

Surface Relocation of $\alpha_6\beta_4$ Integrins and Assembly of Hemidesmosomes in an In Vitro Model of Wound Healing

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Abstract. A transmembrane extracellular matrix receptor of the integrin family, $\alpha_6\beta_4$, is a component of the hemidesmosome, an adhesion complex of importance in epithelial cell–connective tissue attachment (Stepp, M. A., S. Spurr-Michaud, A. Tisdale, J. Ellwell, and I. K. Gipson. 1990. *Proc. Natl. Acad. Sci. USA.* 87:8970–8974; Jones, J. C. R., M. A. Kurpakus, H. M. Cooper, and V. Quaranta. 1991. *Cell Regulation.* 2:427–438). Cytosolic components of hemidesmosomes include bullous pemphigoid (BP) antigens while extracellular components include a 125-kD component of anchoring filaments (CAF) and collagen type VII–containing anchoring fibrils.

We have monitored the incorporation of the $\alpha_6\beta_4$ integrins into forming hemidesmosomes in an in vitro wound-healing explant model. In epithelial cells recently migrated from the edges of unwounded sites over bare connective tissue, $\alpha_6\beta_4$ first appears along the entire cell surface. At this stage, these cells contain little or no cytosolic hemidesmosomal components, at least as detectable by immunofluorescence using BP autoantibodies, whereas they are already positive for laminin and CAF. At a later stage, as cells

become positive for cytosolic hemidesmosome components such as BP antigens as well as collagen type VII, $\alpha_6\beta_4$ becomes concentrated along the basal pole of the epithelial cell where it abuts the connective tissue of the explant.

Polyclonal antibodies to β_4 do not interfere with the migration of epithelial cells in the explant. However, they prevent assembly of hemidesmosomal complexes and inhibit expression of collagen type VII in cells that have migrated over wound areas. In addition, they induce disruption of established hemidesmosomes in nonmigrating cells of the unwounded area of the explant. Monoclonal antibodies to α_6 have a more dramatic effect, since they completely detach epithelial cells in the unwounded area of the explant. Antibodies to CAF also detach epithelial cells in unwounded areas, apparently by inducing separation between epithelium and connective tissue at the lamina lucida of the basement membrane zone. These results suggest a model whereby polarization of $\alpha_6\beta_4$ to the basal surface of the cells, perhaps induced by a putative anchoring filament–associated ligand, triggers assembly of hemidesmosome plaques.

INTEGRINS are a family of transmembrane extracellular matrix receptors (Hynes, 1987; Ruoslahti and Piersbacher, 1987; Humphries, 1990). These heterodimeric glycoproteins are composed of noncovalently associated α and β subunits and have been grouped into three major subfamilies based on the type of β subunit (Hynes, 1987; Hemler et al., 1987; Springer et al., 1987; Ginsberg et al., 1988). Furthermore, some α subunits can complex with more than one β subunit (Cheresh et al., 1989).

In epithelial cells several integrin complexes have been identified. The $\alpha_2\beta_1$ and $\alpha_3\beta_1$ complexes occur on apical and lateral surfaces of basal keratinocytes (Klein et al., 1990; Carter et al., 1990a,b; DeLuca et al., 1990; Larjava et al., 1990). These same complexes also localize to sites of cell–cell contacts in cultured epidermal keratinocytes (Larjava et al., 1990; DeLuca et al., 1990; Kaufmann et al., 1989). In some cases, $\alpha_3\beta_1$ is observed in regions of epi-

thelial cell–connective tissue interactions (Carter et al., 1990a,b) and in cultured keratinocytes both $\alpha_2\beta_1$ and $\alpha_3\beta_1$ are localized to focal adhesions (Carter et al., 1990a,b), which are sites of close attachment between the cell and the underlying substrate as determined by interference reflection microscopy (Burrige et al., 1988). These data support the notion that β_1 integrins are dedicated to cell–cell contacts in epidermis, but in some cases may become involved in cell–matrix adhesion.

In addition to $\alpha_2\beta_1$ and $\alpha_3\beta_1$, a third major integrin expressed in epithelium is the $\alpha_6\beta_4$ complex (DeLuca et al., 1990; Carter et al., 1990b; Klein et al., 1990). Several groups have shown that this complex localizes to the basement membrane zone (Kajiji et al., 1989; Stepp et al., 1990; Carter et al., 1990b; DeLuca et al., 1990; Jones et al., 1991). Most recently immunogold electron microscopy has revealed that $\alpha_6\beta_4$ is specifically associated with hemides-

mosomes, certain specialized epithelial cell components (Stepp et al., 1990; Jones et al., 1991), which are involved in cell-extracellular matrix attachment (Stahelin, 1974).

In cultured human keratinocytes $\alpha_6\beta_4$ colocalizes with a 230-kD plaque component of the hemidesmosome in structures which Carter et al. (1990b) have termed "stable anchoring contacts." These workers suggest that $\alpha_6\beta_4$ /230-kD complexes may mediate cultured cell adhesion to extracellular matrix in addition to $\alpha_3\beta_1$ - and $\alpha_2\beta_1$ -containing focal adhesions. Jones et al. (1991) reported that $\alpha_6\beta_4$ is associated with hemidesmosomes in a novel transformed cell line termed 804G (Riddelle et al., 1991). More importantly, Jones et al. (1991) observed that incubation of 804G cells with antibodies directed against the $\alpha_6\beta_4$ complex inhibited hemidesmosome assembly in 804G cells although these antibodies did not prevent cell-substrate interaction. The latter result is consistent with the work of Carter et al. (1990b, 1991) who presented evidence that adhesion and migration of freshly plated keratinocytes is initially mediated by α_3 while more stable $\alpha_6\beta_4$ -containing stable anchoring contacts form later.

Although the work of Jones et al. (1991) strongly suggests that $\alpha_6\beta_4$ is important for hemidesmosome morphogenesis, the role that this integrin plays in the establishment and maintenance of epithelial cell-connective tissue interactions is unclear. These interactions appear to be mediated by the hemidesmosome and its associated structures in the extracellular matrix such as anchoring filaments and anchoring fibrils (Stahelin, 1974; Kelly, 1966). The former span the basal lamina and connect the plasma membrane to the lamina lucida whereas collagen type VII-containing anchoring fibrils underlie the lamina densa and may act like the roots of a tree to anchor the whole complex into the connective tissue (Kelly, 1966; Sakai et al., 1986). Following the suggestion of Gipson et al. (1987), we will use the term "adhesion complex" to refer to the structure consisting of hemidesmosome, anchoring filaments, and anchoring fibrils.

To begin to answer questions concerning the role of $\alpha_6\beta_4$ in both the assembly and function of the adhesion complex, we have examined the temporal and spatial appearance of $\alpha_6\beta_4$ during the establishment of new epithelial cell-con-

nective tissue interactions in an in vitro epiboly wound model (Kurpakus et al., 1990). In addition, using adhesion assays, we show that antibodies directed against both α_6 and β_4 appear to inhibit hemidesmosome assembly in this system and disrupt formed adhesion complexes. Intriguingly, we show that an antibody preparation directed against an anchoring filament component also disrupts adhesion complexes in epithelial explants. We discuss the possibility that anchoring filaments act as the ligand(s) for $\alpha_6\beta_4$ in the hemidesmosome.

Materials and Methods

Explant Culture

Bovine corneal explants were prepared and maintained in culture as described in Kurpakus et al. (1990). For living explant antibody adhesion assays, corneal explants were maintained for 72 h in culture medium containing a 1:50 dilution of the rabbit antiserum 5710, a 1:50 dilution of the supernatant of the rat monoclonal antibody GoH3 which had been extensively dialyzed against explant medium before addition to the explants, or the mouse monoclonal antibody anti-CAF (component of anchoring filaments)¹ at various concentrations ranging from 100 $\mu\text{g/ml}$ to 1 mg/ml (see below). In certain experiments, the explant medium contained heat-inactivated FBS. For controls, explants were incubated in medium containing a 1:50 dilution of preimmune rabbit serum, normal mouse IgG, a fibronectin receptor antiserum (see below), or irrelevant rat and mouse monoclonal antibodies for 72 h.

