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Monoclonal Antibody AHS-7 Defines a Specific Basement Membrane Antigen Localized to the Hemidesmosome Zone

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MoAb AHS-7 labels the basement membrane of the skin. In this report, we have determined the precise localization and molecular nature of the antigen(s). After epidermal-dermal separation by suction blistering, the antigen(s) were detected on the epidermal side. Immunoelectron microscopy revealed the reaction product observed as a discontinuous line along the plasma membrane of the basal keratinocytes, which corresponded to the distribution of the hemidesmosomes. We extracted the proteins from the normal human epidermis separated from the dermis by suction blistering. After being separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the proteins were electrophoretically transferred to nitrocellulose sheets, and the antigen(s) were identified by immunoperoxidase staining with MoAb AHS-7. We detected 4 protein bands of molecular weights of 82 kd, 104 kd, 120–135 kd, and 140–160 kd when unreduced, in contrast to only two bands of 41 kd and 52 kd when reduced. These data suggest that the antigen(s) recognized by MoAb AHS-7 defines a unique antigen, different from bullous pemphigoid antigen on the hemidesmosome zone. (Received December 12, 1986.)

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The epidermal-dermal junction (EDJ) between basal keratinocytes and dermis can be divided ultrastructurally into 4 areas proceeding from the epidermis toward dermis, i.e., the basal cell plasma membrane with hemidesmosomes, lamina lucida, lamina densa, and sub-lamina densa zone (1). Recently the biochemical characterization of the EDJ has been investigated and several macromolecules identified in specific areas of the EDJ. The presence of laminin (2), fibronectin (3), and bullous pemphigoid antigen (4, 5) in the lamina lucida, and type IV collagen in the basal lamina (6) are confirmed.

Moreover with a recent advance in monoclonal antibody techniques, several monoclonal antibodies, which define unique basement membrane zone antigens, have been obtained, e.g., KF-1, which identifies a noncollagenous constituent of the lamina densa (7), monoclonal antibodies to anchoring fibrils, AF-1 and AF-2 (8), LDA-1 monoclonal antibody, which defines the antigen in the lamina densa and to a much lesser extent, the adjacent sub-lamina densa region (9) and LH 7:2 which also binds the antigen in the lamina densa (10). We have also reported a monoclonal antibody (MoAb AHS-7) which showed linear staining along the basement membrane in the immunofluorescent staining of normal human skin (11). In this paper, we show the precise ultrastructural localization of the antigen(s) defined with MoAb AHS-7 as well as the molecular weights by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Abbreviations: EDJ=epidermal-dermal junction, SDS–PAGE=sodium dodecyl sulfate-polyacrylamide get electrophoresis, DAB=3,3′-diaminobenzidine tetrahydrochloride, PBS=phosphate buffered saline, PLP=periodate-lysin-paraformaldehyde, BSA=bovine serum albumin.

MATERIALS AND METHODS

Immunoreagents

The following immunoreagents were used: normal horse serum; horse biotinylated anti-mouse IgG; avidin DH; biotinylated horseradish peroxidase H (Vector Lab Inc., Burlingame, Calif.); 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Dojin Lab Inc., Kengucho, Kumamoto, Japan); horseradish peroxidase (Sigma Chemical Company, St. Louis, Mo., USA); protein A (Pharmacia Fine Chemicals AB, Uppsala, Sweden).

Immunoperoxidase staining

Normal human skin before and after separation by suction blistering was biopsied from volunteers and snap frozen in liquid nitrogen. Suction blisters were produced according to the method of Kiistala (12). Six-micron cryostat sections were air-dried and fixed in acetone for 10 min, and then stained by the preformed avidin-biotin-peroxidase complex method detailed elsewhere (13). After being blocked with normal horse serum diluted in phosphate buffered saline (PBS) for 30 min at 37°C, sections were incubated for 1 h at 37°C with optimal dilutions of MoAb AHS-7 (IgG2a) or with the culture fluid of mouse myeloma cell line P3×63Ag8.653 as negative controls. Subsequently the sections were followed by 30 min separate incubations with biotin-conjugated horse anti-mouse IgG dilutes in PBS at 37°C and the preformed avidin-biotin-peroxidase complex at 37°C. The reaction product was revealed by incubation in 0.05 M Tris-HCl buffer (pH 7.6), 0.05% DAB, and 0.01% H_2O_2 for 5 min at a room temperature and was followed by a quick rinse in distilled water. Finally, after nuclear staining with hematoxylin, the sections were treated successively from distilled water to xylen and mounted in Eukit.

