

Autoantibodies to Desmoplakin I and II in Patients with Erythema Multiforme

By Dagmar Foedinger, Grant J. Anhalt,[‡] Barbara Boeckscoer, Adelheid Elbe,* Klaus Wolff, and Klemens Rappersberger

From the Department of Dermatology, Division of General Dermatology and the *Division of Immunology, Allergy and Infectious Diseases, Vienna International Research Cooperation Center, University of Vienna Medical School, A-1090 Vienna, Austria; and the †Department of Dermatology, Division of Dermatoimmunology, Johns Hopkins University, Baltimore, Maryland 21205

Summary

Erythema multiforme (EM) represents a syndrome of chronic recurrent inflammatory skin disease. Depending on the severity and extent of skin and mucosal involvement, it is defined either as EM minor or EM major. In this study we demonstrate the presence of autoantibodies (aAbs) against desmoplakin I and II, two major proteins of the desmosomal plaque, in six of six patients with the severe variant of EM, EM major. Light microscopic studies of lesional skin and mucous membranes localized in vivo bound immunoglobulin G (IgG) in a dotted desmosomal pattern along the cytoplasmic membranes of keratinocytes. By immunoelectronmicroscopy, in vivo bound IgG was confined to the desmosomal plaques. These findings were confirmed by indirect immunolocalization studies that demonstrated the presence of IgG aAbs in the serum of patients during active disease. These aAbs did not only bind to desmosomal plaques of epithelial cells where they colocalized with defined murine monoclonal antibodies directed against desmoplakin I and II, but also labeled the intercalated discs of myocardial cells. Biochemical characterization of circulating IgG aAbs revealed desmoplakin I and II as actual target autoantigens. By passive transfer of serum into newborn mice, in vivo binding of serum aAbs to keratinocytes was shown. The findings presented in this study imply a humoral immune response in certain patients with EM major and indicate a potential pathogenetic role of aAbs against desmoplakin I and II in this disease.

Autoantibodies (aAbs)¹ play a crucial role in the pathogenesis of autoimmune bullous diseases such as pemphigus vulgaris, pemphigus foliaceus, and bullous pemphigoid (1, 2). The target autoantigens of pemphigus vulgaris and foliaceus are members of the family of desmosomal cadherins, transmembrane glycoproteins that mediate cell-to-cell adhesion (3–5). In bullous pemphigoid, two distinct antigens have been characterized (6, 7), of which the 230-kD protein is localized within the hemidesmosomal plaque of basal keratinocytes (8). This polypeptide shows extensive sequence homologies with desmoplakin I and II, two major constitutive proteins of the desmosomal plaque (9–12). Desmoplakin I and II have also been identified as components of the antigenic complex characteristic of a recently described bullous autoimmune disease designated paraneoplastic pemphigus (13, 14).

Erythema multiforme (EM) represents a syndrome of inflammatory skin eruptions with a broad spectrum of clinical

manifestations (15). Usually it runs an acute, self-limited, frequently recurrent course characterized by irislike erythemas and papules termed target lesions. This type of disease is designated EM minor. However, certain patients with EM, in addition to target lesions, develop widespread tense blisters and extensive erosions of the oro-genital mucosa and are thus classified as EM major (15). Histopathology characteristically demonstrates single cell death of keratinocytes evolving to focal necrosis of the entire epidermis, a dense mononuclear infiltrate with a pronounced edema of the papillary dermis and subepidermal and very occasional intraepidermal blistering (16).

Previous studies have favored the concept that delayed-type hypersensitivity reactions against various infectious agents (e.g., HSV and mycoplasma) and drugs (e.g., sulfonamides, penicillin derivatives, and phenytoin) (15, 17) trigger the inflammatory cascade. Immunomorphological studies have suggested that the epidermal destruction observed in EM is mainly mediated by cytotoxic T cells (18, 19).

The widespread cutaneous blisters and extensive mucosal erosions of EM major patients often display striking similarities with the clinical features of patients with paraneoplastic

¹ Abbreviations used in this paper: aAb, autoantibody; EM, erythema multiforme; IF, immunofluorescence.

pemphigus (13), an aAb-mediated disease. This prompted us to search for humoral autoimmune phenomena in EM and to test the hypothesis whether humoral immune mechanisms contribute to the devastating condition of these patients.

In this study we present immunomorphological and biochemical evidence that a distinct subset of EM patients, all clinically defined by severe involvement of the skin and mucous membranes, develop characteristic aAbs against desmoplakin I and II. This is only the second human disease in which aAbs against desmoplakins, major proteins of the adhesive plaques of desmosomes, have been detected. Moreover, it is the only one in which there is evidence of binding by passive transfer and temporal correlation with disease activity. Our findings suggest that, in addition to T cell-mediated pathophysiologic events operative in EM, humoral autoimmunity against the desmoplakins may play a crucial role in the pathogenesis of this life-threatening skin disease.

Materials and Methods

Patients

14 patients with characteristic cutaneous target lesions, who clinically and histologically had been diagnosed as EM, entered the study. According to standard clinicopathological criteria, eight patients were classified as EM minor; one of them showed iris lesions with extensive blistering without involvement of the mucous membranes. Six patients, in addition to target lesions, had developed cutaneous blisters and extensive erosions of the oral and genital mucous membranes and were thus defined as EM major (15). Five of them had a history of several recurrences of the mucocutaneous disease, and one experienced the first episode. Extensive clinical and laboratory examination did not reveal the presence of a neoplastic process in any of the patients.

Tissues, Sera, and Antibodies

Tissues. Biopsy specimens of lesional and perilesional skin were available for immunomorphological studies from all patients, except one with EM minor. Normal human skin was obtained from healthy volunteers. Biopsies were divided into three parts and processed for routine histopathology and immunofluorescence studies. For postembedding immunoelectronmicroscopy (IEM) studies, specimens of lesional skin and of normal human skin were placed into PBS, pH 7.4, and chopped into pieces of ~ 0.5 – 1.0 mm³, immersed in 2.3 M sucrose in PBS for 20 min, then mounted on pins, frozen, and stored in liquid nitrogen until further use. BALB/c mice were killed by cervical dislocation, and tongue, liver, and myocardium were excised, snap frozen, and stored at -70°C . Sections of monkey esophagus were purchased from INOVA (San Diego, CA).

Sera. Serum samples were obtained from all 14 patients on several occasions, during active disease and within phases of remission. Blister fluid was obtained from one patient with EM major. We processed sera of patients with pemphigus vulgaris (3), pemphigus foliaceus (1), paraneoplastic pemphigus (1), bullous pemphigoid (4), toxic epidermal necrolysis (2), and sera of 10 healthy volunteers as controls.

Antibodies. Mouse mAb to desmoplakin I and II (desmosomal plaque [dp] 2.15) and a mouse anti-keratin 1, 10/11 mAb were purchased from Progen (Heidelberg, Germany). As second-step reagents for immunofluorescence (IF) microscopy, we used affinity-purified

FITC-conjugated goat F(ab')₂ anti-human IgG, FITC-conjugated affinity-purified anti-human IgM (all from Cappel, West Chester, PA), and FITC-conjugated rabbit anti-mouse IgG (Jaxell, San Diego, CA). For double labeling purposes, TRITC (tetramethylrhodamine isothiocyanate)-conjugated goat F(ab')₂ anti-mouse IgG (Jaxell) was used. IEM was performed with goat anti-mouse IgG conjugated to either 10 nm or—for double labeling experiments—5 nm colloidal gold particles and goat anti-human IgG conjugated to colloidal gold with a particle size of 10 nm (Amersham International, Amersham, Bucks, UK). For immunoblotting we used alkaline phosphatase-conjugated goat anti-human and goat anti-mouse IgG as detector antibodies (Promega Corp., Madison, WI).

