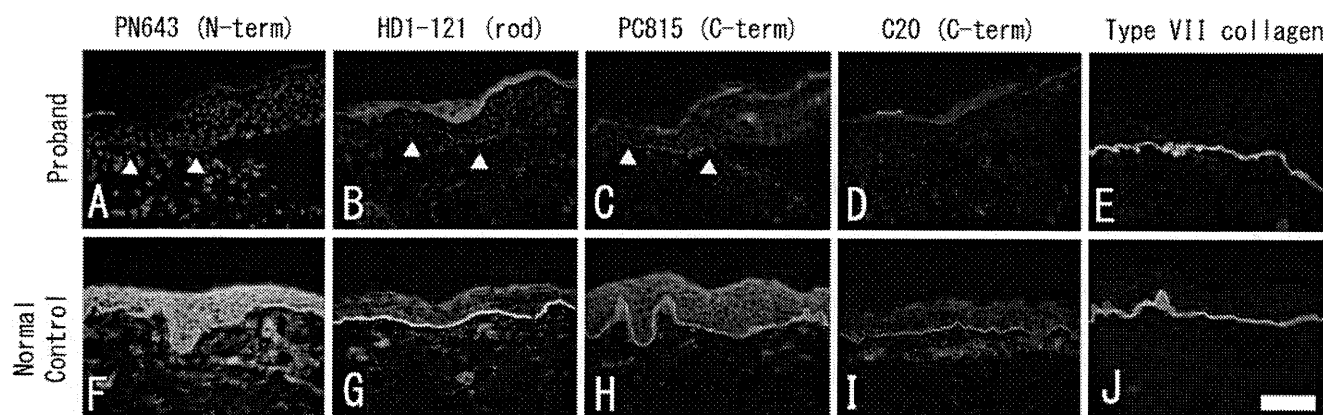


**Figure 3.** The novel *PLEC* mutations detected in the study. Maternal c.10984C>T (p.Gln3662X) (A) and de novo c.11453\_11462del (B) in exon 32 were found in genomic DNA derived from the proband. A thymine substituted for a cytosine in the former mutation is indicated by the red character (A). Deleted nucleotides in the latter mutation are underlined (B). (C) c.10984C>T mutation caused the generation of a site for *BsrI* restriction enzyme. *BsrI* digestion of the 420-bp PCR product with and without the mutation resulted in a single band of 420-bp and in double bands of 246-bp and 174-bp, respectively. c.10984C>T was a maternal mutation. (D) c.11453\_11462del caused the generation of a site for *BbvCI*. The 283-bp PCR product without the mutation was not digested by *BbvCI*. *BbvCI* digestion of the 273-bp PCR product with the deletion mutation showed two bands of 150 and 123-bp. c.11453\_11462del was not detected in the parents' gDNA.

#### Diminished and Truncated Plectin Expression in Skin

We performed immunofluorescence analysis of the skin specimens from the proband using several antibodies that react with molecules of the dermo-epidermal junction (DEJ). To check plectin expression patterns in the skin specimens from the proband, we used four antibodies: PN643 (N-terminal globular domain), HD1-121 (rod domain), PC815 (C-terminal globular domain) and C20 (C-terminal globular domain) (Fig. 1A). Normal human control shows bright DEJ staining of all the antibodies tested (Fig. 4F-I). DEJ labeling of PN643, HD1-121 and PC815 was markedly diminished in the skin specimens from the proband (Fig. 4A-C). Staining of C20 was absent in the proband's skin (Fig. 4D). Immunostaining for type VII collagen (Fig. 4E), laminin 332, type IV collagen, type XVII collagen,  $\alpha 6$  and  $\beta 4$  integrin, and BP230 revealed normal DEJ labeling patterns (data not shown).



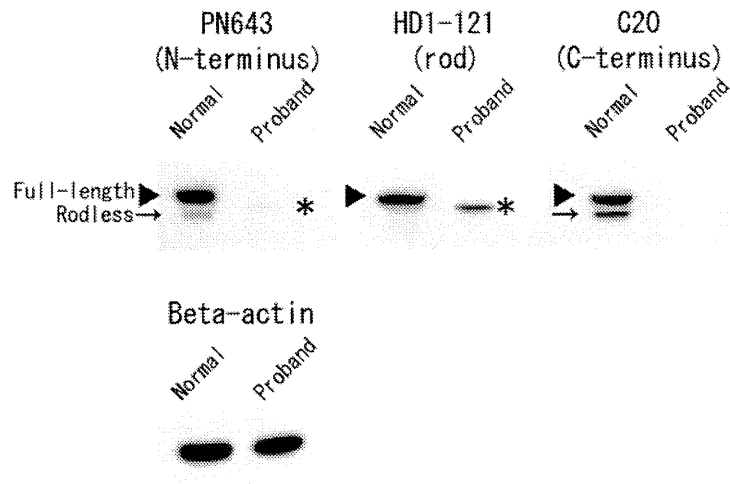
**Figure 4.** Immunofluorescence analysis of the proband's skin sample. In normal human skin, immunofluorescence shows that all of the antibodies against plectin (PN643, HD1-121, PC815 and C20) tested in this study bound to the dermal epidermal junction (DEJ) (F - I). DEJ labeling of PN643, HD1-121 and PC815 are weakly positive in the proband (A - C). In contrast, staining with C20 is negative in the proband's skin sample (D). Type VII collagen shows normal linear labeling in the proband and in the normal control (E, J). Weak labeling is indicated by arrowheads (Bar=100 $\mu$ m).

#### Diminished and Truncated Plectin in Cultured Fibroblasts

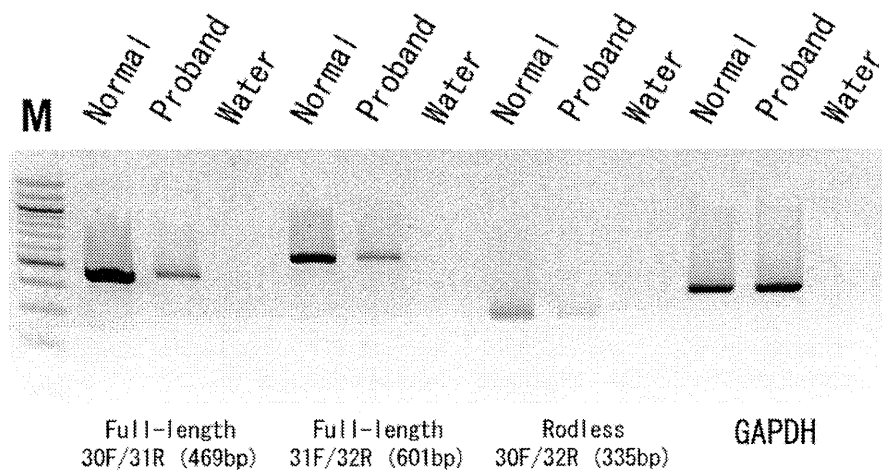
Immunoblot analysis of lysates from normal human cultured fibroblasts revealed that two closely spaced bands, corresponding to two forms of plectin (500kDa full-length and 390kDa rodless), reacted with PN643 and C20 antibodies recognizing the N- and C-termini of plectin (Fig. 5), as previously described (Natsuga, et al., 2010). HD1-121 against the rod domain reacted only with full-length plectin in normal human fibroblasts (Fig. 5). Lysates from cultured fibroblasts from the proband showed a faint band of PN643 and HD1-121 between 500kDa and 390kD, corresponding to truncated full-length plectin. C20 failed to react with lysates from the proband's cells (Fig. 5).

#### Full-length and rodless plectin transcripts are reduced in the proband's cultured fibroblasts

Using RT-PCR, the presence of mRNA that encodes full-length or rodless plectin was demonstrated in the normal human control as well as in the proband's cultured fibroblasts (Fig. 1B, 6). Judging from the PCR analysis results, the quantity of full-length and rodless plectin transcripts was markedly reduced in the proband's fibroblasts compared with those of the normal human control (Fig. 6).



**Figure 5.** Immunoblot analysis of cultured fibroblasts from the normal human control and the proband. Immunoblot analysis of extracts from fibroblasts of the normal control and the proband by using PN643 against the N-terminal actin-binding domain, HD1-121 against the rod domain and C20 against the C-terminal plectin repeats. Rodless plectin (arrows), detected with PN643 and C20, migrates just below full-length plectin (arrowheads) in normal human fibroblasts. Using HD1-121, only full-length plectin is observed in the normal control. In contrast, fibroblasts of the proband contained smaller proteins than 500-kDa full-length plectin, the putatively truncated full-length plectin (asterisks), which was detected with PN643 and HD1-121. C20 did not react with lysates of the proband's fibroblasts. Equal protein loading was confirmed by reprobing with AC15 (anti-beta-actin antibody).



**Figure 6.** Semi-quantitative RT-PCR for full-length and rodless plectin transcripts. The quantity of full-length (30F/31R and 31F/32R) and rodless (30F/32R) plectin transcripts in the proband's cultured fibroblasts is reduced in comparison to those of the normal control. GAPDH mRNA expression was used as the loading control in these experiments. The negative control reaction (DNA-free water instead of cDNA) shows no PCR products. The molecular weight standard (lane M) is a 100-bp ladder.

## DISCUSSION

This is the first report of EB complicated with both MD and PA. Skin detachment within basal keratinocytes was demonstrated by electron microscopy, which indicated the simplex subtype of EB. The proband's skin sample and cultured fibroblasts showed reduced and truncated plectin expression (Fig. 1D).

Both of the premature termination codon (PTC)-causing mutations detected in our case are within exon 32, the last exon of *PLEC*. Nonsense-mediated mRNA decay (NMD) is a quality-control mechanism that selectively degrades mRNAs with PTCs (Holbrook, et al., 2004). When the mRNA has a PTC more than a certain range upstream of one exon-exon junction, the transcript is down-regulated by NMD. In contrast, when PTCs are located in the last exon, NMD does not generally occur and abnormal mRNA is translated into truncated protein. However, some exceptions were described in a study in which transcripts underwent NMD despite having a PTC in the last exon (Chan, et al., 1998). Reduced amounts of full-length and rodless plectin transcripts in the proband's cells are explained by NMD, even though the PTCs of the proband are in the last exon.

