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Abnormal skin development in pupoid foetus (*pf/pf*) mutant mice

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SUMMARY

At 13 days of development the epidermis of mice homozygous for the pupoid foetus (*pf/pf*) mutation varies in thickness between one and ten cell layers. By 16 days of development cells from the dermis have invaded the epidermis and may be found throughout the epidermis and on its surface. Among these cells are nerve fibres and Schwann cells as well as other unidentified cells. Antibodies directed against fibronectin bind to these abnormal groups of cells in the mutant epidermis and on its surface. A basal lamina, as determined by ultrastructure and by the immunofluorescent localization of laminin, was always found at the interface of the mutant epidermis and the invading cell population. By 19 days of development the mutant epidermis is thickened and is permeated by a network of cells including nerve fibres, Schwann cells, blood vessels, and collagen and fibronectin-secreting cells. A basal lamina always separates these groups of invading cells from the epidermal cell population.

INTRODUCTION

The epidermis is an avascular tissue which provides the organism with protection from injury and trauma, such as ultraviolet irradiation and heat and water loss. The maintenance of epidermal integrity is undoubtedly a complex process involving the regulation of epidermal cell mitotic activity and adhesiveness. To achieve adult form, the epidermis must develop in synchrony with the dermis and its extensive nerve and vascular bed. During this process the dermal–epidermal junction of the developing skin remains intact, continuous, and well defined.

The epidermis, as well as other epithelia, is separated from the underlying dermis or connective tissue by a basal lamina. This complex, highly organized extracellular matrix has been implicated in several functions at the dermal–epidermal interface, including mechanical support for the epidermis, barrier function, and mediation of epidermal–dermal adhesion (Daroczy, Feldman & Kiraly, 1979). Bernfield & Banerjee (1978) have suggested that the basal lamina stabilizes epithelial form

Key words: skin development, mouse embryo, basal lamina, *pf/pf* mutant, dermal–epidermal junction.

during embryonic development. In addition, loss of basal lamina components may be associated with the progression from a normal to a neoplastic phenotype (Ingber, Madri & Jamieson, 1981). Little is known of the regulation of basal lamina deposition. Briggaman, Dalldorf & Wheeler (1971) demonstrated that frozen-thawed dermis supports basal lamina formation. Epidermal keratinocytes do not form a basal lamina when cultured on plastic substrates (Hirone & Taniguchi, 1980). However, on collagen substrates basal lamina formation is possible (Mann & Constable, 1977; Hirone & Taniguchi, 1980). Thus a biological substrate and the presence of an extracellular matrix may be permissive for epidermal basal lamina formation.

Epidermal basal laminae are composed of a variety of molecules which are ubiquitous (Stanley, Woodley, Katz & Martin, 1982) and highly conserved throughout evolution. The glycoproteins laminin and Type IV collagen have been identified as components of the basal lamina (Kefalides, Alper & Clark, 1979; Timpl *et al.* 1978) and are deposited by basal cells (Briggaman *et al.* 1971; Dodson & Hay, 1974; Laurie, LeBlond & Martin, 1980; Laurie, LeBlond, Couril & Martin, 1982). Another protein, fibronectin, is a collagen-associated protein which is present in the dermis but is not found in the epidermis above the basement membrane of rodents (Couchman *et al.* 1979) and humans (Stenman & Vahari, 1978).

Mice homozygous for the pupoid foetus (*pf/pf*) mutation develop a markedly thickened epidermis. It has been suggested that this condition is associated with the eruption of nerves into the mutant epidermis (Watson & Ede, 1977). Recently, we described the abnormal development of the dorsal skin of the pupoid foetus mutant mouse (Fisher, Dale & Kollar, 1984) and demonstrated that the mutant epidermis is invaded by a variety of cells from the dermis which establish a network throughout the tissue. To understand the abnormal epidermal development of the *pf/pf* mutant, it is essential to identify the types of cells that invade it; to observe the response of the epidermal cells to the invading cell population; and to determine how the cells from the dermis enter the epidermis. The goal of the present study was to examine, in greater detail, through light and electron microscopy, and through the immunofluorescent localization of laminin, Type IV collagen, and fibronectin, the process of abnormal development of the *pf/pf* mutant mouse.

MATERIALS AND METHODS

Animals

Animals heterozygous for the *pf* mutation were identified by mating tests. Heterozygous males and females were caged together in the evening and successful matings were identified by the presence of a vaginal plug indicating day 0 of pregnancy. Gravid famales were sacrificed on days 13 and 16 of gestation and at the onset of parturition which occurs at 19–20 days of pregnancy.

Foetuses and neonates were dissected in Hanks' balanced salt solution. Dorsal skin was removed from mutants and normal littermates and prepared for light and electron microscopy or immunofluorescence.

Light and electron microscopy

Specimens for light and electron microscopy were fixed in 2 % glutaraldehyde in 0.1 M-cacodylate buffer (pH 7.4) at 4 °C. Following a rinse with buffer, the specimens were postfixed in 2 % OsO_4 in distilled water, rinsed in buffer, and dehydrated through a graded series of ethanol. Following two 5 min changes of propylene oxide, tissue was equilibrated overnight in a mixture of 50 % Epon and 50 % propylene oxide. After 24 h in 100 % Epon, the tissue was polymerized overnight in fresh Epon at 60 °C. Sections 1 μm thick were cut with glass knives on a Sorvall JB-4 microtome and stained with toluidine blue. Thin sections were cut on an LK-B ultramicrotome with a diamond knife (Ladd Research Industries, Inc., Burlington, VT), stained with uranyl acetate and lead citrate, and viewed with a Zeiss EM-10 electron microscope.

Immunofluorescent labelling

Tissue was prepared for immunofluorescent labelling according to the method of Sainte-Marie (1962). Dorsal skin was fixed in 95 % ethanol at 4 °C overnight. Tissue was dehydrated in two 30 min changes of 100 % ethanol at 4 °C. Following two 30 min changes of xylene at 4 °C, the tissue was embedded in paraffin. Serial, 7 μm sections were cut and allowed to adhere to albumin-coated slides for 30 min at 37 °C and stored in a desiccator for 18–24 h at 4 °C. Sections were deparaffinized at room temperature in two 10 sec xylene baths and xylene was removed with two 30 sec washes with 95 % ethanol at 4 °C. The sections were then thoroughly rinsed in 0.02 M-phosphate-buffered saline (PBS). Direct (fibronectin) and indirect (laminin, Type IV collagen) immunofluorescent techniques were utilized to visualize the binding of antibodies.

Antibodies

A fluorescein isothiocyanate (FITC)-conjugated IgG fraction goat anti-human fibronectin antiserum (Cappel, Cochranville, PA) was used for the direct immunofluorescent localization of fibronectin. Sheep anti-laminin (gift of H. Kleinmann, NIH) and rabbit anti-Type IV collagen (gift of G. Martin, NIH) were used for the indirect localization of basement membrane antigens. The preparation of anti-Type IV collagen and anti-laminin has been described previously (Foidart *et al.* 1980; Laurie *et al.* 1982).

FITC-conjugated IgG fractions of rabbit anti-sheep IgG (heavy and light chains) and goat anti-rabbit IgG (heavy and light chains) (Cappel, Cochranville, PA) were used as the secondary antisera for indirect immunofluorescent labelling.

Controls

The following controls were routinely performed along with the antibody-binding localization studies: pre-immune serum (rabbit or sheep) as primary antiserum, elimination of primary antiserum, and examination of specimens for autofluorescence. In the case of the direct localization of binding of antifibronectin, antiserum adsorbed against purified human fibronectin (Collaborative Research, Waltham, MA) was used in the control experiments.