Antibodies

The characterization of rabbit antiserum 5710, directed primarily against the β_4 subunit of $\alpha_6\beta_4$, has been reported (Kajiji et al., 1989; DeLuca et al., 1990; Tamura et al., 1990; Jones et al., 1991). Monoclonal antibody AA3, which also recognizes β_4 , has been characterized (Tamura et al., 1990). Antibodies 5710 and AA3 recognize bovine epithelial hemidesmosomes as determined by immunofluorescence and electron microscopic localization (Jones et al., 1991). A monoclonal antibody directed against α_6 (GoH3) was a gift to V. Quaranta from A. Sonnenberg, (Department of Immunohaematology, Service of the Netherlands Red Cross Central Laboratory of Bloodtransfusion, Amsterdam, The Netherlands) and has been shown to recognize corneal hemidesmosomes (Stepp et al., 1990). The fibronectin receptor antiserum R255 was a gift to V. Quaranta from E. Ruos-

1. Abbreviations used in this paper: BP, bullous pemphigoid; CAF, component of anchoring filaments.

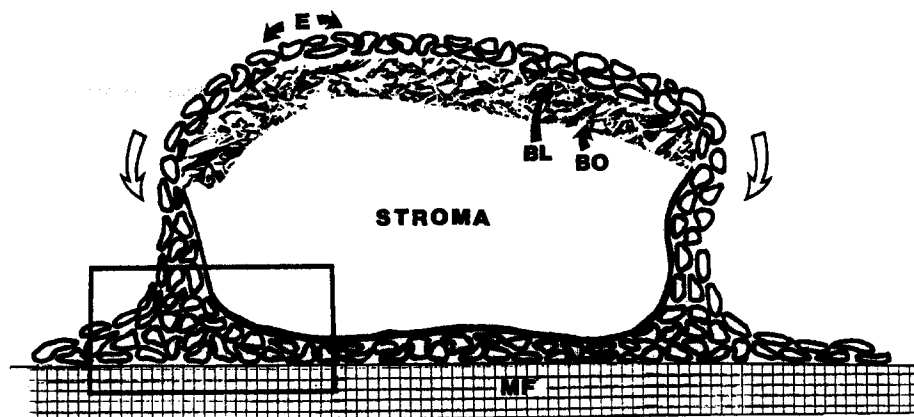


Figure 1. A diagram representing the epiboly model used in this study. A portion of corneal material including epithelium (E), basal lamina (BL), Bowman's layer (BO), which underlies the basal lamina of the cornea, and the stroma are placed in culture on a Millipore filter (MF). Over a period of ~ 24 h epithelial cells migrate from the epithelial sheet on the top of the explant in the direction marked by the curved arrows and eventually by ~ 72 h encapsulate the entire explant. Kurpakus et al. (1990) have shown that adhesion complex formation occurs along the sides and bottom of the explant where epithelial cells have epithelialized previously bare stroma within 72 h in culture. The region marked by the rectangle is that shown in subsequent figures unless otherwise stated.

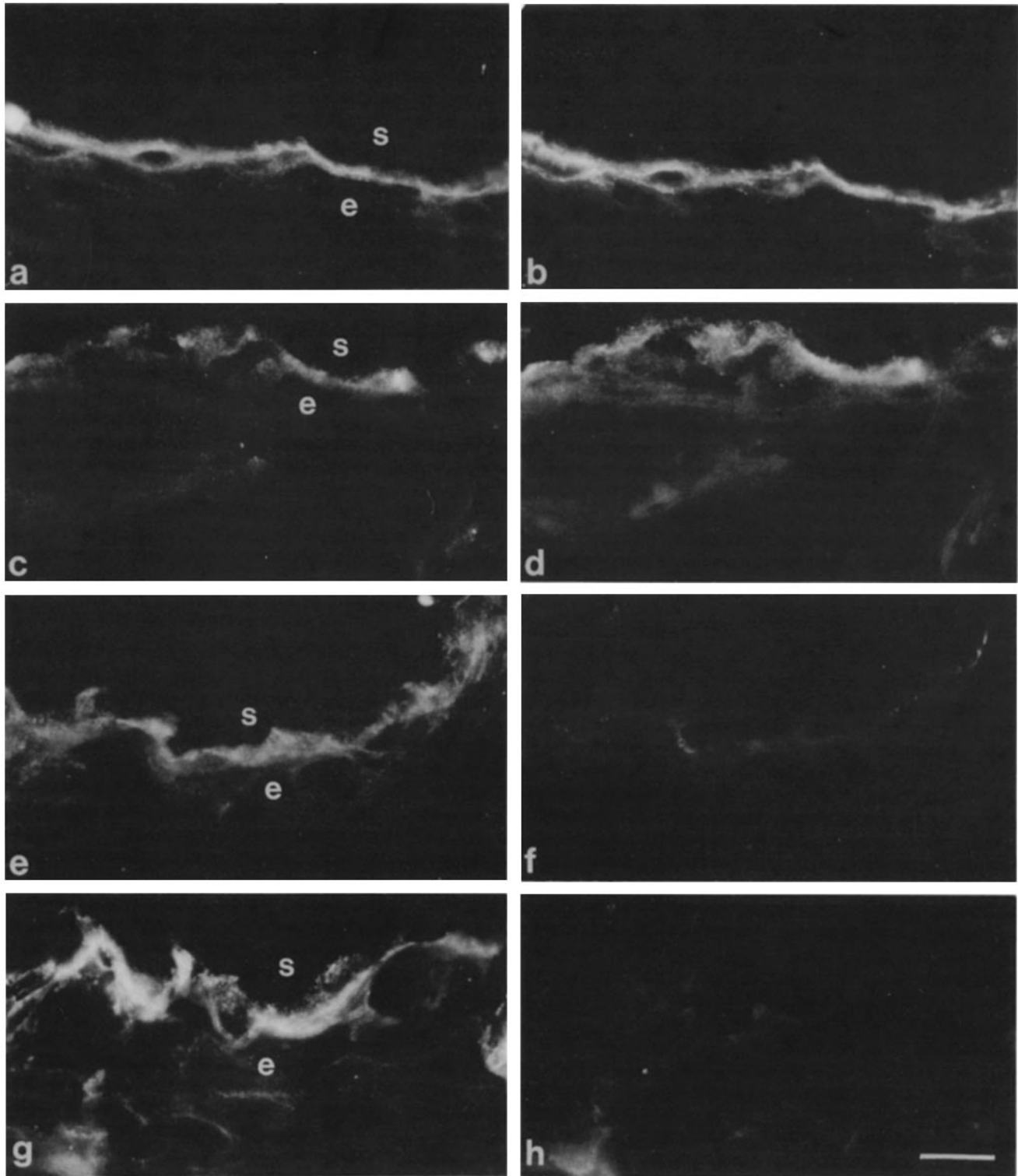


Figure 2. Cryostat sections of the explants at 48 h in culture prepared for double-label immunofluorescence using antiserum 5710 (*a*) and anti-CAF (*b*); AA3 (*c*) and a laminin antiserum (*d*); antiserum 5710 (*e*) and BP autoantibodies (*f*); and antiserum 5710 (*g*) and monoclonal antibodies directed against collagen type VII (*h*). The region of the explant that is shown in these micrographs is indicated in Fig. 1. Note that the antiserum 5710 (*a*, *e*, and *g*), anti-CAF (*b*), AA3 antibodies (*c*), and the laminin antiserum (*d*) generate similar staining along the region of epithelial cell-connective tissue interaction. In contrast, both BP autoantibodies (*f*) and the collagen type VII antibodies (*h*) generate little or no staining along the same surface. *e*, epithelium; *s*, stroma. Bar, 50 μ m.

lahti, (La Jolla Cancer Research Foundation, La Jolla, CA) and has been shown to inhibit cell attachment to fibronectin (Cardarelli and Pierschbacher, 1987).

A human serum sample containing bullous pemphigoid (BP) autoanti-

bodies directed against two hemidesmosomal plaque components of 180- and 230-kD proteins present in cornea and tongue has been previously described and was a gift from R. Freinkel, Department of Dermatology, Northwestern University Medical School, Chicago, IL (Kurpakus and

