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Expression of Basement Membrane Zone Antigens at the Dermo-epibolic Junction in Organ Cultures of Human Skin

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Using the epithelial outgrowth in organ cultures of human skin ("epiboly") as a model system for basement membrane zone neogenesis, the emergence of various antigenic determinants of the junction zone (bullous pemphigoid antigen, type IV collagen and laminin) was studied and the time sequence of their appearance assessed.

All 3 antigens were found at the newly built dermo-epibolic junction; their synthesis, however, followed a distinct time sequence: bullous pemphigoid antigens emerged synchronously with the advancing tip of the migrating epithelium, whereas type IV collagen and to a greater extent, laminin, appeared with considerable delay. At the ultrastructural level, the formation of basal lamina accompanied the emergence of type IV collagen and laminin.

At the light microscopic level, the basement membrane zone of human skin appears as a homogenous, PAS-positive band. Electron microscopy, however, has revealed that it consists of at least four distinct components: (1) the plasma membranes of basal cells, which display regularly arranged hemidesmosomes, (2) the lamina lucida, an electron lucent area traversed by anchoring filaments, (3) the electron dense basal lamina and (4) the subbasal lamina fibrous components including anchoring fibrils, dermal microfibril bundles and collagen fibres [1].

The discovery of distinct antigenic determinants within the human basement membrane zone has shed some light on the nature of its constituents. Using specific antisera to type IV collagen and to laminin, a noncollagenous basement membrane protein, in either an indirect immunofluorescence or immunoperoxidase technique, type IV collagen [2] and laminin [3] were detected in a band-like pattern at the dermo-epidermal interface, in a staining pattern virtually indistinguishable from that observed with bullous pemphigoid (BP) antibodies [4]. However, as opposed to BP antibodies, antibodies to type IV collagen and laminin also stain the basement membranes of the blood vessels. In addition, ultrastructural investigations have revealed that whereas type IV collagen [2] is located mainly within the basal lamina, BP antigen [5] and laminin [3] are found in the lamina lucida of the basement membrane zone.

These distinct basement membrane antigens have also been partially chemically defined. The antigen which binds antibody from bullous pemphigoid sera has been partially characterized as glycoprotein with 2 major bands on SDS gel electrophoresis, one in the 18,000 m.w. region and the second in the 74,000 m.w. region [6]. Type IV collagen is a triple helicle molecule composed of 2 distinct chains of 160,000 and 140,000 m.w. linked by disulfide bonds [7]. Laminin is a large molecular weight noncollagenous glycoprotein composed of 2 distinct chains of 220,000 and 440,000 m.w. linked by disulfide bonds [8].

The purpose of the present study was to determine the kinetics of the emergence of these antigens and their organization into a differentiated basement membrane structure at the dermo-epibolic edge of skin in organ culure. We studied new basement membrane formation under organ culture conditions in which epidermal cells migrate from the cut edges around the dermal aspects of the skin fragments. This process ("epiboly") is, at the ultrastructural level, accompanied by the appearance of basement membrane constituents below the migrating epidermal cells [9].

In this paper we demonstrate that type IV collagen, laminin and BP antigens are synthesized at the dermo-epibolic junction, but that their appearance, as tested by indirect immunofluorescence techniques, follows a distinct time sequence. In analogy, ultrastructural investigations revealed that the extent of epiboly formation is dissociated from the formation of morphologically identifiable basement membrane constituents.

MATERIALS AND METHODS

Organ Culture System

Normal split-thickness skin was obtained from the buttocks of healthy young male volunteers following local anaesthesia with 2% Lidocaine, using a disposable dermatome (Davol, Inc., Providence, R.I.). The skin specimens were cut into square pieces measuring approximately 2 × 2 mm and were then placed on millipore filter rafts and floated in medium 199 containing 20 mM Hepes buffer, 2 mM glutamine, gentamicin 50 μg/ml and supplemented with 20% fetal bovine serum (all tissue culture materials were purchased from Flow Laboratories, Irvine, UK). The organ cultures were kept at 31°C in air. Specimens, obtained in 4 different experiments, were removed from culture at 24-hr intervals up to 7 days of culture; after a brief rinse in PBS, they were embedded in Tissue-Tek II O.C.T. compound (Lab-Tek Products, Division Miles Laboratories, Inc., Naperville, Illinois), snap frozen in liquid nitrogen and processed for immunofluorescent staining. In addition, tissue specimens from one of these experiments were processed for electron microscopic studies.

