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Induction of Complement-Fixing Autoantibodies against Type VII Collagen Results in Subepidermal Blistering in Mice[1]

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Experimental models reproducing an autoimmune response resulting in skin blistering in immunocompetent animals are lacking. Epidermolysis bullosa acquisita (EBA) is a bullous skin disease caused by autoantibodies to type VII collagen. In this study, we describe an active disease model of EBA by immunizing mice of different strains with murine type VII collagen. All mice developed circulating IgG autoantibodies that recognized type VII collagen and bound to the lamina densa of the dermal-epidermal junction. Importantly, subepidermal blisters developed in 82% of SJL-1, 56% of BALB/c mice, and 45% of FcγRIIb-deficient mice, but not in SKH-1 mice. In susceptible animals, deposits of IgG1, IgG2, and complement C3 were detected at the dermal-epidermal junction. In contrast, in the nondiseased mice, tissue-bound autoantibodies were predominantly of the IgG1 subclass and complement activation was weak or absent. This active disease model reproduces in mice the clinical, histopathological, and immunopathological findings in EBA patients. This robust experimental system should greatly facilitate further studies on the pathogenesis of EBA and the development of novel immunomodulatory therapies for this and other autoimmune diseases. ***The Journal of Immunology,*** **2006, 177: 3461–3468.**

The immune system specifically recognizes and eliminates foreign Ags and thus protects the integrity of the host. Tolerance mechanisms that prevent or inhibit potentially harmful reactions to self Ags include clonal deletion of autoreactive T and B cells in the thymus, lymph nodes, and peripheral circulation or their active suppression by regulatory T cells. The breakdown of one or more of these mechanisms may result in autoimmunity (1). Autoimmune diseases are characterized by the activation of autoantigen-specific T and B lymphocytes and by their differentiation into autoreactive effector cells (2). The dissection of mechanisms of autoimmunity induction and tissue damage and the development of more effective therapeutic strategies require adequate disease modeling.

In a group of organ-specific autoimmune diseases, blistering of skin and mucous membranes is associated with autoantibodies against structural epithelial proteins. The blister-inducing capacity of Abs to various Ags, including desmosomal cadherins, acetylcholine receptors, type XVII collagen, laminin 5, BP230, and type VII collagen, has been characterized by passive transfer of (auto)antibodies into experimental animals (3–10). By this approach, significant information has accumulated on the mechanisms effective in the skin that, after binding of autoantibodies, mediate blistering. Interestingly, these mechanisms differ markedly. Although autoantibodies from patients with pemphigus and mucous membrane pemphigoid cause blisters just by binding to their targets, lesion induction by autoantibodies from patients with bullous pemphigoid and epidermolysis bullosa acquisita (EBA)[3] appears to require subsequent activation of inflammation pathways (11–13).

Studies on the induction and modulation of the autoimmune response resulting in blistering have been hampered by difficulties in developing animal models reproducing both autoantibody production and skin pathology (active disease models) (14, 15). Recently, Amagai et al. (16) circumvented this problem by immunizing desmoglein 3-knockout mice with desmoglein 3 and subsequently transferred splenocytes of these mice into *Rag-2*$^{-/-}$ mice expressing this desmosomal cadherin. This led to production of anti-desmoglein 3 Abs in the immunodeficient mice and to suprabasilar cleavage like in pemphigus vulgaris (16, 17). However, active blistering disease has not been induced in immunocompetent mice by immunization with autoantigen (15, 16).

EBA, a severe autoimmune subepidermal blistering disease of skin and mucous membranes, is characterized by tissue-bound and circulating IgG Abs to the dermal-epidermal junction (DEJ) (12). Patients' serum autoantibodies bind to the 290-kDa type VII collagen, the major component of anchoring fibrils (18). Epitopes recognized by the majority of EBA sera were mapped to the noncollagenous (NC) 1 domain of type VII collagen (19–21). The pathogenic relevance of Abs against type VII collagen is supported by compelling evidence. Autoantibodies against type VII collagen from EBA patients were shown to recruit and activate leukocytes in vitro, resulting in dermal-epidermal separation in cryosections of human skin (22, 23). Abs against type VII collagen induce subepidermal blisters when passively transferred into mice (8–10).

In the present investigation, immunization of mice with recombinant murine type VII collagen NC1 induced production of autoantibodies and resulted in a subepidermal blistering phenotype, duplicating the findings in the human disease. Using this animal model, we show that the production of autoantibodies binding to

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[3] Abbreviations used in this paper: EBA, epidermolysis bullosa acquisita; DEJ, dermal-epidermal junction; IF, immunofluorescence; NC, noncollagenous.

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the epidermal basement membrane can be induced in mice of different strains. However, the blistering phenotype is restricted to certain strains. Lesion formation is associated with autoantibodies against type VII collagen that belong to a certain subclass and are capable of activating complement in the skin of the mice.

Materials and Methods

Mice

Seven- to 8-wk-old SJL-1, SKH-1, C57BL/6, and BALB/c female and male mice with a body weight of ~20 g were obtained from Charles River Laboratories. FcγRIIb$^{-/-}$ female mice (on a C57BL/6 background) were obtained from Taconic Farms. All injections and bleedings were performed on mice narcotized by inhalation of isoflurane or i.p. administration of a mixture of ketamine (100 μg/g) and xylazine (15 μg/g). The experiments were approved by local authorities of the Animal Care and Use Committee (Kiel, Germany; No. 6/i/05) and performed by certified personnel.