RESULTS

1) *13- and 14-day embryonic skin*

At 13 and 14 days of development there are distinct histological differences between normal and mutant skin. While normal epidermis is even in thickness with three to four cell layers, the mutant epidermis was found to vary between one and ten cell layers (Fig. 1A, B); the variability in thickness was random. When the thinnest areas of the 13-day mutant epidermis were examined with transmission electron microscopy (Fig. 1C, D), cells that were either viable or degenerating were observed. The viable cells in these areas have irregularly shaped nuclei and hetero-

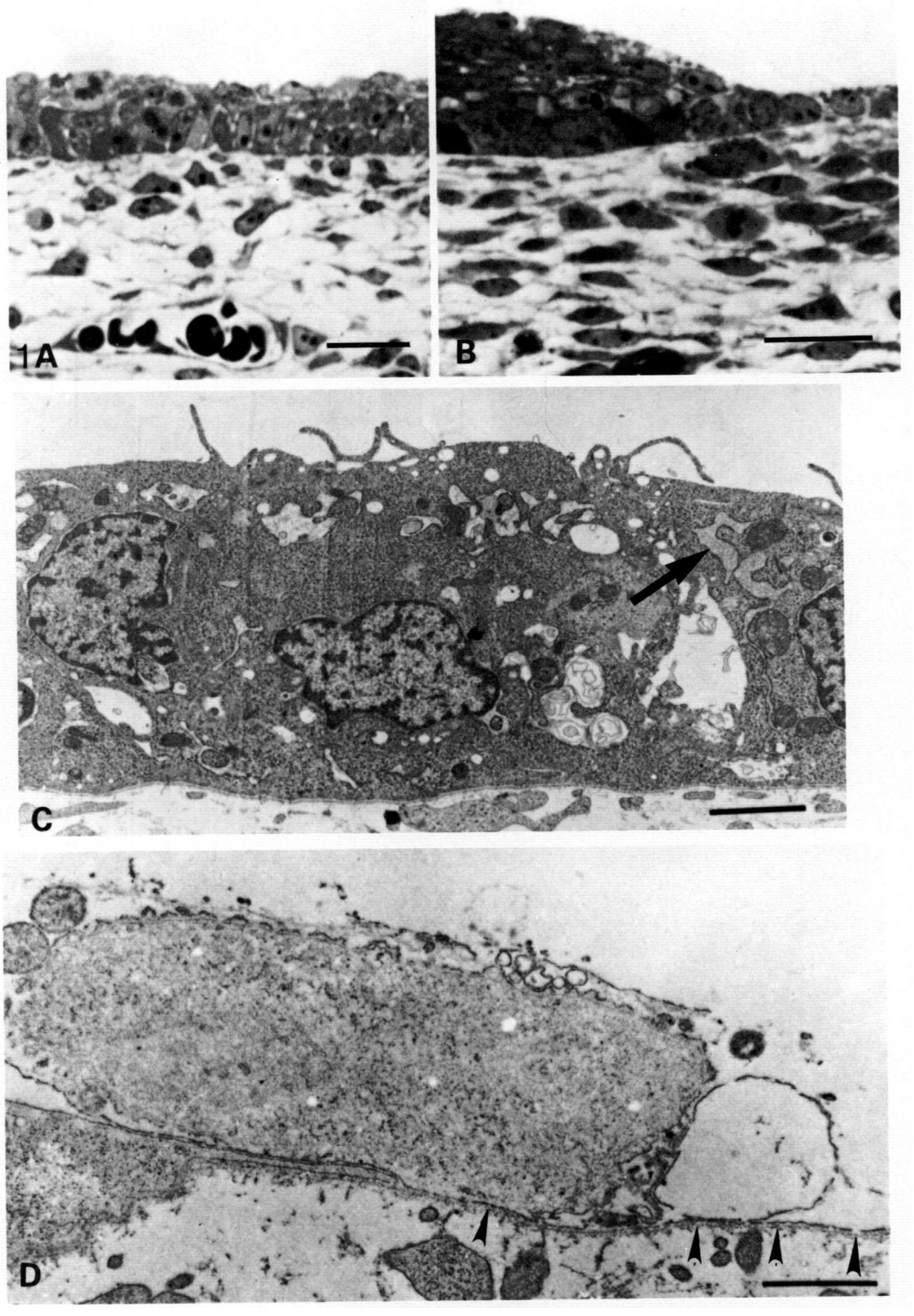

Fig. 1

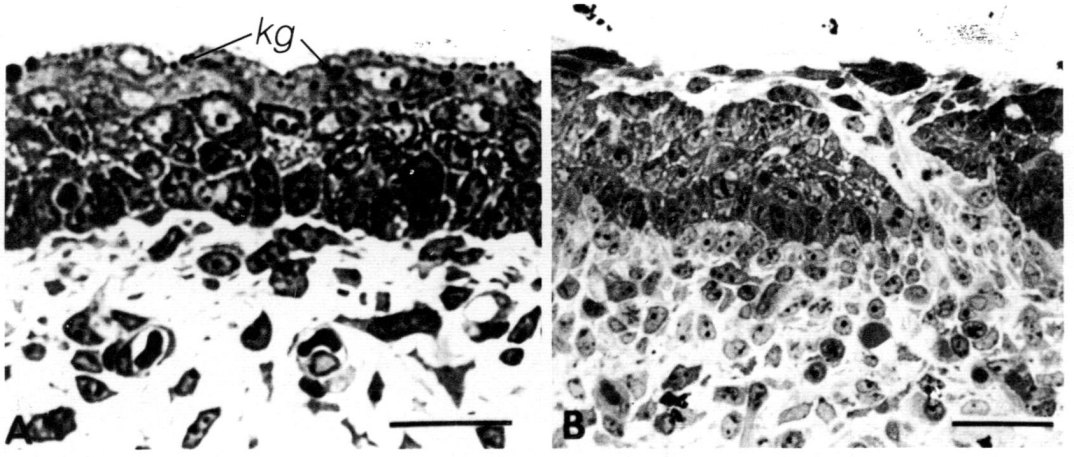

Fig 2A-B. Light micrographs of 16-day normal and mutant skin.
(A) 16-day normal epidermis is even in thickness and keratohyalin granules (*kg*) are just beginning to appear. Bar equals 25 μm.
(B) Cells from the dermis of 16-day mutant skin have aligned along the dermal–epidermal junction. In addition, dermal cells may be seen extending throughout the mutant epidermis or lying upon its surface. Bar equals 25 μm.

chromatin associated with the nuclear membrane. Microvilli extend from these cells into the amnitotic cavity (Fig. 1C). On the other hand, in areas of degeneration the cells are disrupted, cytoplasm has dispersed and few cell membranes remain. Nuclei of degenerating cells are present but show signs of deterioration. The nuclear membranes are discontinuous and heterochromatin is not evident. Despite evidence of degeneration in the epithelium, an intact basement membrane was always noted (Fig. 1D) and the basal lamina was the only structure separating the dermis from the amniotic cavity.

