Monoclonal Antibody to a 35 kD Epidermal Protein Induces Cell Detachment*

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A murine monoclonal antibody (ECS-1) was prepared from BALB/c mice immunized with trypsinized cultured human foreskin keratinocytes. The antibody showed a pattern suggestive of intercellular staining on the nucleated layers of normal human epidermis, adult palm, mouse lip epidermis, and cultured human keratinocytes. ECS-1 stained human fetal skin by 9 weeks estimated gestational age. ECS-1 reacted with a 35 kD protein extracted from neonatal foreskin epidermis and cultured human keratinocytes. The protein required Nonidet P-40 or sodium dodecyl sulfate and mercaptoethanol for solubilization. ECS-1 induced epidermal cell detachment which was enhanced by complement. ECS-1 shares characteristics with human pemphigus antibodies. *J Invest Dermatol 86:634–637, 1986*

The pemphigus family of blistering diseases has been associated with autoantibodies reacting with epidermal cell surface antigens [1]. Differences between pemphigus vulgaris and pemphigus foliaceus antigens were demonstrated by immunoprecipitation of antigens from cultured epidermal cells [2]. We report a mouse monoclonal antibody (ECS-1) with several pemphigus-like properties.

MATERIALS AND METHODS

Materials BALB/c mice were from Dominion Laboratories, Dublin, Virginia; RPMI-1640 and fetal calf serum from K.C. Biological Inc., Kansas City, Missouri; M-199 from Gibco Laboratories Inc., Grand Island, New York; polyethylene glycol-1500 from BDH Chemicals Ltd., England; biotinylated goat antimouse IgM, avidin-type D and biotinylated horseradish peroxidase from Vector Laboratories Inc., Burlingame, California; fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgM (μ chain specific) from Cappel Laboratories, Cochranville, Pennsylvania; FITC-conjugated goat antihuman C3 from Kallestad Laboratories Inc., Chaska, Minnesota; dispase (grade II) Boehringer Mannheim Biochemicals, Indianapolis, Indiana; nitrocellulose paper from Bio-Rad Laboratories, Richmond, California. All other reagents were of the highest grades available. Serum from a patient with classical pemphigus vulgaris with positive direct and indirect titer (1:640) was used as a control for intercellular staining and for blocking studies.

Preparation of Monoclonal Antibody (ECS-1) Confluent cultures of neonatal keratinocytes [3] were rinsed with 0.02% EDTA in phosphate-buffered saline (PBS), treated with 0.25% trypsin in PBS to obtain cell suspension, washed 3 times with PBS, and immediately used for immunization. A female BALB/c mouse was immunized by i.p. injection of keratinocytes (1×10^6 cells) suspended in 0.3 ml of Freund's complete adjuvant. The mouse received 5 additional i.p. injections of 1×10^6 cultured keratinocytes in Freund's incomplete adjuvant at 2-week intervals, and was sacrificed 3 days after the final injection. Spleen cells from the immunized mouse were fused with an immunoglobulin nonproducing mouse myeloma cell line (NS-1), as previously described [4]. Hybrid cells formed colonies after 2 weeks. Hybrids were screened by indirect immunofluorescence with cryostat sections of human foreskin as substrate. The hybrid cells producing antibodies in a pattern suggesting reactivity against the epidermal cell surfaces were cloned by limiting dilution. ECS-1 was obtained after a second subcloning. Antibodies were harvested from the ascites of pristane-primed BALB/c mice injected i.p. with 1×10^6 hybrid cells. Control ascites were prepared from mice injected i.p. with the NS-1 cells or cells from an additional IgM producing hybridoma. The control IgM monoclonal antibody does not stain normal human or mouse epidermis. ECS-1 was determined to be an IgM by immunodiffusion. Its concentration also was determined by immunodiffusion using standards.

