prove invaluable for the study of hemidesmosome structure, assembly, and function.

The hemidesmosome is a component of basal epithelial cells and is considered to play an important role in the attachment of the cell to the basement membrane zone (BMZ) of the underlying connective tissue (Staehelin, 1974). The hemidesmosome was named for its structural similarity to one half of a desmosome (Kelly, 1966). The hemidesmosome, like the desmosome, consists of an electron-dense cytoplasmic plaque, immediately subjacent to the plasma membrane (Kelly, 1966). Furthermore, as is the case with the desmosome, intermediate filaments (IF) attach to the plaque of the hemidesmosome (Kelly, 1966; Shienvold and Kelly, 1976; Ellison and Garrod, 1984). On the extracellular side of the hemidesmosome, certain fine filaments appear to arise from the hemidesmosomal plasma membrane and cross the lamina lucida of the BMZ and attach to the lamina densa (Ellison and Garrod, 1984). Some authors have proposed that these anchoring filaments then come together to form anchoring fibrils that extend down into the connective tissue (Ellison and Garrod, 1984).

Many components of both the plaque and intercellular region of the desmosome have been described (reviewed in Green and Jones, 1989). For example, desmosplakin I and II (240- and 210-kD, respectively) exist in the region of the plaque to which IF attach (Jones and Goldman, 1985); desmoglein I (150-kD) appears to be transmembraneous (Schmelz et al., 1986a,b; Jones, 1988). In contrast, the desmocollins (or desmoglein II of molecular mass 100/115-kD) are located in the intercellular space of the desmosome (Miller et al., 1987; Jones, 1988). Although it has been suggested that certain plaque constituents of the desmosome are also components of the hemidesmosome (Franke et al., 1982; Mueller and Franke, 1983; Cowin et al., 1985; Miller et al., 1987), recent reports have shown that desmoplakins I and II (Jones et al., 1986a), plakoglobin (Cowin et al., 1986) and desmoglein I (Jones et al., 1986a) are not present in the hemidesmosome. Indeed, in contrast to the extensive biochemical characterization of the desmosome, little is known about the individual components of the hemidesmosome, with the exception of hemidesmosome-associated anchoring fibrils, which have been shown to be composed of collagen VII (Sakai et al., 1986).

A number of groups have recently shown that autoantibodies in the serum samples of patients with the autoimmune blistering disease bullous pemphigoid (BP) recognize hemidesmosomal plaque components as determined by immunofluorescence and immunoelectron microscopy (Mutasim et al., 1985; Westgate et al., 1985; Regnier et al., 1985;
Jones et al., 1986a). Thus, these hemidesmosomal autoantibodies should be extremely useful probes for the elucidation of the composition of the hemidesmosome plaque. Indeed, BP autoantibodies are currently being used to monitor hemidesmosome formation in a variety of wound models (Gipson et al., 1989). However, BP serum samples are complex antibody mixtures and it has been reported that the serum of BP patients contains autoantibodies that recognize polypeptides varying in molecular mass from 165 to 240 kD present in, for example, preparations of suction blisters, dermato- mated skin strips and human keratinocyte cell cultures (Stanley et al., 1981, 1984; Labib et al., 1986). Although autoantibodies directed against human polypeptides of 230-kD have been affinity-purified from BP serum samples and have been shown to recognize elements associated with the BMZ, it has yet to be determined whether these autoantibodies recognize hemidesmosomes at the ultrastructural level (Mueller et al., 1989). Thus no data have yet been presented showing which of the autoantibodies in BP serum samples are antihemidesmosomal.

Thus, to characterize the hemidesmosome, we have developed a procedure to enrich for hemidesmosomal proteins of bovine tongue. We detail experiments in which we have characterized two hemidesmosomal plaque elements using BP autoantibodies. Furthermore, we describe an mAb directed against a third hemidesmosomal element. We show that certain cultured epithelial cells express hemidesmosomal components when maintained for prolonged periods on a glass substratum.

**Materials and Methods**

**Cell Culture**

Primary mouse epidermal (PME) cells were prepared as reported (Hennings et al., 1980; Jones et al., 1982). They were maintained at 37°C in keratinocyte growth medium (Clonetics Corp., Boulder, CO). For the preparation of bovine corneal epithelial cell explants, eyes were obtained from a local slaughter house, then rinsed with povidone iodine, then 70% ethanol and finally sterile PBS (6 mM Na+, K+ phosphate, 171 mM NaCl, 3 mM KCl, pH 7.4). Explants were removed from the periphery of the cornea with a 3-mm dermatome punch and placed epithelial side up on poly-L-lysine-coated coverslips (Ebato et al., 1987). The explants were incubated for 30 min at 37°C to allow the corneal stroma to adhere to the coverslip before the culture medium was added. Explants were maintained at 37°C in MEM supplemented with 10% FCS, 50 U/ml of penicillin, and 50 μg/ml streptomycin (Gibco Laboratories, Grand Island, NY).