One reported EBS-MD case was homozygous for a PTC-causing mutation (c.13458\_13473dup) in exon 32 (Schroder, et al., 2002). c.13458\_13473dup is at the downstream of the 6<sup>th</sup> plectin repeat and is predicted to cause a frameshift followed by a premature termination codon. The age of onset for MD in the patient with c.13458\_13473dup was 4 years (Schroder, et al., 2002); in our case, severe muscle weakness was observed immediately after birth. This clinical difference might be explained by the length of truncated proteins identified in each patient. Compound heterozygous mutations of c.10984C>T and c.11453\_11462del encode truncated plectin protein that does not include the intermediate filament (IF) binding site that was mapped to an approximately 50-amino-acid sequence between the 5<sup>th</sup> and 6<sup>th</sup> plectin repeat (Nikolic, et al., 1996; Rezniczek, et al., 2010). Therefore, the truncated plectin in our case might not have bound to IF including desmin in muscle tissues, which might account for the congenital muscle weakness. In contrast, the truncated plectin produced by c.13458\_13473dup harbors the IF-binding site described above. Although the amount of plectin protein was slightly diminished, it may be that substantial amounts of truncated plectin with the residual IF-binding site delayed the development of muscular dystrophy and prevented pyloric atresia in the previous patient (Schroder, et al., 2002).

EBS-MD patients do not have muscular symptoms at birth, but muscle weakness appears later in their life. The type of *PLEC* mutations (PTC-causing mutations or in-frame insertions/deletions) influences the timing of MD onset (Chiaverini, et al., 2010). Also, it may be that, in most EBS-MD cases, the presence of residual rodless plectin resulting from PTC-causing mutations in exon 31 delays the onset of MD because of the remaining IF-binding site in rodless plectin.

It has been postulated that two pathologic elements are involved in the development of PA in EB patients: 1) the integrity of basement membrane and hemidesmosomes, and 2) the control of the normal process of fibrosis in the course of wound healing (Maman, et al., 1998). The sequence of events might be initiated by the separation of the intestinal mucosal layer as a result of disintegration of basement membrane and hemidesmosomes. Inflammatory responses cause massive fibrosis, which might lead to the obstruction of the intestinal lumina, especially in anatomically narrow passages, such as pylorus (Maman, et al., 1998). Previously described EBS-MD patients do not suffer from PA, which suggests that residual rodless plectin can prevent the development of PA. In our case, both full-length and rodless plectin proteins are quantitatively reduced and the shortened plectin might not have functioned normally, which might have led to the PA phenotype.

It had been predicted that some cases of EBS-PA would develop MD, although no such case had been reported in the literature (Natsuga, et al., 2010). One possible explanation is that the poor systemic condition of EBS-PA and the limited observation period due to the patient's very short lifespan prevented the diagnosis of MD. Our data suggests that surgical correction of PA is insufficient to treat EBS-PA patients because they would most likely go on to develop MD even if they survive surgery. Therefore, we should look to develop more fundamental therapeutic options for those patients.

In summary, this study clearly shows that plectin mutations lead to both MD and PA phenotypes in an individual EBS patient.

## ACKNOWLEDGMENTS

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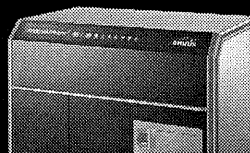
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## Human IgG1 Monoclonal Antibody against Human Collagen 17 Noncollagenous 16A Domain Induces Blisters via Complement Activation in Experimental Bullous Pemphigoid Model

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# Human IgG1 Monoclonal Antibody against Human Collagen 17 Noncollagenous 16A Domain Induces Blisters via Complement Activation in Experimental Bullous Pemphigoid Model

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**Bullous pemphigoid (BP) is an autoimmune blistering disease caused by IgG autoantibodies targeting the noncollagenous 16A (NC16A) domain of human collagen 17 (hCOL17), which triggers blister formation via complement activation. Previous in vitro analysis demonstrated that IgG1 autoantibodies showed much stronger pathogenic activity than IgG4 autoantibodies; however, the exact pathogenic role of IgG1 autoantibodies has not been fully demonstrated in vivo. We constructed a recombinant IgG1 mAb against hCOL17 NC16A from BP patients. In COL17-humanized mice, this mAb effectively reproduced a BP phenotype that included subepidermal blisters, deposition of IgG1, C1q and C3, neutrophil infiltration, and mast cell degranulation. Subsequently, alanine substitutions at various C1q binding sites were separately introduced to the Fc region of the IgG1 mAb. Among these mutated mAbs, the one that was mutated at the P331 residue completely failed to activate the complement in vitro and drastically lost pathogenic activity in COL17-humanized mice. These findings indicate that P331 is a key residue required for complement activation and that IgG1-dependent complement activation is essential for blister formation in BP. This study is, to our knowledge, the first direct evidence that IgG1 Abs to hCOL17 NC16A can induce blister formation in vivo, and it raises the possibility that IgG1 mAbs with Fc modification may be used to block pathogenic epitopes in autoimmune diseases. *The Journal of Immunology*, 2010, 185: 7746–7755.**

**B**ullous pemphigoid (BP) is the most common autoimmune subepidermal blistering disease (1, 2). Circulating autoantibodies target human type XVII collagen (human collagen 17 [hCOL17]), also known as BP Ag 2 (BPAG2) or BP180, which is a major component in hemidesmosome-anchoring filament complexes at the epidermal basement membrane zone (BMZ) (3–9). The major pathogenic epitope of BP autoantibodies is present at the extracellular noncollagenous 16A (NC16A) do-

main, which has distinctive diversity among different species (10–12). Deposition of anti-hCOL17 autoantibodies at the BMZ triggers sequential inflammatory cascades, including complement activation, degranulation of dermal mast cells, infiltration of eosinophils and neutrophils, and subepidermal blister formation elicited by proteinases derived from the inflammatory cells (13–17).

In the inflammatory mechanism of BP, the IgG-dependent classical complement pathway plays an important role in autoimmune blister formation. Complement components including C1q, C3 and C4 are detected at the dermal-epidermal junction (DEJ) in the skins of patients and experimental BP model mice (17–21). BP phenotypes have been abolished by the inhibition of C1q using neutralizing Abs in experimental models (18). C1q-binding amino acid residues underlie IgG–C1q interaction, which forms an initiation complex in the classical complement pathway (22), such as in humoral immunity to pathogens. The pathogens recognized by Abs are cleared by phagocytosis, Ab-dependent cell-mediated cytotoxicity (ADCC), and complement-dependent cytotoxicity (CDC) mechanisms. Interaction of the C1q and IgG Fc region is an initial step in CDC reaction to pathogens (23). The constant 2 (C<sub>H</sub>2) domain is the crucial region for C1q binding to the IgG Fc region (24, 25). Mapping studies of C1q-binding residues have demonstrated that the three spatially close sites (K322, P329, and P331) constitute the binding epicenter in the human IgG1 C<sub>H</sub>2 domain. E318 and K320 have been shown to play a minor role in complement activation by site-directed mutagenesis studies in vitro. Among different species, these residues are relatively well conserved (22, 25–27). However, the precise role of these binding residues has not been determined in the activation of complement cascades in vivo.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: ADCC, Ab-dependent cell-mediated cytotoxicity; BMZ, basement membrane zone; BP, bullous pemphigoid; C1q, complement 1q; CDC, complement-dependent cytotoxicity; C<sub>H</sub>2, constant 2; COL17, type XVII collagen; DB, dilution buffer; DEJ, dermal-epidermal junction; DIF, direct immunofluorescence; Flu, fluorescence; HSC, human serum complement; IIF, indirect immunofluorescence; NC16A, noncollagenous 16A; NHEK, normal human epidermal keratinocyte; PI, propidium iodide; rh, recombinant human; Sf-9, *Spodoptera frugiperda*-9; VH, V region H chain; VL, V region L chain.

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IgG1 is thought to be the predominant subclass of anti-hCOL17 IgG autoantibodies in sera from BP patients. Clinical studies have shown a preferential detection of hCOL17-specific IgG1 and IgG4 autoantibodies in BP sera, with IgG2 and IgG3 being detected in a minority of patients (5, 7, 28). Eighty-five percent and 46% of BP sera contained IgG1 and IgG4 Abs against hCOL17, respectively (6). The presence of IgG1 autoantibodies has been associated with active BP phenotypes (5, 6), and the autoantibody level has been correlated with the area of skin blistering (29). These clinical findings indicate that IgG1 might be the main pathogenic subclass in BP. However, to our knowledge, there has been no direct *in vivo* evidence to demonstrate the pathogenic role of the anti-hCOL17 IgG1 autoantibody until now.

To confirm whether BP IgG1 autoantibodies can induce the BP phenotype *in vivo* and to analyze the importance of various C1q-binding residues in the IgG1 Fc C<sub>H</sub>2 domain in BP, we cloned and constructed a recombinant human (rh)IgG1 mAb against hCOL17 NC16A. By site-directed mutagenesis in the C<sub>H</sub>2 domain of the IgG1 mAb Fc region, alanine substitutions were introduced at various residue sites that were previously verified as C1q binding sites *in vitro*. The blister formation triggered by the non-mutated IgG1 mAb was assessed *in vivo* using our COL17-humanized (*COL17<sup>m-/-</sup>, h<sup>+</sup>*) mouse model, which expresses hCOL17 but not mouse COL17 at the BMZ (17). In contrast, the IgG1 mAbs that were mutated at the P331 and/or P329 residue(s) failed to elicit blister formation. Our findings indicate that IgG1 Abs, as the predominant IgG subclass, can induce subepidermal blister formation via IgG Fc–C1q interaction in BP.