Indirect Immunofluorescence Human tissues were collected after informed consent and stored in OCT at −70°C until examined. Four-micron cryostat sections were cut from the unfixed tissues and allowed to air-dry at room temperature. Sections were reacted with optimal dilutions of ECS-1 (hybridoma culture supernatants, 18.5 μg IgM/ml or diluted ascites, 13.4 μg IgM/ml) in a moist chamber for 30 min and washed twice with PBS. Then,

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Abbreviations:

EGA: estimated gestational age
FITC: fluorescein isothiocyanate
ME: mercaptoethanol
NP-40: Nonidet P-40
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate-buffered saline
PMSF: phenylmethylsulfonyl fluoride
SDS: sodium dodecyl sulfate

the tissues were reacted with 1:40 diluted FITC-conjugated goat antimouse IgM (μ chain specific) for 30 min. After washing with PBS, the fluorescein-labeled sections were mounted and examined under a Zeiss fluorescent microscope using standard filters. Controls for immunofluorescent studies included NS-1 myeloma cell supernatant or ascites and the IgM monoclonal antibody control. Human fetal skins ranging from estimated gestational age (EGA) 7–15 weeks also were examined as previously described [5].

After 31 days of culture, confluent human cultured keratinocytes from neonatal foreskin epidermis were washed with serum-free medium and then 1.2 units/ml of dispase were added. After incubation at 37°C for 30 min, detached cultured keratinocytes were washed gently with serum-free medium, flattened and frozen in OCT embedding medium for indirect immunofluorescence tests.

Experiments attempting to block ECS-1 binding with pemphigus vulgaris serum were performed on sections of human foreskin as follows: sections were incubated in undiluted pemphigus vulgaris serum for 30 min, washed, incubated in ECS-1 culture supernatant (2.75 μg IgM/ml) for 30 min, washed, and finally incubated in a 1:40 dilution of FITC-conjugated goat antimouse IgM (μ chain specific). Another experiment attempting to block pemphigus vulgaris serum binding with ECS-1 was also done. Sections were incubated in culture supernatant (55 μg IgM/ml), washed, incubated in pemphigus vulgaris serum diluted 1:40 for 30 min, washed, and finally incubated in FITC-conjugated goat antihuman IgG diluted 1:40. Slides were mounted and examined as above.

Fixation of human complement to ECS-1 was confirmed with sera from 4 patients with acquired agammaglobulinemia. Four-micron frozen sections of human skin were reacted with ECS-1 as described above. Separate sections were then incubated in a moist chamber for 30 min with agammaglobulinemia serum diluted 1:10 in PBS plus 10 mM calcium chloride and 10 mM magnesium chloride. The sections were then washed twice with PBS before they were reacted with 1:10 diluted FITC-conjugated goat antihuman C3 for 30 min. Negative controls included replacement of ECS-1 with the control IgM hybridoma supernate, heat-treated agammaglobulinemia serum, agammaglobulinemia serum followed by FITC antihuman complement, and FITC antihuman complement alone.

Antigen Extraction and Characterization Epidermis was removed from neonatal foreskins by heat treatment (56°C, 30 s) and extracted with buffer 1 [0.01 M Tris-HCl, pH 7.2, 0.9% NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF) containing 0.5% Nonidet P-40 (NP-40)] [6], or with buffer 2 [0.0625 M Tris-HCl, pH 6.8, containing 2% sodium dodecyl sulfate (SDS) and 5% mercaptoethanol (ME)] [7]. The epidermis then was minced, vortexed in 5 vol of extraction buffer, and centrifuged at 11,600 g for 1.5 min. The supernatants were removed from the pellets and the solubilized proteins heated to 100°C for 5 min with SDS polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Proteins from cultured human keratinocytes (3×10^6 cells) were extracted in the same fashion. Extracted proteins were separated by SDS-PAGE [8] and gels were electrophoretically transferred to nitrocellulose sheets [9]. The blot then was cut into strips; one strip was stained with 0.1% Fast Green FCF for protein staining. The remaining nitrocellulose strips were used for avidin-biotin-peroxidase conjugate immunoperoxidase staining, as recommended by the manufacturer, and developed with 0.05% 4-chloro-1-naphthol in 0.0125% H_2O_2.