Immunizations and evaluation of mice

Mice were immunized at the tail base with 40 μg of recombinant murine type VII collagen (GST-mCOL7C) (8) or human type XVII collagen (GST-NC16A2-5) (24) emulsified in the nonionic block copolymer adjuvant TiterMax (ALEXIS Biochemicals) and boosted at the same location with 40 μg of Ag in TiterMax 3, 6, and 9 wk later. Mice were examined every second day for their general condition and for evidence of cutaneous lesions (i.e., erythema, blisters, erosions, and crusts). Blisters or erosions were counted, and the extent of skin disease was scored as follows: 0, no lesions; 1, <10 lesions or <1% of the skin surface; 2, >10 lesions or 1–5% of the skin surface; 3, 5–10%; 4, 10–20%; and 5, >20% involvement of the skin surface. All mice were observed for at least 12 wk. Twenty of each SJL and BALB/c mice were followed up for 18 wk. For passive transfer studies, 50 μl (1 mg) of IgG purified from diseased SJL and nondiseased SKH mice was injected every second day intradermally into the ears of C57BL/6 mice. After four injections, mice were killed and the ears were prepared for histological and immunopathological examination, as described (8).

Serum samples were obtained from mice at different time points and assayed by immunofluorescence (IF) microscopy and for autoantibody levels by ELISA using recombinant protein. Biopsies of lesional and perilesional skin, esophagus, and colon were obtained at the end of the observation period and prepared for examination by histopathology, electron microscopy, and IF microscopy.

Histological and electron-microscopical studies

Biopsies of lesional and perilesional skin, oral mucosa, esophagus, and colon were fixed in 4% buffered Formalin. Sections from paraffin-embedded tissues were stained with H&E. For electron microscopy, specimens collected from six diseased mice and one control mouse were fixed in 3% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.4) for at least 2 h at room temperature, cut into pieces of 1 mm^3, washed in 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide for 1 h at 4°C, rinsed in water, dehydrated in graded ethanol solutions, transferred in propylene oxide, and embedded in epoxy resin (25). Before examination, ultrathin sections were treated with uranyl acetate and lead citrate.

To evaluate the tissue-bound IgG autoantibodies, ultrathin sections of perilesional skin biopsies from three mice were prepared and immunolabeling was performed, according to the postembedding technique of Tokuyaso and Griffiths (26). Briefly, specimens were immediately fixed in 5% formaldehyde, cryoprotected by 0.03 M polyvinylpyrrolidone/1.6 M sucrose, and frozen in liquid nitrogen. Ultrathin sections (60 nm in thickness) were made at −110°C to −100°C with a Leica Ultracryomicrotome R and covered on Cu/Pd grids (Plano). Prepared sections were incubated with immunogold-conjugated Abs against mouse IgG (12 nm) for 45 min. After incubation with Abs, tissue was contrasted with uranyl acetate and embedded in 2% methylcellulose. Immunogold labeling was visualized and photographed with the Philips electron microscope EM 400T.

To characterize the binding site(s) of anti-type VII collagen Abs ultrastructurally, postembedding immunoelectron microscopy using normal mouse skin was performed, as described (27). In brief, normal murine nasal skin was cryofixed with liquid propane cooled at −190°C, freeze substituted with methanol, and embedded in Lowicryl K11M (Chemische Werke Lowi). Ultrathin sections were incubated with mouse immune sera, followed by 5 nm of gold-conjugated rabbit anti-mouse IgG (Amersham Life Science). Gold labeling was enhanced in size with the InteSE silver enhancement kit (Amersham).

IF microscopy and immunoblot analysis

Tissue-bound autoantibodies were detected by IF microscopy on frozen sections prepared from tissue biopsies using 100-fold diluted FITC-labeled Abs specific to mouse IgG (DakoCytomation), IgG1, IgG2a, IgG2b, IgG3 (all obtained from BD Pharmingen), and murine C3 (Cappel Organon-Teknika). The staining intensity of immunoreactants in the skin of immunized mice was assessed semiquantitatively using a score comprising 0, for no staining; 1, faint staining; 2, medium; and 3, intense staining. Detection of serum autoantibodies in mice followed published protocols with minor modifications (8, 28). Briefly, after incubating with diluted mouse serum, the frozen sections of murine and human skin were treated with 100-fold diluted Abs to mouse IgG and IgG subclasses. Expression and purification of recombinant murine type VII and human type XVII collagen forms followed published protocols (8, 24). Extracts of murine dermis were prepared, as described (8, 29). Recombinant proteins or dermal extracts were fractionated by 12 and 6% SDS-PAGE, respectively, transferred to nitrocellulose, and analyzed by immunoblotting (22). Alternatively, proteins were separated by gradient 4–20% SDS-PAGE. Immunoadsorption of mouse serum with cell lysate of bacteria transformed with wild-type pGEX was performed, as described (8). Rabbit Abs SA8009 and SA2954 generated against rGST fusion proteins containing sequences of human type XVII collagen (GST-NC16A2-4) and murine type VII collagen (GST-mCOL7C), respectively, were described previously (8, 28).

Detection of circulating autoantibody levels by ELISA

ELISA using recombinant murine type VII collagen was performed at room temperature on 96-well microtiter plates. The optimal working conditions of the assay were defined by chessboard titrations with dilutions of Ag and secondary Ab, as described (30). The optimized ELISA was run under the following conditions. Each well was coated with 500 ng of purified protein rGST-mCOL7C or with an equimolar amount of GST in 0.1 M bicarbonate buffer (pH 9.6). After blocking, wells were incubated with a 200-fold dilution of mouse sera for 60 min. Bound Abs were detected using a 10,000-fold dilution of an HRP-labeled rabbit anti-mouse IgG Ab (DakoCytomation) and orthophenylene diamine (Sigma-Aldrich).