Light Microscopic and Ultrastructural Immunomorphology

Direct and Indirect IF. In accordance with routine methods (5), 5- μm cryostat sections of lesional and perilesional skin as well as from mouse tongue, liver, myocardium and urinary bladder, and monkey esophagus were preincubated in PBS, pH 7.4, with 1% BSA (Serva, Heidelberg, Germany). Thereafter, the specimens were exposed to FITC-conjugated goat F(ab')₂ anti-human IgG and IgM diluted 1:100 in PBS, or, for indirect IF, to sera of EM patients diluted 1:20. For control purposes, sera of patients with pemphigus vulgaris, toxic epidermal necrolysis and sera of healthy volunteers (all diluted 1:20 in PBS), as well as a mAb directed against desmoplakin I and II, diluted 1:80 in PBS, were used.

Double Labeling IF on Cultured Human Keratinocytes. For comparative immunolocalization studies, double labeling IF experiments were performed on cultured human keratinocytes. Normal human keratinocytes obtained from surgically removed skin were grown to confluence in chamber slides (LabTek; Nunc, Naperville, IL) in keratinocyte growth medium (Clonetics Corp., San Diego, CA) at low calcium concentration (0.33 mM). These cells and cells grown for 12 h after a switch to high calcium concentration (1.2 mM), which allows the maturation of desmosomes (20, 21), were washed, permeabilized with PBS containing 0.01% saponin (Sigma Chemical Co., St. Louis, MO) and 0.1% sodium azide at pH 7.4, and subsequently incubated with the serum samples of EM patients and the mAb dp 2.15. Bound immunoreactants were visualized with FITC-conjugated goat F(ab')₂ anti-human IgG and TRITC-conjugated goat anti-mouse IgG. After final washing, cells were embedded in Fluoprep (BioMerieux, Marcy l'Etoile, France) and coverslipped.

IEM

Direct IEM. Direct IEM was performed on ultrathin cryosections of the lesional skin of three patients to determine the ultrastructural localization of in vivo bound IgG.

Indirect IEM. The ultrastructural binding sites of circulating human aAbs and murine mAb dp 2.15 were demonstrated on normal human skin.

Preparation of Tissue. We followed methods routinely used in our laboratory (5, 22). To reduce nonspecific binding, the specimens were first incubated in PBS/50 mM glycine (Bio-Rad Laboratories, Richmond, CA)/10% FCS (GIBCO-BRL, Gaithersburg, MD), and after 10 min transferred with forceps to 7–10 μl droplets of the immunoreagents. For direct IEM, the sections were incubated with goat anti-human IgG conjugated to 10 nm colloidal gold particles, diluted 1:10 in PBS/10% FCS for 30 min at room temperature (RT). For indirect IEM, the specimens were incubated with the mAb dp 2.15, diluted 1:100 in PBS/50 mM glycine/5% FCS, and/or the sera of five different patients with EM major, diluted

1:30 in the dilution buffer, for 30 min at room temperature. Thereafter, the sections were rinsed six times for 5 min in PBS and then incubated on small drops of the immunogold reagent diluted 1:10 in PBS/10% FCS. Unbound immunoreagents were removed by washing in PBS for 15 min with three changes of buffer. In double-labeling experiments, we first demonstrated the binding sites of human aAbs by goat anti-human IgG, conjugated to 10 nm colloidal gold particles. The sections were incubated in PBS/10% FCS for 15 min and then consecutively exposed to the mouse mAb dp 2.15 and to the goat anti-mouse IgG conjugated to 5 nm gold particles. Some sections were incubated in goat anti-mouse IgG conjugated to 10 nm colloidal gold particles as controls for direct IEM studies. Sections obtained from shave biopsies of healthy volunteers processed exactly as described above, also served as controls. We used sera of healthy volunteers and sera of patients with pemphigus foliaceus and isotype-matched murine anti-keratin 1, 10/11 antibody as controls for indirect immunolocalization studies.

Biochemical Methods

Immunoblotting of Lysates of Epidermal Sheets. Keratomed split-thickness skin was placed dermal side down in a 1 M sodium chloride (Merck, Darmstadt, Germany)/PBS solution for 12 h at 4°C. Epidermal sheets were peeled from the dermis and homogenized in 2% SDS (BioRad Laboratories) in 0.0625 M tris-buffered saline (TBS), pH 6.8, supplemented with 5% 2-ME (Bio-Rad Laboratories) and 2 mM PMSF (Sigma Chemical Co.) using a motor-driven Teflon type Potter Elvehjem homogenizer (Kontes, Vineland, NJ). After boiling and centrifugation, the supernatant was loaded onto 7% SDS-PAGE slab-gels (23). Separated proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) (24). Under highly stringent conditions, strips of the blotted epidermal proteins were blocked, incubated with patients' sera and the mAb, washed, and then incubated with alkaline phosphatase-conjugated anti-mouse IgG or anti-human IgG, respectively. The enzyme reaction was visualized by nitrobluetetrazolium/bromochloroindolyl phosphatase (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) (25).

Immunoprecipitation of Protein Lysates of Cultured Human Keratinocytes. Immunoprecipitation was performed according to the technique described by Stanley et al. (26, 27). Human foreskin keratinocytes grown to near confluence as described above were incubated overnight with C¹⁴-labeled amino acids (New England Nuclear, Boston, MA). Cells were extracted in 1% NP-40 (Calbiochem-Novabiochem, La Jolla, CA) in TBS with 2 mM PMSF and proteinase inhibitors at a concentration of 5 mM (Antipain, Leupeptin, and Chymostatin; all from Calbiochem-Novabiochem Corp.). A particle-free supernatant was prepared by centrifugation of the cell extracts at 100,000 g for 1 h. Labeled extracts were sequentially preabsorbed with normal human serum and protein A-bearing staphylococci (Pierce, Rockford, IL) and then incubated with the sera of all EM patients and human controls as well as mAb dp 2.15 for 1 h at 4°C. Thereafter, antigen-antibody complexes were precipitated with staphylococcal protein G-Sepharose (Sigma Chemical Co.) and separated by SDS-PAGE, using 5% slab-gels. The precipitated antigens were visualized by autoradiography.

In addition, to confirm the identity of the 210- and 250-kD bands with desmoplakin I and II, 1 ml aliquots of the lysate was preabsorbed with 25 and 75 µg of the mAb dp 2.15 each, followed by precipitation with staphylococcal protein G-Sepharose to deplete them from desmoplakin I and II. Consecutively, these lysates were incubated with the sera of EM major patients and further processed as described above.

Passive Transfer of Serum

To examine whether human aAbs react with epidermal cells in vivo, we performed passive transfers of human serum into neonatal mice according to a modified method of Anhalt et al. (28). Serum of two patients of this study, and for control, of a patient with pemphigus vulgaris and of a healthy volunteer were heated to inactivate complement components. 100 µl serum was injected intraperitoneally or subcutaneously into six 24-h-old BALB/c mice. 18 h later, the mice were killed and tissue of the skin, tongue, liver, and myocardium was obtained for further histopathologic and IF studies.

