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Identification of Bullous Pemphigoid, Pemphigus, Laminin, and Anchoring Fibril Antigens in Human Fetal Skin

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Human fetal skin was evaluated for sequential and regional development of several epidermal antigens. Indirect immunofluorescent methods were used to detect laminin, bullous pemphigoid antigen, pemphigus antigen, and anchoring fibril antigens identified by monoclonal antibodies AF1 and AF2. Eighty-three human fetal skin biopsies from 32 human fetuses were examined. The fetuses examined ranged from estimated gestational age (EGA) of 7–38 weeks. Laminin was present in the basement membrane zone of all the fetal tissues examined. Bullous pemphigoid antigen developed first in the palm and sole, 9 weeks EGA, and was present in all other sites by 17 weeks EGA. Pemphigus antigen was present by 11 weeks EGA. AF1 and AF2 staining was not present until 26 weeks EGA. AF1 and AF2 stained epidermal basal cells in addition to the basement membrane zone area. Comparison of human fetal skin development with basal cell carcinoma identified similarities between basal cell carcinoma and early fetal development.

Human fetal skin follows an orderly morphologic sequence of epidermal and dermal development [1–3]. The initial single-layered epithelium evolves into its final complex organization by cellular differentiation and immigration of cells from other sites. Since little information was known about the sequence of antigenic development of human fetal skin, we evaluated human fetal skin for presence of laminin, bullous pemphigoid antigen, pemphigus antigen, and antigens associated with the development of anchoring fibrils.

MATERIALS AND METHODS

Human Fetal Tissue

Eighty-three human fetal skin biopsies, from 32 human fetuses, were examined. Human fetal tissue collection conformed to current recommendations of the NIH and recommendations of the University of Rochester Committee on Investigation Involving Human Subjects. Signed informed consent was obtained. The fetuses examined ranged from estimated gestational age (EGA) of 7–38 weeks. Fetal tissues under 24 weeks EGA were the products of conception of human abortions. Fetal skin biopsies over 24 weeks EGA were removed from premature infants who had died within 24 h of birth. The gestational age of the infant was established by the EGA taken from the maternal history correlated with the heel-toe length standards [4] when possible. The abortion tissue specimens were collected within 2 h after extraction from the human uterus. The tissue sections were frozen in OCT (Lab-Tek Products, Naperville, Illinois) and stored at −70°C until immunofluorescent examination was completed. Skin from expired premature infants was removed at the time of autopsy. These infants underwent autopsy within 24 h of death, and they were stored at 4°C from death until the time of autopsy. Fetal tissues used in this study could be clearly identified as to their regional site of origin and showed no signs of physical trauma.

Monoclonal Antibodies to AF1 and AF2

AF1 and AF2 are IgG_1 murine monoclonal antibodies which react with human skin basement membrane zone in the region of the anchoring fibrils [5,6]. The hybridoma cell supernatants were concentrated to 1/20 the original volume and stored at −20°C until use. For immunofluorescence the concentrated supernatant was diluted 1:5 in phosphate-buffered saline (PBS) prior to use on fetal skin sections.

Bullous Pemphigoid, Pemphigus, and Laminin Antibodies

After informed consent, human sera was obtained from a patient with bullous pemphigoid and a patient with classical pemphigus vulgaris; both diagnoses were confirmed by direct and indirect immunofluorescence. Indirect immunofluorescent titers on neonatal foreskins for the bullous pemphigoid serum and pemphigus vulgaris serum were 1:160 and 1:320 respectively. For our study, human bullous pemphigoid serum was diluted 1:5 in PBS and pemphigus vulgaris human serum was diluted 1:20 in PBS prior to use on fetal skin sections. These titers were selected as the highest titers with the least nonspecific background on human foreskins. Rabbit antilaminin antisera was purchased from BRL (Gaithersberg, Maryland) and diluted 1:50 in PBS prior to use on fetal skin section.

Indirect Immunofluorescence

The method for indirect immunofluorescence on frozen sections was a modification of those of Beutner et al [7] and Huff et al [8] using frozen 4-μm sections. Control slides using appropriately diluted human serum, rabbit serum, NS1 myeloma cell supernate, and PBS were used in place of primary antibodies. The fluorescein isothiocyanate-conjugated secondary antibody with appropriate specificity (Cappel, Cochranville, Pennsylvania) was diluted 1:20 in 0.01 M PBS, pH 7.2, plus 1% bovine serum albumin, with 0.05% sodium azide added. The secondary antibodies for the human sera, laminin, and monoclonal antibodies were, respectively, goat antihuman IgG Fc specific, goat antirabbit IgG, and goat antimouse $F(ab')_2$. Slides were read on a Zeiss epifluorescence-equipped, standard microscope with excitation filter BP 450-490, beamsplitter FT 510, and barrier filter BP 520-560.