2) *16-day embryonic skin*

a) *Light and electron microscopy* At this developmental stage the thickness of the normal epidermis is uniform and keratohyalin granules can be seen for the first

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Fig. 1A-D. 13-day embryonic normal (A) and mutant (B-D) skin.
(A) Light micrograph of 13-day normal skin. Epidermis is even in thickness with three to four layers. Bar equals 50 μm.
(B) Light micrograph of 13-day mutant skin. Epidermis may vary between one and ten cell layers. Bar equals 50 μm.
(C) Electron micrograph of thin region of 13-day mutant epidermis. Cells have nuclei with dense heterochromatic regions, distended rough endoplasmic reticulum (arrow), and microvilli extending into the amniotic cavity. Bar equals 2 μm.
(D) Electron micrograph from thin region of 15-day mutant epidermis which is degenerating. Nuclei lack chromatin, cytoplasm and cell membranes have dispersed, and basal lamina (arrowheads) is all that separates dermis from amniotic cavity. Bar equals 1 μm.

time. A well-defined dermal–epidermal junction is evident (Fig 2A). The 16-day mutant epidermis, on the other hand, lacks keratohyalin granules and is highly disordered. Dermal cells are palisaded along the dermal–epidermal junction, making this boundary poorly defined. Groups of dermal cells have invaded the mutant epidermis. These cells may be found extending throughout the mutant

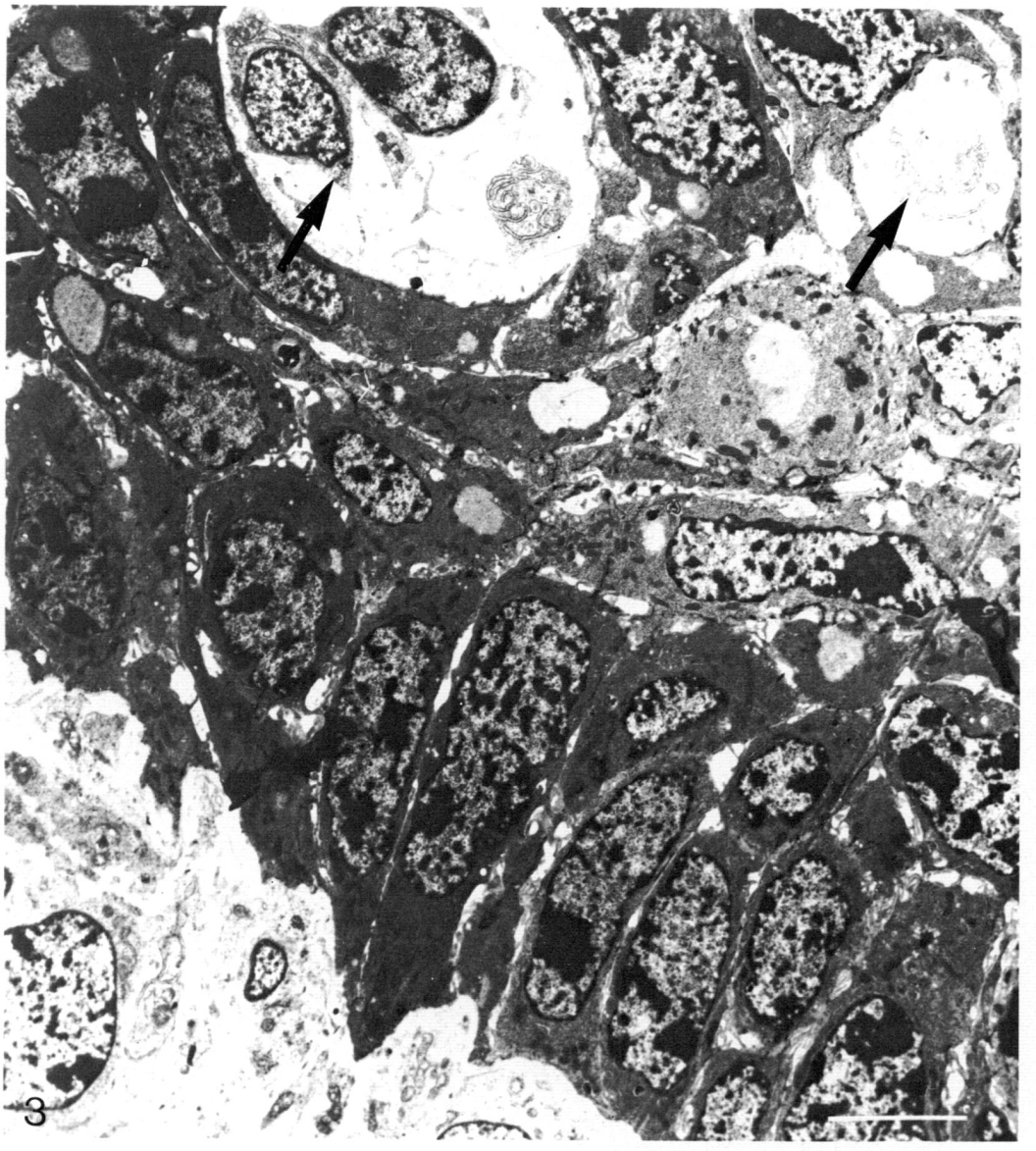


Fig. 3. Electron micrograph of 16-day embryonic mutant skin. At the dermal–epidermal junction cells from the dermis have crowded along the basement membrane. Cells from the dermis may be seen extending into the superficial layers of the epidermis (arrows). Among these cells are nerve fibres, as well as other cells, which are apparently secreting an extracellular matrix. Bar equals 5 μm.

epidermis or lying upon its surface (Fig. 2B). Light microscopy shows no blood vessels or nerves in the mutant epidermis at this stage, although unmyelinated nerve fibres, as well as other cell types, are noted by transmission electron microscopy (Fig. 3). The matrix of extracellular material associated with the invading cells is flocculent and contains some collagen fibres. Since blood vessels and large deposits