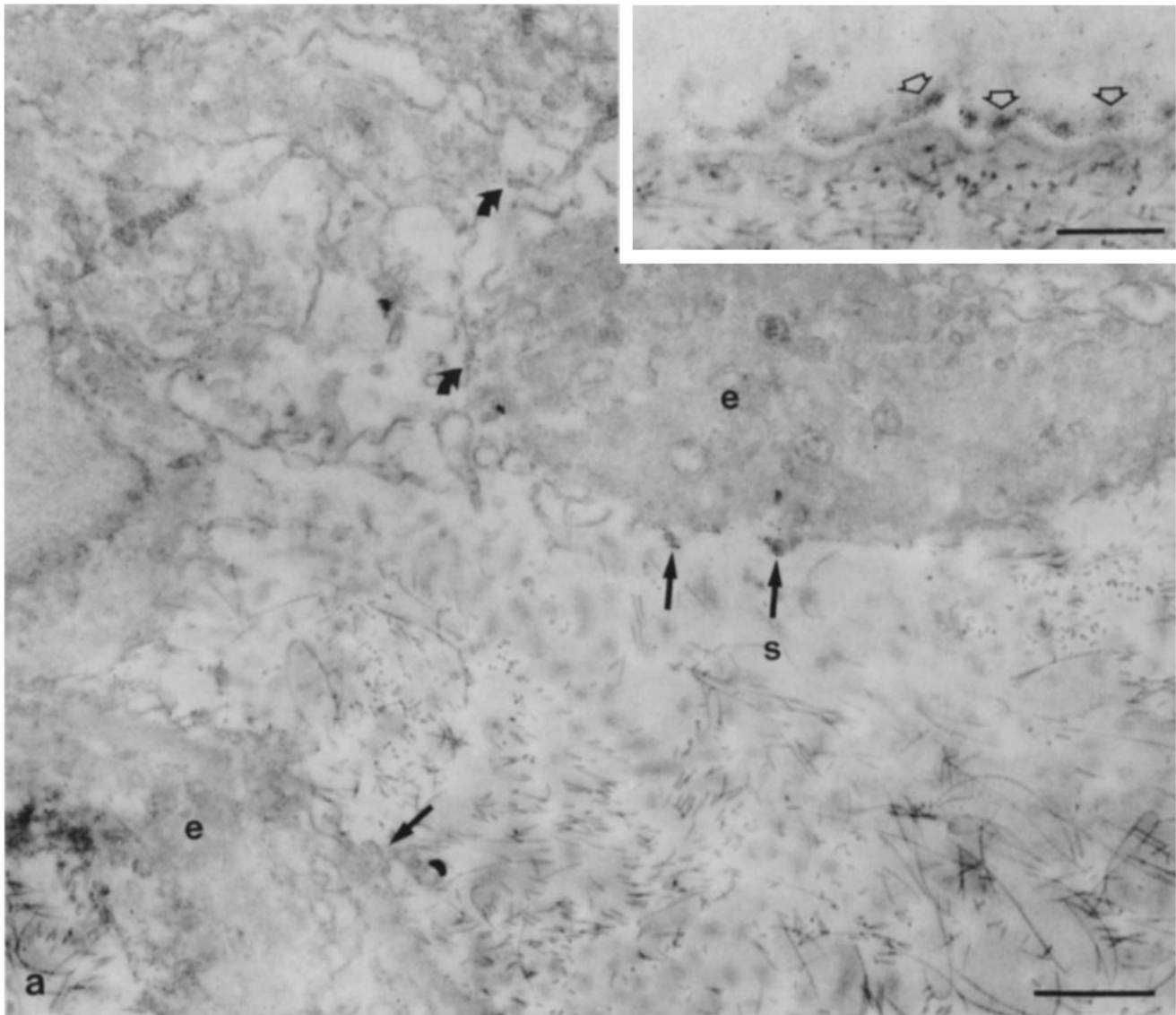


Figure 3.

Jones, 1991). Autoantibodies directed against collagen type VII from a serum sample of a patient with epidermolysis bullosa acquisita were provided by N. Furey, Department of Dermatology, Northwestern University Medical School, and were characterized in Kurpakus et al. (1990). The monoclonal antibody preparation mAbVII, directed against collagen type VII, has been characterized elsewhere (Kurpakus et al., 1990). A rabbit antiserum directed against laminin was obtained from Telios Pharmaceutical, Inc. (San Diego, CA).

A mouse monoclonal IgG antibody, directed against a 125-kD polypeptide CAF, has been described in Klatte et al. (1989) and Kurpakus et al. (1990). Anti-CAF antibodies colocalized with hemidesmosomal plaque components in cultured bovine corneal epithelial cells (Klatte et al., 1989). To prepare anti-CAF for living explant incubations, hybridoma cells were grown in a serum-free medium (PFHMII, Gibco Laboratories, Grand Island, NY; Bethesda Research Laboratories, Gaithersburg, MD) and antibody was isolated and concentrated from the medium by ammonium sulfate precipitation as detailed in Kurpakus et al. (1990). The antibody was subsequently dialyzed extensively against PBS before use.

Immunofluorescence and Immuno- and Conventional Electron Microscopy

After 24–72 h in culture, explants were frozen in liquid N₂ and processed for single- and double-label immunofluorescence microscopy as previously detailed (Kurpakus et al., 1990). Over 20 explants were analyzed at each time point. Alternatively, explants were fixed in 1% glutaraldehyde and then

processed for conventional electron microscopy and embedded in Epon/Araldite, (Tousimis Research Corp., Rockville, MD) (Starger et al., 1978). At least five explants were processed for each time point in this way. For immunoelectron microscopy 8- μ m thick frozen sections of liquid N₂ frozen material were prepared on a cryostat (Tissue-Tek, Miles Labs. Inc., Elkhart, IN) and were processed as previously detailed using appropriate gold-conjugated secondary antibodies (Amersham Corp., Arlington Heights, IL) (Klatte et al., 1989).

Both 1- μ m-thick and ultrathin sections of plastic-embedded material were prepared using an ultramicrotome (LKB Instruments, Inc., Gaithersburg, MD). 1- μ m sections were stained with Toluidine blue, viewed in bright field on a photomicroscope (Model III, Carl Zeiss, Inc., Thornwood, NY) and photographed (Panatomic X film, Eastman Kodak Co., Rochester, NY). Ultrathin sections were stained in uranyl acetate followed by lead citrate. Ultrathin sections were viewed at 60 kV in an electron microscope (model 100CX, JEOL USA, Electron Optics Division, Peabody, MA).

Results

The Appearance of the $\alpha_6\beta_4$ Integrin during Adhesion Complex Formation in an In Vitro Model

A diagram of the in vitro epiboly model used in this study is shown in Fig. 1. In this model, epithelial cells migrate from the top portion of the explant where intact epithelium

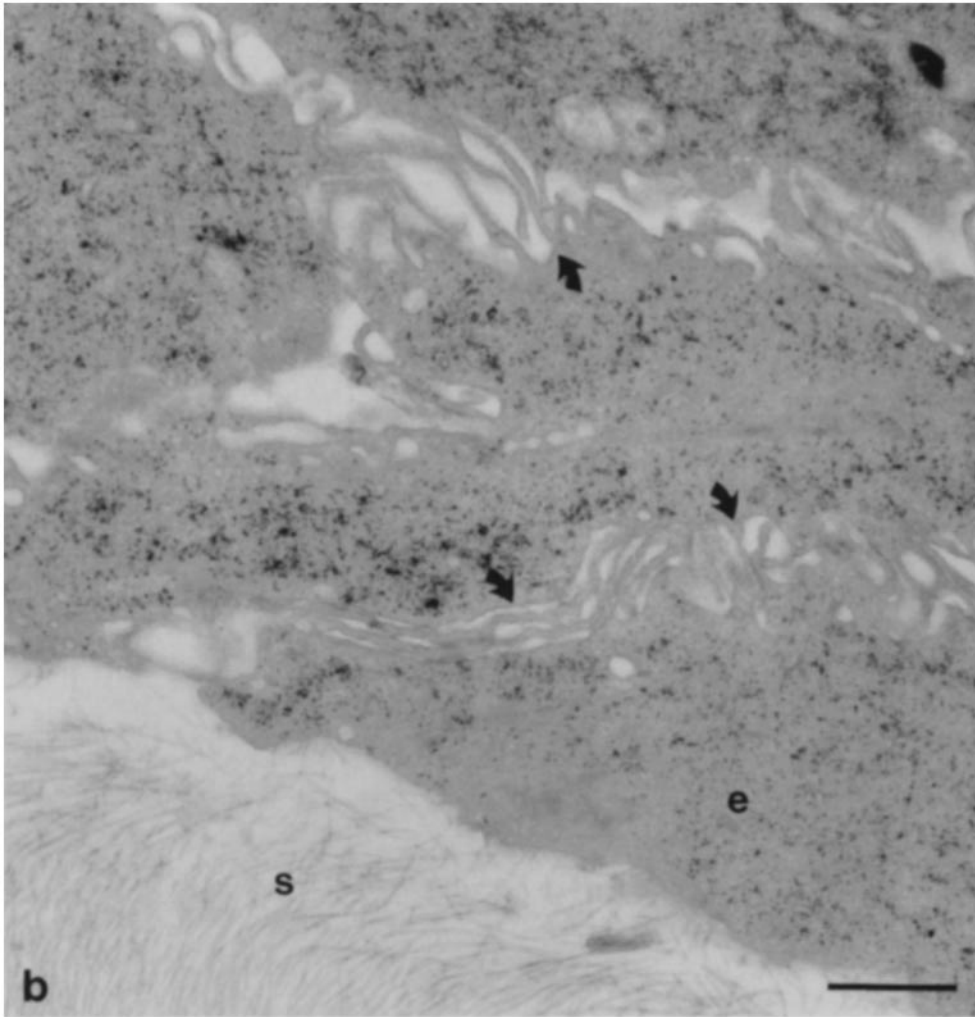


Figure 3. At 24 h in culture a cryostat section of an explant was processed for immunogold ultrastructural localization using antiserum 5710 (a). Note that gold particles are grouped in patches along the region of epithelial cell-connective tissue interaction (a, arrows). Furthermore, gold particles also occur along the highly convoluted apical and lateral surfaces of these cells (a, curved arrows). For comparison, see the convoluted surfaces of epithelial cells in the conventionally prepared electron micrograph of an explant maintained in culture for 48 h that is shown in b (curved arrows). The inset in a shows a region along the top of the explant (where there are intact hemidesmosomes) prepared for ultrastructural localization with antiserum 5710. Note that gold particles are concentrated over hemidesmosomes (open arrows). e, epithelium; s, stroma. Bars: (a and b) 1 μ m; (inset) 0.5 μ m.

and underlying connective tissue are situated (unwounded area) over exposed connective tissue matrix (wounded area) located along the sides and bottom of the explant (Kurpakus et al., 1990). This model allows analysis of the de novo for-

mation of the adhesion complex, consisting of the hemidesmosome and its associated components such as anchoring fibrils and anchoring filaments, in the absence of an intact basement membrane zone.