**Human Sera and Antibody Preparations**

BP, normal human, and pemphigus vulgaris serum samples were generously donated to us by Nancy Furey and Ruth Freinkel (Department of Dermatology, Northwestern University Medical School, Chicago, IL). An mAb preparation directed against collagen VII was kindly provided by Robert Burgeson (Shriners Hospital, Portland, OR). A desmoplakin mAb and an mAb preparation directed against vinculin were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN) and Sigma Chemical Co. (St. Louis, MO), respectively. The rabbit antiserum directed against mouse keratin was described in Jones et al. (1982) and Jones et al. (1985). An antiserum directed against desmoplakin I and II was described in Jones et al. (1987).

**mAb Preparation**

The preparation enriched in hemidesmosomal components (~0.1 mg in SDS/urea buffer) (see Results) was mixed with CPA (Cappel Laboratories, West Chester, PA) and injected into the peritoneal cavity of BALB/C mice. After 2 wk, the mice were boosted intraperitoneally with an additional 0.1 mg of the protein preparation mixed with incomplete Freund's adjuvant and 4 d later, the mouse was killed and its spleen was removed. Spleen cells were fused with an Sp2 myeloma line according to Galfre and Milstein (1981). Cells were plated out in 24-well culture dishes (Costar Corp., Cambridge, MA). Medium from wells containing cell colonies was tested by immunofluorescence on cryostat sections of bovine tongue mucosa and by Western blotting against the protein preparation containing hemidesmosomal components (see below). Hybridoma cells in wells that contained antibodies as detected by these procedures were cloned twice by limiting cell dilution in 96-well plates (Costar Corp.) and medium from resulting colonies as restested by immunofluorescence and Western blotting (see below).

**Immunofluorescence**

Pieces of bovine calf tongue were frozen in liquid nitrogen and embedded in Tissue-Tek OCT compound (Miles Laboratories, Elkhart, IN). Sections of ~5 μm were prepared on a Tissue-Tek cryostat and placed on slides. Sections were fixed for 5 min in ~20°C acetone and air dried. Culture cells grown on glass coverslips were rinsed briefly in PBS and then fixed in ~20°C acetone for 2 min and air dried. In the case of cells processed for localization of antivinculin, cells were prefixed for 5 min in 3.7% formaldehyde in PBS before extraction in ~20°C acetone. Sections or cells on coverslips were incubated in primary antibody (i.e., either whole human serum diluted 1:20 in PBS, affinity-purified antibody or undiluted hybridoma medium in the case of the mouse mAbs) in a moist chamber overnight at room temperature. The slides and coverslips were then extensively washed in PBS. When double labels were desired, the sections or coverslips were incubated overnight in a mixture of primary antibodies as detailed in Jones et al. (1985). In order to minimize nonspecific binding of secondary antibodies, sections or cells on coverslips were incubated for 30 min at 37°C in normal goat serum diluted 1:20 in PBS and then incubated in either fluorochromes conjugated goat anti-rabbit IgG and fluorescein-conjugated goat anti-mouse IgG (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). In the case of double label immunofluorescence, a mixture of rhodamine-conjugated goat anti-mouse IgG and fluorescein-conjugated goat anti-human IgG was used. After extensive washing sections were covered with gelvatol (Monsanto Corp., St. Louis, MO) and mounted with a coverslip. Coverslip preparations were mounted on a slide in gelvatol. Immunofluorescence controls included incubation of sections and cells on coverslips in secondary conjugated antibodies alone and the use of mouse mAbs that do not recognize epithelial cells to determine nonspecific binding sites of primary and secondary antibodies. Stained sections and cells on coverslips were viewed using a Photomicroscope III (Carl Zeiss, Inc., Thornwood, NY) equipped with epifluorescence optics. Fluorescence micrographs were taken using Kodak Plus-X (Eastman Kodak Co., Rochester, NY) film. Films were developed in Diafine (Acufine, Inc., Chicago, IL) two-stage developer.

**Conventional and Immunoelectron Microscopy**

Tissue pieces of bovine tongue mucosa were fixed in 2.5% glutaraldehyde in PBS, postfixed in 1% OsO4 in PBS, rinsed in distilled water, and dehydrated and embedded as previously described (Starger et al., 1978). Thin sections were made on a Reichter Ultracut E (Reichert Instruments, Buffalo, NY) using a diamond knife and were mounted on uncoated copper grids. Thin sections were stained in uranyl acetate and lead citrate and viewed in a JEOL 100CX electron microscope (JEOL USA, Peabody, MA) at an accelerating voltage of 60 kV.