Results

Light Microscopic and Ultrastructural Immunomorphology

Demonstration of In Vivo Bound aAbs in EM Patients. Direct IF studies revealed the presence of in vivo bound IgG restricted to the epidermis of lesional skin in all six patients with EM major and in one with severe EM minor. At low magnification, dense deposits of IgG were found which appeared in an intercellular staining pattern not unlike that seen in pemphigus vulgaris (29). The most intense labeling was found within the spinous cell layer, but sporadically, IgG binding was also seen within the basal and granular cell layer, including the transitional cell layer. At higher magnification, in vivo bound IgG could be clearly confined to the plasma membranes of individual keratinocytes (Fig. 1 A). Moreover, depending on the plane of sectioning, the labeling resolved into distinct fluorescent dots, suggestive of a desmosomal staining pattern. Occasionally, we observed that the intercellular spaces separating IgG-positive keratinocytes were clearly devoid of Ig deposits, indicative of an exclusive binding of the immunoreactants to the cell membrane or to intracellular structures associated with epithelial cell membranes, i.e., desmosomal plaques. As previously described by other investigators (17, 30), IgM deposits were observed along the dermo-epidermal junction in all patients with EM major and in four (out of nine) patients with EM minor; in contrast to in vivo bound IgG, these IgM deposits were not restricted to lesional skin, but were also found in perilesional skin. IgM together with complement C3 was observed in perilesional and lesional skin around the blood vessels of the papillary dermis in all EM patients.

Direct IEM was performed to investigate the ultrastructural localization of in vivo bound IgG in lesional skin. 10 nm colloidal gold particles indicating in vivo bound IgG were detected in keratinocytes, exclusively. The vast majority of the gold particles was associated with the desmosomal plaques (Fig. 1, B and C), where keratin filaments assemble with the desmosome (12). Another notable morphological finding was the detachment of keratin filaments from the desmosomal plaques as has been previously described (31). These filaments formed an irregular meshwork of filament bundles and clumps within the cytoplasm (not shown). However, the desmosomes morphologically appeared well preserved, and neither splitting within the desmosomal glea nor true loss of cell adhesion, acantholysis, was observed. We never saw gold particles within the intercellular spaces, or along the outer sheet

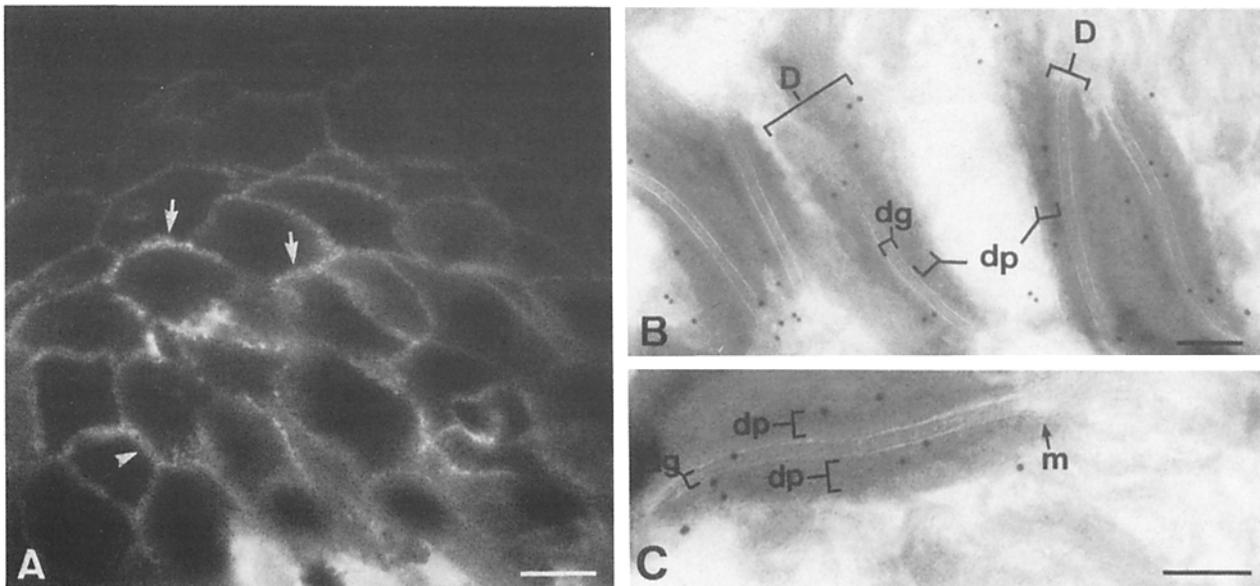


Figure 1. Demonstration of in vivo bound IgG by direct immunofluorescence (A) and direct immunoelectronmicroscopy (B and C). A 5- μm cryostat section of lesional skin from a patient with EM major was incubated with FITC-conjugated goat F(ab')₂ anti-human IgG (A). Note the punctate desmosomal staining pattern of the cell membranes of keratinocytes (arrows). Occasionally, one clearly observes that the intracellular spaces appear devoid of IgG deposits (arrowhead). (B and C) Labeling of a 100-nm ultrathin cryostat section of lesional skin from a patient with EM major with goat anti-human IgG conjugated to 10 nm colloidal gold particles. The gold particles indicating in vivo bound IgG are exclusively localized to the cytoplasmic desmosomal plaques (dp). Other portions of the cell membrane (m), the intercellular spaces, especially the desmoglea (dg), and keratin filaments are devoid of IgG deposits (C). $\times 500$ (A); (D) Desmosome, $\times 30,000$ (B); $\times 70,000$ (C). Bars: 20 μm (A); 0.2 μm (B and C).

of the cell membranes of keratinocytes. In addition, we consistently failed to demonstrate IgG deposits along the dermo-epidermal junction by IEM.

As control, skin of healthy volunteers was processed. Normal human skin did not exhibit any binding of gold particles at specific sites.

Localization of Antigens Recognized by Circulating aAbs of EM Patients in Epithelial and Nonepithelial Tissue. The presence of circulating aAbs in patients with EM was determined by processing several epithelial and nonepithelial tissues for indirect IF. Sera of six (out of six) of the EM major patients and none of eight EM minor patients contained aAbs recognizing antigens in the substrates tested. In normal human skin staining was restricted to epidermal keratinocytes. The labeling was confined to the plasma membrane of keratinocytes throughout the entire epidermis (Fig. 2 A) and at higher magnification, the continuous labeling of the cell membranes resolved into a peculiar punctate fluorescent pattern, typical of desmosomal staining. This distinct desmosomal staining pattern was most clearly evident along the basolateral membranes of basal keratinocytes. A labeling of hemidesmosomes was never observed. Moreover, this characteristic desmosomal staining pattern was consistently seen in all the stratified epithelial tissues of different species tested, such as monkey esophagus and mouse tongue (Fig. 2, B and C). Similarly, in mouse liver, labeling was found at adhesion sites of hepatocytes (Fig. 2 E) and along the bile canaliculi. Epithelial cells of complex epithelia such as mouse urinary bladder revealed a surface staining (Fig. 2 D); in addition, this latter tissue

also showed a delicate labeling along the basement membrane zone. It remains to be clarified whether this reactivity represents labeling of hemidesmosomes or other not yet defined structures of urinary bladder basement membrane. Examination of mouse myocardium, tissue of mesenchymal origin, showed a strong labeling of the intercalated discs (Fig. 2 F), adhesive structures that are known to contain desmoplakin (32). Staining patterns achieved with human EM major serum and with mAb dp 2.15 did not demonstrate essential differences on any of the diverse substrates processed, suggesting that the aAbs indeed bind to desmosomal plaques and similar adhesive structures in mesenchymal tissue.