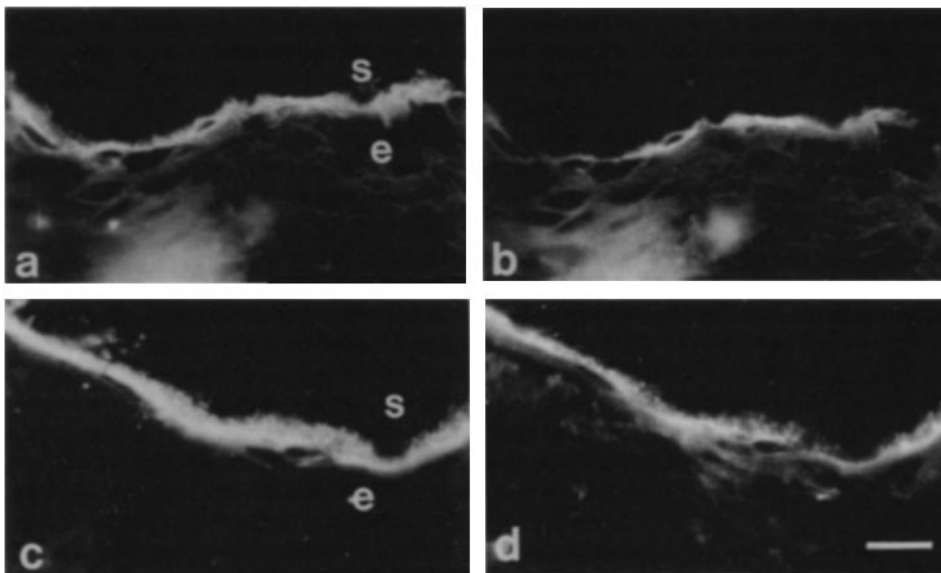


Figure 4. Cryostat section of explants at 72 h in culture prepared for double-label immunofluorescence using antiserum 5710 (a) and BP autoantibodies (b) or antiserum 5710 (c) and monoclonal antibodies directed against collagen type VII (d). Compared with Fig. 2, both BP autoantibodies and the collagen type VII antibodies generate bright fluorescence along the epithelial cell-connective tissue interface (b and d). e, epithelium; s, stroma. Bar, 50 μ m.

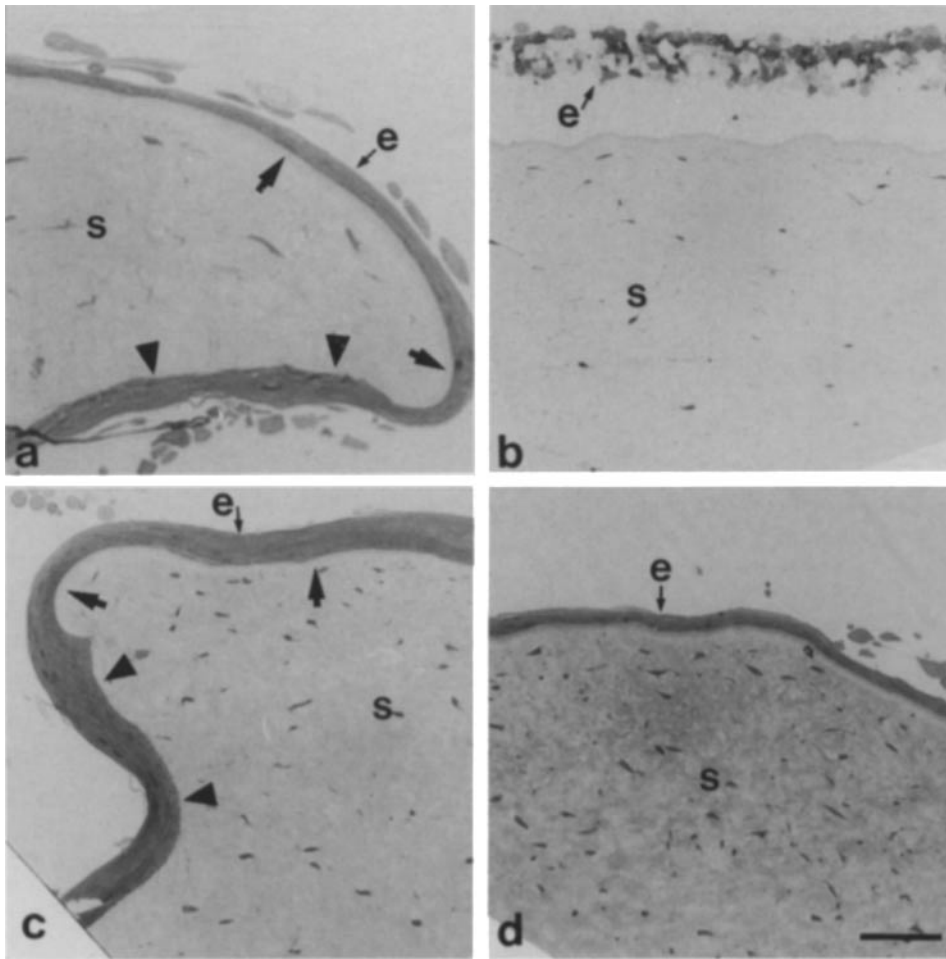


Figure 5. Explants incubated for 72 h in medium containing a 1:50 dilution of antiserum 5710 (*a*), a 1:50 dilution of GoH3 (*b*), a 1:50 dilution of the fibronectin receptor antiserum R255 (*c*), or 100 $\mu\text{g/ml}$ of anti-CAF (*d*) were fixed and embedded in Epon/Araldite and section at 1 μm ; the sections were subsequently stained with Toluidine blue. In *a* and *c* the top of the explant (arrows) and a region along the side (arrowheads) are shown. In *b* and *d* the top of the explants only are shown. Note that antiserum 5710, the R255 antibodies or anti-CAF have no effect either on the integrity of the epithelium on the top of the explant or on the migration of cells over the sides of the explant (*a*, *c*, and *d*). GoH3 antibodies induce complete detachment of the epithelial sheet from the underlying connective tissue (*b*). *e*, epithelium; *s*, stroma. Bar, 250 μm .

Using this epiboly system, Kurpakus et al. (1990) and Kurpakus and Jones (1991) have shown that in those epithelial cells which have migrated over the bare connective tissue both the 125-kD CAF polypeptide (Klatte et al., 1989) and laminin appear before detectable hemidesmosomal plaque components and anchoring fibril components. To compare the temporal and spatial appearance of $\alpha_6\beta_4$ integrins relative to these other elements of the adhesion complex, we undertook a series of double-label immunofluorescence microscopical analyses of frozen sections of corneal explants after various times in culture. Sections from at least 20 explants from different experiments were analyzed with each antibody combination.

Between 24 and 48 h in culture, antiserum 5710, which primarily recognizes β_4 (Kajiji et al., 1989; DeLuca et al., 1990; Tamura et al., 1990; Jones et al., 1991) generated a diffuse labeling in those cells which had migrated over and were in contact with the wound area, i.e., the bare connective tissue matrix along the sides and bottom of the explants (Fig. 2 *a*). Indeed, the staining appeared to envelop those cells that are in contact with the matrix. This type of labeling pattern was comparable to that previously described for anti-CAF (Fig. 2 *b*; Kurpakus et al., 1990) and was also seen using antibody AA3 directed against β_4 (Fig. 2 *c*) and antibody GoH3 which recognizes α_6 (results not shown). At these early time points, the labeling generated by 5710 and anti-CAF colocalized (Fig. 2, *a* and *b*). Laminin antibodies

also colocalized with both anti-CAF (Kurpakus et al., 1990) and α_6 and β_4 integrin antibodies such as AA3 (Fig. 2, *c* and *d*). In contrast, the hemidesmosomal plaque components recognized by BP autoantibodies (Fig. 2 *f*) and collagen type VII (Fig. 2 *h*) were barely detectable by immunofluorescence microscopy at these time points.

To extend these light microscopic results, explants at 24 h in culture were processed for immunogold ultrastructural localization using antibody 5710, followed by 5-nm gold-conjugated secondary antibody. In epithelial cells which had migrated over the wounded area, gold particles were clustered along the region of epithelial cell-stromal interaction (Fig. 3 *a*) and also along the highly convoluted lateral surfaces of the same cells (Fig. 3 *a*; compare with Fig. 3 *b*). On the top side of the explants at 24 h in culture, gold particles were associated primarily with hemidesmosomes on the basal surface of the nonmigrating cells of the unwounded area (Fig. 3 *a*, *inset*).