Cell Detachment Studies with ECS-1 Epidermal keratinocytes from neonatal BALB/c mice prepared according to standard techniques [10] were plated at 2×10^6 cells in 35-mm Corning Petri dishes and incubated 48 h at 32°C in 5% CO_2. The assay for cell detachment was performed using a slight modification of the method by Kawana et al [11]. After 48-h cultivation, the culture medium was gently removed and confluent cultivated epidermal monolayers were incubated in culture medium containing ECS-1 ascites (1.2 mg IgM/ml/dish) for 24 and 48 h in the presence or absence of fresh normal human serum (10%, 400 μl dish) as a source of complement. Fresh normal human serum was obtained from a healthy individual with blood type O. This serum was absorbed with blood type AB red cells at room temperature for 30 min. After centrifugation, the serum from the supernatant was reabsorbed with suspended neonatal mouse epidermal cells at 4°C overnight with gentle rocking. The absorbed serum was collected by centrifugation at 2000 rpm for 20 min, sterilized by Millex-GS filtration (Millipore Corp., Bedford, Massachusetts, pore size 0.22 μm), and used as a source of complement. After incubation the culture dish was gently rinsed by pipetting and the supernatant medium then was recovered. The supernatant collected was centrifuged at 1500 rpm for 5 min and deleted cells were resuspended in 0.5 ml of culture medium. These cells represent detached cells. These aliquots from the resuspended medium were quantitated by a Coulter Counter ZBI and the results were expressed as percent cell detachment over the control value obtained with regular medium (no ECS-1, no complement source). Controls included another IgM monoclonal antibody (0.9 mg IgM/ml/dish) and NS-1 ascites (1.0 mg protein/ml/dish).

RESULTS

Following immunization with cultured human keratinocytes, an IgM monoclonal antibody designated ECS-1 gave a strong pattern suggestive of intercellular staining of the nucleated layers in normal epidermis. The reactivity with granular cell layers was stronger than that seen on basal cells (Fig 1). Similar staining was seen in human adult palm and mouse lip epidermis. Colonies of cultured human keratinocytes, detached with dispase, also showed a similar pattern in addition to diffuse cytoplasmic staining (Fig 2).

The pretreatment of skin sections with sera from a patient with pemphigus vulgaris did not block ECS-1 reactivity, and pretreatment with ECS-1 did not block the binding of the pemphigus vulgaris sera. Indirect immunofluorescence on 8- to 9-week EGA human fetal skin with ECS-1 showed a pattern suggestive of intercellular staining just below the periderm layer (Fig 3). Fetal epidermis is 2–3 layers thick at this age. No staining was found in fetal skin before this age.

Indirect immunofluorescent studies for fixation of human complement to ECS-1 were the same for all 4 agammaglobulinemia sera. ECS-1-treated skin sections demonstrated bright fluorescence in the pattern shown in Fig 1. All of the negative controls showed absent staining in the epidermis.

On immunoblot analysis with ECS-1 using an avidin-biotin peroxidase conjugate as the second antibody, ECS-1 recognized selectively a 35 kD protein extracted from neonatal foreskin and cultured human keratinocytes with NP-40 or SDS and ME (Fig 4).