For immunogold localization on tissue specimens, 5-8-μm-thick cryostat sections of bovine tongue (see above) were placed on glass slides and were fixed for 5 min in acetone (~20°C). The sections were incubated overnight in the appropriate primary antibody at room temperature and were then thoroughly washed in PBS. The sections were preabsorbed for 30 min in normal goat serum diluted 1:20 in Tris-buffered saline containing 1% BSA (20-mM Tris-HCl, 0.9% (wt/vol) NaCl, pH 8.2) at 37°C. An appropriate gold-conjugated (5 nm) secondary antibody (Janssen Pharmaceutica, Beerse, Belgium) was then applied to the sections, which were then incubated for 6–8 h at room temperature. The sections were washed extensively, fixed in 1% glutaraldehyde, postfixed in 1% OsO4, dehydrated and embedded as described by Starger et al. (1978). Controls were similar to those detailed above for immunofluorescence with substitution of gold-conjugated secondary antibodies. Thin sections were prepared and viewed in the electron microscope as described above.
SDS-PAGE and Western Blotting Procedure

SDS-PAGE using 60% acrylamide gels with 4.5% stacking gels was performed on the bovine tongue protein preparations and bovine tongue epithelial desmosomes prepared as described in Jones et al. (1986b) that had been solubilized in 8 M urea, 1% SDS, 5% 2-mercaptoethanol and 64 mM Tris-HCl (pH 6.8) according to the procedure of Laemmli (1970). Approximately 10 μg of protein per lane was used. After SDS-PAGE, separated polypeptides were transferred to sheets of nitrocellulose (Towbin et al., 1979). Immunoblotting was carried out using the described antibodies as detailed elsewhere (Zackroff et al., 1984).

Affinity Purification of Human Autoantibodies

Affinity purification was carried out following the procedure of Olmsted (1981). A curtain polyacrylamide gel of the protein sample containing hemidesmosomal antigens was prepared and then separated polypeptides were transferred to a sheet of nitrocellulose as detailed above. After blocking of the nitrocellulose sheet in 5% nonfat dried milk in PBS, narrow vertical strips were cut off each end of the nitrocellulose sheet and reacted with BP sera. Immunoreactive polypeptides were cut from the remaining nitrocellulose by lining up the end strips and excising a narrow horizontal strip. The strips were cut into small pieces and were then incubated overnight in BP sera. Antibodies bound to the nitrocellulose were eluted and subsequently concentrated as described by Olmsted (1981).

Results

Enrichment of Hemidesmosomal Plaque Components

Thin-section electron microscopical analysis of bovine tongue mucosa reveals that hemidesmosomes occur in abundance along the basal surface of basal epithelial cells (Fig. 1 A). These hemidesmosomes have a typical ultrastructural appearance and are associated on their cytoplasmic side with bundles of IFs and on their extracellular surface with anchoring fibrils (Fig. 1 B). A typical BMZ composed of lamina lucida and lamina densa is evident immediately underlying the hemidesmosomes. (Fig. 1 B).

Tongue mucosa was incubated for 24 h, at room temperature, in an EDTA-containing buffer (6 mM Na+K+ phosphate, 120 mM NaCl, 3 mM KCl, 20 mM EDTA, and 1 mM PMSF, pH 7.0), followed by a 5-min incubation in the same buffer containing, in addition, 1 M KCl. This treatment induces a split between the epithelium and the connective tissue and allows the epithelium and connective tissue to be

Figure 1. Electron micrographs of the basal cells in the epithelium of bovine tongue mucosa. In A, note the hemidesmosomes (arrows) along the basement membrane zone (BMZ). The rectangular area marked in A, is shown enlarged in B. IF bundles (IFB) are observed in association with hemidesmosomal plaques. Underlying the hemidesmosomes, anchoring fibrils are occasionally present (arrowheads). The lamina densa (LD) and the lamina lucida (LL) of the BMZ are evident. Bar in A, 500 nm; bar in B, 250 nm.
mechanically separated (Woodley et al., 1983). As determined by EM, the split appears to occur in the lamina lucida of the BMZ (Fig. 2). Along the basal portion of the separated epithelial piece, intact hemidesmosomal plaques and their associated IFs are observed (Fig. 2 A). Anchoring fibrils remain with the connective tissue underlying the intact lamina densa (Fig. 2 B).

To enrich for the components of hemidesmosomes the stripped epithelium was briefly rinsed in PBS containing 1 mM PMSF. A small aliquot of a urea/SDS sample buffer (see Materials and Methods) was overlayed for 5 min on the basal surface of the stripped epithelium. After this treatment, EM reveals that the basal portion of the basal cells including hemidesmosomes and their associated structures is solubilized (results not shown).

To identify hemidesmosomal components present in the urea/SDS preparation described above we took two approaches. The first involved the use of hemidesmosomal autoantibodies in the serum samples of BP patients as described below. The second was the generation of mAbs after injection of this complex protein mixture into mice.