With increasing times in culture the fluorescence pattern generated by antiserum 5710 or the monoclonals AA3 and GoH3 was more discretely localized to the basal surface of migrating cells along the epithelial cell-explant matrix interface on the sides and bottom of the explants (compare Figs. 2 and 4). This change, which occurred around 72 h in culture, parallels that observed with anti-CAF (Kurpakus et al., 1990). Furthermore, between 48 and 72 h in culture both hemidesmosomal plaque components detected by BP au-

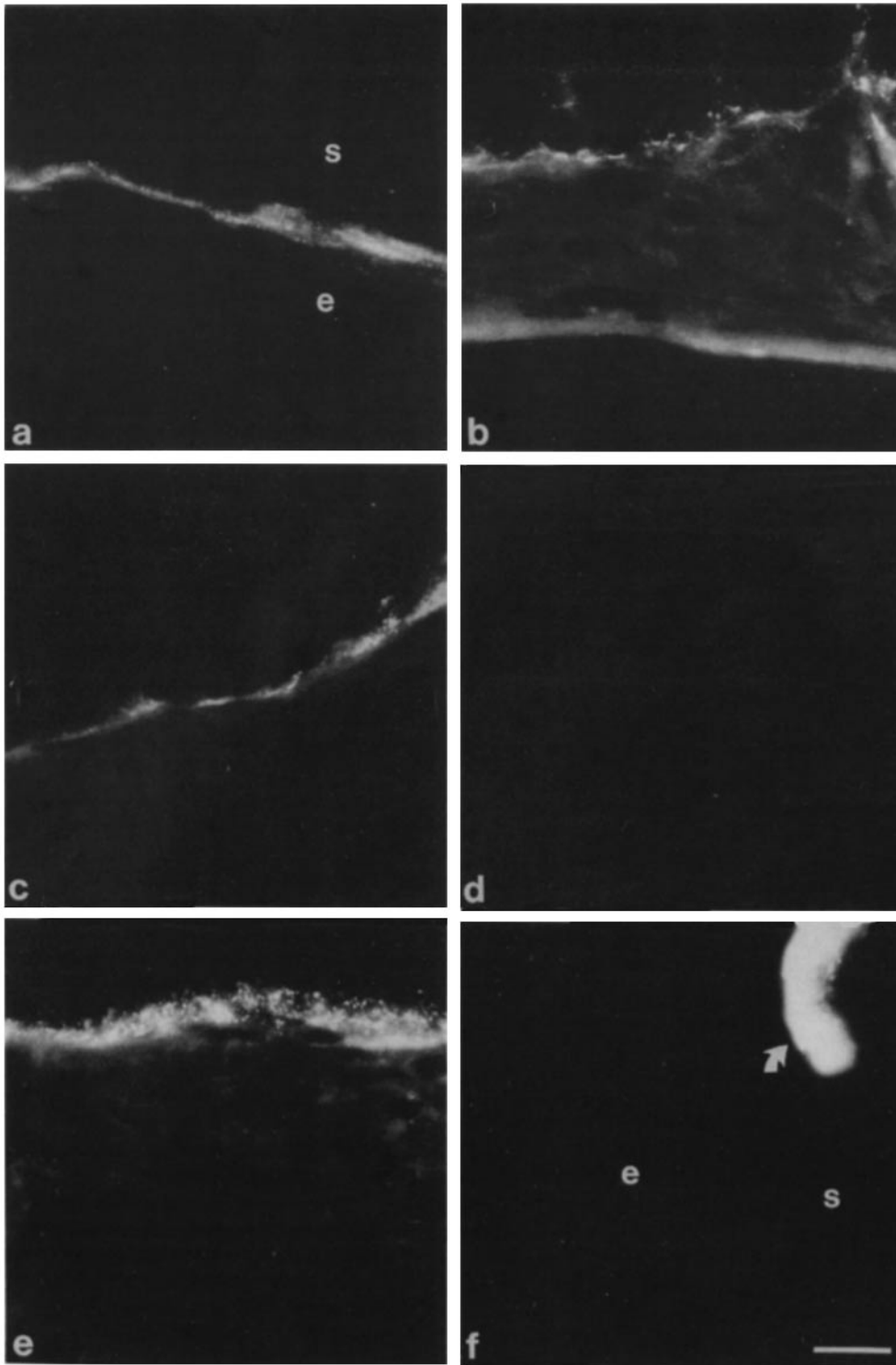


Figure 6. Explants incubated for 72 h in medium containing a 1:50 dilution of antiserum 5710 were processed for immunofluorescence microscopy using anti-CAF (a), a laminin antiserum (b), BP autoantibodies (c), and a monoclonal antibody directed against collagen type VII (d and f). As a control an explant was incubated in medium containing a 1:50 dilution of the preimmune serum 5710 for 72 h and was then processed for immunofluorescence microscopy using the collagen type VII antibody preparation (e). Note that anti-CAF, laminin antibodies, and BP autoantibodies all generate staining along the epithelial cell connective tissue interface in a manner comparable to that observed in Fig. 4. In contrast, collagen type VII antibodies fail to stain the epithelial cell-connective tissue interface along the bottom and side of the antiserum 5710-treated explants compared to the control (compare d with e). The collagen type VII antibodies generate intense staining of the basal lamina region along the top of each antiserum 5710-treated explant but the staining dramatically ends where epithelial cells have covered previously bare stroma (arrow in f). e, epithelium; s, stroma. Bar, 50 μ m.

toantibodies and collagen type VII began to be strongly expressed in cells covering the wounded area (Fig. 4, b and d, respectively). It should be noted that both the BP autoantibodies and the collagen type VII antibodies generated a discrete, localized fluorescence pattern that was always observed in the basal pole of the epithelial cells in direct contact with the stroma (Fig. 4, b and d).

Effect of $\alpha_6\beta_4$ Integrin Antibodies on In Vitro Adhesion Complex Formation

To gain more information about the functional role of $\alpha_6\beta_4$ integrin in the process of adhesion complex assembly, corneal explants were placed into culture for 72 h in medium containing antiserum 5710 or GoH3 antibodies. As controls,