RESULTS

Diffuse background staining of periderm cells was present with all the controls and primary antibodies but was slightly greater with the PBS control. The periderm staining varied from region to region on the individual biopsy showing a bright globular pattern which obstructed visualization of individual cell borders and caused difficulty in interpreting several of the early EGA slides. In order to limit false-positive results, slides were not considered positive unless specific staining was greater than the PBS control.

Table I identifies the fetal tissues sampled. Tissues were divided by EGA and region. Throughout this study, we were unable to identify differences among forearm, arm, leg, or thigh; therefore, these regions are grouped together as extremities. Fig 1 identifies fetal age of first positive fluorescent staining, time sequence of regional differences, and presence of positive staining in all regions examined. By 7 weeks EGA (Fig 2) laminin was present along the basement membrane zone, around blood

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Abbreviations:
EGA: estimated gestational age
PBS: phosphate-buffered saline

TABLE I. *Human fetal skin biopsies studied*

Estimated gestational age (weeks)	Number of different fetuses examined	Location and number				
		Palm	Sole	Extremity	Trunk	Scalp
7	1	1	1	2		
8–9	5	2	3	5	2	
10–11	2	1	1	2	1	1
12–13	1		1			
14–15	4	1	1	4	3	3
16–17	6	3	4	5	4	1
18–19	6	1	5	4	1	1
20–21	1	1	1			1
22–23	1	1	1	1	2	1
26	1		1		1	1
27	1				1	
28	1		1		1	
30	1				1	
38	1		1	1	1	
Total	32	11	21	24	18	9

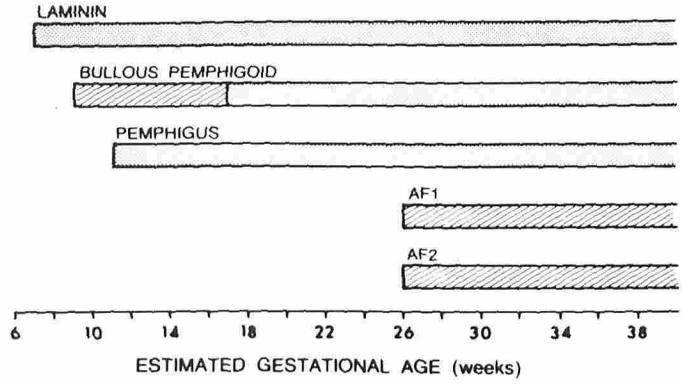

FIG 1. Summary of positive indirect immunofluorescence staining of human fetal tissues. *Hatched bars* identify EGA when variations in regional sites were identified. *Dotted bars* identify EGA when consistent staining of all sites was identified. The regional variation of bullous pemphigoid identified staining on fetal sole and palm prior to other regions. AF1 and AF2 regional variation consisted of variations in intensity of staining and staining patterns.

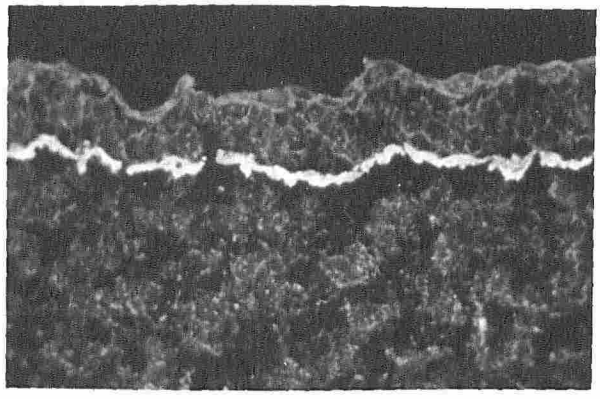

FIG 2. Seven-week EGA fetal sole, stained for laminin shows a linear basement membrane staining pattern (× 400).

vessels, and on the periosteum. The laminin staining consistently showed a bright narrow band along the dermal-epidermal junction and did not show regional variation.

Fetal tissue prior to 9 weeks EGA did not stain with bullous pemphigoid serum. In a single 9-week EGA fetus the sole (Fig 3) and palm showed a thin, interrupted, linear pattern while the arm, thigh, and trunk did not stain. In addition, a faint intercellular staining pattern is visible between basal cells of the sole (Fig 3). Another 9-week EGA fetus demonstrated trace staining of the sole and negative staining of the palm. Eleven weeks EGA and later, fetal soles and palms were always positive for bullous pemphigoid staining. In one 14-week EGA fetus, the sole showed the characteristic mature linear basement membrane staining, (Fig 4) while the palm of the same fetus showed an irregular interrupted pattern with predominant basal cell staining (Fig 5). By 17 weeks EGA, a linear or interrupted linear pattern was seen in all fetal tissues examined.