Statistical analysis

Data are expressed as mean ± SEM of n observations (i.e., the number of animals for the in vivo studies). To compare the weight between diseased and control mice, the independent-samples Student's t test was used. For comparing the frequency of IgG2 autoantibodies and diseased and nondiseased mice, the Fisher's exact test was applied. Differences in disease severity were calculated using the χ^2 test (8). To estimate the correlation between the IF staining intensity at the DEJ and disease activity, the Spearman's rank correlation test was applied (8). To compute these tests, the OpenStat2 free software for Linux (⟨www.agrivisser.com/cgi-bin/English/OpenStat2.htm⟩) was used.

Results

Immunization against autologous type VII collagen induces autoantibody production in mice of different strains

SJL-1 (n = 29), BALB/c (n = 25), FcγRIIb$^{-/-}$ (n = 20), C57BL/6 (n = 10), and SKH-1 (n = 20) mice were immunized against a rGST fusion protein containing a sequence of the type VII collagen NC1 domain. Control SJL-1 (n = 5) and BALB/c (n = 5) mice were immunized with an irrelevant GST fusion protein. Preimmune serum from none of the mice showed IgG reactivity to the DEJ by IF microscopy. One month after the first s.c. injection of autoantigen, circulating IgG binding to the DEJ was detected in all mouse sera by IF microscopy using murine skin as a substrate (Fig. 1*a*). Indirect immunoelectron microscopy analysis demonstrated that mouse IgG Abs labeled the lamina densa of mouse skin (Fig. 1*b*). By immunoblot analysis, IgG Abs from all immune sera, in contrast to preimmune sera, targeted both cell-derived and recombinant forms of type VII collagen (Fig. 1*c*). Levels of IgG autoantibodies specific to type VII collagen were measured at several time points by ELISA using recombinant Ag. Although initially BALB/c mice showed significantly lower levels of autoantibodies to type VII collagen, 12 wk after the first immunization, levels of autoantibodies were similar in all strains

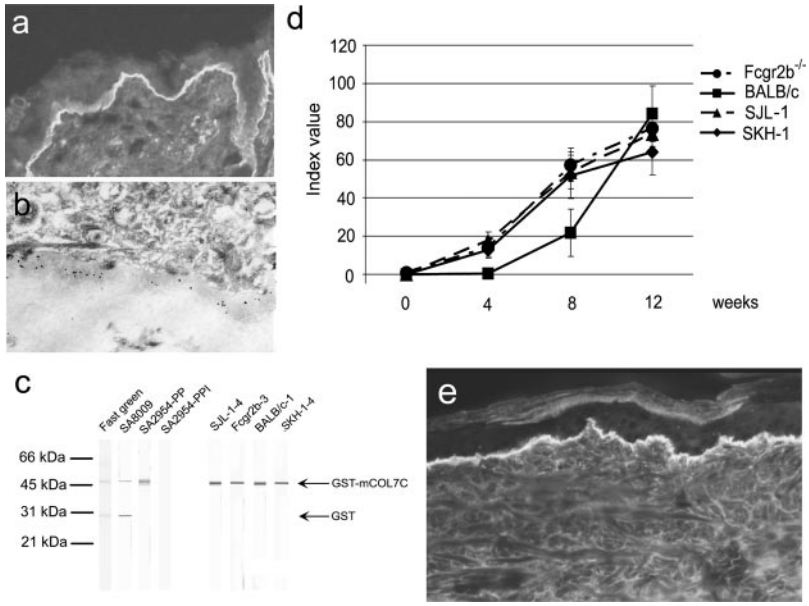

FIGURE 1. Immunization against autologous type VII collagen induces specific autoantibody production in mice. Mice were immunized at the tail base with 40 μg of recombinant murine type VII collagen emulsified in TiterMax and boosted at the same location with 40 μg of Ag in TiterMax 3, 6, and 9 wk later. *a*, By IF microscopy, IgG autoantibodies binding to the DEJ of normal mouse skin are detected in serum from an FcγRIIb$^{-/-}$ mouse 1 mo after immunization (magnification, $\times$400). *b*, By immunoelectron microscopy, serum IgG from an immunized SJL mouse binds to the lamina densa of mouse skin (magnification, $\times$44,000). *c*, Equimolar amounts of GST-mCOL7C and GST were separated by 15% SDS-PAGE (Fast green) and immunoblotted with an Ab specific to an irrelevant GST fusion protein (SA8009), the immune (SA2954-PP), and preimmune serum (SA2954-PPI) of a rabbit immunized against murine type VII collagen, as well as with representative immune mouse sera. After preadsorption against GST, like the specific rabbit Ab (SA2954-PP), the sera from the immunized mice bind to recombinant murine type VII collagen. *d*, Levels of IgG autoantibodies in serum samples of immunized SJL-1 (n = 5), SKH-1 (n = 5), FcγRIIb$^{-/-}$ (n = 5), and BALB/c (n = 5) were measured by an ELISA using recombinant type VII collagen, as described in *Materials and Methods*. Index values, represented as means $\pm$ SE, were calculated based on the OD readings of reference serum samples from an SJL mouse obtained before and 3 mo after immunization against type VII collagen. *e*, IF microscopy, performed on frozen sections of a perilesional skin biopsy from an SKH-1 mouse immunized with GST-mCOL7C, reveals linear deposition of mouse IgG at the epidermal basement membrane (magnification, $\times$400).