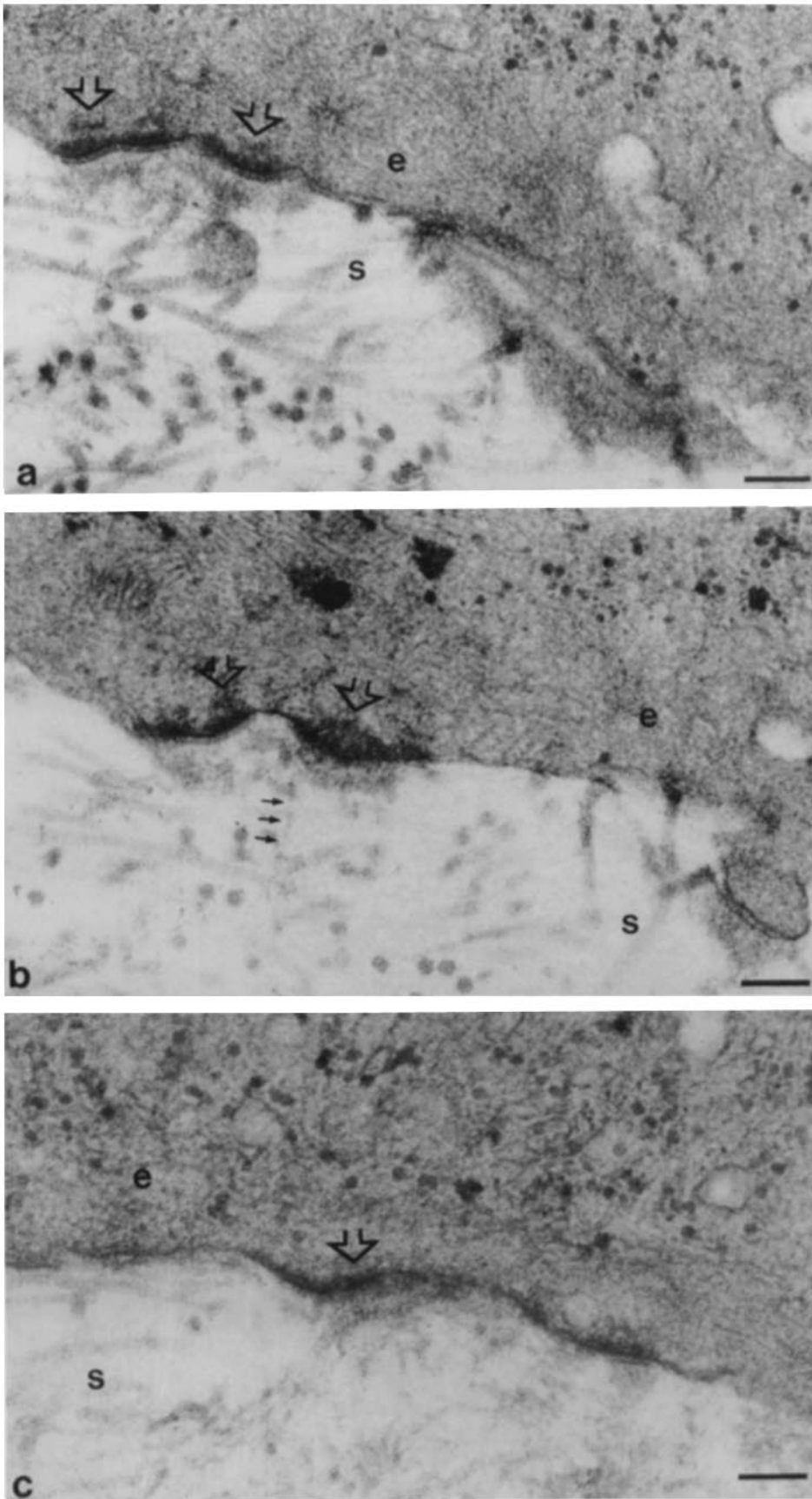
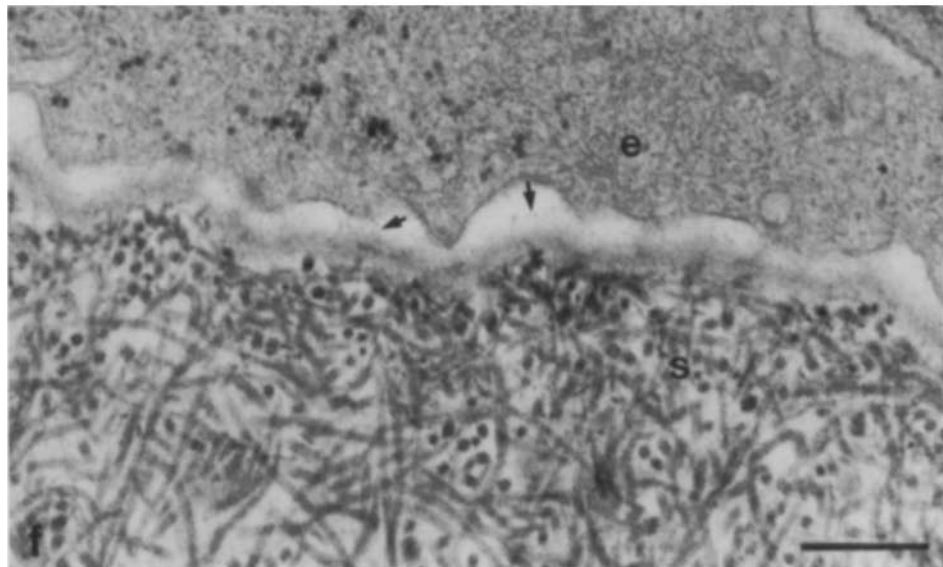
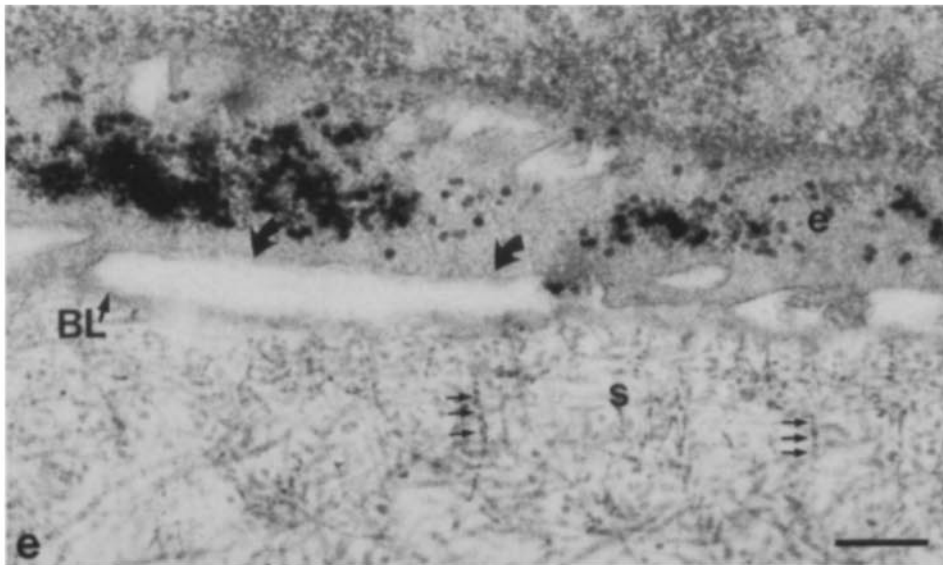
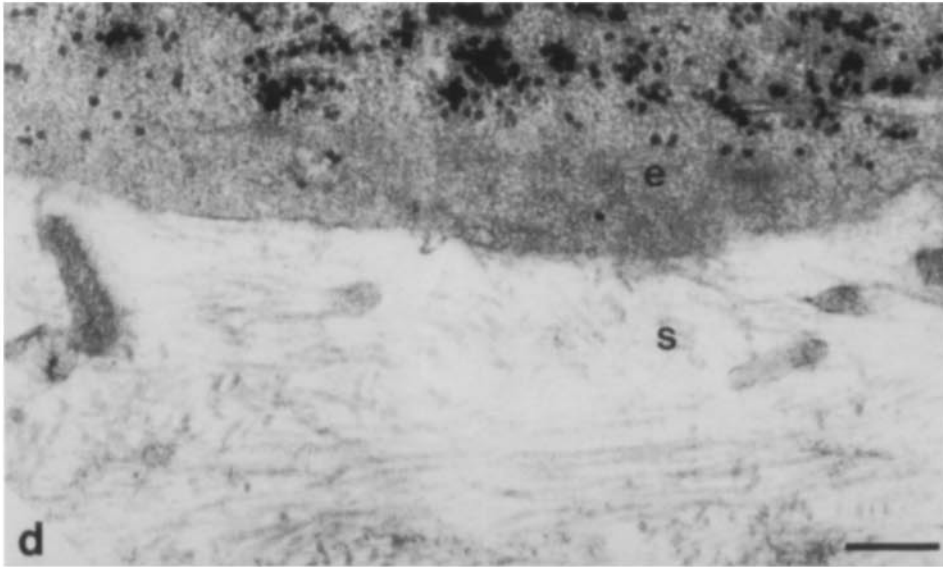


Figure 7. Electron micrographs of explants maintained in culture for 72 h either showing regions of epithelial cell-stromal interaction towards the bottom side of the explant (see Fig. 1 for orientation) (*a-d*) or areas along the top of the explant (*e* and *f*). The explants shown in *a* and *b* were incubated in medium with no added antibodies. In *c* the explant was incubated for 72 h in medium containing a 1:50 dilution of the fibronectin receptor antiserum. The explants in *d-f* were incubated for 72 h in medium containing a 1:50 dilution of antiserum 5710. Note the hemidesmosomal plaques each with an underlying basal dense plate in *a-c* (*open arrows*). The triple arrows in *b* mark a possible immature anchoring fibril. *d* shows that no hemidesmosomal plaques are observed in antiserum 5710-treated explants in regions where epithelial cells have surfaced previously bare stroma. Along the top of the antiserum 5710-treated explants, few if any hemidesmosomal plaques can be seen (*e* and *f*). Furthermore, although there are obvious anchoring fibrils underlying the basal lamina (*BL*) (*e*, *triple arrows*), in certain regions there is a disruption in epithelial cell-connective tissue adherence (*e*, *curved arrows*). In *f*, where there is a loss of attachment of the epithelial cell to the basal lamina, cross sectional profiles of anchoring filaments can be seen (*f*, *arrows*). *e*, epithelial cell; *s*, stroma. Bars: (*a-c*) 0.1 μm ; (*d-f*) 0.25 μm .



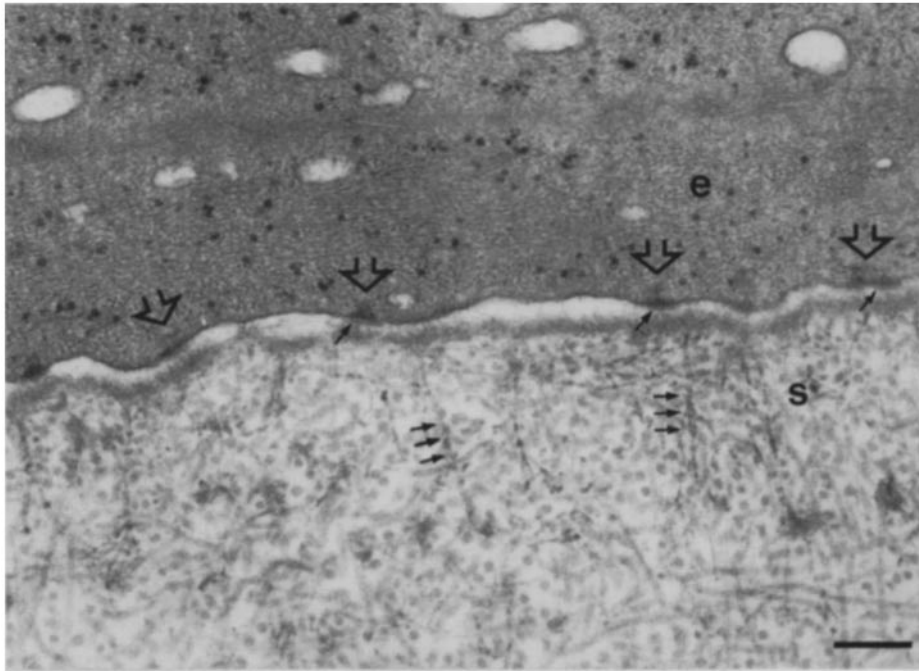


Figure 8. Electron micrograph showing the top portion of an explant maintained in culture of 72 h. Note the hemidesmosomal plaques (*open arrows*), each underlaid by a sub-basal dense plate (*small arrows*). Anchoring fibrils underneath the basal lamina are indicated by the triple arrows. *e*, epithelium; *s*, stroma. Bar, 0.1 μm .

explants were incubated in preimmune serum, normal IgG, and irrelevant rat monoclonal antibodies as detailed in Materials and Methods. More than 20 explants were analyzed for each adhesion assay from two different experiments. We chose 72 h for the incubation period since, in control explants, the majority of epithelial cells in contact with the connective tissue express all of the adhesion complex antigens for which we possess antibodies at this time, and formed hemidesmosomes are observed by electron microscopy (see below; Kurpakus et al., 1990).

