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A Mouse Monoclonal Antibody Against a Newly Discovered Basement Membrane Component, the Epidermolysis Bullosa Acquisita Antigen

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A mouse monoclonal antibody, H3a, directed against the newly described epidermolysis bullosa acquisita (EBA) antigen was obtained using hybridoma techniques. The distribution of the monoclonal antibody is identical to that of the polyclonal serum antibody of patients with EBA. By immunofluorescence, a linear band is seen at the dermal-epidermal junction and, by immunoelectron microscopy, immune reaction products are present in the lamina densa and sublamina densa regions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western immunoblot analysis shows that the monoclonal antibody recognizes 290 and 145 kilodalton proteins present in the immunizing junctional extract, identical with the newly discovered EBA antigen. This monoclonal antibody should be useful in the further isolation and characterization of the EBA antigen.

Epidermolysis bullosa acquisita (EBA) is a subepidermal blistering disease characterized by tissue-bound antibodies and circulating IgG autoantibodies to a normal component of the cutaneous basement membrane zone (BMZ). Immunoelectron microscopic studies have shown that this component is present in the lamina densa and sublamina densa regions of the BMZ [1–3].

In a recent study [4], BMZ proteins were extracted from normal human skin, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and reacted in a Western blot assay with serum samples from EBA patients. IgG antibodies in these sera bound to a major 290 kD protein and a minor 145 kD protein, the EBA antigen(s). Moreover, the antibodies in the patients' sera did not recognize any of the known matrix molecules in identical Western blot assays with purified components. In addition, the EBA antigen migrates differently on SDS-PAGE from other known matrix molecules, including laminin, fibronectin, collagens type I, IV, and V, the bullous pemphigoid antigen, and elastin.

In this study, we have used a human skin BMZ extract as an immunogen to develop a mouse monoclonal antibody directed against the EBA antigen. This monoclonal antibody, H3a, should be valuable in further isolation and characterization of the EBA antigen.

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Abbreviations:
BMZ: basement membrane zone
EBA: epidermolysis bullosa acquisita
FITC: fluorescein isothiocyanate
PBS: phosphate-buffered saline
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

MATERIALS AND METHODS

Preparation of Dermal BMZ Extract

Whole human skin sections were separated at the level of the lamina lucida by incubation in 1 M sodium chloride solution at 4°C for 4 days. The dermal extract was subsequently prepared by extraction for 1 h with 8 M urea and 0.3 M beta-mercaptoethanol in 0.5 M Tris-hydrochloride, pH 8.5, according to the method of Scaletta et al [5].

Preparation of the Hybrid Cell Line and the Hybridoma Antibodies

The H3a hybridoma was produced by conventional techniques with the modifications described by Goldsmith and Briggaman [6]. BALB/c mice (Charles River, Wilmington, Massachusetts) were immunized with an i.p. injection of 2.5 mg of 1-h BMZ extract in 0.25 ml phosphate-buffered saline (PBS) homogenized with an equal volume of complete Freund's adjuvant. Four weeks later a booster of 1 mg BMZ extract in 0.5 ml PBS (without adjuvant) was given i.p., and a final booster of 1 mg BMZ extract in 0.5 ml PBS was injected 3 days before harvesting of the mouse spleen. Nonsecreting P3/NS1 mouse myeloma cells were fused with splenic lymphocytes, and grown in HMT medium over STO cell feeder layers (HGPRT-negative mouse fibroblast lines). Culture supernatants were screened for anti-BMZ antibodies by indirect immunofluorescence using neonatal human foreskin as substrate, and cultures of interest were cloned by limiting dilution.

The supernatants of monoclonal cell cultures were collected and concentrated by ultrafiltration using an XM50 Amicon membrane, molecular weight cut-off 50,000 (Amicon Corp., Danvers, Massachusetts). The antibody was then purified by DEAE Affi-gel Blue chromatography (Bio-Rad Laboratories, Richmond, California) using a Tris-HCl .02 M, pH 7.2 buffer [7], and 70 mM NaCl to elute the IgG. Antibody protein concentration was determined by absorbance spectrophotometry at OD_{280}, and final antibody titer was ascertained by indirect immunofluorescence of serial dilutions.

Assays

Immunofluorescence: Frozen tissues were cut in 8-μm sections and air-dried on glass slides. Sections were overlaid with undiluted hybridoma culture media, or EBA sera diluted 1:20, incubated in a moist chamber at room temperature for 30 min, and then washed with PBS. The tissue was subsequently treated with fluoresceinated goat anti-mouse IgG or antihuman IgG (1:20 dilutions, Cappel Laboratories, Cochranville, Pennsylvania) for 30 min and washed with PBS. The slides were mounted in 90% glycerol-PBS and examined under a Leitz fluorescent microscope.