For immunoelectron microscopic observations, cryostat sections from normal human skin fixed in periodate-lysin-paraformaldehyde (PLP) for 4 h at 4°C were used according to the procedure of McClean & Nakane (14). Immunoperoxidase staining was performed as described above. Ten-micron sections were fixed in osmium tetraoxide and embedded in Epon. Ultrathin sections were examined using JEM 100C electron microscope either with or without counterstaining with lead citrate.

Extraction of normal human skin

Preparations of epidermis were obtained from suction blisters. Approximately 1.0 cm^2 of the epidermal sheet was washed with PBS and then dissolved in 0.15 M NaCl, 0.04 M EDTA, 0.04 M Tris, 0.2 M phenylmethylsulfonyl fluoride (NET buffer), pH 7.0, containing 0.5% NP40 for 1 h at 4°C. Following centrifugation at 105000×g for 1 h, the supernatant was added to equal volume of gel sample buffer (0.0625 M Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS)). The solubilized proteins in the mixture were heated at 100°C for 2 min with or without 5% 2-mercaptoethanol.

SDS–PAGE

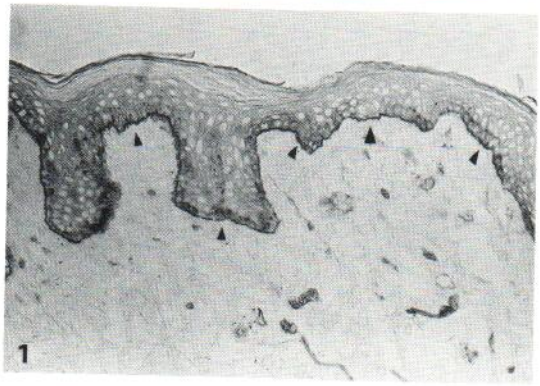
Proteins extracted from normal human epidermis were electrophoresed on slab gels with acrylamide concentration of 7.5% by the method of Laemmli (15). Standards for molecular weight determination were run on each gel.

Electrophoretic transfer of proteins to nitrocellulose

The proteins extracted from normal human epidermis and separated by SDS–PAGE were transferred to nitrocellulose according to the method of Towbin (16). Proteins were transferred overnight at 60 V using KS-8440 GMT gel-membrane transfer apparatus (Marysol Co. Ltd., Tokyo, Japan) with a tank buffer consisting of 25 mM Tris-HCl, 192 mM glycine, 20% methanol at pH 8.3. The nitrocellulose sheet was then cut into strips, each containing one lane of protein. The lanes containing standards for molecular weight determination were stained with 0.1% amido black in 25% isopropylalcohol, 10% acetic acid in distilled water and destained with 25% isopropylalcohol, and 10% acetic acid in distilled water.

Immunoperoxidase staining of transferred proteins

The nitrocellulose strips containing the proteins extracted from the epidermis were used for immunoperoxidase staining to identify the antigen(s) defined by MoAb AHS-7. The strips were incubated in 1% BSA in PBS at a room temperature for 2 h for saturation of free protein-binding sites. The strips were then incubated with MoAb AHS-7 or the culture fluid of mouse myeloma cell line P3×63Ag8.653 at 4°C overnight and washed 3 times with PBS and 3 times with PBS containing 0.05% Tween 80. Thereafter they were incubated stepwise with rabbit anti-mouse Ig and peroxidase-conjugated protein A at appropriate working dilutions in PBS. Peroxidase-labelled protein A was prepared according to



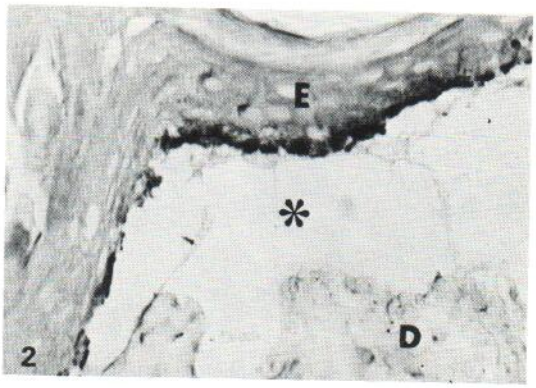


Fig. 1. Indirect immunoperoxidase staining of normal human skin with MoAb AHS-7. Linear staining along the EDJ (arrow-heads) are seen. ×200.