Light Microscopic Analyses. At 72 h in culture, incubation of explants in the presence of antibody 5710 appeared to have no effect on migration of epithelial cells over the bare connective tissue as shown in Fig. 5 *a* in at least 20 explants studied. In addition, the antiserum had no obvious effect on the unwounded region of the explant, at least as far as light microscopical analyses of 1 μm -thick sections of Epon/Araldite-embedded material were able to reveal (see below for ultrastructural analyses).

Expression and localization of CAF, laminin, or BP antigens in those epithelial cells which had epithelialized previously bare connective tissue were similar to that seen in controls; this was revealed by immunofluorescence microscopy of antiserum 5710-treated explants at 72 h in culture (Fig. 6, *a-c*). However, in contrast, in antibody-treated explants collagen type VII expression was dramatically affected (Fig. 6 *d*). No obvious deposition of collagen type VII was observed in epithelial cells interacting with the connective tissue either along the sides or the bottom of the explant (Fig. 6 *d*). This was markedly different from the strong staining generated by collagen type VII antibodies in comparable areas in explants treated with preimmune serum (Fig. 6 *e*). Furthermore, collagen VII staining in the basement membrane zone of the unwounded top area of the treated explant appeared normal (Fig. 6 *f*). In total, 20 treated and 20 control explants were analyzed in this way and similar results were obtained using both a monoclonal antibody preparation

against collagen type VII and human anticollagen type VII autoantibodies.

The effects of α_6 antibodies on adhesion complex assembly were assessed by incubating explants in culture medium containing GoH3 antibody (Fig. 5 *b*). This antibody preparation induced complete detachment of the epithelial sheet along the unwounded top portion of over 20 explants that were examined within 72 h of placing the explants in culture (Fig. 5 *b*). It was remarkable that the detached epithelial sheet remained intact under these conditions. Moreover, similar disruption of epithelial-connective tissue integrity was observed even in explants incubated in GoH3 antibodies for as short a period as 24 h (result not shown).

As an additional control for our living explant assays, we incubated explants in medium containing antibodies directed against the fibronectin receptor for 72 h. This antibody treatment did not affect the ability of the cells to migrate around the explant edges or to deposit adhesion complex components at the cell-connective tissue interface, and cell adhesion to the top portion of the explant was not disrupted at the light microscopic level (Fig. 5 *c*) (see below for electron microscopic examination of such treated explants).

Electron Microscopic Analyses. To determine the effects of the integrin antibodies at the ultrastructural level, at least five control and five antibody-treated corneal explants were processed for electron microscopy for each experiment. Hemidesmosomes comprised of an electron-dense plaque underlaid by a subbasal dense plate occur along the wounded area at 72 h in culture both in untreated explants (Fig. 7, *a* and *b*) and those incubated in preimmune serum, normal IgG, irrelevant rat monoclonal antibodies, and antibodies directed against the fibronectin receptor (see, for example, Fig. 7 *c*).

No hemidesmosomal plaque structures were seen in epithelial cells in wounded areas in five antibody 5710-treated explants examined (Fig. 7 *d*). Moreover, along the unwounded section of the explant, antibody 5710 induced various de-

degrees of disruption of formed adhesion complexes relative to controls (Fig. 7, *e* and *f*). There was complete absence of hemidesmosomal plaques and the epithelial cells appeared to be detached from the basal lamina in antibody-treated explants (Fig. 7 *e*), in marked contrast to that seen along the top of explants maintained in culture for 72 h in the absence of antibody (Fig. 8). In certain areas cross sectional profiles of anchoring filaments were seen in the lamina lucida suggesting that anchoring filament attachment, either to the hemidesmosome or to the lamina densa, had been perturbed (Fig. 7 *f*). In all instances intact anchoring fibrils were observed in treated explants in the unwounded area.

Effect of Anti-CAF on Adhesion Complexes in the Explants

The above results revealed that $\alpha_6\beta_4$ antibodies both inhibited hemidesmosome assembly and perturbed anchoring filaments in formed hemidesmosomes. To extend these observations, we analyzed the effect of anchoring filament antibodies on adhesion complex formation using anti-CAF. This antibody has previously been shown to recognize an extracellular epitope of a 125-kD antigen which is either an associated or structural protein of anchoring filaments as determined by immunogold ultrastructural localization (Fig. 9) (Kurpakus et al., 1990). Indeed, short-term incubation (5 h) of anti-CAF with living explants resulted in binding of antibody to the unwounded top section of the explant, the region where intact adhesion complexes occur in untreated explants (Kurpakus et al., 1990).

Explants incubated in concentrations of anti-CAF ranging from 100 $\mu\text{g/ml}$ to 1 mg/ml for 72 h were fixed and embedded in Epon/Araldite for light/electron microscopy. The antibody treatment did not inhibit cell migration over the bare stroma nor did there appear to be obvious perturbations in the attachment of the epithelial sheet to the connective tissue along the top of each explant, at least at the light microscopic level of resolution, in over 20 specimens examined (Fig. 5 *d*). However, electron microscopic examination of five explants incubated with as little as 100 $\mu\text{g/ml}$ of anti-CAF revealed that in many places there was an absence of hemidesmosomal plaques and defects in epithelial cell-connective tissue interaction along the top of the explants (Fig. 10).

Discussion

In this study we have analyzed the temporal appearance of several hemidesmosomal adhesion complex components during establishment of epithelial-connective tissue interaction in a wound model. Our results reveal that $\alpha_6\beta_4$ integrins, which have recently been shown to be a component of hemidesmosomes (Stepp et al., 1990; Jones et al., 1991), are among the first components of the adhesion complex to appear at sites where migrating epithelial cells associate with the connective tissue. This appearance precedes that of both hemidesmosomal plaque components recognized by BP autoantibodies, a 200-kD plaque component recently characterized by Kurpakus and Jones (1991) and collagen type VII, a component of anchoring fibrils (Sakai et al., 1986). However, $\alpha_6\beta_4$ localization coincides temporally and spatially with laminin and CAF. In addition, antibodies directed against $\alpha_6\beta_4$ appear to block assembly of hemidesmosomal plaque structures in the explant model but do not appear to

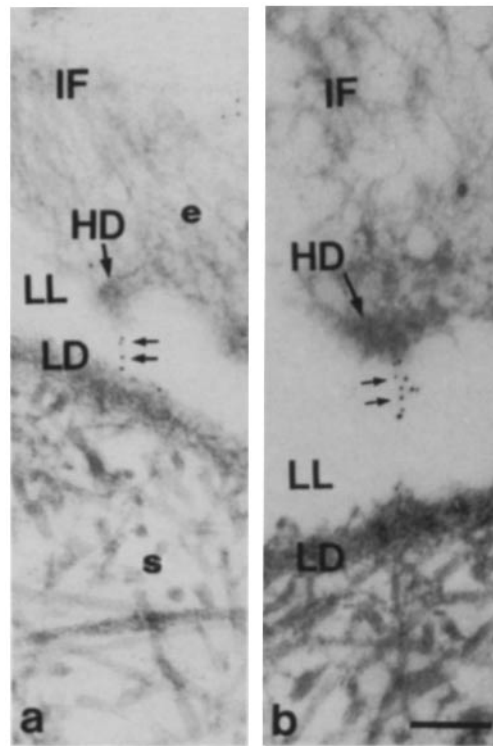


Figure 9. Immunogold ultrastructural localization of anti-CAF on cryostat sections of bovine cornea. Gold particles appear along the lengths of anchoring filaments which span the region between the plaque of the hemidesmosome and the lamina densa region of the basement membrane zone (arrows). HD, hemidesmosomal plaque; IF, intermediate filaments; LD, lamina densa; LL, lamina lucida. *e*, epithelial cell; *s*, stroma. Bar, 0.1 μm .

prevent the appearance of hemidesmosomal plaque elements at the interface between epithelial cells and the connective tissue. This is consistent with a recent study using cultured cells, in which $\alpha_6\beta_4$ antibodies inhibited hemidesmosome assembly but did not block deposition of hemidesmosomal plaque elements at the region of cell-substrate interaction (Jones et al., 1991). Indeed, taken together these results strongly support a role for $\alpha_6\beta_4$ in the nucleation of plaque formation. If this is the case then it is possible that $\alpha_6\beta_4$ together with laminin and CAF forms a template or scaffold which directs the assembly of the remaining complex components (Kurpakus et al., 1990; Jones et al., 1991).