Antisera

Antibodies to type IV collagen and to laminin are those previously characterized [2,3]. Bullous pemphigoid antiserum was obtained from a patient with severe disease.

Immunofluorescence: Frozen tissue specimens were cut into 4 μm cryostat sections (Cryo-cut Microtome, American Optical Corporation) and were air dried for 20 min on albumin coated slides. Serial sections were then incubated for 30 min at 37°C with various antisera dilutions (type IV antiserum 1:80, laminin antiserum 1:80, BP serum 1:320), normal rabbit serum or normal human serum. Antibody binding was detected by a standard immunofluorescence procedure, using either FITC-labeled swine anti-rabbit IgG 1:20 (Nordic Immunological Laboratories, Tilburg, The Netherlands) for the detection of type IV collagen and laminin antibody-binding, or goat anti-human IgG 1:20

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Abbreviations:
BP: bullous pemphigoid

(Kent Laboratories, Inc., North Vancouver, Canada) to reveal BP antibody binding. Optimal working dilutions of antisera were determined by chessboard titration. The plateau-titers of the different antisera were as follows: for laminin and type IV collagen 1:160 and for BP serum 1:640. Slides were viewed under a fluorescence microscope (Orthoplan Universelles Groβfeldmikroskop, Leitz, Wetzlar), equipped for incident illumination.

Electron microscopy: The organ cultures were minced and fixed with Karnovsky's fixative for 5 hr at room temperature, rinsed 3 times in 0,1 M cacodylate buffer, pH 7,4, at 4°C, postfixed at 4°C in 3% aqueous osmic acid, stained en bloque with veronal acetate buffered uranyl acetate, and, after rapid dehydration in a graded series of alcohols, embedded in Epon 812. Ultrathin sections were cut with Reichert OM U2 ultramicrotome, stained with lead citrate and examined with a Philips 400 electron microscope.

RESULTS

1. *Kinetics of Epiboly Formation*

Migration of epidermal cells along the cut edge of the dermis was first observed after approximately 48 hr of culture. There was some individual variation in speed of migration and, consequently, in the extent of the epiboly at the undersurface of the specimen; after 4 days of culture, in most cases the dermal aspects of the explants were largely enclosed by a 2–3-layered epithelium. After 7 days, the epibolic epithelium did not exhibit any morphological alterations whereas some vacuolization and cytolysis were observed in the original epithelium.

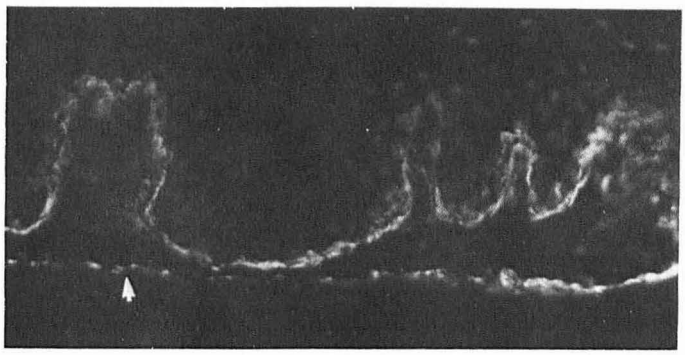

FIG 1. Tissue specimens, maintained in organ cultures for 4 days and exposed to bullous pemphigoid antiserum in an indirect immunofluorescence procedure: Note that bullous pemphigoid antibody binding is seen both along the original dermo-epidermal and the entire dermo-epibolic junction. At the former location a continous linear staining pattern, at the latter location a more spotted staining pattern is observed (*arrow*). Nuclei are counterstained with 0,001% ethidium bromide.