(Fig. 1*d*). In mice immunized with control protein, no autoantibodies to the DEJ or to type VII collagen were detected at any time point by IF microscopy and ELISA, respectively. Serum Ab reactivity against GST was similar in mice immunized against recombinant type VII collagen when compared with mice treated with the control protein (data not shown). In all mice immunized with type VII collagen, but not in mice injected with control protein, linear deposition of mouse IgG was detected at the epithelial basement membrane of skin and esophagus (Fig. 1*e*).

Mice of different strains show distinct susceptibility to the induction of blisters by immunization against type VII collagen

Twenty-four of 29 (82%) SJL-1, 14 of 25 (56%) BALB/c, and 9 of 20 FcγRIIb$^{-/-}$ (45%), but none of the SKH-1 and C57BL/6 mice immunized against type VII collagen developed a blistering phenotype (Table I; Fig. 2). Four to 8 wk after the first s.c. injection of recombinant type VII collagen, all diseased SJL mice showed single blisters on their ears and tails, often accompanied by erythema (Fig. 2*b*). Blisters developed into erosions partly covered by crusts and with longer disease duration, diffuse erythema, and thickening, and scarring of the ears and tails occurred, such that the architecture and shape of these organs began to change (Fig. 2, *a* and *c*). In the majority of diseased mice, 1–2 wk after first lesions had developed, muzzles, periocular skin, and paws, as well as ventral and dorsal aspects of the trunk were also affected. Blisters/erosions on trunk, periocularly, and around the snouts resulted in alopecia after an average time of 8–12 wk after immunization (Fig. 2, *a* and *g*). In BALB/c and FcγRIIb$^{-/-}$ mice immunized against type VII collagen, initial skin lesions were observed 6–8 wk after the first injection (Fig. 2, *f* and *h*). The development and evolution of blistering in these mice had a similar pattern, although with a latency and at a significantly lower scale compared with lesions seen in SJL mice (Fig. 3; Table I). After ceasing s.c. injections of type VII collagen, disease activity decreased in diseased animals (Fig. 3). None of the SKH-1 and C57BL/6 mice or mice injected with control protein (Fig. 2*i*) developed skin lesions. No behavioral alterations or significant weight loss were recorded in immunized mice of different strains compared among each other or with nonimmunized littermates.

Table I. *Mice of different strains show distinct susceptibility to the induction of blisters by immunization against type VII collagen*

Strain	Serum Autoantibodies		Skin Blisters	Skin Immunopathology[c]	
	IF[a]	ELISA[b]		IgG	C3
SJL-1	29/29	29/29	24/29	29/29	29/29
BALB/c	25/25	25/25	14/25	25/25	19/25
Fcgr2b$^{-/-}$	20/20	20/20	09/20	20/20	11/20
SKH-1	20/20	20/20	0/20	20/20	6/20

[a] Reactivity of IgG autoantibodies was detected by IF microscopy on murine skin sections using 10-fold-diluted mouse serum obtained 6 wk after immunization against type VII collagen.

[b] Serum autoantibodies to type VII collagen were detected by ELISA using recombinant murine Ag as described in *Materials and Methods;* serum samples were obtained 6 wk after immunization.

[c] Perilesional skin biopsies, obtained at the end of the experiment, were analyzed for IgG and complement C3 deposits at the epidermal basement membrane by IF microscopy.

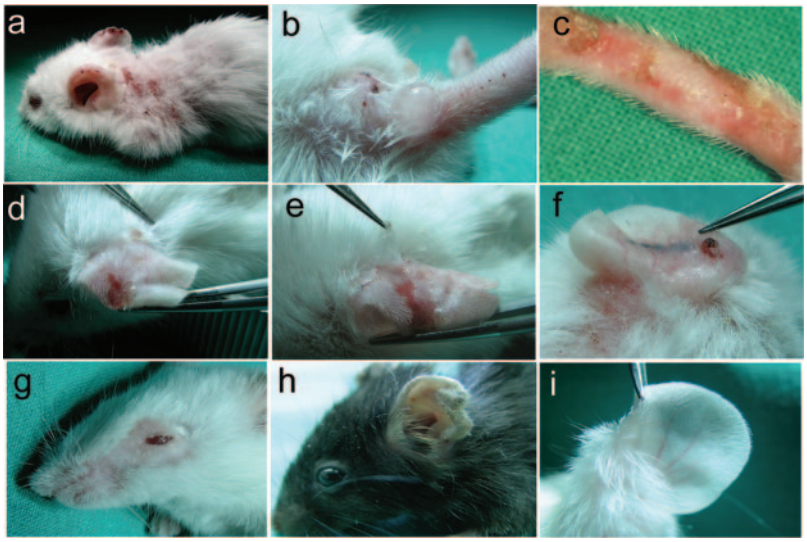

FIGURE 2. Mice of different strains show distinct susceptibility to the induction of blisters by immunization against type VII collagen. *a*, Extensive skin lesions, including blisters, erosions, alopecia, and scarring, developed in an SJL-1 mouse 8 wk after the first immunization against type VII collagen. *b*, Close-up picture of a blister on the tail of an SJL-1 mouse 7 wk after the first immunization against type VII collagen. *c*, Erosions, crusts, skin atrophy, and scarring on the tail of an SJL-1 mouse 12 wk after the first injection of autoantigen. *d*, Erosions and crusts on the right ear of an immunized SJL-1 mouse. *e*, Upon tangential pressure, epidermal detachment could be induced and the epidermis could easily be lifted up from the dermis. *f*, Erosions and crusts on the right ear of an immunized BALB/c mouse. *g*, Periocular scarring and alopecia in an SJL mouse 10 wk after the first immunization against type VII collagen. Erosions and scarring in FcγRII2b$^{-/-}$ mouse immunized against murine type VII collagen (*h*); and no skin lesions in a BALB/c mouse immunized against an irrelevant GST fusion protein (*i*).