Indirect IEM. The exact ultrastructural localization of the antigen(s) recognized by the circulating aAbs was further determined by postembedding immunogold IEM studies on ultrathin cryosections of normal human skin. The major advantage of this method is that the entire surface of the sections including all parts of the desmosome are accessible to antibodies. The electron-dense cytoplasmic desmosomal plaque, with the keratin filaments assembling at its inner surface, was clearly defined. The ultrastructural binding sites of the mAb dp 2.15 and the serum antibodies from the patients with EM major could be clearly localized to the cytoplasmic portion of the desmosomal plaque (Fig. 3, A and B). By double immunogold labeling with serum of patient 1 and the mAb against desmoplakin I and II, a colocalization of desmoplakin and the antigens recognized by the circulating aAbs was confirmed (Fig. 3 D). In contrast, labeling with isotype-matched mAb against keratin 1, 10/11 revealed a distinct

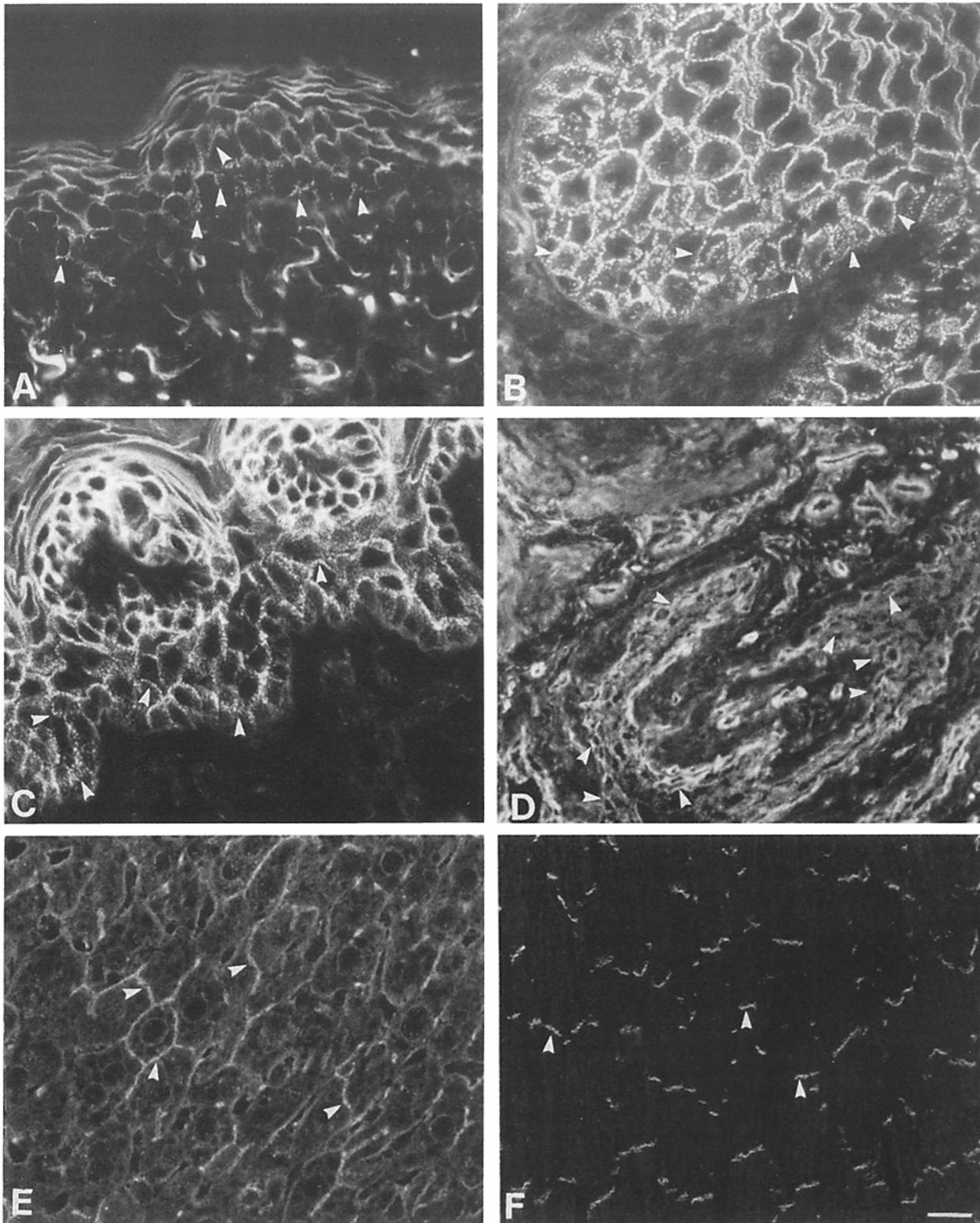


Figure 2. Indirect immunofluorescence: immunomorphological demonstration of circulating aAbs against desmoplakin I and II in sera of EM major patients on various epithelial tissues and mouse myocardium. Note the dotted desmosomal membrane staining of keratinocytes, especially in the basal and suprabasal cell layers of normal human skin (A), monkey esophagus (B), and mouse tongue (C). A more continuous staining of the cell membranes is seen in the upper cell layers. The urothelial cells of mouse urinary bladder show a membrane staining in both cell layers (D). The hepatocytes of mouse liver display distinct labeling of the cell membranes at the junctional sites (E). In mouse myocardium, the labeling is confined to the intercalated discs (F). (Arrowheads) Substrate-specific binding of immunoreactants. $\times 300$; bars, all $20 \mu\text{m}$.

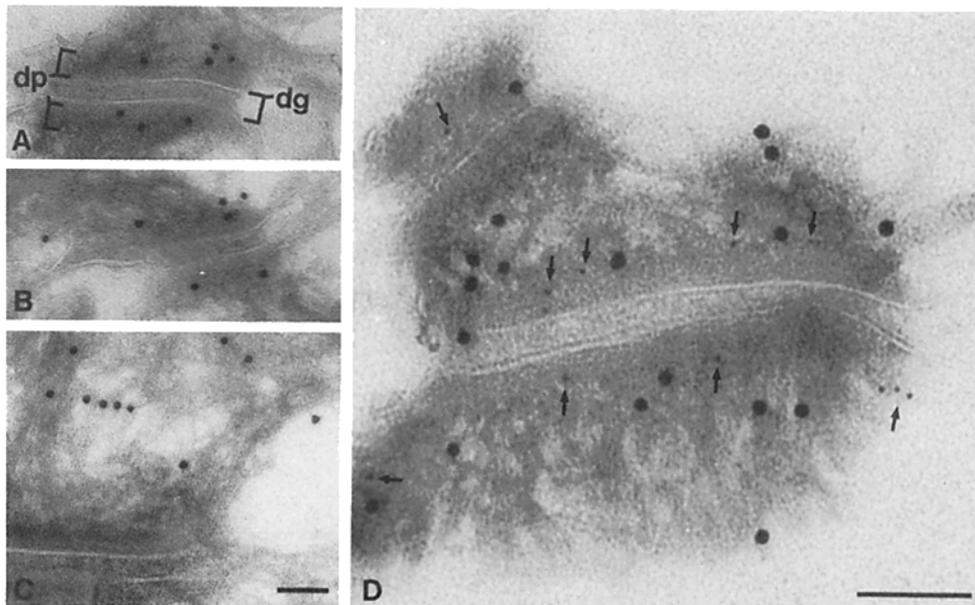


Figure 3. Ultrastructural immunolocalization studies on ultrathin cryosections of normal human skin. Binding of human aAbs against desmoplakin I and II is indicated by 10-nm colloidal gold particles conjugated to goat anti-human IgG (B and D). Labeling with a murine mAb to desmoplakin I and II dp 2.15 is demonstrated with 10-nm gold particles in A and with 5 nm gold particles in D. The mAb and the human aAbs exclusively bind to the inner portion of the electron-dense desmosomal plaque (A and B). Double labeling experiments clearly reveal a colocalization of both, the mAb dp 2.15 (5-nm gold particles, arrows) and the serum aAbs (10-nm gold particles); (D). The desmoglea and the keratin filaments are devoid of immunoreactants. In contrast, binding of a mouse monoclonal anti-keratin 1, 10/11 antibody is confined to the intermediate size filaments as demonstrated by 10-nm gold particles in C. (dg) Desmoglea (B); $\times 100,000$ (A–C), $\times 200,000$ (D). Bars 0.1 μm .

labeling of intermediate size filaments (Fig. 3 C) but not of desmosomal plaques. Normal human serum did not show any labeling.