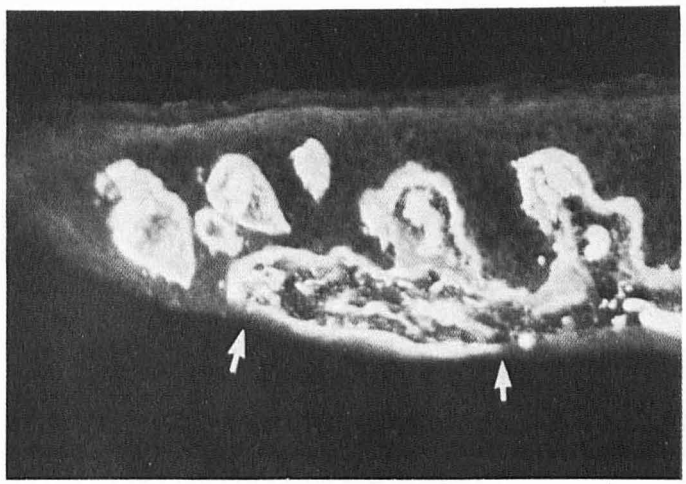

FIG 2. Tissue specimens, maintained in organ cultures for 4 days and reacted with rabbit anti-type IV collagen antiserum in an indirect immunofluorescence procedure: antibodies bind in a linear fashion along the dermo-epidermal and also at a large portion of the dermo-epibolic junction (between 2 *arrows*). In addition, the presence of type IV collagen antibodies within the basement membrane of blood vessels is revealed.

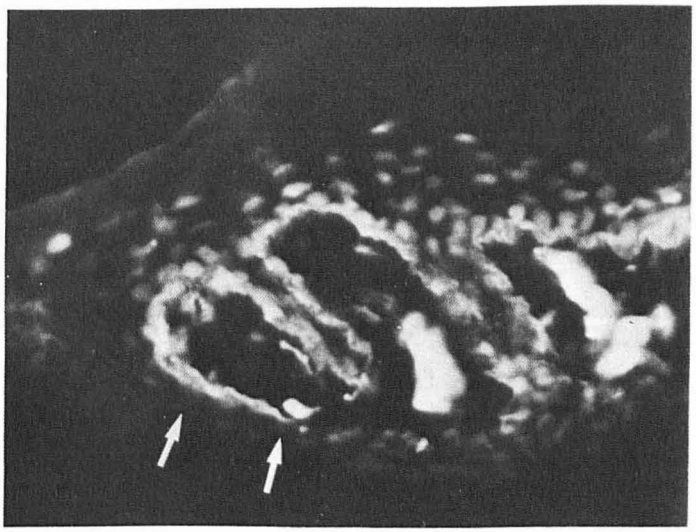

FIG 3. Rabbit antibodies to laminin produced a positive band-like staining along the length of the dermo-epidermal junction and also for a short distance along the most proximal area of the dermo-epibolic junction (between 2 *arrows*). Laminin antibody reactivity within the basement membrane of blood vessels is visible. Nuclei are counterstained with 0,001% ethidium bromide.

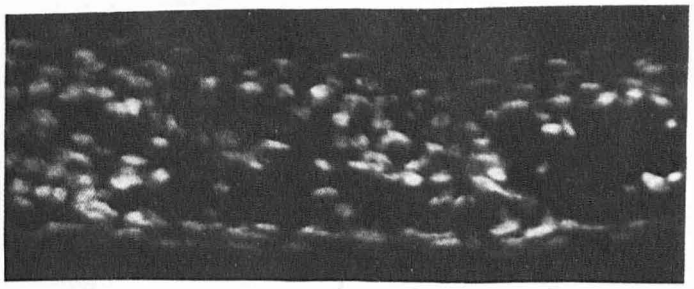

FIG 4. Control: Using normal rabbit serum in the first step of the identical indirect immunofluorescence procedure, no staining was visible at either the dermo-epidermal or the dermo-epibolic junction. Nuclei are counterstained with 0,001% ethidium bromide.

2. *Immunofluorescence Findings on the Dermo-epidermal Junction*

In all experiments, the characteristic continuous linear staining pattern along the dermo-epidermal junction was found after processing with BP serum and type IV collagen and laminin antisera; with the latter 2 sera, intense staining of the basement membranes of dermal vessels was observed. The staining remained unchanged in pattern and intensity throughout the whole observation period.