## Materials and Methods

### Establishment of the EBV-transformed B cell clones

PBMCs were prepared from seven active nontreated BP patients by density gradient using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). The diagnosis of BP was made by the typical clinical and histological manifestations as well as by laboratory data including anti-hCOL17 ELISA and indirect immunofluorescence (IIF). The clinical and immunological characteristics of the BP patients are summarized in Supplemental Table I. B cells were isolated by using microbeads conjugated to anti-human CD19 mAb (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by seeding at 15 cells/well in 96 U-bottom microplates (BD Biosciences, San Jose, CA) in complete RPMI 1640 medium containing 2.5 μg/ml ODN2006 (InvivoGen, San Diego, CA) and incubation for 2 wk with irradiated cord mononuclear cells as the feeder layer cells (50,000/well) in the presence of EBV (30% supernatant of B95.8 cells). The culture supernatants were tested for the presence of anti-hCOL17 NC16A Abs by ELISA coated with rhCOL17 NC16A peptide as reported previously (17). The index values of ELISA were defined by the following formula: index = (OD of tested supernatant – OD of negative control)/(OD of positive control – OD of negative control) × 100 (30–32). As a positive control, we used a standard BP serum supplied with the hCOL17 NC16A-ELISA kit (MESACUP BP180 test; Medical and Biological Laboratories, Nagoya, Japan) as reported previously (17, 33). Adjustment of the values relative to the positive control allows comparison of results from different plates, even if performed under different conditions. Positive wells were cloned by limiting dilution in the presence of ODN2006 and irradiated cord mononuclear cells.

### Construction of IgG1 expression vector

L chain (κ and λ) and Fd (V region + constant domain 1) fragments were amplified by RT-PCR from monoclonal B cell clones using general primers for the Ab library (Table I), cloned into pCR2.1 vectors (Invitrogen, Carlsbad, CA), and sequenced by ABI 3100 genetic analyzer (Applied Biosystems, Foster, CA). After introducing one cutting site by the restriction endonucleases EcoRV at the 3' end, the L chain was inserted into the human IgG1 expression vector pAc-κ-Fc or pAc-λ-Fc (Progen, Heidelberg, Germany) at the SacI and EcoRV cutting sites (Supplemental Fig. 1). Then the Fd sequence was inserted into the vector at the XhoI and SpeI cutting sites. The constructed baculovirus vector containing Fd and V<sub>κ</sub> or V<sub>λ</sub> genes was transfected to *Spodoptera frugiperda*-9 (Sf-9) insect cells using the BaculoGold Transfection Kit (BD Biosciences). Recombinant baculovirus was

harvested from the supernatant of Sf-9 cell culture medium TNM-FH (Grace's insect medium, 10% FBS, and penicillin-streptomycin) (Invitrogen) at 4–5 d after the transfection. Secreted rhIgG1 Abs were detected in the supernatant using an hCOL17 NC16A-ELISA kit (MBL, Nagoya, Japan). The whole procedure followed the manufacturers' recommendations.

### Production and purification of the rhIgG1 mAb

The recombinant virus inocula, via three rounds of plaque purification, infected Sf-9 cells at a multiplicity of infection of 0.5–10. The cells were grown in SF-900 II SFM (Life Technologies, Carlsbad, CA) serum-free suspension culture using a spinner 1-L flask with a vertical impeller (Corning, Lowell, MA) at 27°C. One week later, supernatant was harvested and clarified by centrifugation and filtered through 0.45 μm filters (Millipore, Bedford, MA). Purification was performed on HiTrap Protein G column (GE Healthcare). The eluted IgG in 1 M glycine-HCl (pH 2.7) was dialyzed in PBS for at least 48 h and then quantified by spectrophotometer (Pharmacia Biotech, Uppsala, Sweden) at 280 nm.

### Generation of the mutated IgG1 mAbs

Alanine substitutions were incorporated into the human IgG1 pAc-λ-Fc vector by site-directed mutagenesis using a Quick Change Mutagenesis Kit (Stratagene, La Jolla, CA), according to the manufacturer's instructions. We constructed seven mutants: five single-site ones and two multisite ones. The mutagenic oligonucleotide primers containing target sites are listed in Table I. Primers were purified by PAGE to prevent the primers from experiencing a significant decrease in mutation efficiency. Sequences were verified by ABI 3100 genetic analyzer (Applied Biosystems). The different constructs were expressed in the Sf-9 cells described above. The IgG was directly purified from the filtered serum-free supernatant using 5 ml HiTrap Protein G column (GE Healthcare).

### Establishment of hCOL17-293 cells

To establish the hCOL17-293 cell line, FlpIn 293 cells (Invitrogen) were first cotransfected with the constructed plasmid pcDNA5/FRT (Invitrogen) that had been inserted with the hCOL17 gene (a gift from Dr. K. B. Yancey, University of Texas Southwestern Medical Center, Dallas, TX) and pOG44 (Invitrogen) and were then cultured in selective medium (DMEM, 100 μg/ml hygromycin B [Invitrogen], and 10% FBS). Second, the expression levels of hCOL17 were detected by Western blot analysis using BP serum Abs (1:20) as the first Ab. Eventually, the positive clones were maintained in DMEM containing 50 μg/ml hygromycin B and 10% FBS. The procedures were handled according to the manufacturers' recommendations.

### Epitope mapping of the IgG1 mAb

We synthesized the N terminus half (hNC16A 1–3, aa 490–534) of the hCOL17 NC16A domain as a GST-fusion protein using the expression vector pGEX2-T (Amersham Biosciences, Uppsala, Sweden) and bacteria B12 (Amersham Biosciences), as reported previously (17, 34). Other peptides including hNC16A 1 (aa 490–506), hNC16A 2 (aa 506–520), hNC16A 2.5 (aa 514–532), and hNC16A 3 (aa 520–534) were expressed in the same way and then purified with a GSTrap FF affinity column (GE Healthcare). The purified IgG1 mAb 3.B6 (0.25 μg/ml) was incubated with the 0.5 μg/lane GST-fusion proteins that were transferred to a 0.2-μm nitrocellulose membrane (Bio-Rad, Richmond, CA) for storage at 4°C overnight. Immunoblots were probed with anti-human IgG polyclonal Abs conjugated with HRP (1:1000; DakoCytomation, Glostrup, Denmark). BP serum from patient 3 (1:30) was acted as the positive control.

### Competitive-inhibition assay

Competitive-inhibition ELISA was performed in triplicate as described previously (35). Inhibitor solutions (five subpeptides of the hNC16A, full-length hNC16A, and GST protein) were double serially diluted in 0.5% BSA and mixed with equal volumes of the IgG1 mAb 3.B6 (2.5 μg/ml), then incubated at 37°C for 1 h. One hundred microliters of the mixtures was added to the wells coated with hCOL17 NC16A. The remainder of the assay was performed according to standard procedures. OD was measured at 450 nm.

### C1q-binding assay

The C1q-binding activity of the mutated IgG1 mAbs was determined by C1q ELISA-binding assay. The mAbs with double serial dilution (starting concentration: 20 μg/ml) in 0.05 M sodium carbonate buffer (pH 9) were coated in a 96-well plate (Nalge Nunc International, Rochester, NY) and left overnight at 4°C. The plates were washed three times with PBS containing 0.05% Tween 20 and blocked for 1 h at room temperature with

ELISA diluent buffer (BD Biosciences), then incubated for 2 h with 100  $\mu$ l 4  $\mu$ g/ml human complement component C1q (Sigma-Aldrich, St. Louis, MO) in ELISA diluent buffer. Then, 100  $\mu$ l of a 1:400 dilution of sheep polyclonal to C1q (HRP) (Abcam, Tokyo, Japan) was added after washing, and incubation was done for 1 h. Plates were washed five times and displayed in tetramethylbenzidine-soluble reagent (ScyTek, Logan, UT) and then stopped by the addition of 50  $\mu$ l 20% H<sub>2</sub>SO<sub>4</sub> (Wako, Osaka, Japan). The OD was determined at 450 nm. To correct for background, the OD at 450 nm was subtracted from the OD at 620 nm. Binding activity was calculated by the following formula: index value = (OD<sub>test</sub> - OD<sub>blank</sub>) / (OD<sub>St.</sub> - OD<sub>blank</sub>), where St. is standard human IgG1 (BD Biosciences) and blank is the diluent buffer.

#### CDC assay

Serum complements from human (Quidel, San Diego, CA) and mouse (Innovative, Novi, MI) were used for cytotoxicity assay. The mAbs (20–0.04  $\mu$ g/ml) were diluted with dilution buffer (DB) (DMEM [Life Technologies] [pH 7.2], 2 mM glutamine, 0.1% BSA, and 50  $\mu$ g/ml hygromycin). The hCOL17-293 cells were washed in DB and resuspended at a density of 10<sup>6</sup> cells/ml. In a typical assay, 50  $\mu$ l of the mAbs, 50  $\mu$ l diluted complement, and 50  $\mu$ l cell suspension were added to flat-bottom tissue culture 96-well plates. The mixture was incubated for 2 h at 37°C in 5% CO<sub>2</sub> incubator to facilitate cell lysis. Then, 50  $\mu$ l Alamar Blue (Invitrogen) diluted in the DB was added to each well and incubated overnight at 37°C in 5% CO<sub>2</sub> incubator. Fluorescence (Flu) value was monitored at 530-nm excitation wavelength and 630-nm emission wavelength using a 96-well Fluorometer (Berthold, Tokyo, Japan). Reduced Flu units exhibit proportionally to the number of viable cells. The activity of the various mutants was examined by plotting the percent CDC activity against the log of working concentration of the mAb. The Flu value of triplicates was used to calculate the percent cytotoxicity: percent CDC activity = 100  $\times$  (1 - [Flu<sub>no complement</sub> - Flu<sub>test</sub>] / [Flu<sub>no complement</sub> - Flu<sub>total lysis</sub>]), where Flu<sub>total lysis</sub> reading from positive control well was incubated for an additional 15 min with lysis solution (Roche, Mannheim, Germany).