Immunoelectron microscopy: Frozen specimens were cut into 10-μm sections and air-dried. The tissue was incubated with undiluted H3a supernatant or control media for 30 min in a moist chamber at room temperature and washed with PBS. The tissue was subsequently treated with fluorescein isothiocyanate (FITC)-goat antimouse IgG diluted 1:20 in PBS, FITC-rabbit antigoat IgG diluted 1:1 in PBS with 50% normal rabbit serum, goat anti-horseradish peroxidase diluted 1:1 in PBS with 50% normal rabbit serum (Cappel Laboratories, Cochranville, Pennsylvania) and finally, horseradish peroxidase 1 mg per 20 ml PBS (Sigma, St. Louis, Missouri), all steps separated by washing in PBS twice. Tissues were subsequently fixed in 2.5% glutaraldehyde, stained with Hanker-Yates reagent (Polysciences, Warrington, Pennsylvania) in 0.1 M Tris buffer, pH 7.5, with 1% H_2O_2 and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer. The specimen then was serially dehydrated in 50–100% ethanol, embedded in Epon, sectioned with a diamond knife, and examined in a JEM 100B electron microscope.

SDS-PAGE and Western immunoblot analysis: The extracted BMZ proteins were separated by SDS-PAGE as described by Laemmli [8] under reducing conditions using 5% separating gels and 3% stacking

gels. The separated proteins were electrophoretically transferred from the acrylamide gels to nitrocellulose paper (Western blot) overnight at 60 mA using a Hoefer transblot apparatus (Hoefer Scientific Instruments, San Francisco, California) [9]. The nitrocellulose sheet was cut into strips of one lane each. A high-molecular-weight lane and a BMZ extract lane were stained with 0.1% amido black. The remaining nitrocellulose strips were saturated with 3% bovine serum albumin in Tris-buffered saline for 1 h, then washed and incubated for 4 h with undiluted H3a supernatant, medium, or EBA serum, diluted 1:200. The strips were washed 8 times, and subsequently incubated for 3–4 h with ^{125}I-labeled affinity-purified rabbit antimouse IgG F(ab')$_2$ (for H3a supernatant and media) or [^{125}I]staphylococcal protein A (for EBA serum) prepared by the method of Hunter and Greenwood [10]. Strips were washed twice, then overnight, exposed to Kodak X-Omat film for up to 72 h at −70°C, and developed.

The rabbit antiserum to mouse IgG F(ab')$_2$ fragments (Cappel Laboratories, West Chester, Pennsylvania) was purified by precipitation at 45% ammonium sulfate and chromatography on DEAE-cellulose [11]. The IgG fraction was affinity purified on mouse IgG Sepharose which was produced by 45% ammonium sulfate precipitation of mouse IgG, purification on DEAE-cellulose [11], and coupling to Sepharose 4B by the method of Parikh et al [12]. The affinity-purified rabbit antimouse IgG fraction was subsequently eluted with 1.0 M acetic acid into tubes containing 1.0 M unbuffered Tris base, and dialyzed. This affinity-purified rabbit antimouse IgG F(ab')$_2$ was labeled with [^{125}I] NaI (Amersham, Arlington Heights, Illinois) to a specific activity of 4.9×10^4 cpm per ng [10]. The ability of the labeled antibody to detect mouse antibody was tested with a monoclonal antibody to epithelial cells, and was confirmed by fluorescence studies conducted in parallel.

Characterization of the Monoclonal Antibody

Antibody isotyping: Standard Ouchterlony double-diffusion techniques were employed for typing the monoclonal antibody using immunoglobulin class-specific rabbit antimouse antisera against the following determinants: G1, G2a, G2b, G3, M, A, kappa light chain, and lambda light chain (Bionetics, Charleston, South Carolina).

Complement fixation: Complement fixation was assessed by incubation of neonatal human foreskin for 30 min with H3a, then with human C3 in a moist chamber at 37°C for 45 min. After washing with PBS, we added goat antihuman C3 (1:10 dilution, Cappel Laboratories, Cochranville, Pennsylvania) for 30 min and washed with PBS. Slides were mounted in 90% glycerol-PBS.

Staphylococcal protein A binding: Tissue treated with H3a was overlaid with undiluted fluorescein-conjugated staphylococcal protein A for 30 min, washed, and examined. In addition, [^{125}I]staphylococcal protein A labeling of H3a-treated nitrocellulose papers with BMZ extracts was compared with [^{125}I]rabbit antimouse labeling of Western immunoblots.