3. *Immunofluorescence Findings on the Dermo-epibolic Junction*

a. BP Antibody: binding was consistently found throughout the whole length of the epibolic epithelium extending to the foremost migrating cells at any stage of epithelial migration (Fig. 1).

b. Type IV collagen: In 3 of 4 experiments type IV collagen was not detectable at any portion of the dermo-epibolic junction when tissue specimens were reacted with the appropriate antiserum after 2 or 3 days of culture. In only one experiment did

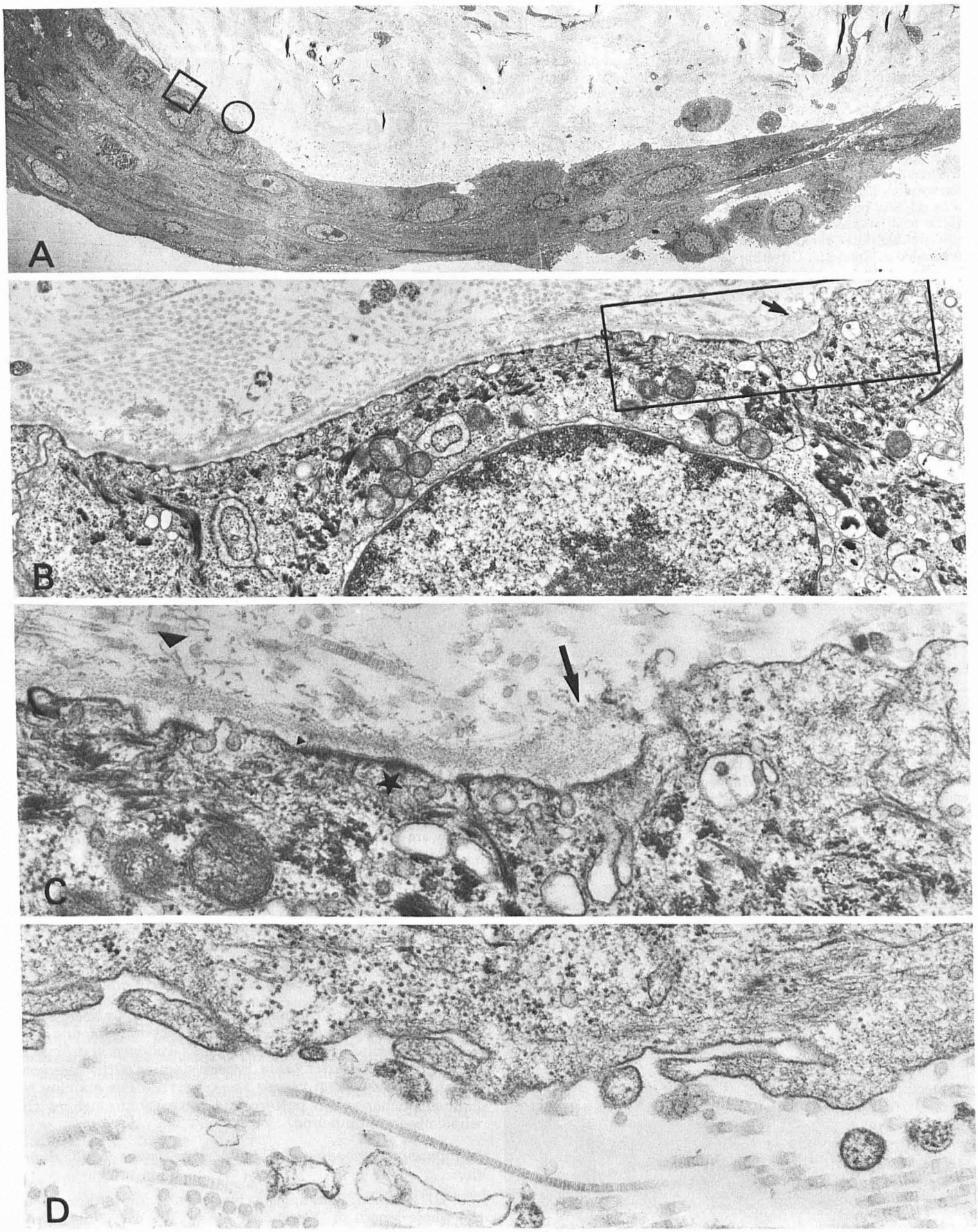

FIG 5*A*. Electronmicrograph revealing epibolic epithelium at the undersurface of an organ culture at 7 days. *Rectangular* and *encircled areas* will be disclosed in further detail in figures 5*B* and *D* (reduced from × 1,000). *B*, Higher magnification of area enclosed by rectangle in Fig 5*A*, showing basal lamina and end point of basal lamina (*rectangular area*) (reduced from × 17,000). *C*, Higher magnification of cut-off point of basal lamina structure (*arrows*) (reduced from × 50,000). Large ▼ anchoring fibrils, ★ hemidesmosomes, small ▲ anchoring filaments. *D*, Higher magnification of the area encircled in Fig 5*A*. Note that epithelium rests on dermal ground substance and collagen without an intervening basal lamina structure (reduced from × 50,000).

type IV antibody reactivity extend to epibolic areas directly adjoining the basement membrane zone of the original epidermis whereas the more distant portions were completely devoid of antibody binding. From day 4 to day 7 a consistently increasing portion of the basement membrane zone below the epibolic epithelium expressed type IV collagen antigenic determinants, slowly proceeding from the original dermo-epidermal junction, but never reaching the advancing tip (Fig 2).

c. Laminin: Similar to the appearance of type IV collagen antigenic determinants, laminin was first detected in the dermo-epibolic junction on the fourth day of organ culture, but was always confined to the most proximal areas of epiboly (Fig 3). In the subsequent days we observed the continuing extension of laminin antibody binding along the dermo-epibolic junction. However, areas which displayed laminin antibody reactivity never extended as far along the newly formed basement membrane zone as did the areas exhibiting type IV staining, which suggests that laminin may be formed more slowly than type IV collagen.