Desmosomal IF Staining Pattern on Cultured Human Keratinocytes. Double labeling immunolocalization studies were performed with serum from all patients with EM major and the murine mAb dp 2.15 on cultured human keratinocytes. To do so we processed monolayers of human keratinocytes grown at low calcium concentration and cells 12 h after a shift to high calcium concentration. Only after a gentle permeabilization of cells with saponin could staining be achieved with both the human serum samples and the mAb. Cells at low calcium displayed an irregularly distributed punctate staining pattern throughout the entire cytoplasm. Cells grown

in high calcium showed bright fluorescent dots along their cytoplasmic membranes, especially at adhesion sites with neighboring keratinocytes, that clearly colocalized in double labeling experiments (Fig. 4, A and B) (14, 33).

Biochemical Characterization of aAbs of EM Major Patients

Immunoblotting. The specificity of human aAbs against desmosomal structures was examined by immunoblotting of epidermal lysates. The aAbs present in the sera of EM major patients were reacted with the electrophoretically separated proteins of epidermal extracts. Sera of all six patients with EM major but none of the eight patients with EM minor bound to polypeptides of 250 and of 210 kD (Fig. 5, lanes 3–8). These two polypeptides precisely comigrated with des-

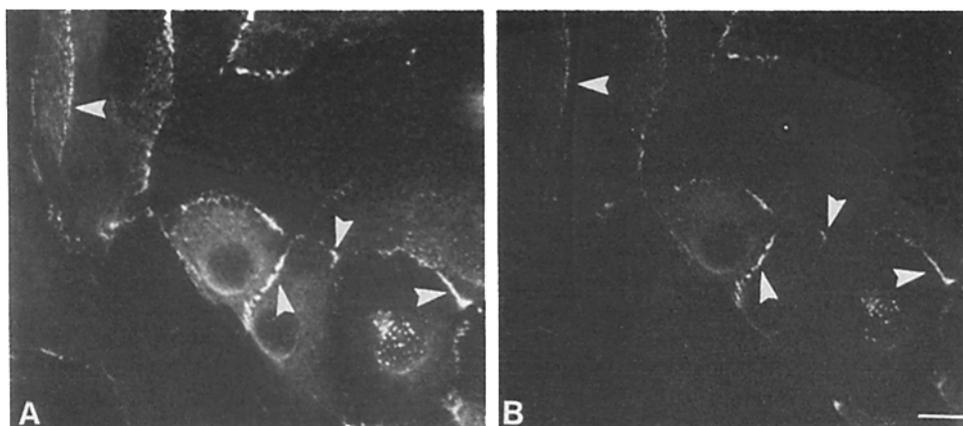


Figure 4. Double immunofluorescence labeling of cultured human keratinocytes grown in high calcium. Cells were gently permeabilized with saponin and incubated with serum of an EM major patient (A) and the mAb to desmoplakin I and II (B). Bound antibodies were visualized by FITC-conjugated anti-human IgG and TRITC-conjugated anti-mouse IgG. Both reactants display a distinct dotted staining of the cytoplasmic membranes of cultured human keratinocytes. Note the dominant staining and colocalization of serum aAbs and mAb dp 2.15 at the binding sites of neighboring keratinocytes (arrowheads). $\times 300$, bar 20 μm .

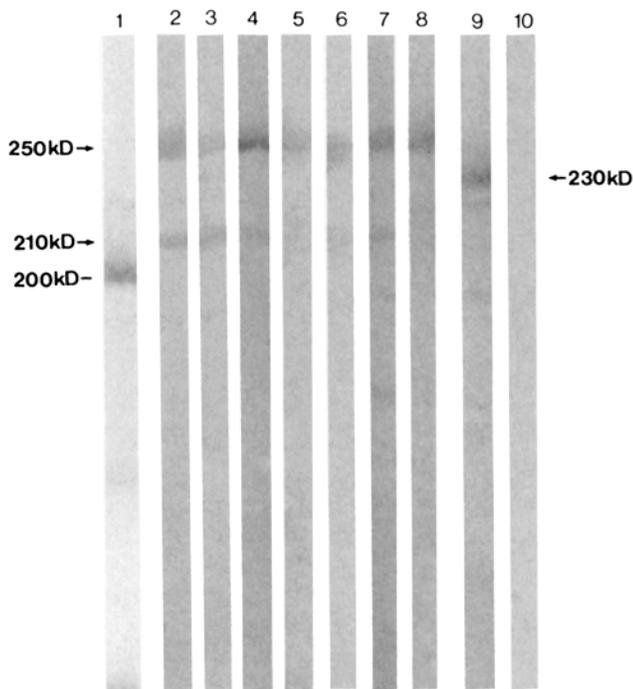


Figure 5. Immunoblotting. Lysates of normal human epidermis were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with sera of EM major patients (lanes 3–8), and the mAb dp 2.15 (lane 2). Human aAbs of all EM major patients (lanes 3–8) bind to polypeptides of 250- and 210-kD molecular mass, that comigrate with desmoplakin I and II (lane 2). As controls, labeling with serum of a patient with bullous pemphigoid (230-kD band in lane 9) and normal human serum (lane 10) is shown. Molecular weight standard indicates 200 kD (lane 1).

moplakin I and II as demonstrated by staining with mAb dp2.15 (Fig. 5, lane 2). Additional controls with sera of 10 healthy volunteers and two patients with toxic epidermal necrolysis did not stain any specific band. Serum samples of two patients with EM major were obtained 9 mo after the last EM episode. Interestingly, after processing these sera in

dilution identical to that present during active disease (1:500), no specific labeling of epidermal extracts was found.

Immunoprecipitation. Immunoprecipitation studies of C^{14} -labeled human keratinocytes demonstrated the presence of aAbs in the sera of five (out of six) EM major patients, whereas the sera of all EM minor patients were devoid of precipitating IgG. These aAbs precipitated two polypeptides of 250 and 210 kD (Fig. 6, lanes 5, 8–11), that precisely comigrated with desmoplakin I (250 kD) and desmoplakin II (210 kD), as indicated by precipitation with the mAb dp 2.15 (Fig. 6, lane 4). In addition, blister fluid of one patient precipitated the identical complex of polypeptides (Fig. 6, lane 6). These findings were further confirmed by processing a serum of a patient with paraneoplastic pemphigus, which revealed the presence of aAbs against the characteristic antigen complex (250 kD/230 kD/210 kD/190 kD/170 kD) (Fig. 6, lane 2). The identity of the 210 and 250-kD bands with desmoplakin I and II was additionally confirmed by a consecutive immunoprecipitation of the lysates with the mAb dp 2.15 and the patients' sera. Preabsorption of the lysates with mAb dp 2.15 strongly reduced the reactivity with serum aAbs (Fig. 6, lane 13) and even completely depleted the autoantigens (desmoplakin I and II) from the lysate (Fig. 6, lane 14). Serum samples of two patients with EM major obtained 9 mo after the last EM recurrence failed to precipitate any specific band (Fig. 6, lane 7). We also consistently failed to detect circulating aAbs in all patients with EM minor and healthy volunteers (Fig. 6, lane 12), whereas serum of a patient with bullous pemphigoid precipitated the characteristic polypeptide of 230 kD (Fig. 6, lane 3).