RESULTS

Immunofluorescence and Immunoelectron Microscopic Studies

Indirect immunofluorescence staining of normal human skin with H3a showed a linear band of fluorescence at the dermal-epidermal junction without staining of vascular BMZ (Fig 1). Sections treated with HMT medium rather than H3a were devoid of fluorescence. Sections treated with serum samples from EBA patients showed a linear pattern of fluorescence identical to that seen with H3a, except that the background nonspecific fluorescence was much greater when polyclonal EBA sera were used.

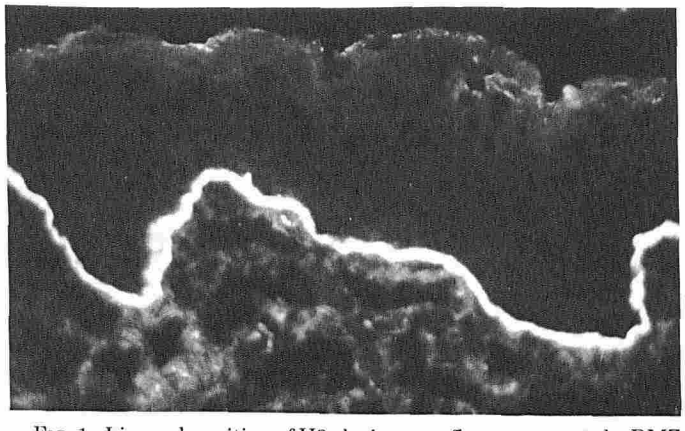

FIG 1. Linear deposition of H3a by immunofluorescence at the BMZ of human neonatal foreskin (× 870).

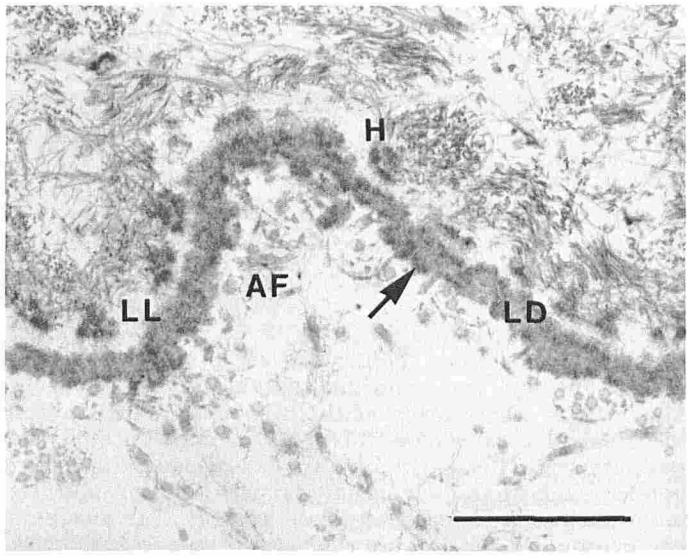

FIG 2. Immunoelectron micrograph prepared with H3a antibody shows the localization of reaction product (*arrow*) in and below the lamina densa (*LD*). Lamina lucida (*LL*) is free of reaction products. Hemidesmosomes (*H*) were stained equally on both control and antibody (H3a)-treated specimens. *AF* = anchoring fibrils. Uranyl acetate and lead citrate. *Calibration bar* = 1 μm.

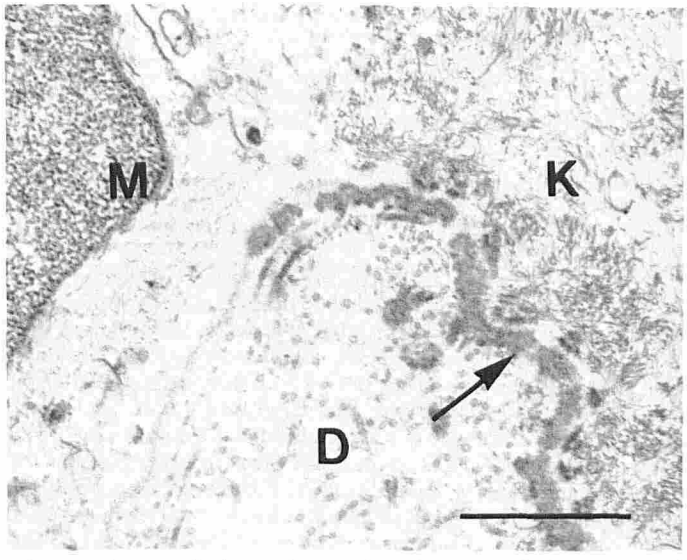

FIG 3. Immunoelectron micrograph with H3a antibody. An area of dermal-epidermal junction is seen in which a basal keratinocyte (*K*) and melanocyte (*M*) are adjacent, with dermis (*D*) beneath. Reaction products (*arrow*) are present beneath the keratinocyte but absent under the melanocyte. Uranyl acetate and lead citrate. *Calibration bar* = 1 μm.