d. Controls: Substituting normal rabbit serum for rabbit antiserum to type IV collagen and laminin or normal human serum for BP serum produced no staining by indirect immunofluorescence at either the dermo-epidermal or the dermo-epibolic junction (Fig 4).

4. *Basal Lamina Neogenesis*

On the first 2 to 3 days of culture, the epidermal cells were observed migrating directly on the cut surface of the dermal collagen (Fig 5*A*); basal lamina was not present underneath the epibolic epithelium. Basal lamina formation along the dermo-epibolic junction was first seen on day 4 (5*B*, 5*C*) but by day 7 was still lagging far behind the advancing tip of the migrating epithelium (5*D*). Hemidesmosomes, anchoring filaments and anchoring fibrils were plentiful at the newly formed junction zone (Fig 5*c*).

DISCUSSION

In organ cultures of human skin, epidermal cells begin to migrate from the cut edges around the free dermal aspect of the explant within 1-2 days [10]. During subsequent days of organ culture the dermis is partially or completely encircled by the migrating epithelium [11]. This *in vitro* process corresponds to the *in vivo* process of wound healing. The time sequence of morphologic changes taking place during both processes have been studied at both the light and electron microscopic level: in wound repair Odland and Ross [12, human system] and Krawczyk [13, mouse system] observed the appearance of new hemidesmosomes and, at the same time, the formation of basement membrane components beneath the migrating epidermal cells. Extending these investigations, Briggaman, Dalldorf, and Wheeler [14] demonstrated in a series of recombination experiments that the newly formed basal lamina is primarily derived from the epidermal cells. Similar events have been reported to occur during epiboly formation. Newly formed basal lamina below the migrating epithelium was found by Sarkany and Gaylarde [9] and Fritsch et al (unpublished data 1972).