By 11 weeks EGA the intermediate and basal cells showed positive epidermal intercellular staining with pemphigus serum.

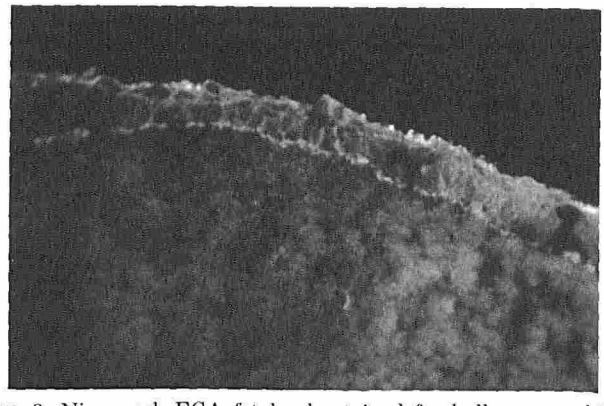

FIG 3. Nine-week EGA fetal sole stained for bullous pemphigoid antigen identifies a thin, interrupted, linear basement membrane staining pattern in addition to intercellular staining of basal cells (× 250).

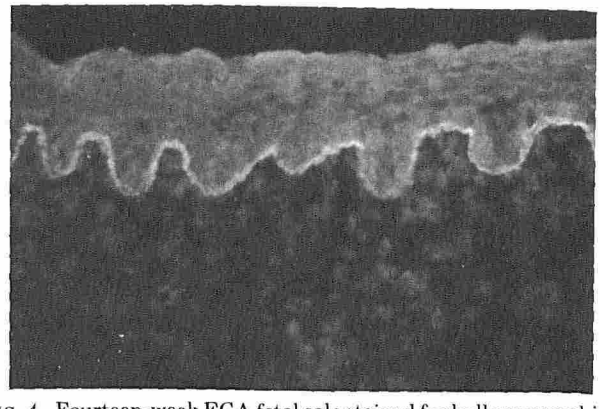

FIG 4. Fourteen-week EGA fetal sole stained for bullous pemphigoid antigen identifies a linear basement membrane staining pattern (× 250).

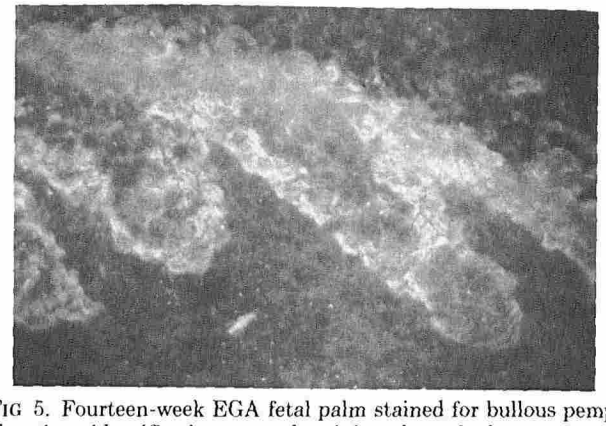

FIG 5. Fourteen-week EGA fetal palm stained for bullous pemphigoid antigen identifies interrupted staining along the basement membrane zone with diffuse basal cell staining (× 400).

The staining was consistently positive (14 positive, 3 trace positive, and 2 negative) on more mature fetal tissues regardless of biopsy location up to 15 weeks EGA. After 15 weeks EGA all tissues were positive for pemphigus. On many of the tissues, pemphigus antiserum absorbed on human group AB red blood cells [9] was compared to serum before absorption and differences were not seen.

All fetal tissues prior to 26 weeks EGA were negative for AF1 and AF2 staining. Fetal biopsies from 26 through 38 weeks EGA demonstrated positive fluorescence for AF1 and AF2, but not in the same linear pattern previously described [5,6]. Although both AF1 and AF2 stained alike, the pattern varied from a diffuse basement membrane zone and basal cell fluorescence to bright staining of the basal cell-basement membrane area. The pattern was consistent within an individual biopsy and on repeat examination of that biopsy. However, on different regions examined on the same fetus, regional intensity of staining was present.