#### ADCC assay

The normal PBMCs were fractionated by Ficoll-Paque PLUS gradient and resuspended in assay reaction buffer (RPMI 1640, 10 mM HEPES, 1% FBS, and 100  $\mu$ g/ml gentamycin). The hCOL17-293 cells were washed and resuspended in the assay reaction buffer. Double-serial-diluted mAb in a 50- $\mu$ l assay reaction buffer was incubated with 50  $\mu$ l target cells (hCOL17-293 cells) (10,000 cells/well) for 30 min at 37°C. Then 50  $\mu$ l effector cells (normal PBMCs) in a cell suspension (100,000 cells/well) was dispensed into the wells, and incubation was continued for 4 h at 37°C. The activity of lactate dehydrogenase was determined by using the Cytotoxicity Detection PLUS (lactate dehydrogenase) Kit (Roche), according to the manufacturer's instructions. The absorbance (450 nm) of triplicates was used to calculate the percent cytotoxicity: percent cytotoxicity = 100 - 100  $\times$  (OD<sub>test</sub> - OD<sub>background</sub>) / (OD<sub>total lysis</sub> - OD<sub>background</sub>), where OD<sub>total lysis</sub> reading from the positive-control well was incubated for an additional 15 min by lysis solution (Roche).

#### Passive-transfer models

The Abs including the nonmutated, mutated, BP and healthy serum IgGs were individually injected into 1-d neonatal COL17-humanized (COL17<sup>m-/-.h+</sup>) mice at a dose of 25–200  $\mu$ g/g body weight as described previously (17). At 48 h after i.p. injection of the IgG Abs, skin blister formation was assessed by gentle skin friction. Back skin was used for histological examination (Genetic-Lab, Sapporo, Japan) and direct immunofluorescence (DIF) test

using FITC-conjugated polyclonal Ab to human IgG (1:100) (DakoCytomation), murine C1q (1:40) (MBL), and murine C3 (1:200) (Abcam).

#### Immunofluorescence

Immunofluorescence analysis using the nonmutated or mutated IgG1 mAbs was performed on adult COL17<sup>m-/-.h+</sup> mouse tail, neonatal COL17<sup>m-/-.h+</sup> mouse skin, and normal human skin as described previously (17). Flu labeling was performed with FITC-conjugated secondary Abs (1:100) (DakoCytomation), followed by 10  $\mu$ g/ml propidium iodide (PI) (Sigma-Aldrich) to counterstain the nuclei. The stained samples were observed and photographed under a confocal laser scanning microscope (Olympus Fluoview FV1000; Olympus, Tokyo, Japan).

#### Ethical considerations

This study was approved by the Institutional Review Board of Hokkaido University (Sapporo, Japan) and fully informed consent from all patients was obtained for use of human material. All animal operations were approved by and performed in accordance with the Institutional Lab Animal Care and Use Committee of Hokkaido University.

#### Statistics

Data values were shown as means and/or percentages. We determined statistical significance using Student *t* test or Pearson  $\chi^2$  test. One-way or two-way ANOVA test was used for comparing the C1q-binding, CDC, and ADCC activities of the nonmutated and mutated mAbs. A *p* value <0.05 or 0.01 was considered statistically significant. Analysis was carried out using the statistical software SPSS 10.0 (SPSS, Chicago, IL).

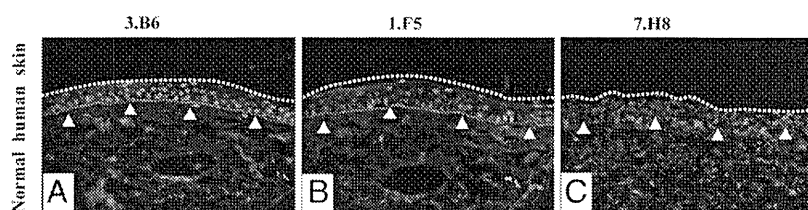
## Results

#### Establishment of immortalized B cell clones derived from BP patients

By means of EBV transformation and limiting dilution (36), three immortalized B cell clones (3.B6, 1.F5, and 7.H8) were established from PBMCs of the seven active BP patients (3.B6 from patient 3, 1.F5 from patient 1, and 7.H8 from patient 7). These three clones stably grew and secreted the IgG mAbs against hCOL17 NC16A. ELISA analysis showed that all the supernatants from the three clones (3.B6, 1.F5, and 7.H8) recognized the hCOL17 NC16A protein (respective index values [mean  $\pm$  SD]: 82.99  $\pm$  4.32, 40.18  $\pm$  2.53, and 30.79  $\pm$  1.61). The IgG subclasses of the three clones are IgG1, IgG1, and IgG4, respectively, as determined by gene sequencing. IIF analysis using normal human skin revealed that the supernatants of both 3.B6 and 1.F5 showed linear IgG deposition at the BMZ (Fig. 1A, 1B). In contrast, the supernatant of clone 7.H8 failed to recognize the BMZ (Fig. 1C). We then generated rhIgG1 mAbs containing the Fab regions of these three immortalized B cell clones.

#### Generation of rhIgG1 mAbs against hCOL17 NC16A

To construct rhIgG1 mAbs, we amplified and cloned the cDNA that encodes IgG L and H chain variable domains (V<sub>K</sub> or  $\lambda$  and Fd) from the immortalized B cell clones by RT-PCR using Ab library general primers (Table I). The 700-bp L/H chain variable genes (VL/VH) of clones 3.B6, 1.F5, and 7.H8 were cloned and ampli-



**FIGURE 1.** Characterization of immortalized B cell clones. IIF analysis was used for detecting the immunoreactivity of the IgG supernatant collected in the first week with normal human skin. Rabbit polyclonal Abs against the H and L chains of human IgG (DakoCytomation) conjugated with FITC were used as secondary Abs (1:100). IgG in the diluted supernatant (1:1) from 2 clones 3.B6 (A) and 1.F5 (B) deposits linearly (green, FITC) at the DEJ (white arrowheads) (skin surface, white dotted line). Negative staining is observed from clone 7.H8 (C). Cell nuclei (red) were counterstained with PI. Original magnification  $\times$ 200 (A–C).

Table I. General RT-PCR primers for cloning variable genes (V) and the mutagenic primers for substituting alanines at C1q binding sites

|                               |  |
|-------------------------------|--|
| Primers for $\kappa$ -chain   |  |
| HK5                           | GAMATYGAGCTCACSCAGTCTCCA                             |
| HK3                           | GCGCCGTCTAGAACTAACACTCTCCCTGTTGAAGCTCTTTGTGACGGGCAAG |
| Primers for $\lambda$ -chain  |  |
| HL5                           | CASYTGAGCTCACKCARCCGCCCTC                            |
| HL3                           | GAGGGATCTAGAATTATGAACATTCTGTAGG                      |
| Primers for Fd                |  |
| H <sub>1,3</sub>              | CAGGTGCAGCTGGTGSAGTCTGG                              |
| H <sub>2</sub>                | CAGGTCAACTTGAAGGAGTCTGG                              |
| H <sub>4</sub>                | CAGGTGCAGCTGCAGGAGTCTGGG                             |
| V <sub>H5</sub>               | CAGGTGCAGCTCGAGSAGTCTGG                              |
| HG3                           | GCATGTACTAGTTTTGTACAAGA                              |
| Single-site mutagenic primers |  |
| E318→A                        | TGGCTGAATGGCAAGGCGTACAAGTGAAGGTC                     |
| K320→A                        | GCTGAATGGCAAGGAGTACCGGTGCAAGGTCTCCAACAAA             |
| K322→A                        | GCAAGGAGTACAAGTGCAGCGGTCTCCAACAAAGCCC                |
| P329→A                        | CAACAAAGCCCTCGCAGCCCCATCGA                           |
| P331→A                        | AGCCCTCCAGCCGCCATCGAGAAAACC                          |
| Multisite mutagenic primers   |  |
| E318A K320A K322A             | GACTGGCTGAATGGCAAGGCGTACCGGTGCGCGGTCTCCAACAAAGCCCTC  |
| P329A P331A                   | CAACAAAGCCCTCGCAGCCGCCATCGAGAAAACC                   |

General primers for V<sub>L</sub> and Fd chains (M = A, C, Y = C, T; S = G, C; K = G, T; R = A, G). Mutagenic primers at C1q binding sites (only showing the sense primer; the underlined codons are substituted into alanines).

fied by the V <sub>$\lambda$</sub> /VH<sub>1,3</sub>, V <sub>$\kappa$</sub> /VH<sub>2</sub>, and V <sub>$\kappa$</sub> /VH<sub>1,3</sub> subfamily primers, respectively (Table II). Details of the variable gene sequence from the clones are summarized in Supplemental Table II.

Both the VL and VH genes were successively cloned into XhoI/SpeI and SacI/EcoRV sites of the pAc- $\kappa$ -Fc or pAc- $\lambda$ -Fc baculovirus IgG1 expression vectors (Supplemental Fig. 1) (37). Recombinant baculovirus was produced by transfecting the recombinant vectors that contained VL/VH genes into Sf-9 insect cells. Postinfection with the purified recombinant baculovirus, the Sf-9 cells secreted rhIgG1 mAbs in the culture supernatant. ELISA analysis showed that the rIgG1 mAb from the clone 3.B6 recognized hCOL17 NC16A (index value [mean  $\pm$  SD]: 161  $\pm$  5.37) but not the unrelated control protein, human type VII collagen (index value: 2.2  $\pm$  0.04). The human COL17 NC16A ELISA index values of mAbs derived from clones 1.F5 and 7.H8 were 4.23  $\pm$  0.02 and 1.74  $\pm$  0.01, respectively, which are significantly lower than that of clone 3.B6 ( $p < 0.01$ ). This result indicates that the reactivity and/or affinity of IgG Abs secreted from insect cells was altered from those produced by human B cells, possibly as a result of differences in three-dimensional structure, to glycosylation or to other factors, even if the Abs had the same gene sequences as the variable regions (38, 39). For example, N-deglycosylation was even observed to affect the m.w. of the H or L chains of Ch-K20-sf9 (37). IIF analysis demonstrated that the rIgG1 mAb 3.B6 reacted with the BMZ of normal human (Fig. 2A) and COL17-humanized (COL17<sup>m-/+</sup>, h<sup>+</sup>) mouse skin (Fig. 2B) but not with the wild-type mouse skin (Fig. 2C).