Blisters localize at the sublamina densa of the basement membrane zone

From each mouse, three skin biopsies were obtained for histopathological examination at the end of the observation period. In all mice that showed skin lesions at the time of tissue collection, light microscopic analysis of skin biopsies revealed extensive dermal-epidermal separation and different degrees of inflammatory infiltrates dominated by neutrophils (Fig. 4*a*). Histological examination of skin biopsies from mice immunized with control protein (Fig. 4*b*) and from nondiseased mice immunized against type VII collagen demonstrated no blisters and no inflammatory infiltrate at the DEJ. In one SKH mouse, dermal-epidermal separation and a low inflammatory infiltrate were observed. In five SJL mice with skin lesions, biopsies were also obtained from oral mucous membranes, esophagus, and colon. In all five mice, subepithelial blisters were found in mucous membranes, whereas no blisters were detected in esophagus and colon (data not shown). Electron microscopy of lesional skin biopsies of diseased SJL-1 mice ($n = 5$) demonstrated split formation in the uppermost dermis. The basal lamina was found in the blister roof (dermolytic blister formation) and adhered to basal keratinocytes that showed intact hemidesmosomes (Fig. 4*c*). By immunoelectron microscopy of a perilesional skin biopsy, IgG deposits localized to the sublamina densa of the DEJ. IF analysis of lesional skin of diseased mice demonstrated IgG deposits predominantly on the epidermal side of the cleavage (Fig. 4*d*). Based on the binding of IgG to the sublamina densa, this IF analysis confirms the dermolytic split formation.

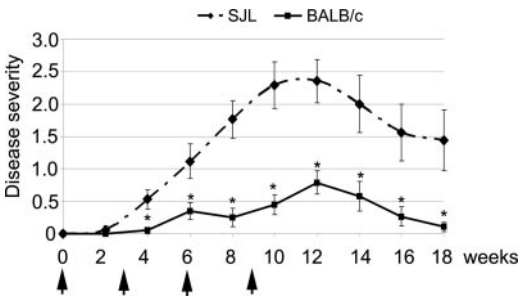

FIGURE 3. SJL mice develop an earlier and more severe blistering disease compared with BALB/c mice. SJL and BALB/c mice were given primary and booster injections (arrowheads) of murine type VII collagen and evaluated for skin lesions, as described in *Materials and Methods*. Disease activity is represented as means ± SEM in 17 SJL and 20 BALB/c mice. *, $p < 0.01$ represents significant difference of disease severity between the two groups.

The blistering phenotype in mice is associated with local deposits of complement

IF microscopy of perilesional mouse skin revealed linear deposits of mouse C3 at the DEJ in all diseased animals that were immunized with type VII collagen (Table I; Fig. 5*b*). No or weak deposits of murine C3 were found by direct IF microscopy at the DEJ of nondiseased animals (Table I; Fig. 5, *d* and *f*). Grading of the relative intensity of C3 deposits in 41 diseased and 31 nondiseased mice demonstrates that complement deposition was significantly higher in diseased compared with nondiseased mice (1.65 ± 0.12 and 0.37 ± 0.13; $p < 0.001$). The relative intensity

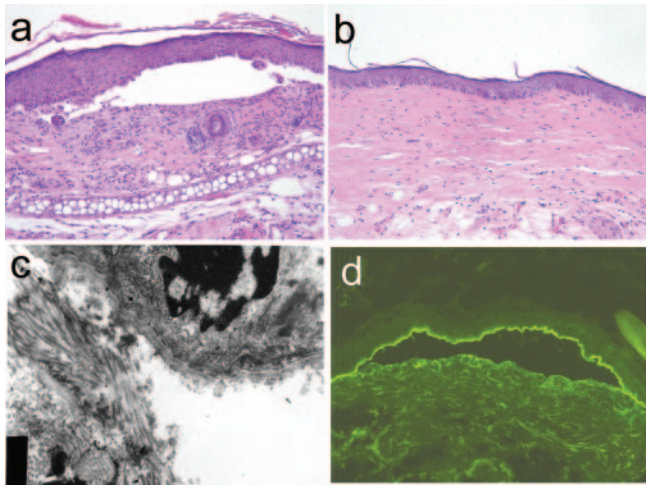

FIGURE 4. Blisters localize below the lamina densa of the epidermal basement membrane. *a*, Histological examination of skin biopsies from a diseased SJL-1 mouse reveals extensive subepidermal cleavage (magnification, ×400). *b*, No histological changes in the skin of a mouse immunized against the control protein (magnification, ×200). *c*, Electron-microscopic examination of a lesional skin biopsy from a diseased mouse demonstrates that the blister roof contains the lamina densa bordered by basal keratinocytes with hemidesmosomes. Dermal connective tissue represents the blister floor (magnification, ×11,000). *d*, IF analysis of lesional skin of diseased mice demonstrated IgG deposits predominantly on the epidermal side of the cleavage.