Characterization of Hemidesmosomal Plaque Components Using BP Autoantibodies

The five BP serum samples we have characterized by indirect immunofluorescence microscopy contain autoantibodies that generate typical hemidesmosomal staining in stratified squamous epithelia (see below). However, immunoblotting analyses of these five BP serum samples using the urea/SDS preparation containing hemidesmosomal components described above reveals that there are considerable differences in reactivity from one patient to another (Fig. 3). For example, autoantibodies in the serum of one patient recognize a 140-kD polypeptide, whereas little or no such reactivity is seen with the other four BP serum samples (Fig. 3, lane 4). However, there are a number of polypeptides recognized by autoantibodies in three or more of the sera samples that we have so far analyzed. Four of the BP serum samples contain autoantibodies which recognize an 180-kD protein (Fig. 3, lanes 2, 3, 4, and 6). Three of our serum samples contain autoantibodies which recognize a 240-kD polypeptide (Fig. 3, lanes 2, 5, and 6). Neither the 180- nor the 240-kD poly-
BMZ as those generated by autoantibodies in the whole serum from which they were affinity purified (results not shown). Furthermore, immunoelectron microscopical analyses of the localization of the 180- and 240-kD autoantibodies reveals that they localize to the hemidesmosomal plaque region (Fig. 5, A and B).

The SDS/urea preparation containing hemidesmosomal antigens also contains desmosomal antigens, for example the desmoplakins as shown by immunoblotting (Fig. 6). Thus, to rule out the possibility that the antihemidesmosomal autoantibodies recognize desmosomal antigens at the immunochromatographical level, we also analyzed the affinity purified autoantibody preparations by immunoblotting on enriched preparations of bovine tongue epithelial desmosomes. Both the anti-180- and 240-kD autoantibodies fail to recognize components present in the desmosome preparation (Fig. 6).

An mAb Preparation Directed against a Hemidesmosomal Plaque Component

Mice were immunized with the preparation containing hemidesmosomal components described above. After the fusion of spleen cells of immunized mice with the Sp2 myeloma line, putative hemidesmosomal mAb-producing clones were

peptides are recognized by autoantibodies in a pemphigus vulgaris serum sample or a normal human serum sample (Fig. 3, lanes 7 and 8, respectively). Thus, both the autoantibodies directed against the 180- and the 240-kD are likely candidates for those BP autoantibodies that generate the hemidesmosomal staining pattern that we detect by immunofluorescence. To determine whether this is the case, autoantibodies were affinity purified to the 240- and 180-kD polypeptides using pieces of nitrocellulose to which the 180- or 240-kD polypeptides had been transfected as described in Materials and Methods. For the affinity purification we chose to use the serum samples whose immunoblots are shown in Fig. 3, lanes 2 and 5, because the blotting pattern of lane 2 shows strong reactivity with the 180-kD polypeptide, whereas the blotting pattern in lane 5, shows little reactivity with the 180-kD polypeptide but a strong reactivity with the 240-kD polypeptide. Fig. 4 shows our success in affinity purifying both the 180- and 240-kD autoantibodies. These antibody preparations were then analyzed by indirect immunofluorescence using bovine tongue mucosa (results not shown). This reveals that the affinity-purified antibodies generate similar immunofluorescence patterns along the
Figure 5. Cryostat sections of bovine tongue were processed for immunogold localization using anti-180-kD autoantibodies (A), anti-240-kD autoantibodies (B), the mAbHD (C), and a collagen VII antibody preparation (D). Small arrows mark the position of hemidesmosomes. In A and B, gold particles are associated with the plaque of the hemidesmosome. In C, gold particles are observed close to the BMZ (arrowhead). The open arrow marks the lamina lucida side of a hemidesmosome. In D, collagen VII localizes to the lamina densa (open arrow) underlying the hemidesmosome marked by the arrow. The arrow head marks an accumulation of gold particles in association with amorphous material in the connective tissue. Bar, 250 nm.

selected by determining by indirect immunofluorescence whether they generate the type of pattern in stratified squamous epithelia which would be expected for an antibody directed against a hemidesmosome, i.e., a punctate immunofluorescence staining pattern along the BMZ similar to that generated by BP autoantibodies. The immunofluorescence pattern generated by one such mAb (an IgG termed mAbHD) is shown in Fig. 7. As in the case of the BP autoantibodies the mAbHD did not recognize sites of desmosomes in the epithelium (Fig. 7).

The mAbHD fails to stain mouse, human, and guinea pig tissues, suggesting bovine specificity (results not shown).

Immunoblotting analysis of the mAbHD antibody reveals that it recognizes a 125-kD polypeptide present in the protein preparation containing hemidesmosomal components and fails to recognize polypeptides present in an enriched preparation of bovine tongue epithelial desmosomes (Figs. 4 and 6).