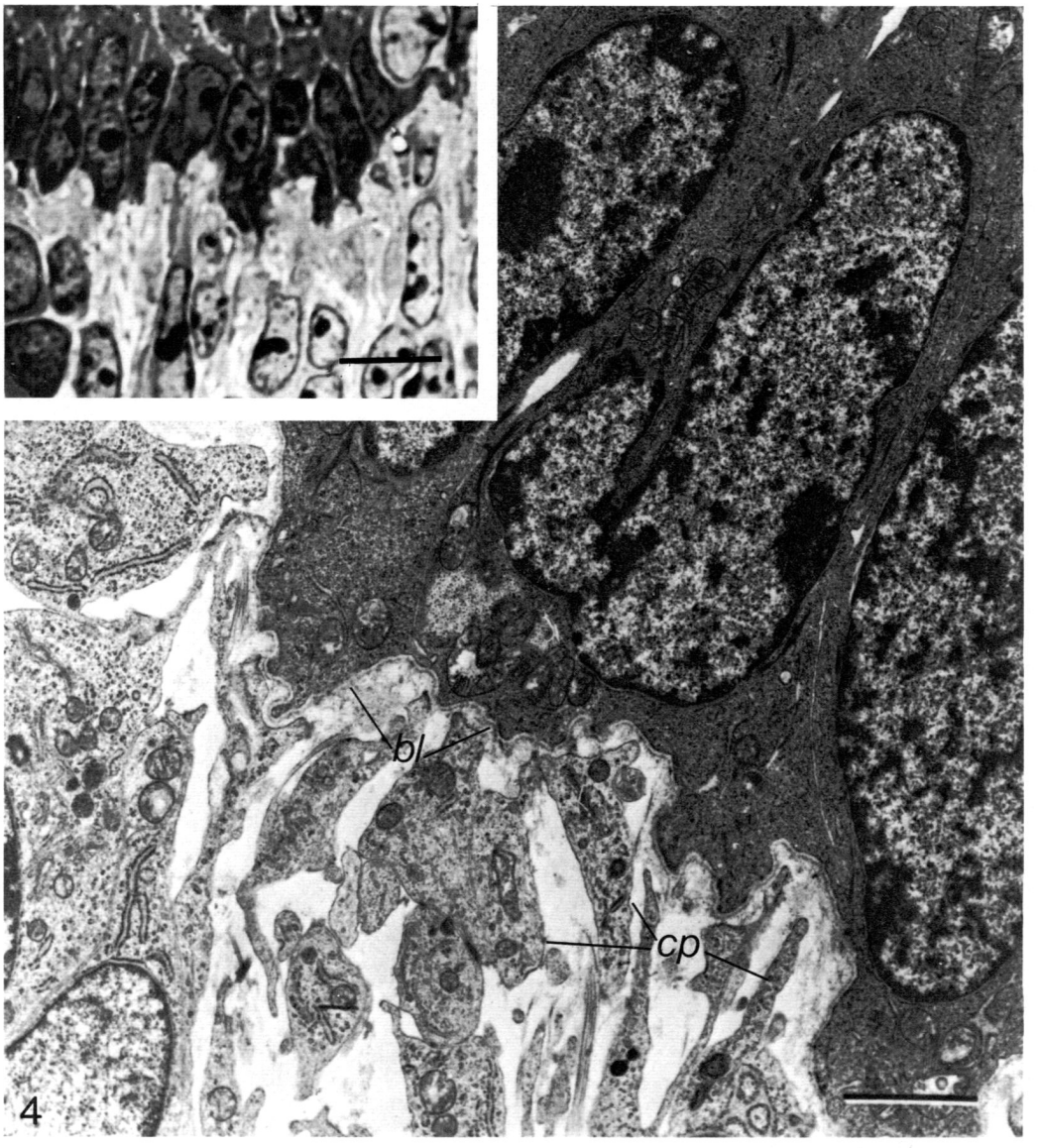


Fig. 4. Dermal–epidermal junction of 16-day *pf/pf* mutant skin. Cells in dermis are palisaded beneath epidermis (inset). The processes of these cells (*cp*) approach and sometimes contact the basal lamina (*bl*) which always remains intact. Bar equals 2μm; inset bar equals 10μm.

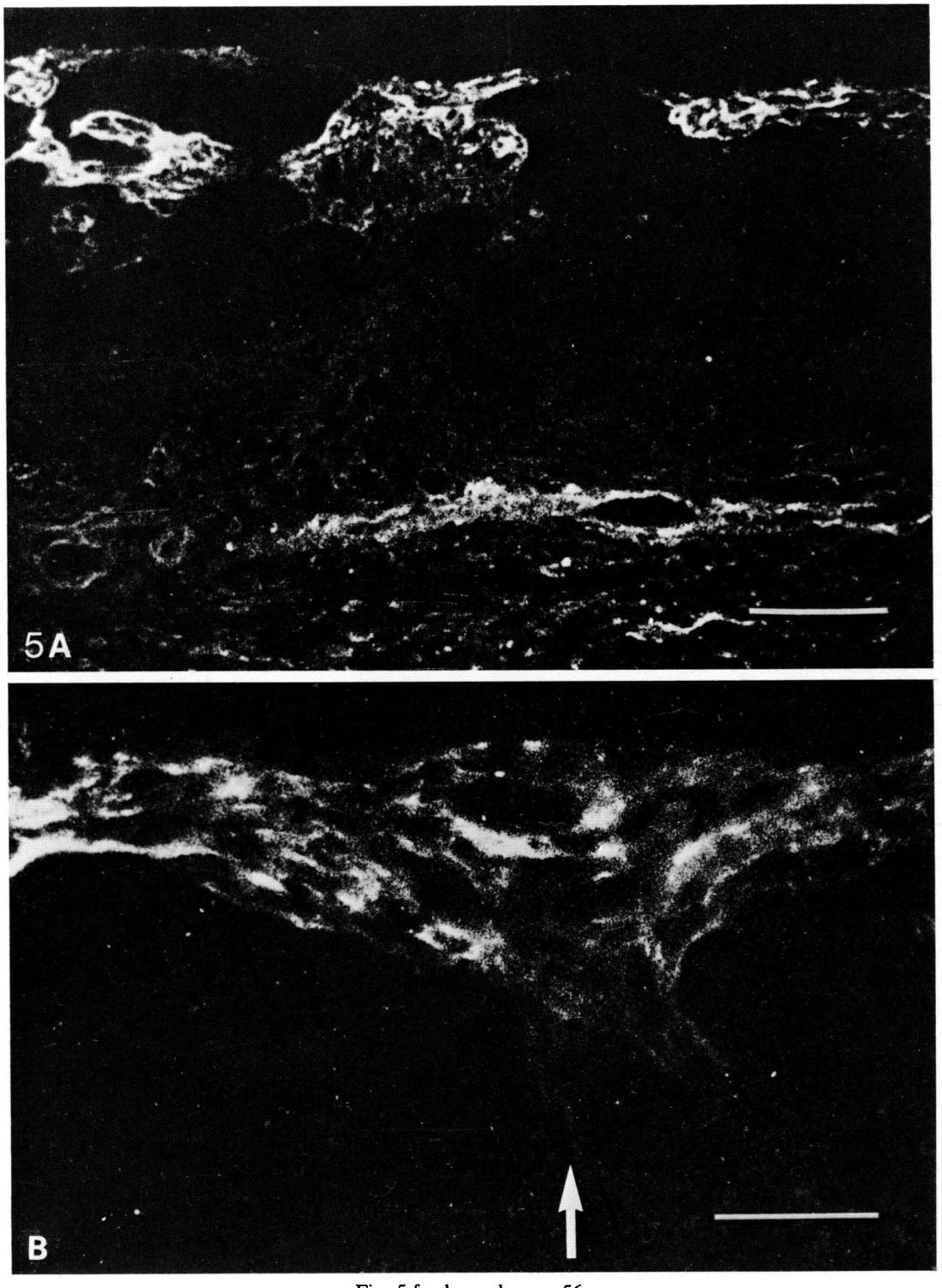
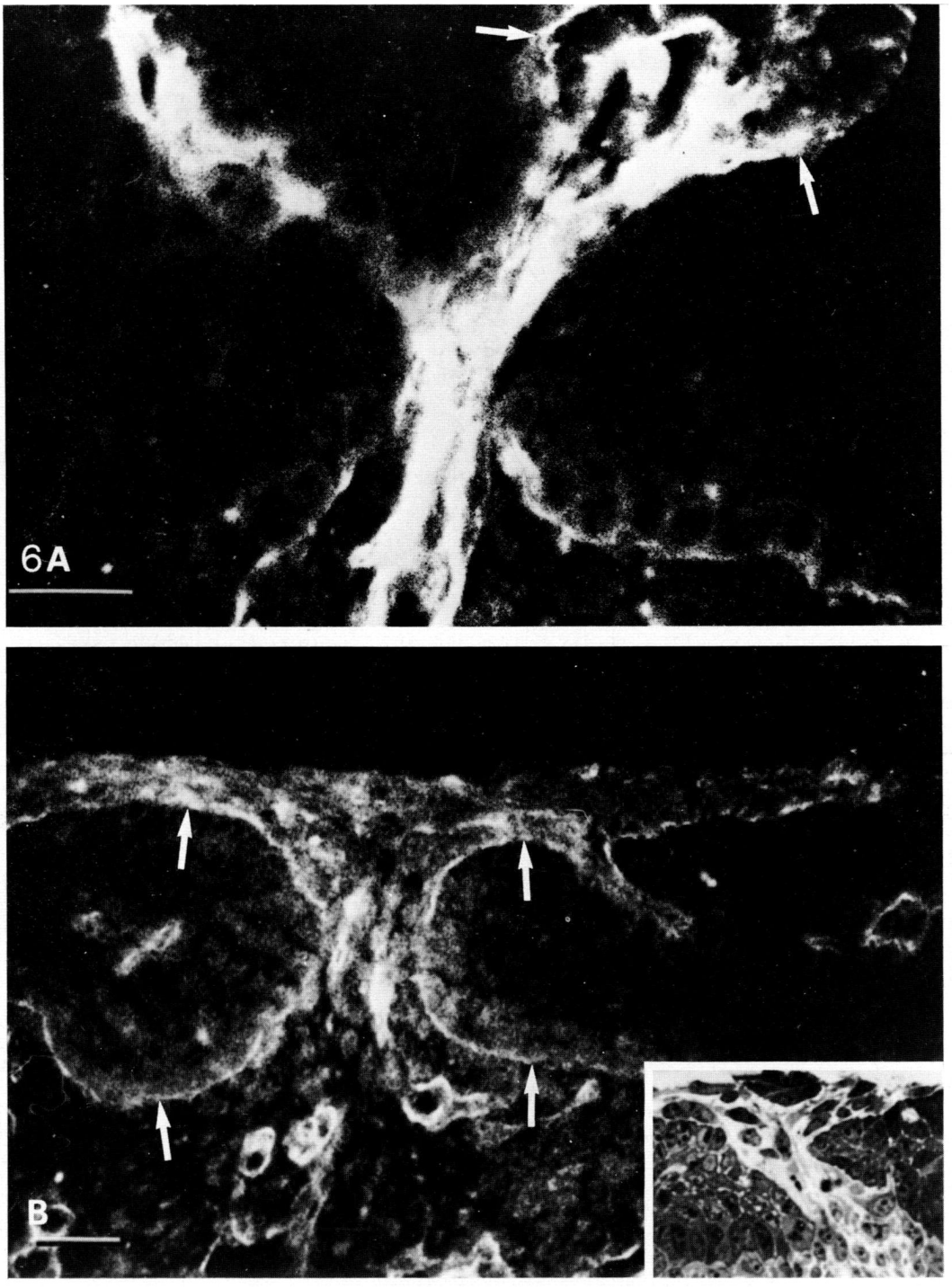