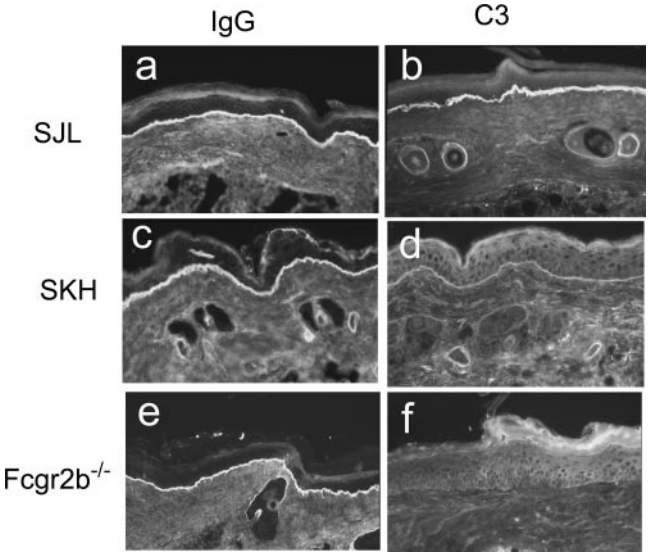

FIGURE 5. The blistering phenotype in mice is associated with local deposits of complement. Skin biopsies from mice immunized against type VII collagen were assessed by IF microscopy for the presence of IgG and C3 deposits. Strong IgG deposition was found at the epidermal basement membrane in an SJL (*a*), SKH (*c*), and FcγRIIb$^{-/-}$ (*e*) mouse. Staining for complement C3 was strong in SJL (*b*), weak in SKH-1 (*d*), and almost absent in FcγRIIb$^{-/-}$ (*f*) mice. The patterns of reactivity are representative of the patterns revealed in the other mice, as shown in Table I.

of C3 deposits correlates with the extent of the skin disease ($r = 0.88$; $p < 0.01$).

Subepidermal blistering is associated with IgG2 autoantibodies against type VII collagen

The IgG subclass of autoantibodies bound at the DEJ was assessed by IF microscopy of perilesional skin biopsies from immunized mice. Results of this analysis are summarized in Table II, and patterns of immunoreactivity are depicted in Fig. 6. In SJL mice, in addition to IgG1 deposits, strong deposition of IgG2a and IgG2b Abs was detected. In some of BALB/c and FcγRIIb$^{-/-}$ mice, IgG1 deposition was accompanied by IgG2a and IgG2b Abs. SKH-1 mice predominantly showed linear IgG1. In 2 and 4 of 20 mice, weak IgG2a and IgG2b deposits were found, respectively; no IgG3 deposition was detected. In the immunized mice, tissue deposits of IgG2 autoantibodies were more frequent in diseased compared with nondiseased animals using the Fisher's exact test ($p < 0.001$), and the relative intensity of IgG2a/b deposits correlates with the extent of the skin disease ($r = 0.68$; $p < 0.01$). To study the blister-inducing potential of autoantibodies from diseased and nondiseased mice, IgG purified from 3 diseased SJL and 3 nondiseased SKH mice was injected into the ears of a total of 6 C57BL/6 mice. Blistering was observed in mice injected with IgG purified from SJL (Fig. 7*a*), but not with IgG from SKH mice (Fig. 7*b*). In addition, IF microscopy of perilesional skin revealed linear deposition of C3 at the DEJ in mice injected with IgG purified from SJL (Fig. 7*c*), but not SKH mice (Fig. 7*d*). Deposition of IgG1 at the DEJ was found in mice injected with IgG from both SJL (Fig. 7*e*) and SKH mice (Fig. 7*f*). However, deposits of IgG2a and IgG2b were observed in mice injected with IgG purified from SJL (Fig. 7*g*), but not from SKH mice (Fig. 7*h*).

Table II. *IgG subclass distribution of autoantibodies bound at the epidermal basement membrane in immunized mice*[a]

Mouse Strain	IgG1	IgG2a	IgG2b	IgG3
SJL	20/21	7/21	21/21	3/21
BALB/c	22/22	9/22	6/22	1/22
Fcgr2b$^{-/-}$	20/20	10/20	12/20	1/20
SKH	20/20	2/20	4/20	0/20

[a] Perilesional skin biopsies were analyzed for deposition of IgG isotypes at the epidermal basement membrane by IF microscopy.

Discussion

Animal models replicating the active disease are needed to study the regulation of autoreactive T and B cells in autoimmune blistering diseases. Our study describes an active disease model for EBA by immunization of mice against autologous type VII collagen. These mice developed blisters of skin and oral mucous membranes that were associated with circulating autoantibodies against type VII collagen as well as deposits of IgG and complement at the epithelial basement membrane. Dermal-epidermal separation localized to the sublamina densa. Thus, the blistering phenotype induced in the mice reproduces the clinical, histological, immunopathological, and ultrastructural features of human EBA. In addition, the disease in these animals mimics subepidermal blistering induced in mice by passive transfer of Abs against type VII collagen (8–10).