To confirm that the putative hemidesmosomal antibody mAbHD recognizes a bona fide hemidesmosomal component, immunoelectron microscopy was undertaken. Although gold particles were found associated with the HD plaque, in most instances gold particles appear in the region of the HD immediately subjacent to the HD plasma mem-
Figure 6. The extract of bovine tongue basal epithelial cells (lanes 1, 3, 5, 7, and 9) and an enriched preparation of bovine tongue desmosomes (lanes 2, 4, 6, 8, and 10) were subjected to SDS-PAGE simultaneously and then transferred to nitrocellulose. Lanes 1 and 2 show amido black stains of transferred protein. Molecular weights are indicated by circles. From top to bottom: myosin, β-galactosidase, phosphorylase B, and BSA. Although the affinity purified anti-240- and anti-180-kD autoantibody preparations recognize 240- and 180-kD polypeptides present in the basal cell extract (lanes 3 and 5, respectively, arrowheads) they fail to recognize polypeptides in the desmosome preparation (lanes 4 and 6, respectively). The mAbHD, which recognizes a 125-kD polypeptide in the basal cell extract (lane 7, arrowhead), also does not recognize components in the desmosome preparation (lane 8). An antiserum directed against desmoplakin recognizes a 250-kD polypeptide in the basal cell extract (lane 9) and 250- and 220-kD polypeptides in the desmosome preparation (lane 10). The latter (desmoplakin I and II) are indicated by arrows in the desmosome preparation (lane 2).

Figure 7. A cryostat section of bovine tongue mucosa processed for double-label indirect immunofluorescence using a BP serum sample (A) and the mAbHD (B). Note the punctate stain along the dermal–epithelial border. The epithelium is at the left. Bar, 50 μm.
brane, the region where anchoring filaments are located (Fig. 5 C). This type of localization is quite distinct from that observed using an antibody preparation directed against collagen VII, which immunogold localization reveals is located along the lamina densa and in certain electron amorphous areas in the connective tissue (Sakai et al., 1986) (Fig. 5 D).

**Expression of Hemidesmosomal Antigens in Cultured Epithelial Cells**

PME were grown on glass coverslips and maintained in KGM for up to 3 wk. The level of Ca\(^2+\) in the KGM is 0.15 mM. At this level of Ca\(^2+\) PME cells stratify and, furthermore, the cells possess desmosomes as determined by EM (results not shown). Such cultured cells were then processed for indirect immunofluorescence using whole BP serum samples and in addition the affinity-purified 240- and 180-kD autoantibody preparations. Approximately 50% of the cells are stained by the BP autoantibodies 1 wk after plating them onto glass coverslips. This percentage of positively stained cells does not appear to change if cells are maintained for up to 3 wk.
2 wk in culture. Both the whole serum sample and the 180-kD antibody preparation, which was affinity purified from the whole BP serum, generate a spotty fluorescence pattern in PME cells maintained in culture for 1 wk (Fig. 8). By carefully focussing through cells, the bright fluorescence spots appear to be located along the substratum surface of the cell. The 240-kD antibody preparation fails to generate any obvious staining in the PME cells even though the whole serum sample from which these autoantibodies were purified generates a similar staining pattern to that shown in Fig. 8.

Because the whole BP serum sample and the affinity-purified anti-180-kD autoantibodies generate similar immunofluorescence staining patterns in the cultured cells for the remaining studies that we describe we use the whole BP serum whose immunoblot is shown in Fig. 3, lane 2.

Because the immunofluorescence patterns generated by the BP autoantibodies in PME cells may represent rudimentary hemidesmosomes, we wondered whether there was a relationship between the organization of the fluorescence spots generated by the BP autoantibodies and certain other adhesive sites of cultured cells termed focal contacts (Geiger, 1982). For this analysis we used an anti-vinculin mAb as a marker for such focal contacts (Geiger, 1981). Because this antibody preparation only works on formaldehyde-fixed preparations, double-label immunofluorescence analyses of cultured cells using the anti-vinculin mAb and BP autoantibodies was not possible because formaldehyde fixation greatly reduces the ability of BP autoantibodies to bind to cultured cells. Nevertheless, the type of fluorescence pattern generated by the BP autoantibodies in PME cells and that generated in comparable cells by the vinculin antibody preparation are distinct (compare Figs. 8 and 9).

Similar spotty fluorescence is observed in PME cells maintained in low levels of Ca\(^{2+}\) and processed for indirect immunofluorescence using antibodies directed against desmoplakin, a plaque element of desmosomes (Jones and Goldman, 1985). Therefore, to rule out the possibility that the autoantibodies were staining similar structures to those stained by desmoplakin antibodies, we undertook double-label immunofluorescence using BP autoantibodies and an
anti-desmoplakin mAb (Fig. 10). Although both the desmoplakin antibody preparation and BP autoantibodies generate spotty fluorescence in PME cells, the patterns do not colocalize (Fig. 10).