The amount of the mAb 3.B6 in the supernatant of the infected Sf-9 cells ranged from 5 to 16  $\mu$ g/ml, values that were determined by sandwich ELISA using sheep anti-human Fab Abs. Kinetic analysis using the Biacore system demonstrated that the mAb 3.

B6 had high affinity for  $K_D = 5.3 \times 10^{-9}$  M ( $K_d = 1.9 \times 10^{-4}$  1/s;  $K_a = 3.6 \times 10^4$  1/Ms;  $K_D = K_d/K_a$ ). The purified mAb 3.B6 stained the cell surface of the normal human epidermal keratinocytes (Fig. 2D) and 293 cells that had been transfected with the hCOL17 gene (Fig. 2E), suggesting that this mAb recognizes the extracellular domain of hCOL17.

Epitope mapping by Western blot analysis using the rNC16A subpeptides revealed that the mAb 3.B6 specifically reacted with the full-length hNC16A, hNC16A 1–3, hNC16A 2, and hNC16A 2.5 (Fig. 3A, 3B). Binding inhibition assays demonstrated that two peptides (hNC16A 2 and hNC16A 2.5) sharing seven amino acids inhibited the binding of mAb 3.B6 to the hNC16A in a dose-dependent manner (Fig. 3C). Thus, the mAb 3.B6 specifically recognized an epitope present at subregion 2 of the hNC16A domain, which was also recognized by the serum derived from the same donor (patient 3) (Fig. 3B).

#### Generation of rhIgG1 mAbs with mutations at C1q binding sites and characterization of C1q-binding activity

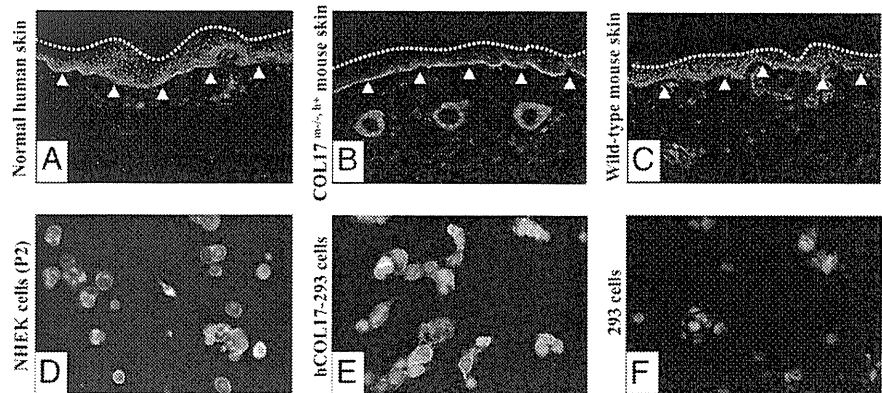
After the binding activity of the IgG1 mAb 3.B6 was verified, site-directed mutagenesis was introduced at the C1q binding sites in the C<sub>H</sub>2 domain of the rhIgG1 Fc region. Five independent residues (E318, K320, K322, P329, and P331) were substituted with alanine by single-site or multisite (E318 K320 K322 or P329 P331) mutation in the C<sub>H</sub>2 domain. Both IIF and Western blot analysis demonstrated that all the mutated mAbs reacted to the hCOL17 NC16A as specifically as the nonmutated mAb did (data not shown). Kinetic analysis also demonstrated that the mutated mAbs had similar affinity with the nonmutated IgG1 mAb 3.B6 (data not shown).

In contrast, all the mutated mAbs demonstrated significantly reduced binding ability to C1q compared with that of the nonmutated mAb ( $p < 0.01$ ) (Fig. 4). The binding abilities of E318A and K320A were slightly lower than that of the nonmutated mAb. No significant difference was observed between the two mutated mAbs ( $p > 0.05$ ). The K322A, E318A K320A K322A, and P329A mAbs showed half the binding ability of the nonmutated mAb. There were no significant differences among these three mAbs ( $p > 0.05$ ). Alanine substitution at position P331 or P329 P331 seemed to demonstrate the lowest C1q-binding capacity of

Table II. Usage of Ab general primers for cloning variable genes from the B-cell clones

| B Cell Clone | L Chain                           | VL Subfamily | H Chain           | VH Subfamily |
|--------------|-----------------------------------|--------------|-------------------|--------------|
| 3.B6         | V <sub><math>\lambda</math></sub> | VL1-JL3b     | VH <sub>1,3</sub> | VH1-D4-JH6   |
| 1.F5         | V <sub><math>\kappa</math></sub>  | VK1-JK3      | VH <sub>2</sub>   | VH2-D6-JH4   |
| 7.H8         | V <sub><math>\kappa</math></sub>  | VK1-JK2      | VH <sub>1,3</sub> | VH3-D5-JH6   |

**FIGURE 2.** Characterization of rhIgG1 mAb 3.B6. Analysis on the specificity of mAb by IIF assay shows that the mAb (green, FITC) deposits linearly at the DEJ (white arrowheads) (skin surface, white dotted line) of normal human (A) and COL17-humanized (*COL17<sup>m-h/+</sup>*) mouse skin (B), but does not deposit in the wild-type mouse skin (C). At the cellular level, the mAb binds to the plasma membrane (green) of second-passage normal human epidermal keratinocytes (NHEKs, P2) (D) and hCOL17-293 cells (E), but does not bind to 293 cells (negative control) (F). Red shows nuclear staining by PI. Original magnification  $\times 100$  (A–C);  $\times 400$  (E, F).



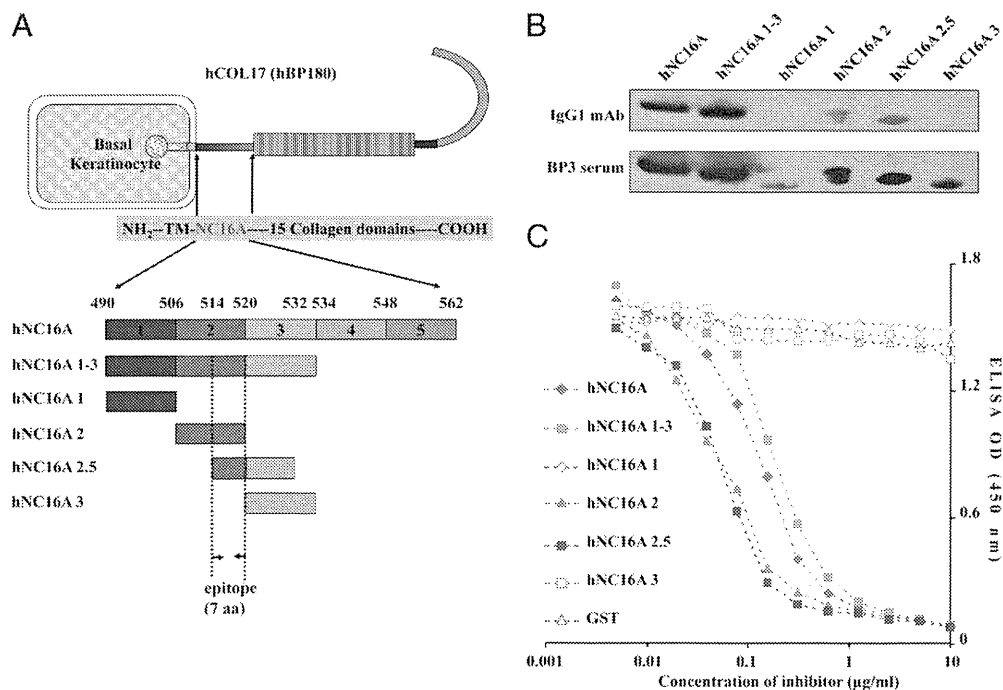
any of the mutated mAbs. These data indicate that P331 is the most important residue for IgG1 Fc–C1q interaction, followed by the P329, K322, K320, and E318 binding sites in the C1q-binding motif of the Fc C<sub>H</sub>2 domain.

*In vitro characterization of the CDC and ADCC activities of the mutated IgG1 mAbs*

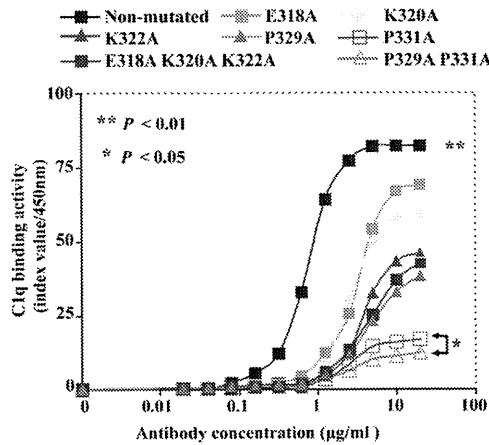
To test the ability of the mutated IgG1 mAbs to activate complement cascade in vitro, CDC assay was carried out using the hCOL17-293 cells as target cells. At 1:20 dilution of human serum complement (HSC), the nonmutated mAb showed the highest CDC activity (Fig. 5A). The CDC activity of the two mutated mAbs (E318A and K320A) was nearly half that of the nonmutated mAb, followed in decreasing order by K322A, E318A K320A K322A, and P329A. Their CDC activity was about one-third that of the nonmutated mAb. The mutants P331A and P329A P331A showed

very low CDC activity. When the HSC concentration was increased, the percentage of CDC activity increased in the mutants E318A, K320A, K322A, P329A, and E318A K320A K322A (Fig. 5B, 5C). In contrast, no obvious change was observed in the CDC activity for P331A or P329A P331A, even at the highest HSC concentration (Fig. 5C). Similar results were shown when mouse serum complement was used instead of HSC (Fig. 5D–F). Taken together, these results indicate that all five residues of the IgG1 C<sub>H</sub>2 domain play at least moderate roles and that P331 is the most important residue for activating complements in vitro.