A major difficulty in developing animal models of autoimmune diseases has been to overcome self-tolerance. However, for some autoimmune conditions, experimental models reproducing active disease have been successfully generated by immunizing animals with the (auto)Ag (31–37). In our study, we immunized BALB/c, C57BL/6, and SJL animals that are inbred strains commonly used to study autoimmunity in mice. In addition, we used outbred SKH-1 mice because their hairless condition facilitates skin evaluation. FcγRIIB is thought to be an epistatic modifier of autoimmunity; its deletion results in a strain-specific enhancement of systemic lupus erythematosus (38, 39) or in the development of arthritis and Goodpasture disease on nonpermissive H-2 backgrounds (40, 41). Based on these data, we hypothesized that FcγRIIb$^{-/-}$ mice on C57BL/6 background might be prone to develop clinically overt autoimmunity against type VII collagen too.

Interestingly, we were able to induce an autoimmune response to type VII collagen in all mice immunized against autologous protein. However, although all immunized mice exhibited both comparable levels of circulating autoantibodies and tissue-bound IgG, skin blistering was restricted to SJL, BALB/c, and FcγRIIb$^{-/-}$ mice. In contrast, SKH-1 and C57BL/6 animals showed no blistering clinically or histologically. This strain dependence strongly suggests a genetic predisposition to the induction of skin blistering in mice by autoantibodies to type VII collagen. In addition, our observations demonstrate that the presence of circulating autoantibodies as well as the mere binding of autoantibodies to the Ag are not sufficient to induce skin blisters. A similar phenomenon has been observed in other models for autoimmune diseases. Experimental animals, either passively transferred with specific autoantibodies or immunized against the autoantigen, despite in vivo IgG deposition, failed to acquire a disease phenotype (42–45). Although local deposition of Igs has been considered a major criterion for the diagnosis of an autoimmune disease, our present findings and previous data demonstrate that tissue-bound autoantibodies may well be detected in the absence of specific clinical findings.

In the skin of EBA patients, deposition of complement components is found with an incidence ranging from ~40 to 100% (46–48). However, in patients included in these studies, diagnosis of EBA was based only on clinical and histological features, while

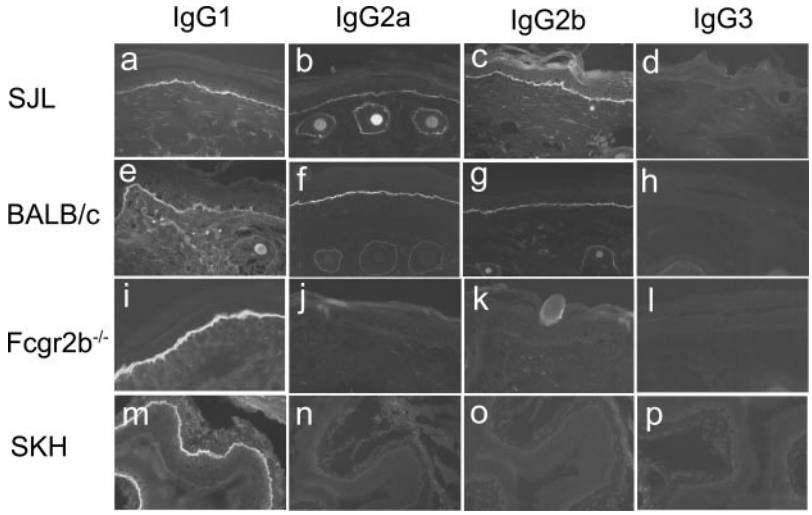

FIGURE 6. Subepidermal blistering is associated with IgG2b and IgG2a autoantibodies against type VII collagen. Depicted are the results of IF microscopy for IgG subclass distribution of autoantibodies in four mice immunized against type VII collagen. Strong IgG1 deposition was found at the epidermal basement membrane in SJL (*a*), BALB/c (*e*), FcγRIIb$^{-/-}$ (*i*), and SKH (*m*) mice. IgG2a and IgG2b deposits were found in the SJL (*b* and *c*) and BALB/c (*f* and *g*), but not in the FcγRIIb$^{-/-}$ (*j* and *k*) and SKH (*n* and *o*) mice. *d*, *h*, *l*, and *p*, None of the mice demonstrated cutaneous deposits of IgG3 (magnification, ×400). Analysis of the IgG subclass distribution of autoantibodies at the DEJ in immunized mice is summarized in Table II.

reactivity to type VII collagen has not been confirmed (47). Therefore, in these patients, EBA may have been confounded with other subepidermal autoimmune blistering diseases (e.g., anti-p200 pemphigoid or anti-epiligrin cicatricial pemphigoid). In the active disease model described in this study, we found IgG deposition at the DEJ in all mice immunized against type VII collagen. In contrast, staining for complement C3 was significantly increased in diseased compared with nondiseased mice, suggesting that activation of complement is important for blister formation. To further explore this hypothesis, we analyzed the IgG subclass distribution of tissue-bound and circulating autoantibodies in mice. IgG1 autoantibodies were detected in all animals immunized against type VII collagen. However, deposits of complement-fixing IgG2a and IgG2b autoantibodies were significantly more frequent and intense in diseased compared with resistant mice. In addition, IgG from diseased, but not resistant mice induced skin blistering by passive transfer into mice. The resistance of mice to skin blistering is therefore most likely related to the lack of induction of complement-fixing autoantibodies; this resulted in absence/major reduction of complement activation in nondiseased mice. Consistent with this hypothesis are the observations that rabbit Abs to murine type VII collagen are not pathogenic in C5-deficient mice (8) and that $F(ab')_2$ of pathogenic Abs against type VII collagen fail to induce subepidermal blisters in mice (8, 10). Nevertheless, a direct, Fc-independent, pathogenic role of autoantibodies against type VII collagen (e.g., by interfering with its ligand function) cannot be excluded. However, in vivo experimental evidence supporting this hypothesis is still lacking.