The spots of fluorescence generated by the BP autoantibodies appear to be aligned in rows and we wondered whether they were associated with elements of the cytoskeleton. Double-label immunofluorescence analysis of PME cells processed with BP autoantibodies and an antiserum directed against keratin reveals that the fluorescent spots localize along keratin containing IF bundles (Fig. 11).

The mAbHD antibody fails to stain PME cells, as would be expected because our tissue immunofluorescence analysis of the mAbHD suggests that the mAbHD is bovine specific. However, using a procedure described by Ebato et al. (1987) cultures of bovine corneal epithelial cells were prepared from explants of cornea. Within 2 d of plating, epithelial cells migrate from the explant onto the coverslip (Ebato et al., 1987). These cultured cells are epithelial in nature as indicated by their positive staining by antibodies directed against corneal keratins (Kurpakus et al., 1989). Within 1 wk in culture, these corneal epithelial cells stratify. As in the case of the PME cells, there is a gradual increase in the number of corneal epithelial cells stained by the BP autoantibod-
ies during the first week of culture until ~30% are positively stained at day 7. Furthermore, BP autoantibodies and the mAbHD generate similar spotty fluorescence at the substratum attached surface of these cells as determined by double-label immunofluorescence at day 7 (Fig. 12). The spots appear to be aligned in rows in a manner similar to that observed in the PME cells (compare Figs. 8 and 12).

Discussion

Hemidesmosomes appear to play an important role in the attachment of basal epithelial cells to the underlying connective tissue. However, there is little information concerning the composition of these important cell junctions. The latter is in part due to an inability to purify the hemidesmosome. This contrasts with the wealth of information on other cell junction types, i.e., desmosomes, gap junctions, and adherens junctions, for which isolation schemes have been described (Skerrow and Matoltsy, 1974; Goodenough and Revel, 1971; Hertzberg et al., 1979; Tsukita and Tsukita, 1989). Although we have not yet successfully purified hemidesmosomes, we have prepared a basal epithelial cell extract from bovine tongue epithelium which contains plaque elements of the hemidesmosome. Specifically in this study we have used BP autoantibodies to characterize two hemidesmosomal components of 240- and 180-kD using the basal cell preparation. Furthermore, we have produced an mAb preparation that recognizes a 125-kD component of the hemidesmosome. Indeed, our basal cell extract appears to be an excellent source of hemidesmosomal components and we are currently undertaking characterization of several other putative hemidesmosomal mAbs derived following injection of this protein mixture into mice. Furthermore, now that we have identified three hemidesmosomal proteins in the crude protein preparation, we are in a position to purify these polypeptides and undertake more detailed biochemical character-
Figure 12. Bovine corneal cells processed for double-label indirect immunofluorescence using BP autoantibodies (A) and the mAbHD antibodies (B). Both autoantibodies and the mAbHD stain similar linear rows of spots. Bar, 5 μm.

ization of them. Such studies should enhance our ability to gain new insights into the structure, function, and assembly of the hemidesmosome.

All three of our hemidesmosomal antibody preparations fail to recognize desmosome components. This provides further evidence that the hemidesmosome and the desmosome are immunologically distinct (Jones et al., 1986a). As discussed in Jones et al. (1986a) it is quite remarkable that although the hemidesmosome bears considerable resemblance to one-half of a desmosome, hemidesmosomal components appear to be absent from desmosomes and vice versa. This also suggests that the components of the hemidesmosome that are involved in IF–hemidesmosomal plaque interaction are distinct from desmosomal plaque–IF linkers. The characterization of hemidesmosomal plaque components should now enable us to begin to identify which, if any, of these components mediate IF–plaque interaction.

Our results support the idea of heterogeneity in BP serum samples, at least as far as their immunoblotting reactivity with epithelial cell extracts is concerned. Other workers have shown that BP serum samples recognize a variety of polypeptides in various preparations of skin and cultured keratinocytes (see, for example, Labib et al. [1986] and Bernard et al. [1989]). Recently, Mueller et al. (1989) have purified 230-kD BP autoantibodies from BP sera and have shown that these autoantibodies localize to the BMZ. However, no ultrastructural localization data by these authors were presented. It is tempting to speculate that the 240-kD autoantibodies that we have characterized are identical to the 230-kD purified autoantibodies described by Mueller et al. (1989). These 230-kD autoantibodies have been claimed to be the major reactive autoantibodies in BP sera (Mueller et al., 1989). Furthermore, the 180-kD autoantibodies that we have characterized may be the same as those detected by Labib et al. (1986) and Bernard et al. (1989) in certain BP serum samples. However, it is possible that there are species differences in the immunoblotting reactivity of BP serum samples on bovine versus human tissue specimens and therefore the above remains to be clarified. Nevertheless, our results reveal that BP serum samples may contain multiple antibodies that recognize hemidesmosomes (for example, in Fig. 3 the serum samples whose immunoblots are shown in lanes 2 and 6 contain both the 240- and 180-kD autoantibodies).