Cell detachment studies showed ECS-1 ascites (1.2 mg IgM/ml) caused some cell detachment at 48 h compared with an identical

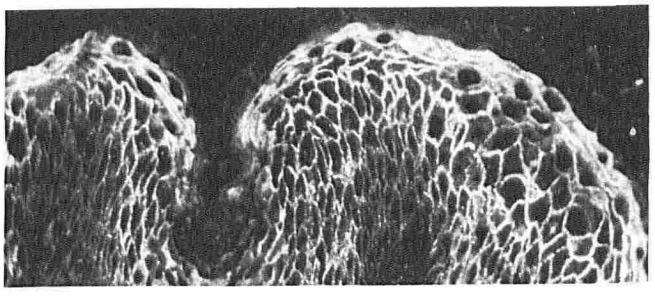

Figure 1. Foreskin stained with ECS-1. Indirect immunofluorescent staining patterns of normal human neonatal foreskin epidermis by ECS-1 (ascites, 13.4 μg IgM/ml). Selective staining is seen in a pattern similar to pemphigus foliaceus (× 400).

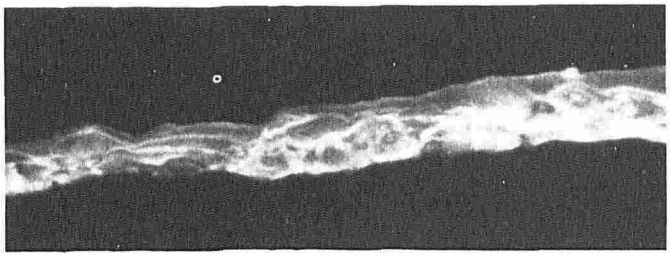

Figure 2. Colony of epidermal keratinocytes showing a pattern suggestive of intercellular staining and some cytoplasmic staining (× 400). Hybridoma supernatant concentrated to 18.5 μg IgM/ml.

volume of control ascites (Table I). When cells were maintained in media with ECS-1 (1.2 mg IgM/ml) and complement, the cell detachment was about 3.5 times higher than that obtained with ECS-1 ascites alone, and 5 times greater than that of the control ascites plus complement ($p < 0.01$ by t-test).

DISCUSSION

On indirect immunofluorescent sections ECS-1 produced a pattern suggestive of intercellular staining of the nucleated layers of normal epidermis. The reactivity of ECS-1 with increasing fluorescent intensity from the basal layer to the upper layers appears more like that of pemphigus foliaceus autoantibodies than pemphigus vulgaris autoantibodies [1]. Colonies of dispase-treated cultured human keratinocytes showed a similar pattern in addition to intracellular staining.

ECS-1 staining was compared with pemphigus staining in frozen sections of human fetal skin. The age of onset of the antigen defined by ECS-1, 8- to 9-week EGA, was similar to that of pemphigus antigen [5,12].

The 35 kD antigen recognized by ECS-1 is similar in molecular weight and extracted under the same conditions as the pemphigus antigen previously purified by Peterson and Wuepper [13]. The estimated molecular weight of this antigen by urea-SDS-PAGE was 33,000. Recently, Stanley, Yarr, Hawley-Nelson et al [6] reported the characterization of a pemphigus antigen extracted from a cultured mouse keratinocyte cell line (PAM) and human epidermal cell cultures by immunoprecipitation. An antigen with a molecular weight of 130,000 was specifically precipitated from both human and mouse cell extracts by sera from patients with pemphigus vulgaris [6]. They also demonstrated the distinction between epidermal antigens binding pemphigus vulgaris and pemphigus foliaceus autoantibodies using an immunoprecipitation technique [2].

Injections of ECS-1 into newborn mice did not produce blistering disease or epidermal staining. This could be due to the inability of ECS-1, an IgM antibody, to pass the basement membrane zone. When the antibody was directly added to cultured mouse keratinocytes, it caused cell detachment which was enhanced by complement. The inability of ECS-1 to block the binding of the pemphigus vulgaris and vice versa suggests antibody to the ECS-1 epitope is absent or at a low concentration in the polyclonal pemphigus serum. Further experiments with other polyclonal pemphigus antibodies will be necessary to address that finding more precisely.