Fig. 5 for legend see p.56



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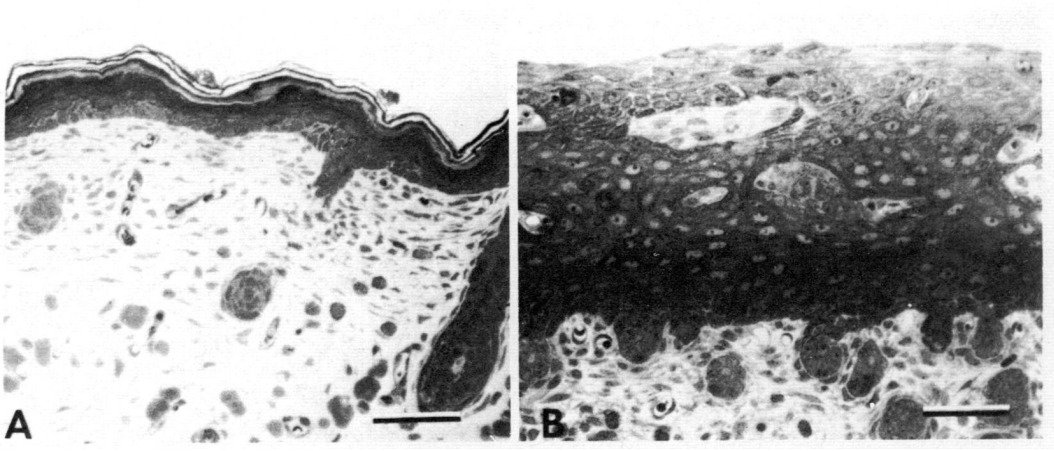

Fig. 7A-B. Light micrographs of normal and mutant newborn skin.

(A) Normal epidermis is even in thickness and is keratinized with well-developed granular and cornified layers. Bar equals 50 μm.

(B) Mutant epidermis is much thicker than normal. Foreign cells extend throughout the mutant epidermis and the complete absence of keratinization is apparent. An abnormal granular layer containing small, sparse keratohyalin granules is present. Bar equals 50 μm.

of collagen are detected in these pockets of cells by the time of birth, endothelial cells and fibroblasts are most likely present in the invading population of cells.

Examination of the mutant dermal–epidermal junction by transmission electron microscopy reveals long cellular processes of the palisading dermal cells that approach and sometimes contact the basal lamina (Fig. 4). Extensive lengths of the basal lamina in these areas have been examined and are always intact – the palisading cells were not found to penetrate the basal lamina. The pockets of foreign cells within the mutant epidermis are separated from the epidermal cells by a basement membrane.

Fig. 5. Detection of anti-fibronectin binding in 16-day mutant skin by direct immunofluorescence.

(A) Extensive binding of anti-fibronectin antibodies is noted throughout the 16-day mutant epidermis and on its surface. Bar equals 50 μm.

(B) Binding of anti-fibronectin antibodies is associated with cells that extend throughout the embryonic mutant epidermis and onto its surface. These cells are often noted to be continuous with the dermis (arrow). Bar equals 25 μm.

Fig. 6. Binding of anti-laminin to 16-day mutant skin as detected by indirect immunofluorescence.

(A) Vessel from dermis as it enters the 16-day mutant epidermis. Anti-laminin binds not only to the epidermal basal lamina at the dermal–epidermal junction but also to the epidermal basal lamina that is laid down adjacent to the foreign cell population (arrows). Bar equals 15 μm.

(B) Cells from the dermis extend through the thickness of the 16-day mutant epidermis and onto its surface. A basal lamina (arrows) has been deposited by the basal cells at the normal dermal–epidermal junction and also by the superficial cells of the epidermis, wherever they encounter the invading cell population. A similar region from a toluidine-blue-stained, semi-thin section is depicted in the inset. Bar equals 25 μm; inset × 370.

b) *Fibronectin localization*. Pockets of fibronectin binding were found throughout the thickness of the 16-day embryonic mutant epidermis (Fig 5A, B). These ectopic dermal pockets were often extensive and located either on the surface of the mutant foetal epidermis or within the epidermis (Fig. 5A, B). Often they could be traced in serial sections and were found to be continuous with the underlying dermis (Fig. 5B).