One of the interesting findings of our study relates to the pathogenicity of BP autoantibodies in the disease. As in the case of another autoimmune blistering disease of the skin termed pemphigus, BP autoantibodies may be causative factors in the disease (Anhalt et al., 1981; Haustein et al., 1984). However, this remains controversial (for a discussion of this see Gammon and Briggaman, 1988). Indeed, whereas pemphigus autoantibodies appear to bind to the cell surface and are thereby able to induce cell separation, the BP autoan-
tibodies that we have characterized here bind to cytoplasmic elements of the hemidesmosome. Thus, it is difficult to imagine how these hemidesmosomal autoantibodies are able to induce disruption of basal cell-connective tissue interaction. Rather, these hemid-smosomal autoantibodies may not be causative in the disease and may result as a consequence of the disruption of basal cells and their components which occurs in blistering areas of the skin of diseased patients.

We also show that both the 180-kD autoantibodies and the mAbHD localize in cultured cells maintained on glass coverslips. We have not detected the 240-kD antigen in cultured cells using affinity-purified 240-kD BP autoantibodies even though the whole serum, from which these autoantibodies were purified, does. The lack of staining of cultured cells by the 240-kD purified BP autoantibodies may result from too low a titer of purified 240-kD autoantibodies for detection by immunofluorescence. Alternatively, the cultured cells may not contain the 240-kD antigen or it may be masked in some way. If this is the case, then the serum sample from which the 240-kD autoantibodies were purified must contain other autoantibodies yet to be characterized that generate immunofluorescence staining patterns in cultured cells.

We have not as yet been able to determine whether the hemidesmosomal antigens expressed in cultured cells are organized into bona fide hemidesmosomes along their substratum-attached surface. Even though other authors have observed hemidesmosome-like structures in cultured corneal cells (see, for example, Billig et al., 1982), in preliminary studies we have been unable to detect hemidesmosome-like structures in our cultured cells by conventional electron microscopy (Jones, J. C. R., unpublished results). Our immunofluorescence observations suggest that the hemidesmosomal antigens are distributed towards the substratum surface of the cells, the site expected for formed hemidesmosomes. Furthermore, the hemidesmosomal antigens are associated with IF bundles in cultured cells as is the case in basal epithelial cells in situ (Staehelin, 1974). Thus these data tempt us to speculate that cultured cells may indeed possess at least rudimentary hemidesmosomal structures. Because < 50% of the cultured cells express hemidesmosomal antigens, it is possible that in our electron microscopical analyses hemidesmosomes were present in cells but we were unsuccessful in locating them.

In summary, we present characterization of three hemidesmosomal plaque elements using a combination of human autoantibodies and mAbs. The hemidesmosome is the last of the known epithelial cell junctions, i.e., desmosomes, adherens junctions, and gap junctions, to be characterized. Our hemidesmosomal antibodies should provide excellent probes for studying hemidesmosome assembly in a number of in vitro systems, including wound models and reassociation of cultured epithelial cell sheets with denuded connective tissue as elegantly described by Gipson et al. (1983). Furthermore, these probes will be of use in studying the fate of hemidesmosomes in a variety of disease states in which there are abnormalities in basal cell-connective tissue interactions such as basal cell carcinomas (Jones et al., 1989).

This work was supported by a grant from National Institutes of Health (GM-38470) to J. C. R. Jones and was aided by Basil O'Connor Starter Scholar Research Award No. 5-682 from the March of Dimes Birth Defects Foundation. J. C. R. Jones is a Junior Faculty Research Awardee of the American Cancer Society (JFRA-232).

Received for publication 13 June 1989 and in revised form 4 September 1989.

References

Anhalt, G. J., C. F. Bahn, R. S. Labib, J. J. Voorhees, A. Sugar, and L. A. Diaz. 1981. Pathologic effects of bullous pemphigoid autoantibodies on rabbit corneal epithelium. J. Clin. Invest. 68:1097-1101.

Bernard, P., L. Didierjean, F. Denis, J. -H. Saurat, and J. -M. Bonnetblanc. 1989. Heterogeneous bullous pemphigoid antibodies: detection and characterization by immunoblotting when absent by indirect immunofluorescence. J. Invest. Dermatol. 92:173-177.

Billig, D., A. Nicoll, R. Mcintyre, P. Cowin, J. Morgan, and D. Garrod. 1982. The cytoskeleton and substratum adhesion in chick embryonic corneal epithelial cells. J. Cell Sci. 57:51-71.

Cown, P., H. -P. Kapprell, and W. W. Franke. 1985. The complement of desmosomal plaque proteins in different cell types. J. Cell Biol. 101:1442-1454.