Passive Transfer of Serum

A slight erythema was observed at the sites of subcutaneous injection of biochemically characterized serum of two patients with EM major into newborn mice, 18 h after injection. Skin biopsies did not display any histopathological features of EM. However, by direct IF an intense labeling of

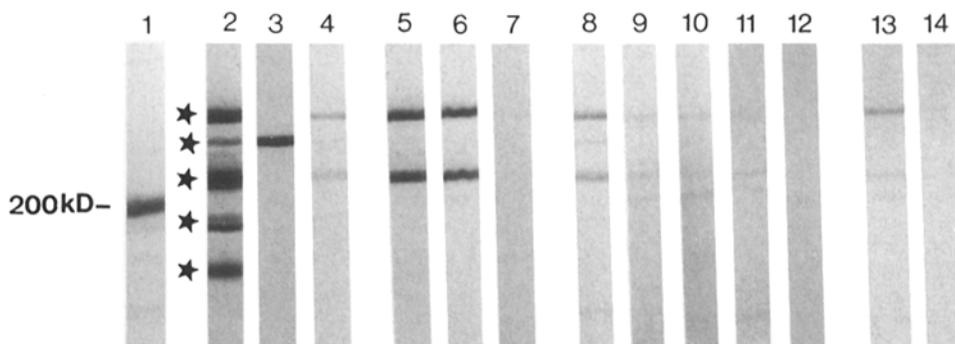


Figure 6. Immunoprecipitation. Immunoprecipitation of protein extracts of radiolabeled cultured human keratinocytes with sera of EM major patients 1–5 (lanes 5, 7, 8–11) and blister fluid of patient 1 (lane 6). All sera and the blister fluid precipitate two major bands of 250- and 210-kD molecular weight. Bands of identical mobility were precipitated by the murine mAb to desmoplakin I (250 kD) and II (210 kD) (lane 4). A serum sample obtained from an EM major patient 9 mo after the last clinical eruption (lane 7),

as well as serum samples from a healthy volunteer (lane 12) failed to immunoprecipitate any specific polypeptide. Lane 2 represents the characteristic antigen complex precipitated by serum of a patient with paraneoplastic pemphigus and lane 3 shows the precipitation of the 230-kD antigen with serum of a bullous pemphigoid patient. (Lane 1) Molecular weight standard indicating 200 kD. Asterisks mark from the top to bottom: 250, 230, 210, 190, and 170 kD. Preabsorbing the lysates with the mAb dp 2.15 in a concentration of 25 μ g/1 ml lysate strongly reduced the reactivity with serum aAbs (lane 13), and higher concentration of mAb dp 2.15 (75 μ g/1 ml lysate) almost completely depleted the autoantigen (lane 14) (same serum as shown in lane 8).

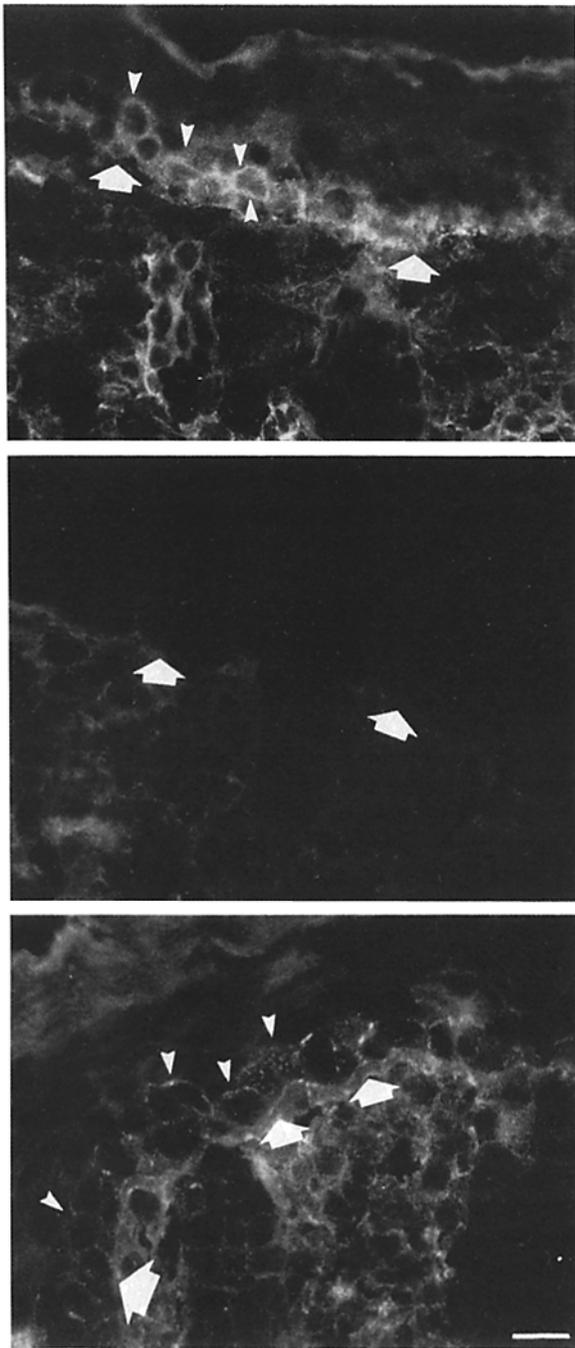


Figure 7. Antibody binding after passive transfer of serum from an EM major patient into newborn BALB/c mice. 18 h after subcutaneous injection direct immunofluorescence using FITC-conjugated goat F (ab')₂ anti-human IgG revealed in vivo-bound human IgG decorating the cytoplasmic membranes of murine epidermal keratinocytes (*arrowheads, top*). Most intense labeling was detected within the basal and suprabasal cell layers overlying the sites of injection. As control, injection of normal human serum did not result in intraepidermal IgG deposits (*middle*). Passive transfer of a serum from a patient with pemphigus vulgaris leads to the characteristic staining of the intercellular spaces (*arrowheads, bottom*). (*Arrows*) Basal membrane zone. $\times 300$, bar 20 μm .

the cell membranes of basal and suprabasal keratinocytes was detected (Fig. 7, *top*). These IgG deposits labeled the cytoplasmic membranes of mouse keratinocytes, and occasionally, this staining pattern was reminiscent of cytoplasmic labeling. A delicate punctate labeling was seen after subcutaneous and intraperitoneal injection of pemphigus vulgaris serum (Fig. 7, *bottom*). We consistently failed to demonstrate in vivo deposits of human IgG in the murine epidermis after intraperitoneal injection of the serum of EM major patients. In addition, other murine tissues such as tongue, liver, and myocardium were also devoid of in vivo bound human antibodies. Injection of control sera of healthy volunteers consistently yielded negative results by direct IF (Fig. 7, *middle*).

Discussion

In this study we provide immunomorphological and biochemical evidence for the presence of circulating IgG (IgG1 and IgG4) aAbs to desmoplakin I and II in patients with EM major but not in patients with the minor variant of this disease and not in control sera of healthy volunteers. Direct IF studies of lesional and clinically uninvolved skin revealed in vivo bound IgG along the cell membranes of keratinocytes of lesional skin. On the other hand, when normal human skin of healthy volunteers was incubated with the serum of such patients, indirect IF revealed a similar staining pattern of the cytoplasmic membranes of keratinocytes. This suggested the presence of circulating serum aAb that bind to epidermal proteins. Moreover, at the light microscopic level these immunoreactants were distributed in a punctate pattern along the cell membranes of keratinocytes indicating a binding to desmosomes, major structures of cell adhesion. To prove the validity of this assumption, several epithelial and mesenchymal tissues that contain desmosomes and desmosomal proteins such as mouse tongue, liver, bladder, and myocardium, as well as monkey esophagus, were labeled with the sera of patients and, for comparison, with a mAb directed against the defined desmosomal proteins desmoplakin I and II. Both, patients' sera and mAb revealed a clearcut punctate, desmosomal staining pattern of epithelial cells from tongue and esophagus, as well as hepatocytes, and the intercalated discs of mouse myocardium, a tissue of mesenchymal origin that contains desmoplakins (32).