To further investigate whether these mutations affect the Fc–FcγR–mediated ADCC activity of the IgG1 mAbs, we analyzed the mutated mAbs for ADCC activity using the hCOL17-293 cells as target cells and normal PBMCs as effector cells. The mutated mAbs (E318A, K320A, K322A, and E318A K320A K322A) exhibited ADCC activity similar to that of the nonmutated mAb



**FIGURE 3.** Epitope mapping of the IgG1 mAb 3.B6. A schematic map of hCOL17 (hBP180) showing the location of the hNC16A domain and the subpeptides synthesized for epitope mapping studies (A). Western blot analysis reveals that the IgG1 mAb recognizes the following hNC16A subpeptides: hNC16A full-length (aa 490–562), hNC16A 1–3 (aa 490–534), hNC16A 2 (aa 506–520), and hNC16A 2.5 (aa 514–532) (B). Two subpeptides (hNC16A 1 and 3) other than the abovementioned four are also recognized by the BP serum from the same donor (patient 3). In ELISA inhibition assay (C), the IgG1 mAb 3.B6 (2.5  $\mu\text{g/ml}$ ) was incubated with the five subpeptides as inhibitor (10–0.005  $\mu\text{g/ml}$ ), with GST protein acting as negative control and hNC16A acting as positive control. The index value of the binding of mAb 3.B6 to the full-length hNC16A was detected by ELISA at OD 450 nm. The three subpeptides (hNC16A 1–3, hNC16A 2, and hNC16A 2.5) almost completely inhibited the binding activity of the IgG1 mAb 3.B6 in a dose-dependent manner. No significant difference in mean inhibitory activity was observed among the three subpeptides ( $p > 0.05$  by two-way ANOVA test).



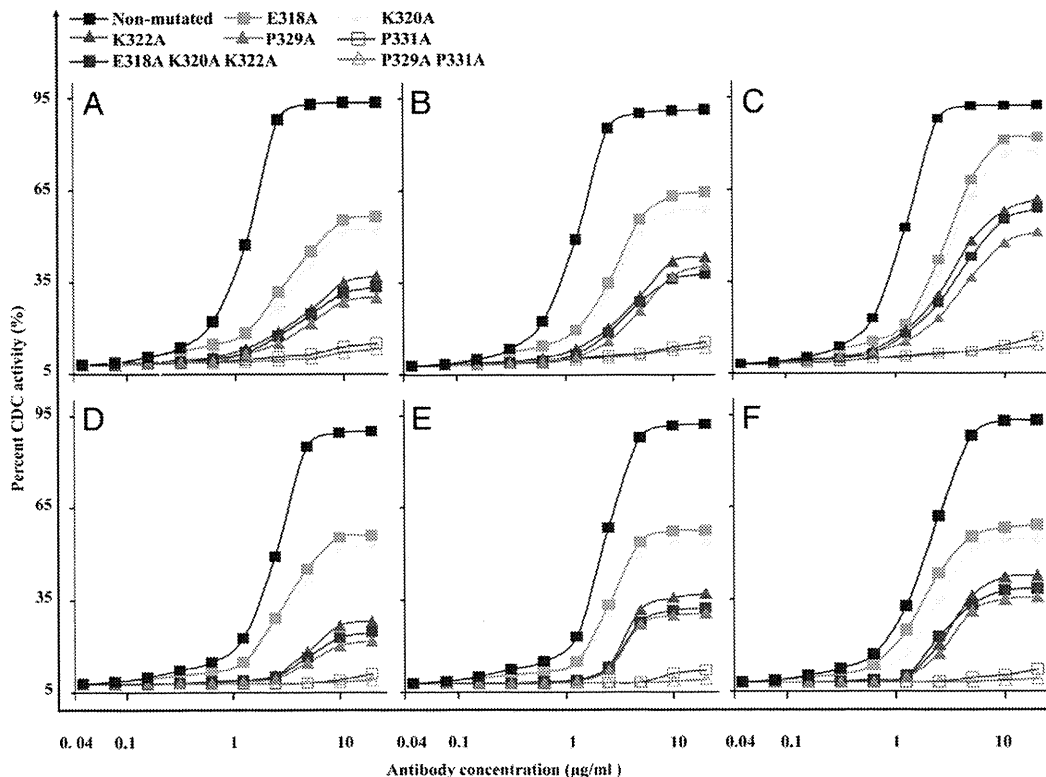
**FIGURE 4.** C1q-binding activity of the mutated IgG1 mAbs. The ELISA Index value of C1q binding increases gradually with increases in the IgG1 Ab. Maximal binding activity in descending order is nonmutated, then E318A, K320A, K322A, E318A K320A K322A, P329A, P331A and P329A P331A mutated mAbs. Mean index value is higher for the non-mutated mAb than for any other mutated mAbs ( $p < 0.01$  by two-way ANOVA test). There is no significant difference in mean index value for E318A versus K320A or for K322 versus E318A K320A K322A or P329A ( $p > 0.05$  by one-way ANOVA test). Significant difference is seen between P331A and P329A P331A ( $p < 0.05$  by one-way ANOVA test).

(Fig. 6). In contrast, the substitutions at P329 or P329 P331 almost completely eliminated the ADCC activity of the mAbs ( $p < 0.01$ ). Alanine substitution at the P331 site partially decreased the ADCC

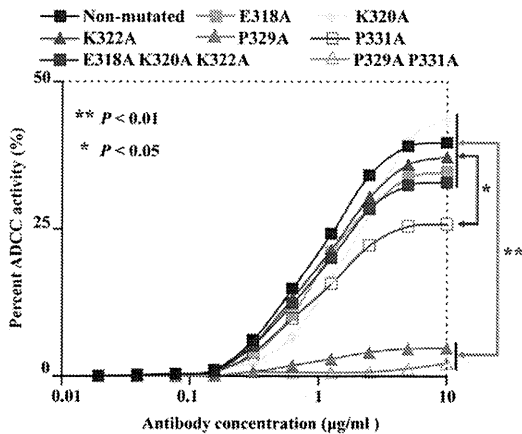
activity ( $p < 0.05$ ). Altogether, these results suggest that P329 is a critical residue for IgG1-FcγR interaction in vitro.

*In vivo functional characterization of the nonmutated and mutated IgG1 mAbs using a COL17-humanized (COL17<sup>m-/-</sup>, h<sup>+</sup>) BP mouse model*

To investigate the pathogenic activity of the nonmutated and mutated IgG1 mAbs in vivo, we performed a passive-transfer experiment using neonatal COL17-humanized (COL17<sup>m-/-</sup>, h<sup>+</sup>) mice that we had established recently (17). Forty-eight hours after i.p. injection of the nonmutated IgG1 mAb (200 µg/g body weight), eight of nine neonatal mice became erythematous and showed BP-like skin blistering by gentle skin friction (Fig. 7A, Table III). Histopathologic examination of the recipient mouse skin confirmed the formation of subepidermal blistering (Fig. 7B), infiltration of lymphocytes and neutrophils (Fig. 7C), and degranulation of mast cells (Fig. 7D). DIF examination revealed the linear deposition of human IgG (Fig. 8A) at the BMZ, as well as of mouse C1q (Fig. 8B) and C3 (Fig. 8C). Administration of the lower dose of the nonmutated IgG1 mAb (100, 50, or 25 µg/g body weight) resulted in a lower frequency of phenotypic changes (five of seven, four of seven, or zero of five mice, respectively) (Table III). ELISA analysis of the sera of recipient mice revealed that the mean index value of the circulating anti-hCOL17 NC16A IgG1 mAbs declined from 55.39 (200 µg/g body weight) to 43.74, 36.91, and 26.53 (100, 50, and 25 µg/g body weight, respectively). Similar results were observed in positive control models with BP patients' IgG autoantibodies (data not shown) in which the BP phenotype was induced as described previously (17).



**FIGURE 5.** Characterization of in vitro CDC activity for the mutated IgG1 mAbs. The nonmutated mAb has the highest percent CDC activity ( $p < 0.01$  by one-way ANOVA test) at HSC dilution of 1:20 (A), 1:10 (B), and 1:5 (C), as well as at murine serum complement dilutions of 1:20 (D), 1:10 (E), and 1:5 (F). E318A and K320A have significantly higher CDC activity than K322A, P329A, and E318A K320A K322A ( $p < 0.01$  by two-way ANOVA test). No significant difference in the mean percent activity is seen for E318A versus K320A or for K322A versus P329A or versus E318A K320A K322A ( $p > 0.05$  by one-way ANOVA test). P331A and P329A P331A show no CDC activity. Significant differences are seen for the two mAbs versus the other mAbs ( $p < 0.01$  by two-way ANOVA test).



**FIGURE 6.** Characterization of in vitro ADCC activity for the mutated IgG1 mAbs. In IgG1 mAb-dependent cell-mediated cytotoxicity (ADCC) assay, normal human PBMCs were applied as the effector cells, and hCOL17-293 cells were the target cells. No significant differences in ADCC percent activity are shown for the E318A, K320A, K322A, and E318A K320A K322A versus the nonmutated mAbs ( $p > 0.01$  by one-way ANOVA test). ADCC activity is higher for these four mAbs than for P331A ( $p < 0.05$  by two-way ANOVA test). The mAbs P329A and P329A P331A show almost no ADCC activity, and significance differences are observed versus the others and nonmutated mAb ( $p < 0.01$  by two-way ANOVA test).