Fig. 2. Indirect immunoperoxidase staining of the skin after suction blistering with MoAb AHS-7. The linear staining is seen only along the roof of the blister, i.e., the base of the epidermis. ×400. (E=epidermis, D=dermis, *=blister.)

the method of Nakane (17). After each incubation, the strips were washed 3 times with PBS and 3 times with PBS containing 0.05% Tween 80. Finally, the strips were developed with 0.05% DAB, 0.01% H_2O_2 in 0.05 M Tris-HCl, at pH 7.2.

RESULTS

Immunoperoxidase staining in light microscopy

Indirect immunoperoxidase staining of normal human skin with MoAb AHS-7 showed linear staining along the EDJ (Fig. 1). The staining along the EDJ was absent from sections incubated with the control culture fluid of myeloma cell line P3×63Ag8.653. Indirect immunoperoxidase staining of the skin after suction blistering revealed that the antigen(s) defined by MoAb AHS-7 were present only along the roof of the blister, i.e., the base of the epidermis but not on the dermal side of the separation (Fig. 2).

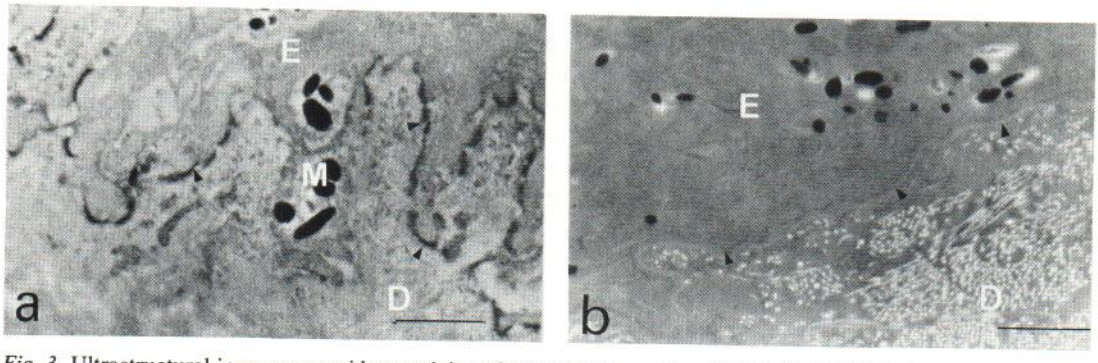


Fig. 3. Ultrastructural immunoperoxidase staining of normal human skin with MoAb AHS-7 (without counterstaining of lead citrate). (*a*) The electron dense deposits are recognized as a broken line along the EDJ except for the melanocyte-dermal junction. (*b*) No reaction product is seen after incubation with non-reactive mouse monoclonal antibody. (Arrow-heads show the EDJ. E = epidermis, D = dermis, M = melanocyte.) Bars =1 μm.