In addition to morphologic studies, the kinetics of basement membrane formation can be studied by determining the time sequence of the appearance of the various chemical constituents of the basement membrane. When studying the process of epiboly formation, Marks, Abell, and Nishikawa [15] had observed the binding of BP antibodies within the dermo-epibolic junction on some of their cultured tissue specimens. Very recently, Stenn, Madri, and Roll [16] reported that in a 48 hr organ culture of mouse skin, migrating epithelium contains type AB_2 collagen, a newly described collagen subtype [17], but not the collagen types I, III and IV. In this study we present evidence that (1) 3 separate antigenic determinants can be revealed along the dermo-epibolic junction: BP antigen, type IV collagen and laminin and (2) that their appearance in organ cultures follows a distinct time course. BP antigen emerges synchronously with the advancing tip of the migrating epithelium, though it cannot be deduced from this study whether this antigenic determinant is newly synthesized by the migrating epithelial cells or is just "carried along" by those cells. Type IV collagen and laminin, in contrast, are synthesized with considerable delay. They were first detectable at day 3 and 4, respectively, for a short distance in epibolic areas directly adjoining the original basement membrane zone; during subsequent days they extended to emerge in a consistently increasing portion of the dermo-epibolic junction though never reaching the advancing tip of the migrating column. Pattern and time course of the emergence of these antigens, therefore, resemble closely those observed for the appearance of the basal lamina. Obviously, no direct comparison can be made between immunofluorescence and electron microscopy data without immunoelectron microscopy; it nevertheless appears safe to assume that these 2 processes are linked to each other.

At first glance, these results seem to contradict those of Stenn, Madri, and Roll [16]. It has to be considered, however, that our experimental conditions differ from their work in 2 very important aspects: First, they worked in the mouse rather than in the human system; and secondly, they maintained skin in organ culture for only 48 hr. It is obvious from our studies that longer than 48 hr is required for the emergence of type IV collagen.

We do not know at present time what mechanisms are operative in controlling the rigorous time schedule of antigen emergence at the newly built junction zone. The different speed of emergence may simply reflect different synthesis rates of the antigens by the epidermal cells. Alternatively, junction zone neogenesis may well represent a multistep process, one step being dependent on and triggered by the previous ones.

It is interesting to note that BP antigen is the first antigen detected below the migrating epidermal cells. This antigen is restricted to squamous epithelia as opposed to type IV collagen and laminin which are present in all basement membranes. The presence of BP antigen along the dermo-epibolic junction does not parallel the deposition of the basement membrane differentiated structure (basal lamina) by electron microscopy. It is conceivable that BP antigen serves some cohesive bond between the basal cells and the dermis whereas the type IV collagen and laminin may subserve the function of forming a scaffolding on which the epidermis is anchored. Each of these may also be important in differentiation, for barrier function or in other processes.

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Program Announcement

Occupational Cutaneous Hazards and Diseases

Application receipt dates: March 1, July 1, and November 1

The National Institute for Occupational Safety and Health (NIOSH) announces exciting new opportunities for dermatologic researchers. NIOSH is currently inviting research and demonstration grants which will study a variety of issues and problems surrounding occupational cutaneous hazards and diseases. The Institute anticipates that studies generated as a result of this Program Announcement will help support NIOSH's mission to reduce the incidence and prevalence of occupationally-related skin disorders.

The goal of this announcement is to encourage *high quality* research and demonstration grants in specific domains.

Data Systems Research

Projects to identify the potential *extent* and *societal impact* of occupational skin disease

Biochemical and Physiological Research

Studies to elucidate the *nature* of occupational skin disease

Control Technology Research

Studies to develop new and improved *methods* to prevent occupational skin disease

Test Methodology Research

Projects to improve *test methodologies* and facilitate early *identification* of cutaneous hazards

Epidemiological Research

Studies which consider the *epidemiology* of occupational cutaneous diseases

Health Education Research

Projects to increase *awareness* of the importance of occupational skin diseases

Questions related to this announcement should be addressed to: Roger A. Nelson, Research Grants Program Officer, National Institute for Occupational Safety and Health, 5600 Fishers Lane, Room 8–63, Rockville, Maryland 20857, Telephone: (301) 443-4493, or Mr. Joseph West, Grants Management Officer, NIOSH, 5600 Fishers Lane, Room 8–35, Rockville, Maryland 20857, Telephone: (301) 443-3122.