Cown, P., H. -P. Kapprell, W. W. Franke, J. Tamkun, and R. O. Hynes. 1986. Plagkoglobin: a protein common to different kinds of intercellular adhesion junctions. Cell. 46:1063-1073.

Ebatu, B., J. Friend, and R. A. Thoft. 1987. Comparison of central and peripheral human corneal epithelium in tissue culture. Invest. Ophthalmol. & Vis. Sci. 28:1450-1456.

Ellis, J., and D. R. Garrod. 1984. Anchoring filaments of the amphibian epidermal-dermal junction traverse the basal lamina entirely from the plasma membrane of hemidesmosomes to the dermis. J. Cell Sci. 72:163-172.

Franke, W. W., R. Moll, D. L. Schiller, E. Schmid, J. Kartenbeck, and H. Moller. 1982. Desmosplakins of epithelial and myocardial desmosomes are immunologically and biochemically related. Differentiation. 23:115-127.

Galfre, S., and C. Milstein. 1981. Preparation of monoclonal antibodies: strategies and procedures. Methods Enzymol. 73:3-46.

Gammnon, W. R., and R. A. Briggsman. 1988. Absence of specific histologic changes in guinea pig skin treated with bullous pemphigoid antibodies. J. Invest. Dermatol. 90:495-500.

Geiger, B. 1981. Involvement of vinculin in contact-induced cytoskeletal interactions. Cold Spring Harbor Symp. Quant. Biol. XLVI:671-682.

Gipson, I. K., S. M. Grill, S. J. Spurr, and S. J. Brennan. 1983. Hemidesmosomal antigens in formation in vitro. J. Cell Biol. 97:849-857.

Gipson, I. K., S. Spurr-Michaud, A. Tisdale, and M. Keough. 1989. Reassembly of the anchoring structures of the corneal epithelium during wound repair in the rabbit. Invest. Ophthalmol. & Vis. Sci. 30:425-434.

Goodenough, D. A., and J. P. Revel. 1971. The permeability of isolated and in situ mouse hepatic gap junctions studied with enzymatic tracers. J. Cell Biol. 50:81-91.

Green, K. J., and J. C. R. Jones. 1989. Interaction of intermediate filaments with the cell surface. In Intermediate Filaments. R. D. Goldman, and P. M. Steinert, editors. Alan R. Liss, New York. In press.

Haumore, U. F., I. Lohrisch, and K. Herrmann. 1984. Pathogenetic studies in bullous pemphigoid: review of the literature and our own results. J. Dermatol. 11:508-518.

Hennings, H., M. D. Cheng, P. M. Steinert, K. A. Holbrook, and S. H. Yusp. 1981. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. J. Cell. 19:245-254.

Hertzberg, E. L., and N. G. Bilulu. 1979. Isolation and characterization of gap junctions from rat liver. J. Biol. Chem. 254:2138-2147.

Jones, J. C. R. 1988. Characterization of a 125K glycoprotein associated with bovine epithelial desmosomes. J. Cell Biol. 89:207-216.

Jones, J. C. R., and R. D. Goldman. 1985. Intermediate filaments and the initiation of desmosome assembly. J. Cell Biol. 106:501-516.

Jones, J. C. R., A. E. Goldman, P. M. Steinert, S. Yusp, and R. D. Goldman. 1982. Dynamic aspects of the supramolecular organization of intermediate filament networks in cultured epidermal cells. Cell Motil. 2:197-213.

Jones, J. C. R., A. E. Goldman, H. -Y. Yang, and R. D. Goldman. 1983. The organizational fate of intermediate filament networks in two epithelial cell types during mitosis. J. Cell Biol. 100:93-102.

Jones, J. C. R., H. K. Steineman, and B. A. Goldsmith. 1989. Hemidesmosomes, collagen VII and intermediate filaments in basal cell carcinoma. J. Invest Dermatol. In press.

Jones, J. C. R., K. L. Vikstrom, and R. D. Goldman. 1987. Evidence for heterogeneity in the 160/165 x 10-8 M, glycoprotein components of desmosomes. J. Cell Sci. 88:513-520.

Jones, J. C. R., K. M. Yokoo, and R. D. Goldman. 1986a. Is the hemidesmosome a half desmosome? An immunological comparison of mammalian desmosomes and hemidesmosomes. Cell Motil. Cytoskel. 6:560-569.

Jones, J. C. R., K. M. Yokoo, and R. D. Goldman. 1986b. Further analysis of pemphigus autoantibodies and their use in studies on the heterogeneity, structure and function of desmosomes. J. Cell Biol. 102:1109-1117.

Kelly, D. E. 1965. Fine structures of desmosomes, hemidesmosomes, and an adependral globular layer in developing newt epidermis. J. Cell Biol.