Complement-fixing autoantibodies are potent mediators of tissue damage. Th1 cells have been implicated in the pathogenesis of most Ab-mediated autoimmune diseases, because they help B cells to produce complement-fixing autoantibodies (49, 50). In our study, autoantibodies that bound in the skin of diseased mice were essentially of IgG1 and IgG2a/b isotypes. These findings demonstrate that the autoimmune response in diseased mice rather shows a Th1/Th2 polarization than a pure Th2 or a T-independent Ag-driven reaction. IgG2 autoantibodies have been implicated as mediators of tissue injury also in other autoimmune diseases, including myasthenia gravis, experimental autoimmune encephalomyelitis, and vasculitis (44, 51, 52). In contrast, in the active mouse model for pemphigus, disease expression is not dependent on complement activation. In desmoglein 3$^{-/-}$ mice, following immunization against desmoglein 3, Ag-specific B cells developed. When transferred into Rag-2$^{-/-}$ mice, these B cells predominantly produced IgG1 Abs that bound to the Ag

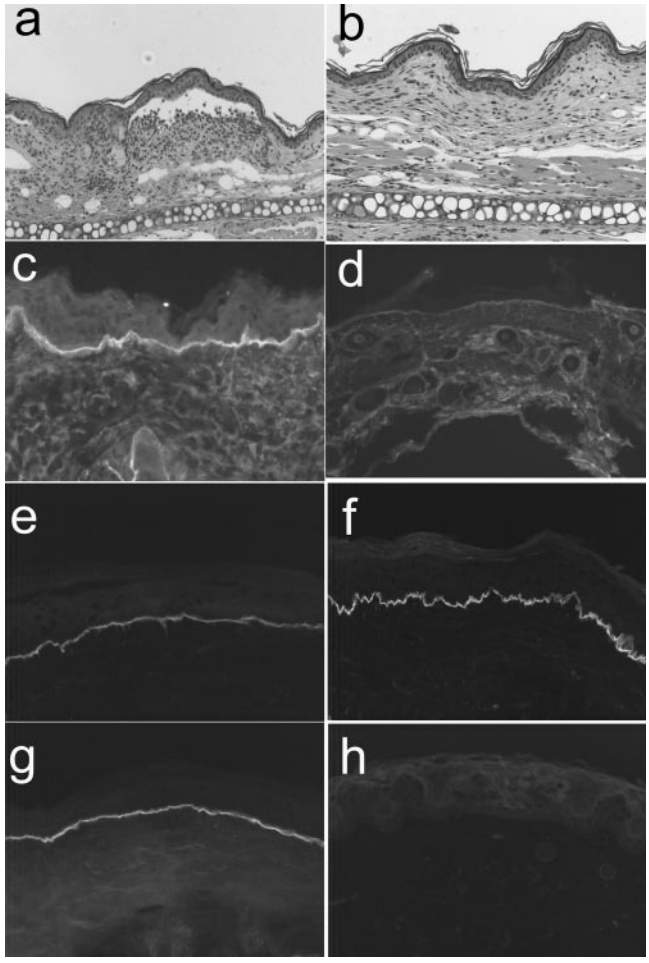

FIGURE 7. IgG purified from diseased mice induces dermal-epidermal separation when passively transferred into mice. A total of 50 μl (1 mg) of IgG purified from three diseased SJL and three nondiseased SKH mice was injected every second day intradermally into the ears of a total of six C57BL/6 mice. After four injections, histopathological analysis shows subepidermal blisters in mice injected with IgG purified from SJL (*a*), but not from SKH (*b*) mice. IF microscopy of perilesional skin reveals linear deposition of C3 at the DEJ in mice injected with IgG purified from SJL (*c*), but not SKH (*d*) mice. Deposition of IgG1 was observed in mice injected with IgG from both SJL (*e*) and SKH (*h*) mice, whereas deposits of IgG2b were found only in mice injected with IgG purified from SJL (*g*), but not from SKH (*h*) mice.

in the skin of the mice and induced acantholytic blisters. These Abs did not activate complement or recruit leukocytes (16, 53). These data are strengthened by work using the passive transfer model of pemphigus showing susceptibility of C5-deficient mice to the experimental pemphigus (54) and induction of blisters in mice by $F(ab')_2$ (54), Fab (55), and single-chain variable fragment (56) fragments of pemphigus autoantibodies. In contrast, the active animal model of EBA described in this report will facilitate studies on autoantibody-induced tissue damage mediated by complement and inflammatory cells.

Our animal model allows for the evaluation of the autoimmune response and skin blistering by simple serological assays and clinical inspection, respectively. Therefore, this robust experimental system can be used as a model for organ-specific autoimmunity to investigate the immunogenetics of EBA and the contribution of T and B lymphocytes for both the initiation and regulation of autoantibody production. In addition, this active disease model should greatly facilitate the development of new therapeutic strategies modulating the autoimmune response.

In conclusion, this study demonstrates that an autoimmune response against type VII collagen can be readily induced by immunization of mice, resulting in induction of skin blisters. The blistering phenotype of the mice recapitulates the clinical, histological, and immunopathological changes in the skin of patients with EBA. This experimental system should greatly facilitate the further dissection of the cellular and molecular pathogenesis of EBA. In addition, it should help in the development of novel, more specific therapies for EBA, which will also have implications for the management of other autoimmune diseases.

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Disclosures

The authors have no financial conflict of interest.

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