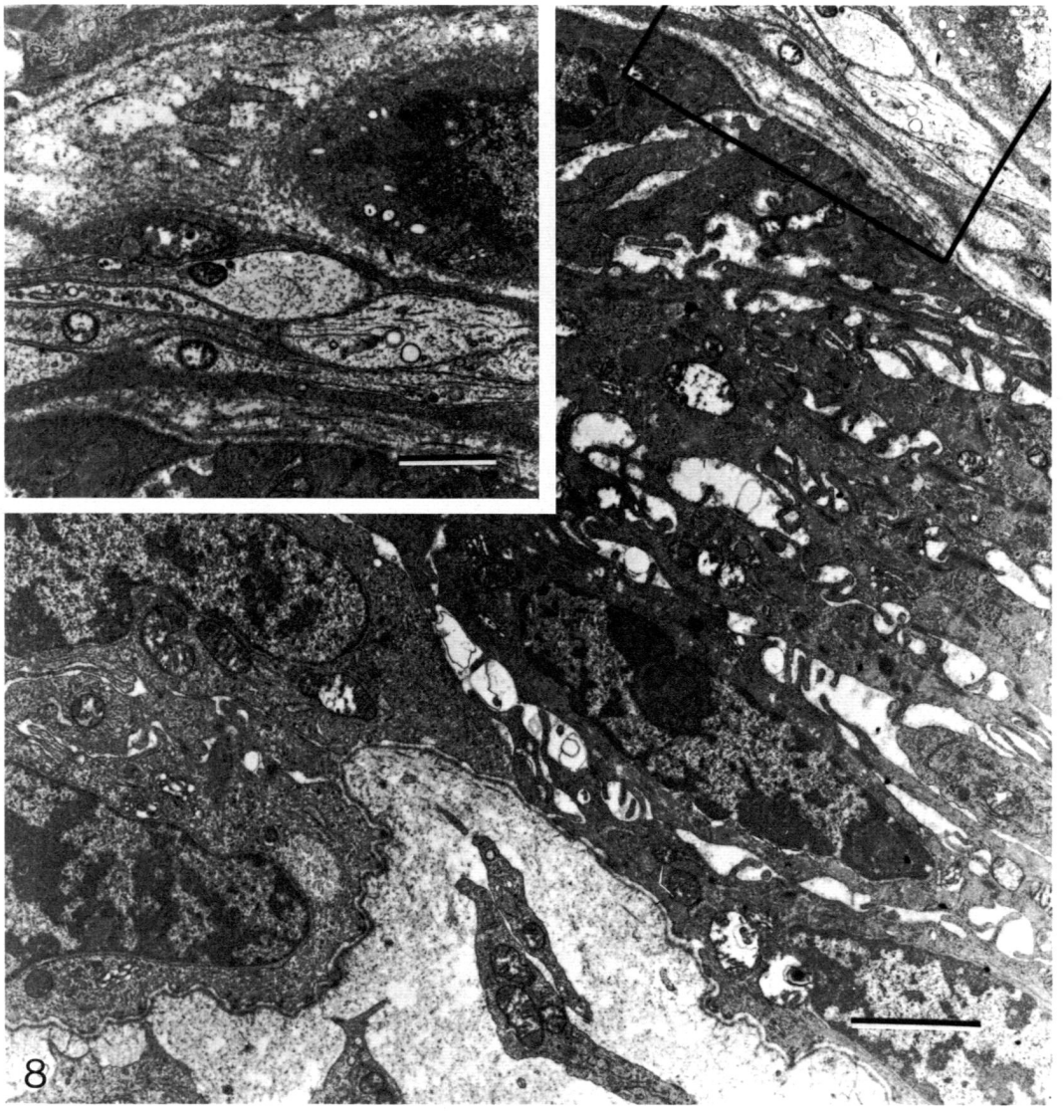

Fig. 8. Newborn *pf/pf* mutant skin. The numbers and types of cells invading the skin are variable. A bundle of nerve fibres is seen coursing through the epidermis. *Inset*: These fibres contain synaptic vesicles and are associated with collagen. Bar equals 2 μm; *Inset*, bar equals 1 μm.

c) *Laminin localization.* Antibodies directed against laminin were found to bind to the embryonic mutant epidermal basement membrane and to the basement membranes of vessels in the dermis (Fig. 6). In addition, laminin localized in an unusual fashion within mutant skin. The antibody outlined the areas of cellular invasion into the mutant epidermis and these areas correlated with anti-fibronectin binding (Fig. 5). Areas containing dermal cells or vessels extending into the

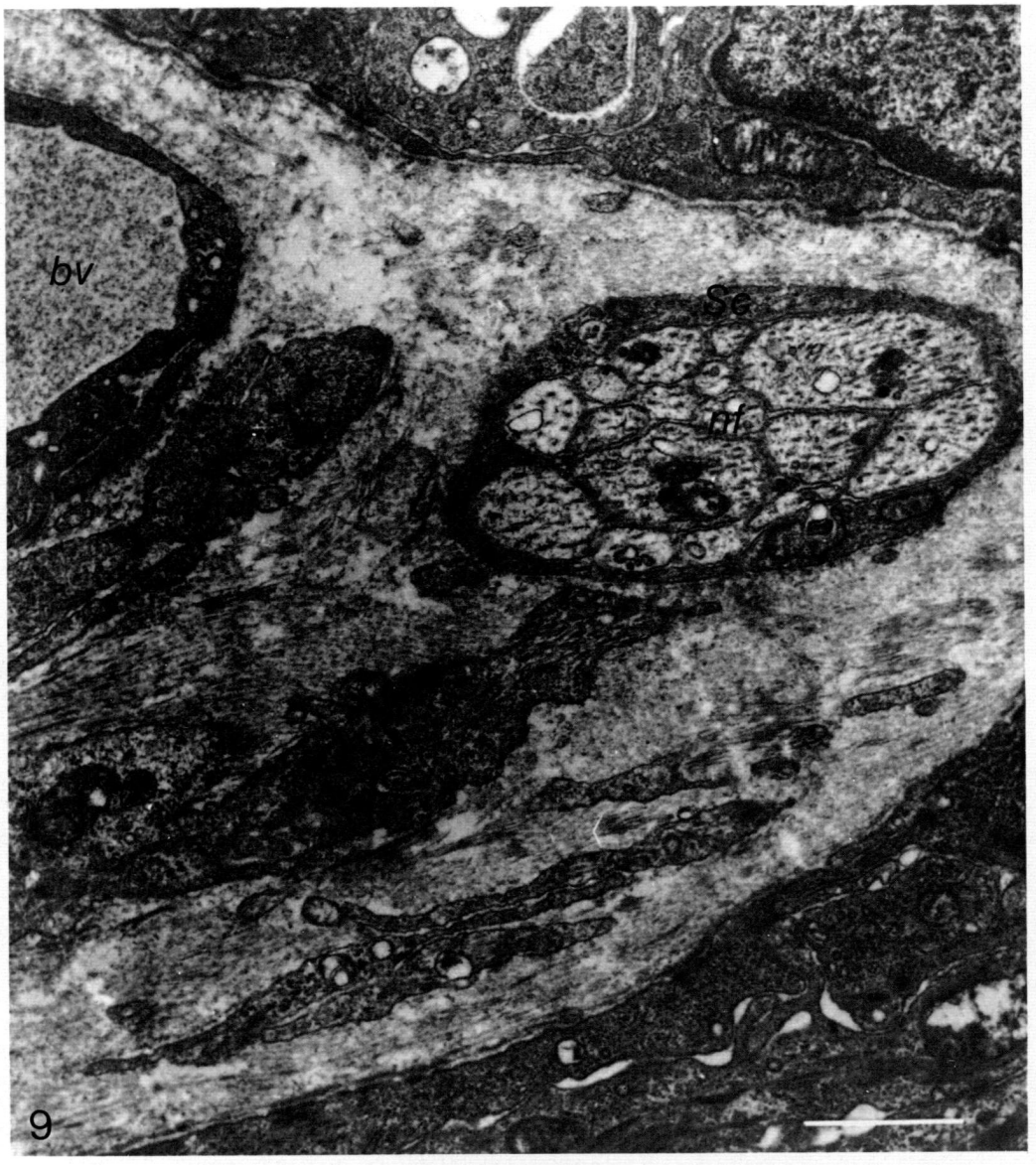

Fig. 9. Newborn *pf/pf* mutant skin. Blood vessels (*bv*) have formed throughout the mutant epidermis. Also present are nerve fibers (*nf*) associated with a Schwann cell (*Sc*) and extensive collagen deposits. Bar equals 1 μm.

epidermis were observed (Fig. 6A). Large areas where cells from the dermis pushed into the epidermis and spread upon its surface were also common (Fig. 6B). Wherever the invading cells were located within foetal mutant epidermis, they were surrounded by laminin-secreting epidermal cells.

3) *Newborn skin*

a) *Light and electron microscopy.* At the onset of parturition the normal epidermis is fully keratinized and is approximately 50 μm in thickness (Fig. 7A). The mutant epidermis is very thick and permeated by a network of cells that are continuous with the dermis (Fig. 7B). By electron microscopy, the content of the network of cells was found to vary. In some cases the foreign cells may consist of not more than a bundle of nerve fibres (Fig. 8). The nerves, while associated with supporting cells, are unmyelinated and may contain synaptic vesicles. In other areas extensive collagen deposits, as well as blood vessels and nerves, are noted (Fig. 9). In all cases, epidermal cells are separated from the foreign cell population by a basal