Some pemphigus antibodies will fix complement to normal human skin [14,15], and recently, Kawana, Jordon, and Janson [16] have demonstrated that pemphigus antibodies will fix complement to organ cultured skin explants and to epidermal monolayers in tissue culture. Moreover, they showed pemphigus antibody-induced epidermal cell detachment was enhanced by complement [11]. Using their methods, we have shown that ECS-1 induces complement-enhanced cell detachment.

Recently, a monoclonal antibody with pemphigus-like staining, which had an antigen that could not be detected by immunoblotting and which was not studied for its ability to cause cell detachment, has been described. The relation between the antigen defined by that antibody and ECS-1 is unknown [17].

Since several pemphigus antigens with cell membrane and desmosomal locations have been described [18] it is reasonable to

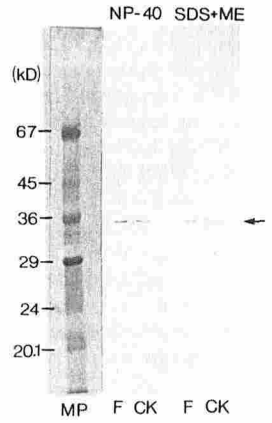

Figure 4. Molecular weight of the antigen defined by ECS-1. Proteins extracted from neonatal foreskin epidermis (*F*) and cultured human keratinocytes (*CK*) with Nonidet P-40 (*NP-40*) or SDS and mercaptoethanol (*SDS + ME*) were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose sheets. The antigen was identified by avidin-biotin immunoperoxidase staining using ECS-1 ascites (13.4 μg IgM/ml) and NS-1 ascites as a control. ECS-1 specifically identified a band of approximately 35K (*arrow*), as estimated from molecular weight standard marker proteins (*MP*). NS-1 ascites did not stain this band.

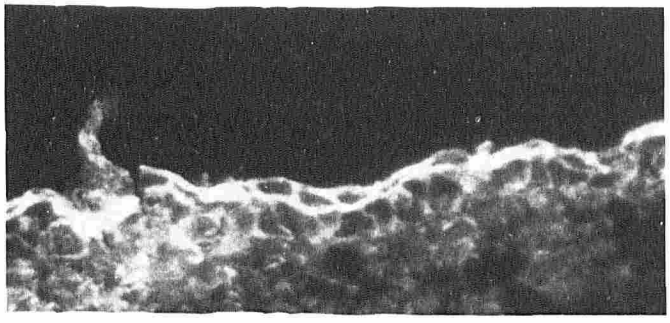

Figure 3. Fetal epidermis stained with ECS-1. Eight- to nine-week human fetal skin stained for ECS-1 (hybridoma supernatant concentrated to 18.5 μg IgM/ml) showed intercellular staining pattern just below the periderm layer (× 400).

Table I. Complement-Mediated Detachment of Epidermal Cells

	Complement[a]	Percent Cell Detachment[b] (mean ± SEM) 24 Hours	48 Hours
ECS-1	+	14.9 ± 4.1	47.5 ± 3.0
(1.2 mg IgM/ml/dish)	−	7.8 ± 3.1	13.0 ± 2.8
IgM monoclonal	+	5.2 ± 4.7	8.1 ± 2.6
antibody control	−	0.6 ± 0.4	10.1 ± 1.8
(0.9 mg IgM/ml/dish)			
NS-1	+	4.0 ± 0.7	10.9 ± 2.1
(1.0 mg protein/ml/dish)	−	3.3 ± 0.2	10.0 ± 4.9

[a]Ten percent preabsorbed normal fresh serum as a source of complement; + = present, − = absent.
[b]Three plates for each point.

assume that the process of antibody combination with more than one membrane protein can trigger acantholysis. Using monoclonal probes such as ECS-1, this process can be studied more critically.

It is intriguing that under the experimental conditions described, there was borderline cell detachment by a cell membrane antibody which could be significantly accentuated with complement. ECS-1-like IgG antibodies could be derived from ECS-1 by gene switching mechanisms [19] and would be extremely useful in more detailed studies of the role of complement in pemphigus-induced acantholysis.

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