Ultrastructural immunoperoxidase staining of normal human skin incubated with H3a revealed a heavy continuous band of electron-dense reaction product localized to the lamina densa and immediate sublamina densa regions without preferential aggregation at sites of anchoring fibrils or hemidesmosomes (Fig 2). This pattern was also seen with immunoelectron microscopy of tissue sections treated with sera from patients with EBA. The band of electron-dense deposition followed the contour of the basal keratinocytes but was diminished or absent beneath the plasma membrane of melanocytes (Fig 3).

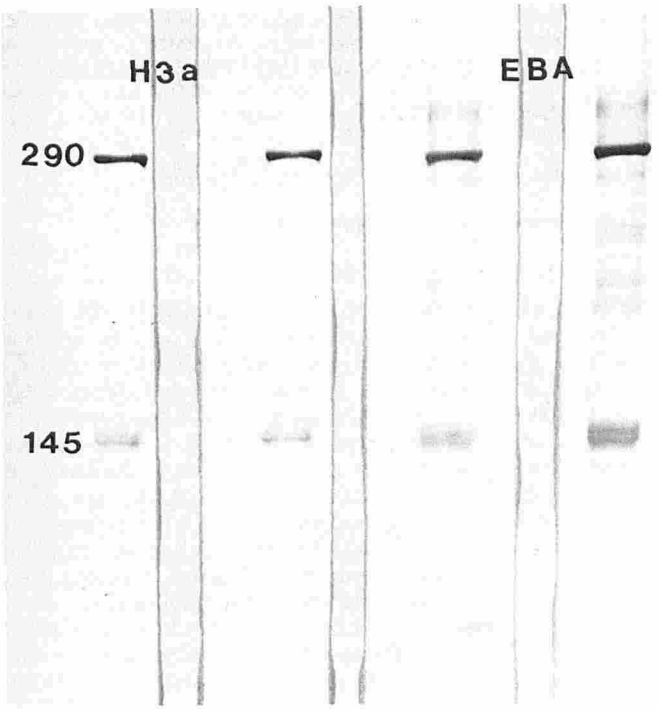

FIG 4. Identical binding patterns of H3a and polyclonal EBA serum antibodies to 145 and 290 kD proteins of junctional extract by Western immunoblot analysis. The *left two lanes* were treated with H3a antibody (*H3a*), and the *third and fourth lanes* with sera from 2 different patients with EBA (*EBA*).

SDS-PAGE and Western Immunoblot Analysis

Lanes of the BMZ extract transferred to nitrocellulose paper and stained with amido black revealed more than 100 protein bands. When identical unstained lanes were incubated with H3a antibody, the antibody bound to 2 bands: a major band of 290 kD and a minor band of 145 kD (Fig 4). These protein bands were also bound by antibodies in serum samples from 7 patients with EBA, but not with serum samples from normal individuals or patients with other blistering diseases. H3a did not bind in the Western immunoblots to known matrix components, including elastin, laminin, fibronectin and collagens type I, IV, and V.

Characterization of the H3a Monoclonal Antibody

By isotype analysis, the H3a was an IgG_1, kappa antibody. The monoclonal antibody showed no binding to complement or staphylococcal protein A. The protein concentration of purified antibody ranged from 5.7–20 mg/ml with a limiting dilution titer of 1:400 to 1:3200.

DISCUSSION

In this study, we have described a new monoclonal antibody, H3a, which is specifically directed against the EBA antigen. This monoclonal antibody binds to an antigen localized to the lamina densa and sublamina densa region, a pattern which matches that of polyclonal antibodies from patients with EBA. Even more convincing are the data from the Western immunoblot experiments in which both H3a and sera from EBA patients bound to 290 kD and 145 kD proteins in extracts of human skin basement membrane. The monoclonal nature of H3a is supported by finding only one immunoglobulin subclass (IgG_1, kappa) produced by this hybridoma.

When compared with immunofluorescent staining using the polyclonal EBA serum antibodies, the H3a monoclonal antibody stains the dermal-epidermal junction of normal human skin with minimal nonspecific background fluorescence. H3a promises to be useful in assessing the distribution of the EBA antigen in various human and animal tissues, as well as in studying its appearance in gestational development and relative concentration in different human tissue sites. Finally, the monoclonal antibody in its unlimited supply should be valuable in isolating and subsequently further characterizing the EBA antigen itself.

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