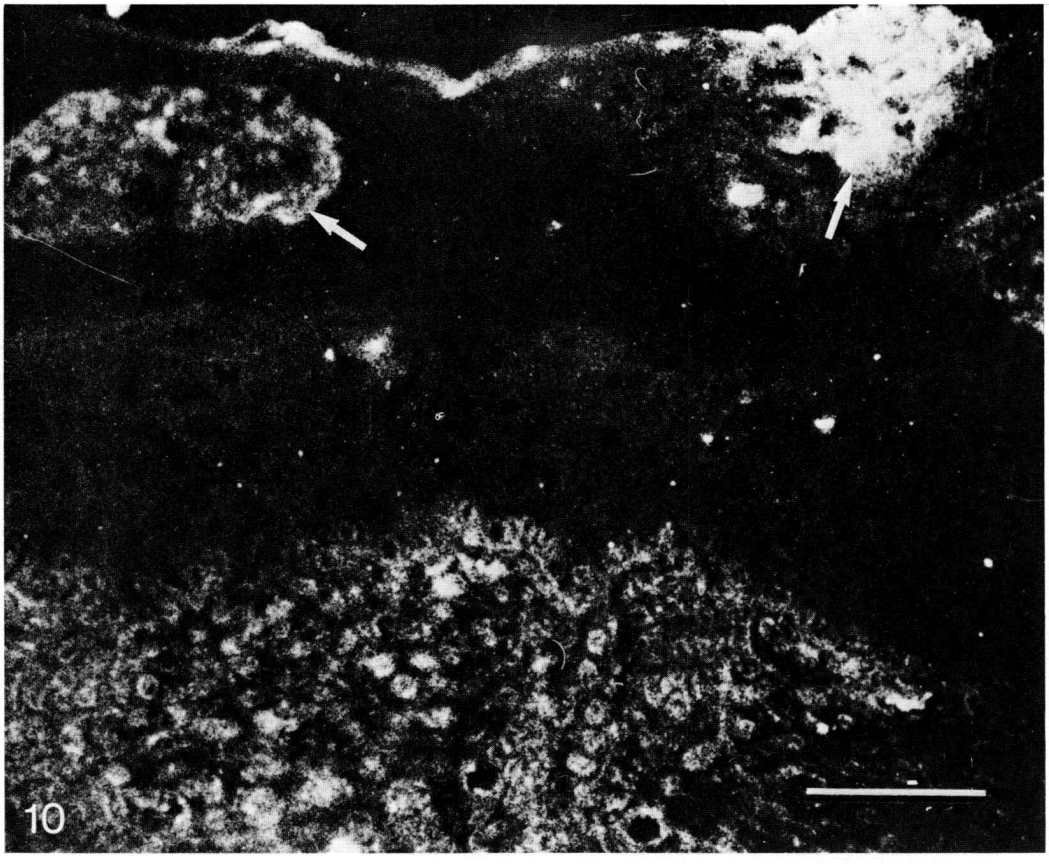

Fig. 10. Binding of anti-fibronectin to newborn mutant skin. Discrete foci of binding are detected throughout the mutant epidermis and on its surface (arrows). Bar equals 25 μm.

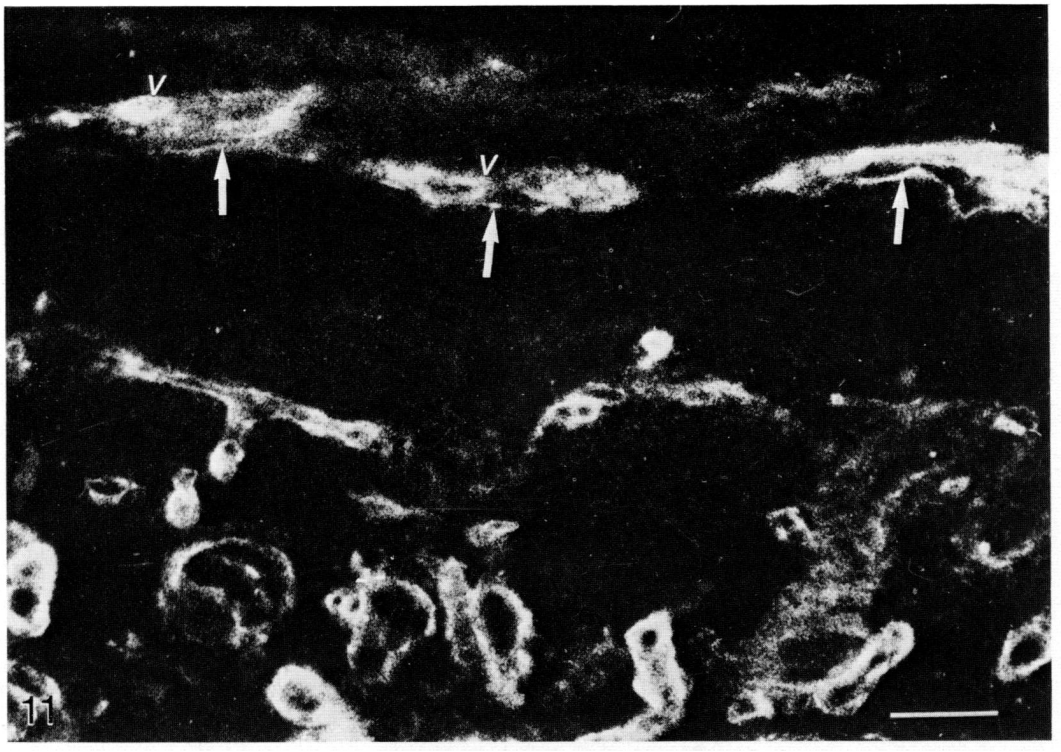

Fig. 11. Binding of anti-laminin to newborn *pf/pf* mutant skin as detected by indirect immunofluorescence. Antibodies outline the circumference of discrete foci of ectopic cells (arrows) found throughout the epidermis and on its surface. Within these areas vessels (*v*) are highlighted by anti-laminin fluorescence. Bar equals 25 μm.

lamina which has well-developed hemidesmosomes. Anchoring fibrils are present (Fig. 9).

b) *Fibronectin localization.* Binding of anti-fibronectin antibodies to newborn mutant skin was also found to be abnormal and correlated with light microscopic observations. The foci of anti-fibronectin binding appeared more discrete than those in mutant foetal skin and were localized within the thickened mutant epidermis or on its surface (Fig. 10).

c) *Laminin localization.* In newborn mutant skin, anti-laminin binding outlined foci which were found throughout the thickness of the epidermis. These foci were more condensed than those in the 16-day foetal mutant tissue. Areas of antibody binding were detected either within the thickened, hyperplastic epidermis or on its surface (Fig. 11). Basal laminae associated with the blood vessels and nerves were detected within these foci (Fig. 11).

d) *Type IV collagen localization.* The immunofluorescent localization of Type IV collagen in normal and mutant skin was found to be identical with that of laminin. In normal tissue, Type IV collagen was localized to the epidermal basal laminae as well as to the basal laminae of hair follicles and dermal blood vessels.

Dermal vessels were seen to travel subjacent to the epidermal basement membrane and to branch away from the epidermis (Fig. 12A). The mutant skin bound the antibody in a manner consistent with anti-laminin binding. Foci of foreign cells within the epidermis were outlined by Type IV collagen, and blood vessels and