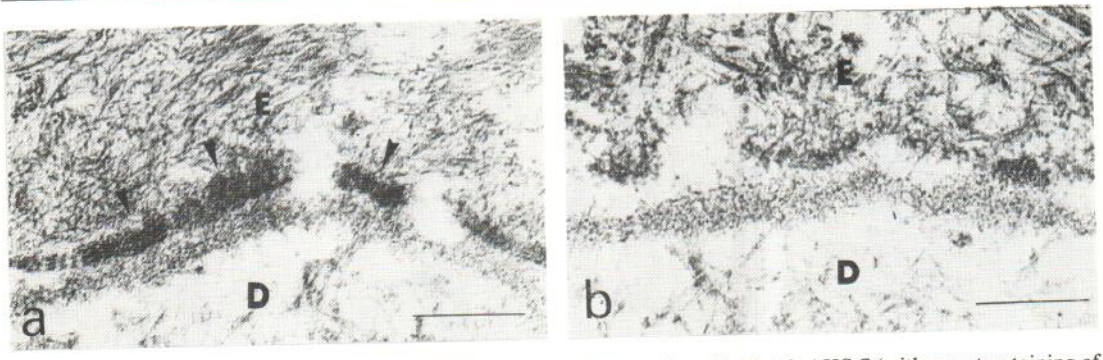


Fig. 4. Ultrastructural immunoperoxidase staining of normal human skin with MoAb AHS-7 (with counterstaining of lead citrate). (*a*) The electron dense deposits are present on the hemidesmosomes (arrow-heads). (*b*) No electron dense deposits are recognized after incubation with non-reactive mouse-monoclonal antibody. (E = epidermis, D = dermis.) Bars =0.25 μm.

Immunoelectron microscopy

At a low magnification, the electron-dense reaction products following staining with MoAb AHS-7 are recognized as a broken line along the EDJ (Fig. 3*a*), which were revealed to be localized along the plasma membrane of the basal cells, mainly at the hemidesmosomes at a much higher magnification (Fig. 4*a*). Again no such electron dense deposits were observed when the sections were incubated with the control culture fluid of myeloma cell line instead of MoAb AHS-7 (Figs. 3*b* and 4*b*).

Identification of the antigen(s) extracted from the epidermis

Immunoperoxidase staining of the nitrocellulose containing non-reduced solubilized proteins from the epidermis revealed 4 bands consisting of approximately 82 kd, 104 kd, 120 to 135 kd, 140 to 160 kd. When the extracted proteins were reduced and electrophoresed, proteins were resolved into 2 bands consisting of approximately 41 kd (minor subcomponent) and 52 kd (major subcomponent) (Fig. 5). No positive stained bands were recognized in immunoperoxidase staining using the control culture fluid of myeloma.

DISCUSSION

Recent progress in techniques for identifying and characterizing basement membrane zone macromolecules confirmed the presence of several kinds of molecules in the EDJ. There are laminin (2), fibronectin (3), bullous pemphigoid antigen (4, 5), and other as yet poorly defined antigens identified by in vivo bound and circulating antibodies in the sera of patients with herpes gestationis (herpes gestationis antigen) (18) and scarring pemphigoid (scarring pemphigoid antigen) (19, 20) in the lamina lucida, and type IV collagen (6), heparan sulfate proteoglycan (21) and KF-1 antigen (7) in the lamina densa. Furthermore AF-1, AF-2 (8), and epidermolysis bullosa acquisita antigen(s) (19, 20) are detected in the sublamina densa zone. Woodley et al. (23) showed that after suction blistering, only bullous pemphigoid antigen is present at the base of the epidermis among 5 basement membrane components consisting of bullous pemphigoid antigen, laminin, heparan sulfate proteoglycan, type IV collagen, and type V collagen. The antigens defined by the 5 monoclonal antibodies, KF-1, AF-1, AF-2, LDA-1, and LH 7:2 are also thought to be present on the dermis after suction blistering, since they are recognized ultrastructurally below the lamina lucida. In this study, the immunoperoxidase staining with MoAb AHS-7 showed a linear staining pattern only at the base of the epidermis of suction blisters, which