Biochemical studies support our hypothesis that circulating aAbs of patients with EM major actually bind to desmoplakin I and II and not to any other desmosomal/cytoplasmic protein. By immunoprecipitation of extracts of C¹⁴-labeled human keratinocytes two major bands of 250 and 210 kD were detected that precisely comigrated with desmoplakin I and II and Western blot experiments clearly showed a binding of serum aAbs to desmoplakin I and II. These experiments exclude the possibility that desmoplakins are simply coprecipitated with another protein but show that they are true targets of the aAbs. Because desmoplakin I and II are localized within the cytoplasm and do not have any extracellular/transmembrane domains, the question arose whether they actually represent in vivo binding sites. We have addressed this question

by direct IEM studies and demonstrate that in vivo bound IgG is mostly confined to the innermost portions of the desmosomal plaques which excludes the possibility that another not yet defined (homologous) protein with an extracellular localization is recognized by the aAbs. These findings are further corroborated by indirect ultrastructural immunolocalization studies with ultrathin cryosections of normal human skin which reveal that serum aAbs and mAbs against desmoplakin I and II colocalize to the desmosomal plaques.

Taken together, these findings clearly show the presence of a new subtype of human aAbs that appears to be a characteristic feature of certain patients with EM major, and that obviously binds to a cytoplasmic antigen. The findings also indicate that a humoral immune response occurs in such patients, not unlike that seen in patients with defined bullous autoimmune diseases. In the recent past, one of us (13, 14) has described a new mucocutaneous blistering disease that occurs in association with certain internal neoplasms and that was therefore designated "paraneoplastic pemphigus". Patients with paraneoplastic pemphigus develop aAbs against a characteristic subset of five different polypeptides of 250, 230, 210, 190, and 170 kD. The 250- and 210-kD polypeptides were identified as desmoplakin I and II and the 230-kD protein as the bullous pemphigoid antigen I, whereas the 190- and 170-kD polypeptides still have to be characterized. All patients with EM major as defined in this study, who were shown—by several laboratory examinations and extensive routine diagnostic procedures—not to have a concurrent neoplastic disorder, obviously develop aAbs against a distinct antigenic complex that consists of desmoplakin I and II.

Desmoplakins are major constitutive proteins of desmosomes that are exclusively located intracellularly and contribute, in part, to the formation of desmosomal plaques. Desmoplakins consist of an α -helical coiled coil rod domain of 130-nm length with heptad repeats similar to the two chained coiled coil rod domains of all intermediate filaments. On either end of the rod there are globular subdomains. More recent molecular investigations have revealed that the COOH-terminal globular domain which includes three subdomains composed of series of 38-residue repeats, and which does not incorporate into the plaque, mediates the assembly of keratin filaments to the desmosomal plaque (12, 34, 35). Our observations by IEM that human aAbs to desmoplakin I and II bind to those portions of the desmosomal plaque, where keratin filaments assemble, open the attractive possibility that they bind to the COOH-terminal domain and thus interfere with the assembly/adhesion of keratin filaments with desmosomes. This hypothesis is further strengthened by the ultrastructural observation of a disassembly of keratin filaments from desmosomes, by the subsequent clumping of filaments into an electron-dense meshwork within the cytoplasm (31), and finally by the appearance of cytologic features similar to dyskeratosis. In accordance with this are our ultrastructural studies that revealed morphologically well preserved desmosomes also in pathologically affected keratinocytes, and this explains the

complete absence of true acantholysis. These observations may be seen in line with the findings of Klymkowsky et al. (36, 37) who observed similar ultrastructural changes of intermediate filament clumping and persistence of desmosomes after microinjection of mAb against keratin filaments into Ptk2-cells. They also indicate that neither the integration of desmoplakins into desmosomes nor the adhesive properties of these structures is involved in the pathogenetic events operative in EM. Any speculation about a pathogenic role of aAbs against desmoplakins seems to be tempered by the fact that they are intracellular proteins. How should serum aAbs bind to and presumably compromise the function of such intracellular antigens? We addressed the question whether aAbs to desmoplakin I and II may play a role in the pathogenesis of EM in a set of experiments with cultured human keratinocytes and neonatal mice. By IF cultured cells displayed a clearcut desmosomal staining with EM sera and murine mAb to desmoplakin I and II after mild permeabilization with saponin. In addition, subcutaneous injection of serum into neonatal BALB/c mice resulted in a binding of human IgG to mouse keratinocytes within the epidermis overlying the sites of injection, in a pattern similar to that of lesional skin of EM patients. However, after intraperitoneal injection we consistently failed to demonstrate in vivo bound IgG within the epidermis. These findings suggest that additional events, such as mild damage of keratinocytes as induced by permeabilization of cells in culture or edema by injection in vivo, are required to provide access of aAbs to the cytoplasm.

Our findings have to be seen in context with previous investigations on EM that have demonstrated a preponderance of cytotoxic T cells within the lesional epidermis (18, 19). One could further speculate that the infiltration of such cells into the epidermis may cause the damage that is required for desmoplakin I and II aAbs to gain access to and bind to their intracellular targets. Bound antibodies might then interfere with the assembly of keratin filaments with the desmosomal plaque resulting in a disassembly of keratin filaments, keratin filament clumping, single cell necrosis, and focal necrosis of the entire epidermis. Circumstantial evidence that aAbs might be causally involved in the pathogenesis of EM major comes from the observation of circulating aAbs during the first disease episode in one patient. Alternatively, it has to be considered that aAbs are only raised secondarily as a consequence of T cell-mediated keratinocyte injury and that they are therefore not primarily involved in the pathogenesis of EM. At this time, therefore, we currently do not have convincing evidence that these aAbs contribute to the pathology of EM major.

This study shows that aAbs directed to desmoplakin I and II characterize and are possibly involved in epidermal pathology of EM major. This provides us with a new disease model with which we can further investigate how serum aAbs may gain access to, bind with, and compromise the function of important cytoplasmic epithelial adhesion molecules.

We are indebted to Werner W. Franke (German Cancer Research Center, Institute of Cell and Tumor Biology, Heidelberg, Germany) for stimulating our interest in desmoplakins, and to Gerold Schuler (Department of Dermatology, University of Innsbruck, Innsbruck, Austria) and Georg Stingl (Department of Dermatology, Division of Immunology, Allergy and Infectious Diseases, University of Vienna) for their advice, personal contributions, valuable discussion, and critical reading of the manuscript.

This work was supported in part by U.S. Public Health Service grants RO1-AR 32490 and RO1-AR 40018 (to G. J. Anhalt) and by a grant from the Austrian Science Foundation JO 426-MED (to K. Rappersberger).

Address correspondence to Dr. Klemens Rappersberger, Division of General Dermatology, Department of Dermatology, University of Vienna Medical School, Währinger Gürtel 18-20, A-1090 Vienna, Austria.

Received for publication 14 June 1994 and in revised form 6 September 1994.