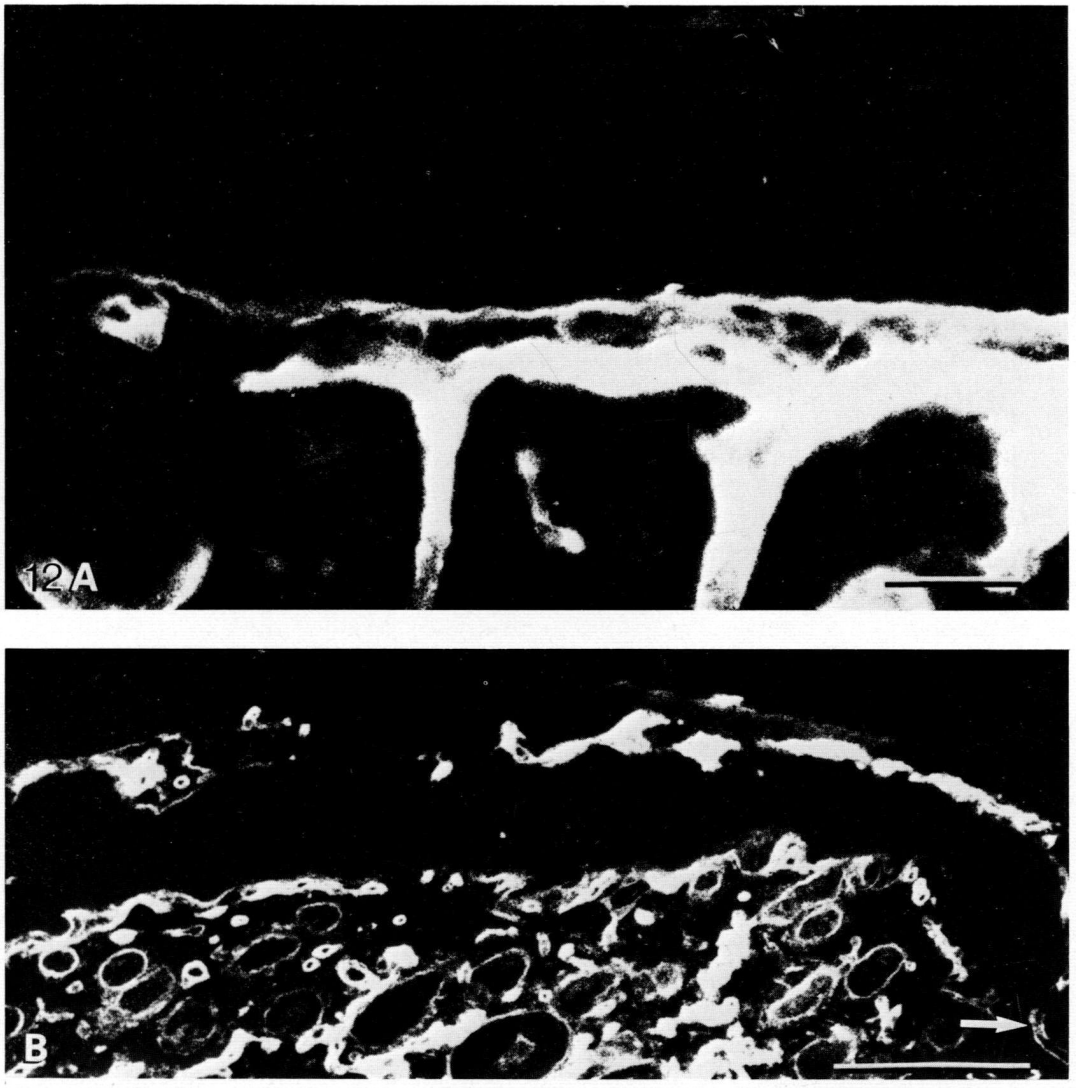

Fig. 12. Binding of anti-Type IV collagen to normal and mutant newborn skin as detected by indirect immunofluorescence.

(A) The basal lamina of normal epidermis is bound by anti-Type IV collagen antibodies. A blood vessel branching away from the epidermis is detected immediately subjacent to the epidermis. Bar equals 25 μm.

(B) A blood vessel may be seen entering the mutant epidermis (arrow). Anti-Type IV collagen binds extensively throughout the mutant epidermis to the basal lamina that surrounds the pockets of foreign cells. Vessels within these areas are also highlighted by the fluorescence. Bar equals 100 μm.

nerves located within the network of foreign cells were delineated by anti-Type IV collagen staining (Fig. 12B).

4) *Controls*

Control studies including pre-immune serum, absence of primary antiserum, and anti-fibronectin antibodies absorbed against purified fibronectin were routinely negative.

DISCUSSION

Normal epidermis is avascular and contains few nerve fibres. How these conditions are maintained in developing and adult skin is unknown. Mice homozygous for the pupoid foetus (*pf/pf*) mutation are unusual in that the epidermis of these animals is invaded by cells from the dermis. These cells include cells which secrete fibronectin and collagen. In addition, endothelial cells and nerve fibres are seen throughout the network of foreign cells established within the epidermis (Fisher *et al.* 1984; Figs. 6, 7, 9). At 13 to 14 days of development, when the mutant skin varies between one and ten cell layers, the mutant epidermal cells in the thinnest areas appear to degenerate. In some areas a naked basement membrane is all that separates the mutant dermis from the amniotic cavity. It seems likely that the dermal cells which push into the mutant epidermis enter through these regions. It has been suggested that the release of protease inhibitors from avascular tissue such as cartilage (Kuettner & Pauli, 1981) and urinary bladder epithelium (Waxler, Kuettner & Pauli, 1982) protects these tissues from vascularization. The relevance of such findings to the etiology of *pf/pf* defects is unknown.

By 16 days of embryonic development cells from the dermis of the *pf/pf* mutant have become aligned along the dermal–epidermal junction (see Fig. 4, inset). While the appearance of these cells suggests an active cellular invasion, ultrastructural observations of these areas indicate that the basal lamina remains intact. Thus, it appears that the individual cells subjacent to the epidermis do not digest their way through the basal lamina. This observation also supports the notion that it is the degenerating areas of the 13-day mutant epidermis which admit the dermal cells into the epidermis.

Where foreign cells extend into the mutant epidermis, the adjacent epidermal cells secrete a basal lamina which restores the natural border between the two populations of cells. This observation has been confirmed by ultrastructural observations and by the immunofluorescent localization of the basal lamina antigens laminin and Type IV collagen. Thus, even though the mutant epidermis becomes permeated throughout by cells from the dermis, it remains separated from them by a basal lamina. From these observations it appears that a constant interaction responsible for the maintenance of a basal lamina is occurring between the dermis and epidermis.

It has been consistently demonstrated that local increases in fibronectin concentration are associated with involved areas of diseased skin. While the significance

of these observations is unclear, they are consistent with the notion of a scaffolding effect upon which local tissue repair could occur (for review see Akiyama & Yamada, 1983). We have noted local fibronectin increases associated with large intrusions of cells in the 16-day *pf/pf* mutant epidermis. As this invasion obviously elicits much change in the mutant skin, our observations are consistent with the notion of fibronectin as a scaffolding substrate.

The network of cells located throughout the thickness of the mutant epidermis are undoubtedly providing the epidermal cells with abnormal cues. Epidermal cells, even the most superficial ones, respond to contact with these foreign cells by depositing a basement membrane. It remains to be demonstrated whether these cells display basal cell phenotype in other respects such as their mitotic activity and molecular composition.

The absence of normal keratinization in the mutant epidemis can be related to the lack of synthesis of the epidermal protein filaggrin (Fisher *et al.* 1984). It is likely that filaggrin synthesis is retarded due to the hyperplasia, the aberrant stratification, and the confusion of epidermal polarity that is created by the presence of the invading cells. The mutant epidermis does not possess a simple basal layer of cells bordering a dermis. Instead, it is penetrated throughout by cells which create a network of dermis including blood vessels and nerve fibres, as well as fibroblasts that secrete fibronectin and collagen.

A previous report (Fisher *et al.* 1984) indicates that the *pf* gene is not expressed in the mutant epidermis. Mutant skin attains a normal, keratinizing epidermis when grafted to normal hosts. Thus, we have suggested that the *pf* gene acts systemically (Fisher *et al.* 1984). One of the primary events in the development of the mutant phenotype appears to be the loss of continuity of the mutant epidermis at 13 days of development, followed by the invasion of cells from the dermis into the mutant epidermis at 14–16 days of development. Surely, during embryonic development barriers exist which protect epithelia against vascularization and cellular invasion. If the *pf/pf* mutation is expressed systemically then the mutant will be a valuable tool in which to study systemic effects upon the maintenance of the epidermal barrier to cellular invasion.

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