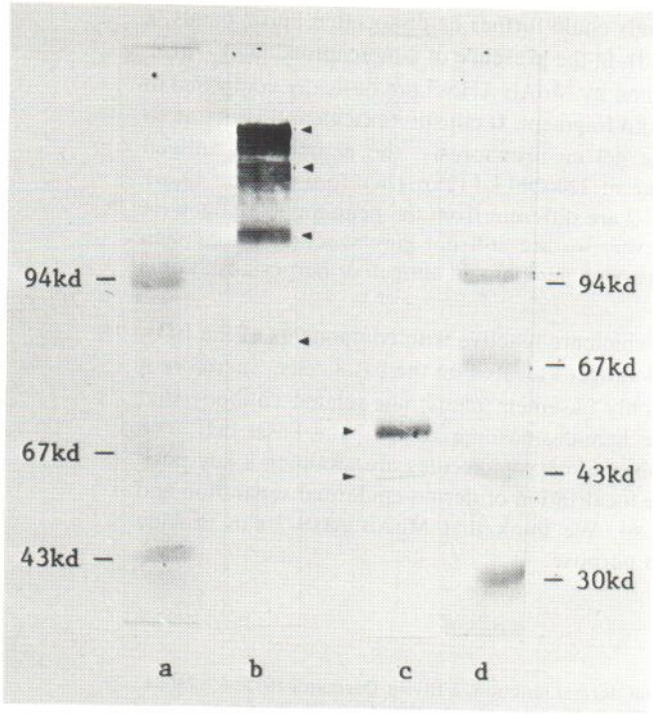


Fig. 5. Identification of the MoAb AHS-7-related antigens extracted from normal human skin. Proteins extracted from suction blister-derived epidermis or the standard proteins for molecular weight were either reduced or unreduced, separated by SDS–PAGE, and electrophoretically transferred to nitrocellulose strips. Lane (*a*): The standard proteins (unreduced) are stained by amido black protein staining. Lane (*b*): MoAb AHS-7-related antigens (unreduced) were specifically identified by immunoperoxidase staining of the strips using MoAb AHS-7. Four protein bands, 82 kd, 104 kd, 120–135 kd, and 140–160 kd are recognized (arrowheads). Lane (*c*): MoAb AHS-7-related antigens (reduced) were also identified. A minor band, 41 kd and a major band, 52 kd are recognized (arrowheads). Lane (*d*): The standard proteins (reduced) are stained by amido black protein staining. No positive-stained bands were observed in immunoperoxidase staining of epidermal protein (unreduced or reduced) with the control fluid of mouse myeloma.

suggests that the antigen(s) defined by MoAb AHS-7 are present at a site similar to bullous pemphigoid antigen. Although herpes gestationis antigen and scarring pemphigoid antigen might also be located at a site near to that of the antigen(s) defined by MoAb AHS-7, their localization in the EDJ has not been elucidated yet after suction blistering .

Immunoelectron microscopic observation revealed that the reaction products were deposited along the plasma membrane of the basal cells like a broken line at a low magnification. Higher magnification demonstrated that this discontinuity along the basement membrane zone is caused by the fact that the reaction products are localized to hemidesmosomes, although it is not clear whether the MoAb AHS-7-related antigens are present on the plasma membrane or on the attachment plaque of the hemidesmosome. This immunoelectron microscopic staining pattern resembles that of bullous pemphigoid antigen recognized by Yamasaki & Nishikawa (24). Therefore this finding suggests that the antigen(s) defined by MoAb AHS-7 is located at a site near to bullous pemphigoid antigen.

We further identified the MoAb AHS-7-reactive antigen(s) among the proteins extracted from the suction blister-derived epidermis by SDS–PAGE. MoAb AHS-7 stains 4 bands which correspond to the molecular weights of approximately 82 kd, 104 kd, 120–135 kd,

and 140–160 kd, respectively. Those 4 bands could further be dissociated into 2 bands, a minor band (41 kd) and a major band (52 kd), in the presence of 2-mercaptoethanol. These data might suggest that the antigen(s) defined by MoAb AHS-7 are basically composed of disulfide linked 41 kd fragments and/or 52 kd fragment. It constitutes a clear distinction to the finding that, by SDS–polyacrylamide gel electrophoresis, the pemphigoid antigen could be resolved as a doublet in the range of 220–240 kd (25). Therefore it seems likely that the antigen(s) defined by MoAb AHS-7 are different from the pemphigoid antigen on the basis of the molecular weight. However, we are still not sure whether the antigen defined by MoAb AHS-7 is the same as scarring pemphigoid antigen or herpes gestationis antigen.

Among various monoclonal antibodies which are reactive with components of the EDJ, there is no other antibody that detects the antigens located on the basal cells. Therefore it is noteworthy that MoAb AHS-7 is the only basement membrane-related antibody that reacts with the antigens localized to the hemidesmosome zone of the basal cell. The antibodies which identify basement membrane macromolecules are attaining a key position as a tool in understanding the precise localization of dermo-epidermal separation and diagnosis of mechanobullous diseases (26). We think that MoAb AHS-7 can provide another important and useful tool for this purpose.

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