References

- Stanley, J.R. 1993. Cell adhesion molecules as targets of autoantibodies in pemphigus and pemphigoid, bullous diseases due to defective epidermal cell adhesion. *Adv. Immunol.* 53: 291-325.
- Stanley, J.R. 1989. Pemphigus and pemphigoid as paradigms of organ specific, autoantibody-mediated diseases. *J. Clin. Invest.* 83:1443-1448.
- Amagai, M., V. Klaus-Kovtun, and J.R. Stanley. 1991. Autoantibodies against a novel epithelial cadherin in pemphigus vulgaris, a disease of cell adhesion. *Cell.* 67:869-877.
- Stanley, J.R., L. Koulu, V. Klaus-Kovtun, and M.S. Steinberg. 1986. A monoclonal antibody to the desmosomal glycoprotein desmoglein I binds the same polypeptide as human autoantibodies in pemphigus foliaceus. *J. Immunol.* 136:1227-1230.
- Rappersberger, K., N. Roos, and J.R. Stanley. 1992. Immunomorphologic and biochemical identification of the pemphigus foliaceus autoantigen within desmosomes. *J. Invest. Dermatol.* 99:323-330.
- Labib, R.S., G.J. Anhalt, H.P. Patel, D.F. Mustasim, and L.A. Diaz. 1986. Molecular heterogeneity of the bullous pemphigoid antigens as detected by immunoblotting. *J. Immunol.* 136: 1231-1235.
- Mutasim, D.F., L.H. Morrison, Y. Takahashi, R.S. Labib, J. Skouge, L.A. Diaz, and G.J. Anhalt. 1989. Definition of bullous pemphigoid antibody binding to intracellular and extracellular antigen associated with hemidesmosomes. *J. Invest. Dermatol.* 92:225-230.
- Thacher, S.M., and P.L. Hefti. 1991. Characterization of 230kD bullous pemphigoid antigen associated with the detergent-insoluble fraction of cultured keratinocytes. *J. Invest. Dermatol.* 96:139-143.
- O'Keefe, E.J., H.P. Erickson, and V. Bennet. 1989. Desmoplakin I and desmoplakin II purification and characterization. *J. Biol. Chem.* 264:8310-8318.
- Green, K.J., M.L.A. Virata, G.W. Elgart, J.R. Stanley, and D.A.D. Parry. 1992. Comparative structural analysis of desmoplakin, bullous pemphigoid antigen and plectin: members of a new gene family involved in the organization of intermediate filaments. *Int. J. Biol. Macromol.* 14:145-153.
- Tanaka, T., D.A.D. Parry, V. Klaus-Kovtun, P.M. Steinert, and J.R. Stanley. 1991. Comparison of molecularly cloned bullous pemphigoid antigen to desmoplakin I confirms that they define a new family of cell adhesion junction plaque proteins. *J. Biol. Chem.* 266:12555-12559.
- Green, K.J., D.A.D. Parry, D.M. Steinert, M.L.A. Virata, R.M. Wagner, B.D. Angst, and L.A. Nilles. 1990. Structures of the human desmoplakins: implications for function in the desmosomal plaque. *J. Biol. Chem.* 265:2603-2612.
- Anhalt, G.J., K. Soo Chan, J.R. Stanley, N.K. Korman, D.A. Jabs, M. Kory, H. Izumi, H. Ratrie III, D. Mutasim, L. Ariss-Abdo, and R. Labib. 1990. Paraneoplastic pemphigus: an autoimmune mucocutaneous disease associated with neoplasia. *N. Engl. J. Med.* 323:1729-1735.
- Oursler, J.R., R.S. Labib, L. Ariss-Abdo, T. Burke, E. O'Keefe, and G.J. Anhalt. 1992. Human autoantibodies against desmoplakins in paraneoplastic pemphigus. *J. Clin. Invest.* 89: 1775-1782.
- Huff, J.C., W.L. Weston, and M.G. Tonnesen. 1983. Erythema multiforme: a critical review of characteristics, diagnosis criteria, and causes. *J. Am. Acad. Dermatol.* 8:763-775.
- Bedi, T.R., and H. Pinkus. 1976. Histopathological spectrum of erythema multiforme. *Br. J. Dermatol.* 95:243-250.
- Howland, W.W., L.E. Golitz, W.L. Weston, and J.C. Huff. 1984. Erythema multiforme: clinical, histopathologic, and immunologic study. *J. Am. Acad. Dermatol.* 10:438-446.
- Tonnesen, M.G., T.J. Harrist, B.U. Wintroub, M.C. Mihm, and N.A. Soter. 1983. Erythema multiforme: microvascular damage and infiltration of lymphocytes and basophils. *J. Invest. Dermatol.* 80:282-286.
- Margolis, R.J., M. Tonnesen, T.J. Harrist, A.K. Bhan, B.U. Wintroub, M.C. Mihm, and N.A. Soter. 1983. Lymphocyte subsets and Langerhans cells/intermediate cells in erythema multiforme. *J. Invest. Dermatol.* 81:403-406.
- Duden, R., and W.W. Franke. 1988. Organization of desmosomal plaque proteins in cells growing at low calcium concentrations. *J. Cell Biol.* 107:1049-1063.
- Stanley, J.R., and S.H. Yuspa. 1983. Specific epidermal protein markers are modulated during calcium-induced terminal differentiation. *J. Cell Biol.* 96:1809-1814.
- Tokuyasu, K.T. 1985. Application of cryoultramicrotomy to immunocytochemistry. *J. Microscop.* 143:139-149.
- Hashimoto, T., M.M. Ogawa, A. Konohana, and T. Nishikawa. 1990. Detection of pemphigus vulgaris and pemphigus foliaceus antigens by immunoblot analysis using different antigen sources. *J. Invest. Dermatol.* 94:327-331.
- Towbin, H.T., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad.*

- Sci. USA.* 76:4350-4354.
25. Blake, M.S., K.H. Johnston, G.J. Russel-Jones, and E.C. Gotschlich. 1984. A rapid, sensitive method for the detection of alkaline phosphatase-conjugated anti-antibody on Western blots. *Anal. Biochem.* 136:175-179.
 26. Stanley, J.R., L. Koulo, and C. Thivolet. 1984. Distinction between epidermal antigens binding pemphigus vulgaris and pemphigus foliaceus autoantibodies. *J. Clin. Invest.* 74:313-320.
 27. Stanley, J.R., M. Yaar, P. Hawley-Nelson, and S.I. Katz. 1982. Pemphigus antibodies identify a cell surface glycoprotein synthesized by human and mouse keratinocytes. *J. Clin. Invest.* 70:281-288.
 28. Anhalt, G.J., R.S. Labib, J.J. Voorhees, T.F. Beals, and L.A. Diaz. 1982. Induction of pemphigus in neonatal mice by passive transfer of IgG from patients with the disease. *N. Engl. J. Med.* 306:1189-1196.
 29. Beutner, E.H., R.E. Jordon, and T.P. Chorzelski. 1968. The immunopathology of pemphigus and bullous pemphigoid. *J. Invest. Dermatol.* 51:63-80.
 30. Bushkell, L.L., S.E. Mackel, and R.E. Jordon. 1980. Erythema multiforme: direct immunofluorescence studies and detection of circulating immune complexes. *J. Invest. Dermatol.* 74:372-374.
 31. Orfanos, C.E., G. Schaumburg-Lever, and W.F. Lever. 1974. Dermal and epidermal types of erythema multiforme. *Arch. Dermatol.* 109:682-688.
 32. Angst, B.D., L.A. Nilles, and K.J. Green. 1990. Desmoplakin II expression is not restricted to stratified epithelia. *J. Cell Sci.* 97:247-257.
 33. Ma, A.S.P., and A.L. Lorincz. 1988. Immunofluorescence localization of peripheral proteins in cultured keratinocytes. *J. Invest. Dermatol.* 90:331-335.
 34. Stappenbeck, T.S., and K.J. Green. 1992. The desmoplakin carboxyl terminus coaligns with and specifically disrupts intermediate filament networks when expressed in cultured cells. *J. Cell Biol.* 116:1197-1209.
 35. Stappenbeck, T.S., E.A. Bornslaeger, C.M. Corcoran, H.H. Luu, M.L.A. Virata, and K.J. Green. 1993. Functional analysis of desmoplakin domains: specification of the interaction with keratin versus vimentin intermediate filament networks. *J. Cell Biol.* 123:691-705.
 36. Klymkowsky, M.W., R.H. Miller, and E.B. Lane. 1983. Morphology, behaviour, and interaction of cultured epithelial cells after the antibody-induced disruption of keratin filament organization. *J. Cell Biol.* 96:494-509.
 37. Klymkowsky, M.W. 1981. Intermediate filaments in 3T3 cells collapse after intracellular injection of a monoclonal anti-intermediate filament antibody. *Nature (Lond.)* 291:249-251.