One interesting aspect of our studies concerns the distribution of staining generated by $\alpha_6\beta_4$ antibodies during the epithelialization of wounded regions of the explants. Although $\alpha_6\beta_4$ antibody binding appears always limited to those epithelial cells in contact with the connective tissue, these antibodies stain along the entire surface of these migrating cells. Later the $\alpha_6\beta_4$ complex disappears from the lateral and apical surfaces of the cells and becomes primarily concentrated along the basal surfaces in regions of epithelial cell-connective tissue interaction. This phenomenon occurs during the same period that hemidesmosomal plaque components, CAF, and collagen type VII are detectable at the immunofluorescence level along the basal poles of the same cells. The polarization of hemidesmosomal plaque components may trigger in some way the change in distribution of $\alpha_6\beta_4$ integrins. Once this has occurred, $\alpha_6\beta_4$

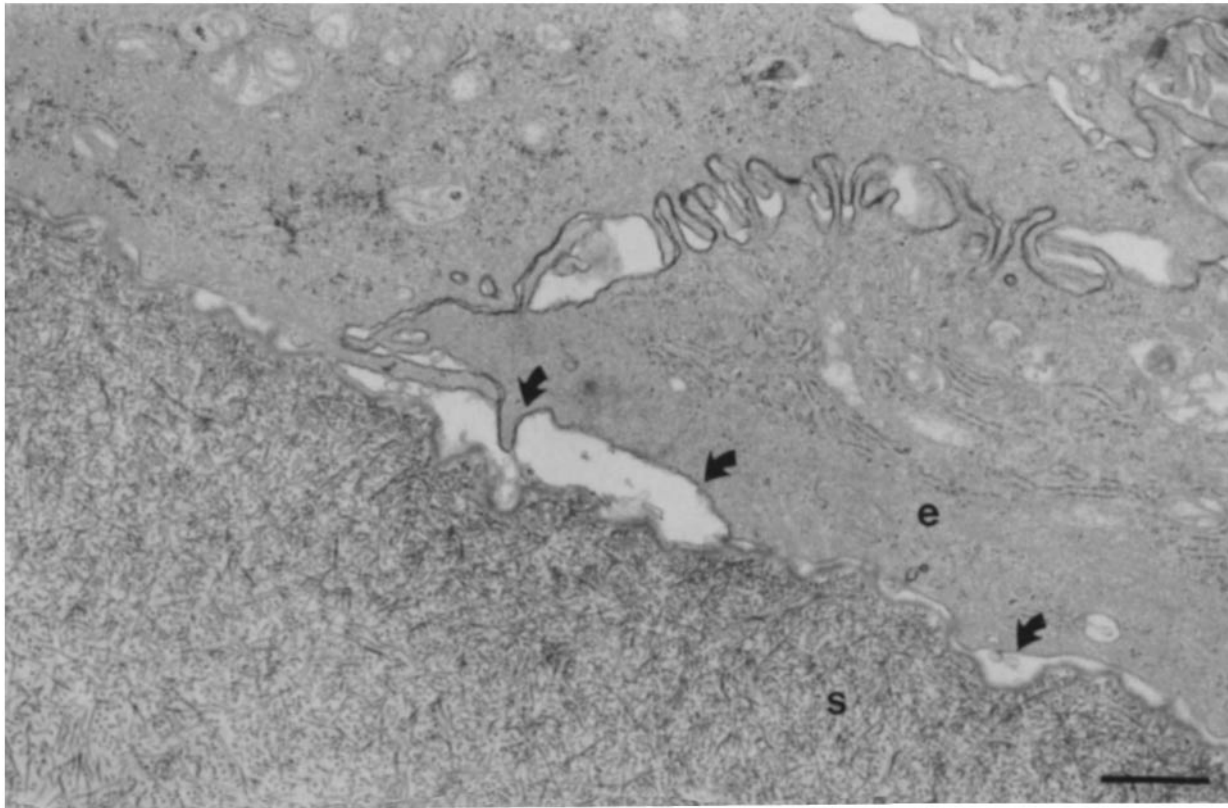


Figure 10. An electron micrograph of the top region of an explant which had been incubated for 72 h in medium containing 100 $\mu\text{g/ml}$ of anti-CAF. Note that few hemidesmosomal plaques are observed and there are regions where the epithelium has come away from the underlying connective tissue (*curve arrows*). *e*, epithelial cell; *s*, stroma. Bar, 1 μm .

could then nucleate assembly of the hemidesmosomal plaque from its cytoplasmic constituents, evidence for which we have already discussed above. These results emphasize the complex interplay among the cytoplasmic, transmembrane, and extracellular components of the adhesion complex during its assembly.

Recent reports have presented evidence that collagen type VII is made by epithelial cells and is not a product of connective tissue cell components (Regauer et al., 1990). Our preliminary data reveal that incubation of explants in medium containing antiserum 5710 dramatically changes the expression of collagen type VII by those cells which epithelialize the bare stroma. Indeed, our results suggest that antiserum 5710 induces inhibition of collagen type VII synthesis and/or instability of collagen type VII protein or even down regulation of collagen type VII message. However, we cannot rule out the possibility that prevention of hemidesmosome assembly by the 5710 antiserum may result in diffusely localized collagen type VII which may be difficult to resolve by immunofluorescence microscopy.

In addition to the effects of $\alpha_6\beta_4$ integrin antibodies on adhesion complex formation we also present evidence that the $\alpha_6\beta_4$ antibody preparations perturb established adhesion complexes in the explants. In antiserum 5710-treated explants, electron microscopical analyses reveal that anchoring filament attachment, either to the lamina densa region of the basal lamina or to the plasma membrane of an epithelial cell, may be disrupted. In α_6 -treated explants there are dramatic perturbations in epithelial cell-connective tissue interaction.

These results certainly suggest that $\alpha_6\beta_4$ integrins are involved in maintenance of the integrity of epithelial-connective tissue interaction via adhesion complexes. Furthermore, the effects of the antiserum 5710 on anchoring filaments support a notion that anchoring filaments may bind to $\alpha_6\beta_4$. Since the ligand for $\alpha_6\beta_4$ has not yet been identified, our results raise the possibility that anchoring filaments, possibly CAF, may act as the $\alpha_6\beta_4$ ligand. This would be consistent with ultrastructural localization of CAF and $\alpha_6\beta_4$ (Kurpakus et al., 1990; Stepp et al., 1990; Jones et al., 1991). However, it should be emphasized that our studies do not exclude that the ligand for $\alpha_6\beta_4$ is laminin. This is supported by our data concerning the coincident expression and localization of laminin and $\alpha_6\beta_4$ during adhesion complex formation and the occurrence of certain epitopes of laminin in the lamina lucida (Schittny et al., 1988). If laminin is in fact the ligand for $\alpha_6\beta_4$, then the binding site on laminin is a novel one as Sonnenberg et al. (1990) have discussed.

The presence of α_6 antibodies in the explant culture medium results in complete separation of the epithelial sheet from the underlying connective tissue along the top portions of every explant we have analyzed. The detached epithelial sheet remains intact suggesting that the α_6 antibodies have only minimal effects on cell-cell interaction within the epithelium. These results imply that α_6 plays an essential role in maintaining the adhesion of an epithelial sheet to the underlying connective tissue.

Antiserum 5710, which primarily recognizes β_4 , induces only partial detachment of epithelial cells from the connec-

tive tissue in treated explants. This is not the same result we observed in comparable studies using α_6 antibodies where the epithelium is completely lost from each explant. These data may first appear to be contradictory. Certainly, when we started these experiments we assumed that antibodies to β_4 and α_6 would have the same effects on epithelial cell-connective tissue integrity. However, one can hypothesize, for example, that epithelial cell-connective tissue adhesion involves both $\alpha_6\beta_4$ and additional epithelial cell receptor(s) which involve α_6 . In other words, α_6 integrin complexes not only with β_4 but also other β subunits. To date, the only β integrins known to complex with α_6 are β_4 and β_1 (Hemler et al., 1989). We have been unable to detect β_1 integrin along the basal surface of basal epithelial cells in a variety of stratified epithelial tissues by immunofluorescence microscopy using a β_1 -specific antiserum (kindly provided by Dr. Ken Yamada, National Institutes of Health, Bethesda, MD) (Kurpakus, M., and J. Jones, unpublished observations). Rather, β_1 is distributed along the apical and lateral surfaces of these same cells (Larjava et al., 1990; DeLuca et al., 1990). Since we therefore have no evidence that $\alpha_6\beta_1$ plays a role in epithelial cell-connective tissue interaction, we would have to invoke an as yet unidentified β . Another explanation for our results is that α_6 plays a more pivotal role than β_4 in regulation of extracellular binding of the $\alpha_6\beta_4$ complex to its ligand. Thus, perturbation of the $\alpha_6\beta_4$ complex with α_6 antibodies may have more deleterious effects on epithelial cell-connective tissue interaction than β_4 antibodies.

Like α_6 antibodies, anti-CAF, which recognizes anchoring filaments, induces detachment of the epithelial sheet from the underlying connective tissue on the top of the explants. This is circumstantial evidence that anchoring filaments act as extracellular ligands for $\alpha_6\beta_4$, a possibility that we have already discussed. It is now important to assess more rigorously this point at the biochemical level, particularly as we have now identified a component of the anchoring filament, namely the 125-kD polypeptide (Kurpakus et al., 1990). Furthermore, it will be of interest to determine the relationship between our 125-kD polypeptide and the recently described complexes of proteins termed epiligrin and kalinin (Carter et al., 1991; Rousselle et al., 1991). In cultured cells epiligrin may act as the extracellular ligand of both $\alpha_3\beta_1$ and $\alpha_6\beta_4$ (Carter et al., 1991). Kalinin may be the same complex of proteins as epiligrin and has been proposed to be a structural component of anchoring filaments (Rousselle et al., 1991).

In summary, our results in this model system support a role for $\alpha_6\beta_4$ in both the establishment of adhesion complexes during wound healing and maintenance of adhesion complex integrity in unwounded areas of epithelial-connective tissue interaction. It remains to be seen to what extent the in vitro epiboly system corresponds to physiopathological situations. To this end, our goal is to characterize the protein-protein interactions of $\alpha_6\beta_4$ integrins, both in the extracellular matrix and in the cytoplasm of an epithelial cell.

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