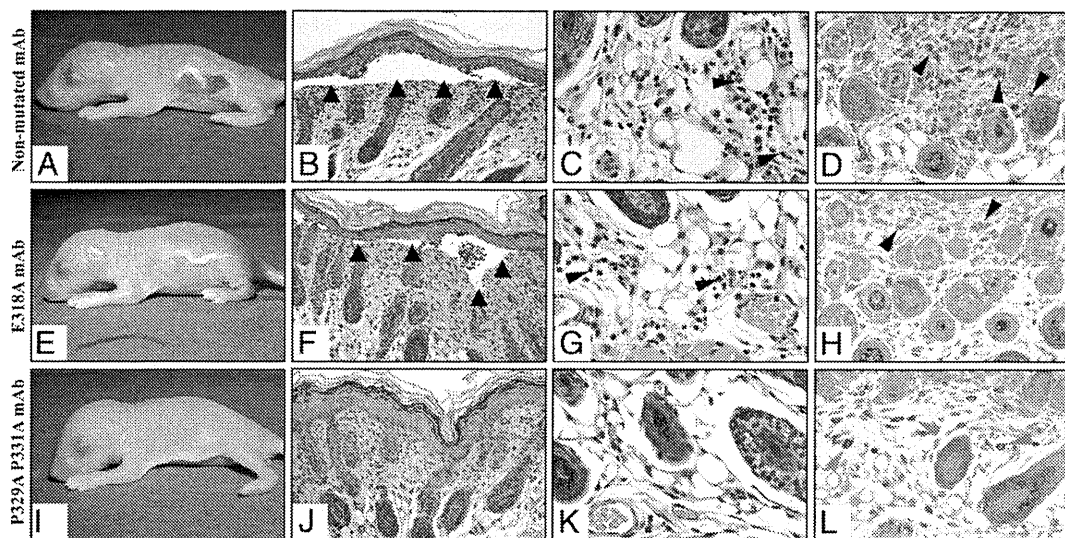
In contrast, IgG1 mAbs with the double-site mutation (P329A P331A) completely failed to induce the BP phenotype (zero of eight mice) at the highest dose (200  $\mu\text{g/g}$  body weight), whereas IgG1 mAbs with single-site mutation (P329A or P331A) showed significantly reduced pathogenic capability (one of seven or one of eight mice, respectively) compared with nonmutated IgG1 mAb ( $p < 0.01$ ) (Figs. 7I–L, 8G–I, Table III). The other three IgG1 mAbs with a single-site mutation (E318A, K320A, or K322A) that showed high or moderate complement activity in vitro elicited skin detachment in the majority of mice at the highest dose (200

$\mu\text{g/g}$  body weight) and demonstrated pathogenic activities in a dose-dependent manner as was true for the non-mutated IgG1 mAb (Figs. 7E–H, 8D–F). There were no significant differences in positive ratio of skin detachment between these three mAbs and the nonmutated mAb ( $p > 0.05$ ) (Table III). No significant differences were observed in the hCOL17 NC16A-ELISA index values (mean values) of the circulating human IgG Abs in the recipient sera among the mutated or nonmutated IgG1 mAb models at the dose of 200  $\mu\text{g/g}$  body weight ( $p > 0.01$ ).

## Discussion

This study provides, to our knowledge, the first direct evidence that human IgG1 Abs against hCOL17 NC16A can induce subepidermal blisters in vivo. BP phenotypic features, including dermal-epidermal separation, deposition of IgG1 Abs C1q and C3, recruitment of neutrophils, and degranulation of mast cells, were successfully reproduced in COL17-humanized (*COL17<sup>m-/-</sup>. h<sup>+</sup>*) mice by the administration of human IgG1 mAbs against human COL17 NC16A, although the components of the immune system, such as complements and inflammatory cells, are still of murine origin in this experimental model.

It is well known that various IgG subclasses have distinct functional properties. IgG1 and IgG3 are the most effective complement activators, whereas IgG4 is not capable of fixing complements (40). Previous clinical studies demonstrated that IgG1 and IgG4 autoantibodies are major IgG subclasses of BP patient autoantibodies and that IgG2 and IgG3 are minor subclasses of BP patient autoantibodies (5, 7, 28). In vitro analysis using cryosections of human skin and leukocytes from healthy volunteers demonstrated that IgG1 autoantibodies purified from BP sera showed much stronger pathogenic activity than that of IgG4 autoantibodies (41). However, there has been no direct evidence of pathogenic activity of IgG1 autoantibodies in a BP model in vivo. Our group and others have successively reproduced BP phenotypes in COL17-humanized mice by administering IgG autoantibodies prepared from BP patients (15, 17). However, these autoantibodies were poly-



**FIGURE 7.** Pathogenic activity in vivo of the nonmutated and mutated IgG1 mAbs. The neonatal COL17-humanized (*COL17<sup>m-/-</sup>. h<sup>+</sup>*) mice were i.p. injected with the nonmutated IgG1 mAb (200  $\mu\text{g/g}$  body weight) or the mutated IgG1 mAbs E318A and P329A P331A (200  $\mu\text{g/g}$  body weight). Forty-eight h later, pathogenic activity was determined by skin blistering test and histology biopsy of back skin including H&E and toluidine blue staining. Skin blistering is positive for the nonmutated and E318A mAbs models (A, E) and negative for the P329A P331A model (I). H&E staining shows epidermal detachment (black arrowheads) at the DEJ in the nonmutated (B) and E318A (F) mAbs models but not in the P329A P331A model (J). The numbers of neutrophils (black arrowheads) and degranulated mast cells (black arrowheads) infiltrating the dermis are more greatly reduced in the P329A P331A (K, L) model than in the nonmutated (C, D) and E318A (G, H) models. Red arrowheads indicate normal undegranulated mast cells. Original magnification  $\times 100$  (B, F, J);  $\times 200$  (D, H, L);  $\times 400$  (C, G, K).



Table III. Pathogenic activity of the non-mutated and mutated IgG1 mAbs against hCOL17 NC16A in the COL17-humanized (COL17<sup>m-/-</sup>, h<sup>+</sup>) BP mouse model

| Abs                       | Mice with Skin Detachment <sup>a</sup> , for Each Injection Dosage (μg/g Body Weight) |        |       |       |
|---------------------------|---|--------|-------|-------|
|                           | 200 μg  | 100 μg | 50 μg | 25 μg |
| Nonmutated                | 8/9   | 5/7    | 4/7   | 0/5   |
| Mutated                   |   |        |       |       |
| E318A                     | 4/6   | 2/5    | 0/5   | 0/4   |
| K322A                     | 5/8   | 2/6    | 1/6   | 1/5   |
| E318A K320A K322A         | 3/7   | 2/6    | 0/5   | 0/5   |
| P329A                     | 1/7   | 1/5    | 0/4   | 0/5   |
| P331A                     | 1/8   | 0/7    | 0/6   | 0/5   |
| P329A P331A               | 0/8   | 0/5    | 0/4   | 0/6   |
| Normal human <sup>b</sup> | 0/6   | —      | —     | —     |

<sup>a</sup>Skin blistering test performed at 48 h in neonatal mice.

<sup>b</sup>Human IgG purified from serum in single normal volunteer.

clonal IgG containing all four IgG subclasses. In this study, we have directly demonstrated that anti-hCOL17 NC16A IgG1 mAb has pathogenic activity in vivo by using COL17-humanized (COL17<sup>m-/-</sup>, h<sup>+</sup>) BP model mice. As previous clinical studies on BP (5, 7, 28), our results further support the notion that IgG1 autoantibodies against hCOL17 are potent and possibly the main pathogenic IgG autoantibodies in BP.

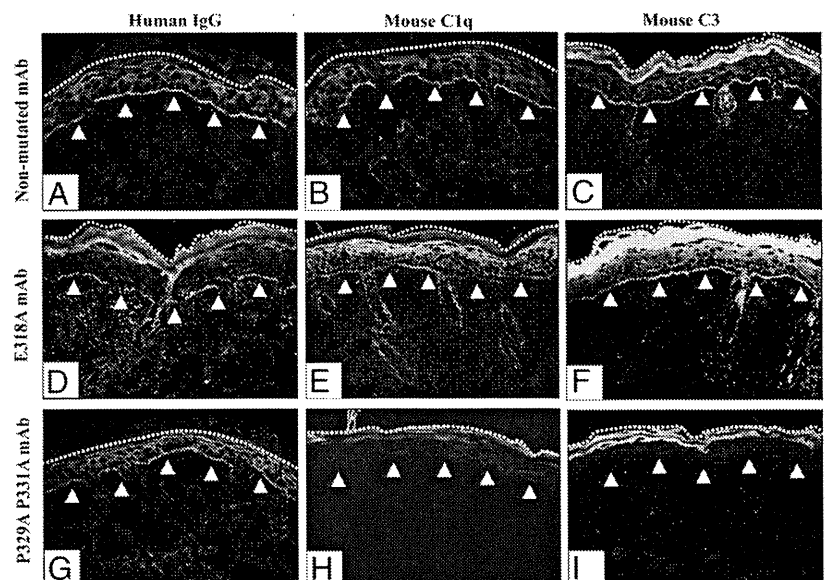
Previous studies have demonstrated that activation of the complement pathway is a pivotal step in the BP pathomechanism (17, 18, 42). By comparing nonmutated and/or mutated IgG1 mAbs in terms of pathogenic activity, we have precisely analyzed the roles of C1q-binding residues of the IgG1 Fc region (E318, K320, K322, P329, and P331) in initiating complement activation both in vitro and in vivo. These results are consistent with previous mutagenesis studies that showed that these binding residues are required for complement activation in vitro (22, 25, 27). The alanine substitution at these five single-residue sites variously decreased the CDC activity of the IgG1 mAb against hCOL17 NC16A. The IgG1 mAb that was mutated at P331 showed low CDC activity in vitro, and it failed to produce a BP phenotype in the COL17-humanized (COL17<sup>m-/-</sup>, h<sup>+</sup>) mice. In contrast, the IgG1 mAbs mutated at K322 or at E318 K320 K322 showed moderate CDC activity in vitro and high (K322) to moderate (E318 K320 K322) pathogenic activity in vivo. The other mutated

mAbs (E318A and K320A), which showed high CDC activity in vitro, showed high pathogenic activity in vivo. The results of in vivo pathogenic activity and in vitro CDC and ADCC activities are summarized in Supplemental Table III. The findings suggest that the moderate CDC activity of mutated mAbs is sufficient to induce skin detachment in neonatal COL17-humanized mice and that the P331 residue is the key residue for complement activation and is required for the pathogenic activity of IgG1 mAb in vivo. Thus, complement activation ability seems to correlate with the pathogenic effects of the mutated IgG1 mAbs in COL17-humanized mice (Fig. 5, Table III). Taken together, these findings further suggest that IgG1 Fc-dependent complement activation plays a major pathogenic role in subepidermal blister formation in BP.