DISCUSSION

The development of the human epidermis begins in the 4th to 6th week of gestation with a single layer of keratinocytes associated with a basal laminin [1,2]. Laminin, a noncollagenous basement membrane glycoprotein, is present in the basement membrane of animal and human tissue, localizing to the lamina lucida [10]. Sequential examination of developing mouse embryo identified laminin in the cytoplasm of the 16-cell morula prior to basement membrane formation. As basement membrane formed, it contained laminin [11]. Extrapolating mouse data to human suggests that laminin may be present in human epidermal basement membrane by 4–6 weeks EGA. We were unable to examine epidermal basement membrane before 7 weeks EGA, but laminin was consistently present by that time.

Previous authors have evaluated human fetal epidermis for the presence of pemphigus and bullous pemphigoid antigen by indirect immunofluorescent methods. Muller et al identified weak intercellular pemphigus staining by 9 weeks EGA and strong staining after 12 weeks EGA [12]. Varelzidis et al did not identify pemphigus antigen until 16 weeks EGA and noticed inconsistent staining up to 38 weeks EGA [13]. Our results are consistent with Muller with the additional findings that regional sites were evaluated and differences were not detected on multiple regions of the same fetus.

Muller et al [12] noted bullous pemphigoid antigen to be weak and patchy at 12 weeks EGA, whereas Varelzidis et al [13] did not identify bullous pemphigoid antigen until 16 weeks EGA. Neither group saw consistent bright linear immunofluorescence before 23 weeks. Our studies noted specific regional differences, suggesting that the presence of bullous pemphigoid antigen is related to or associated with epidermal maturation. Previous electron microscope studies identified foot epidermal development to be more advanced in thickness and state of differentiation during the first trimester. Palm development was not evaluated in that study [14]. Our data suggest that bullous pemphigoid antigen expression is dependent on maturity of overlying epidermis, being expressed at an earlier gestational age in the palm and sole. Bullous pemphigoid antigen developed in 3 patterns. Most commonly bullous pemphigoid antigen was identified in a thin, interrupted linear pattern, but it was also present in a thin interrupted linear pattern associated with intercellular staining between basal cells, or in a basal cell cytoplasmic pattern. With increasing gestational age the pattern remained linear but became brighter and continuous. Fetal basal cell staining with bullous pemphigoid antibody is consistent with data identifying bullous pemphigoid antigen in the cytoplasm and on the cell membrane of cultured human epidermal cells [15,16]. Overall, our studies identified regional development and earlier development of bullous pemphigoid antigen than previous studies. Heterogeneity of bullous pemphigoid antigen as defined by different pemphigoid sera, has been recognized [17]. If truly heterogeneous, different bullous pemphigoid antigens may develop at different gestational ages.

Absent staining for AF1 and AF2 prior to 26 weeks EGA was unexpected. Anchoring fibrils are present in the dermis by 72 days EGA by their electron microscopic appearance [2], yet the antigens associated with AF1 and AF2 did not appear until 16 gestational weeks later. AF1 and AF2 identify a linear basement membrane zone staining pattern in human adult tissue but the fetal tissues characterized a diffuse basal cell basement membrane zone staining pattern. Both AF1 and AF2 antibodies are products of mice immunized with epithelial components [6]. In the developing fetus, we identified AF1 and AF2 antigens first within the epidermis. This sequence of development suggests that AF1 and AF2 antigens are produced and expressed in fetal epidermis and then are translocated to the anchoring fibrils with time or associated with maturity.

Characterization of basal cell carcinoma has shown persistence of laminin [18,19] and type IV collagen at the tumor-dermal junction, with faint or undetectable bullous pemphigoid antigen [19]. Electron microscopy studies revealed areas with persistence or disappearance of the basal lamina [20] and irregular narrowing of the lamina lucida [21]. Studies on fetal skin show basal lamina and laminin present by 7 weeks EGA, yet bullous pemphigoid antigen does not develop until 9–17 weeks EGA or later. The time between 7 and 17 weeks EGA suggests that in one way basal cell carcinoma may recapitulate fetal skin development showing basal lamina development without bullous pemphigoid antigen. In recent studies, we have evaluated the basement membrane zone in basal cell carcinoma and shown absent or weak staining for AF1, AF2, and bullous pemphigoid antigen, while laminin staining was undiminished [22]. This is the same pattern characteristic of early fetal development.

In future studies, epidermal cell culture systems may offer an in vitro method for recapitulating the ontogeny of fetal skin. Epidermal cells cultured on collagen-coated plastic dishes form a basement membrane zone, but no anchoring fibrils [23]. Human epidermal keratinocytes synthesize both laminin [24] and bullous pemphigoid antigens in culture [15,24]. Perhaps through environmental control of epidermal cells with growth factors, hormones, or specially prepared culture plates, a more complete recapitulation of fetal development will be obtained. Such recapitulation of skin culture will offer new modes of therapy for large wounds, ulcers, and burns.

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