In the classical complement pathway, C1q binding to IgG1 triggers proteolytic cascades, which result in the generation of a large amount of C3b. This explains why the immunofluorescence intensity of mouse C1q was weaker than that of mouse C3 in DIF examinations of mouse models, as shown in Fig. 8. In addition to C3b being capable of generating membrane attack complexes as the opsonin, it induces various inflammatory reactions by binding to the complement receptors expressed on the effector cells, such as granulocytes, monocytes, neutrophils, or mast cells (43–45). In our BP mouse model, inflammatory cells, such as mast cells, neutrophils, and lymphocytes, are observed at the dermis. It has been demonstrated that C3 binding to complement receptors stimulates activation and chemotaxis of neutrophils and/or mast cells (46). Mast cells can produce various mediators, such as leukotrienes, platelet-activating factor, and cytokines, that contribute directly or indirectly to neutrophil recruitment (47, 48). The neutrophils recruited into skin then release elastase and gelatinase B, damaging the BMZ (49, 50). These data suggest that subepidermal blistering is mediated by a complement-dependent cellular (mast-cell and neutrophil) cytotoxicity pathway.

In addition to IgG1 Abs activating complement cascades, they may directly recruit and activate effector cells via the interaction between the Fc region and reciprocal FcγRs. In this study, we analyzed the Fc-FcγR-mediated ADCC activity of IgG1 mAbs. That activity was fully defective in the IgG1 mAb that was mutated at P329, and the mutation at P331 moderately reduced the activity of the IgG1 mAb (Fig. 6). In contrast, the other mutated mAbs (E318A, K320A, K322A, and E318A K320A K322A)

**FIGURE 8.** Capability of activating complement in vivo for the non-mutated and mutated IgG1 mAbs. In these three models (Fig. 7), 48 h after injection of the IgG1 mAbs, the complement activation capability was determined by DIF of human IgG and mouse complement components C1q and C3. All three of the mAbs (nonmutated IgG1, mutated E318A, and mutated P329A P331A) deposit at the DEJ (white arrowheads) (skin surface, white dotted line) of mouse skin (A, D, G). Mouse complement components C1q and C3 (green, FITC) are activated by the nonmutated (B, C) and E318A mAbs (E, F) but not by P329A P331A (H, I) at the DEJ (white arrowheads). Original magnification  $\times 200$  (A–I).



showed similar ADCC activity to that of the nonmutated IgG1 mAb. These results indicate that P329 is a key residue in IgG1-dependent ADCC reaction and that P331 plays a minor role in that process. A site-directed mutagenesis study also demonstrated that alanine substitution at the P329 residue causes the dual deficiencies of ADCC and CDC activity in vitro (25). If we focus on the pathogenic activity of IgG1 mAbs in BP, our mutagenesis studies suggest that Fc-Fc $\gamma$ R-mediated ADCC activity may have contributed to the blister formation. In this study, in vitro CDC analysis showed that P329A and K322A had similarly moderate CDC activity (Fig. 5) but mutually distinct ADCC activity (Fig. 6). K322A showed normal ADCC activity, and P329A was incapable of triggering the ADCC reaction. Our in vivo results showed almost no skin detachment for the P329A-mutated mAb (one of seven mice) but some skin detachment for the K322A mAb (five of eight mice) (Table III). These results suggest that the ADCC (Fc $\gamma$ R-dependent immune reaction) plays a minor but augmenting role in IgG1-dependent blister formation. This concept is further supported by a previous report by Mihai et al. (41) in which the pathogenicity of BP IgG1 and IgG4 autoantibodies was amplified by the additional recruitment of neutrophils and mast cells via Fc-Fc $\gamma$ R interaction.

Recently, our group has used a phage display technique to develop a monoclonal Fab Ab against hCOL17 NC16A from BP patients (51). The monoclonal Fab Ab lacked pathogenic activity when administered to neonatal COL17-humanized (COL17<sup>m-/-</sup>, h<sup>+</sup>) mice. Furthermore, the monoclonal Fab Ab competitively protected the COL17-humanized (COL17<sup>m-/-</sup>, h<sup>+</sup>) mice from the blister formation that would normally be induced by IgG autoantibodies prepared from BP patients. These results are consistent with our current study, and they further suggest the crucial role of the IgG1 Fc region in BP pathomechanism. In addition, complete IgG1 Ab has a longer half-life than Fab Ab in vivo (52, 53). Therefore, our current study raises the possibility that complete IgG1 mAbs with mutation at the C1q binding sites of the Fc region would protect the pathogenic epitopes from attack by the autoantibodies not only in BP but also in other autoimmune diseases in which complement activation plays an essential role.

In conclusion, to our knowledge, this study is the first in vivo evidence that IgG1 Abs targeting hCOL17 NC16A alone can trigger blister formation, and it suggests that IgG1-dependent complement activation via IgG1 Fc-C1q interaction plays a crucial role in BP.

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## Disclosures

The authors have no financial conflicts of interest.

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## Correspondence

### Two cases of cutaneous sporotrichosis in continental/microthermal climate zone: global warming alert?

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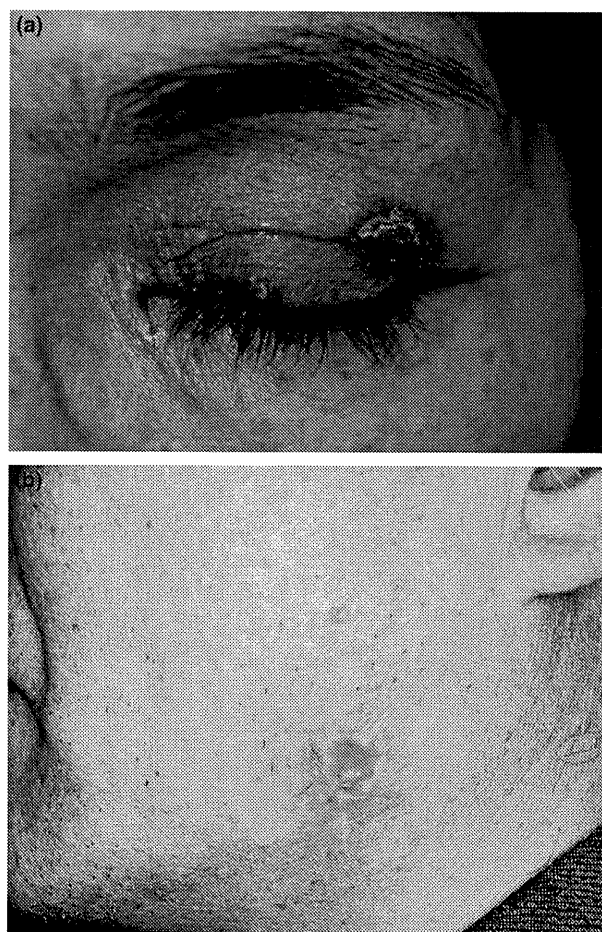
Sporotrichosis is commonly encountered in tropical and subtropical areas, but rarely in continental/microthermal climate zones as defined by the Köppen–Geiger Climate Classification.<sup>1</sup> We report two cases of cutaneous sporotrichosis in Hokkaido, an island in the continental/microthermal climate zone in Japan.

Patient 1 was a 55-year-old Japanese woman, who presented in 2009 with a 6-month history of two dark-red, crusted, infiltrated skin lesions measuring about 10 mm and 2 mm, respectively, on the left upper eyelid (Fig. 1a). She was working as a farmer in Hokkaido and had never lived in any other part of Japan. Histopathological examination of haematoxylin and eosin-stained specimens showed a prominent epidermal hyperplasia and abundant inflammatory infiltration in the dermis. Periodic-acid-Schiff (PAS) and Grocott's methenamine silver (GMS) stains revealed a few round and budding yeast-like cells scattered in the dermis, occasionally within a giant cell. Cultures of the tissue sections of lesion on Sabouraud dextrose agar and potato dextrose agar grew dark-brown velvety colonies. Slide cultures from the colonies contained septate branching hyphae, with slender, tapering conidiophores arising at right angles. A sporotrichin skin test gave a positive reaction.

Patient 2 was a 55-year-old man, who presented in 2002 with a chronic erosive nodule measuring 20 × 10 mm on the left mandible, which had been present for over 1 year on 2002 (Fig. 1b). He was working as a carpenter in Hokkaido, and had never lived in any other part of the country. Histopathological analysis found prominent epidermal hyperplasia and a chronic granulomatous inflammatory cell infiltrate. PAS and GMS stained round yeast-like cells scattered throughout the dermis. Cultures of the biopsied tissue samples grown on Sabouraud dextrose agar produced dark-brown velvety colonies. Slide cultures from the colonies contained septate, branching hyphae. Sporotrichin skin test gave a positive reaction.

Cutaneous sporotrichosis is a fungal infection commonly encountered in tropical and subtropical areas.<sup>1</sup> In Japan, > 3500 cases of cutaneous sporotrichosis were reported as

of 2001 on Honshu Island, which falls in the temperate/mesothermal climate zone.<sup>2</sup> By contrast, no case reports were reported from Hokkaido in English journals before 2004. Similarly, few cases have been reported in other continental/microthermal climate zone around the world,<sup>3</sup> suggesting that cutaneous sporotrichosis is extremely rare in that zone. This geographical difference in reported cases may be due to the fact that *Sporothrix schenckii*, the pathogenic fungus that causes sporotrichosis, prefers moderate temperatures (around 22 °C).<sup>1</sup> The yearly temperature in Hokkaido



**Figure 1** (a) Patient 1. Nodule and papule on the left upper eyelid. (b) Patient 2. Nodule on the left mandible.

averaged for the years 2000–2008 was 9.1 °C, so these results further suggest that *S. schenckii* rarely occurs in Hokkaido. Interestingly, three cases of cutaneous sporotrichosis, including our two, have been reported from Hokkaido in the Japanese literature since 2000, whereas only one case was recorded before 2000. We suggest that the prevalence of cutaneous sporotrichosis in Hokkaido may be increasing as a result of recent global warming.

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### The Fas/Fas ligand system, rather than granzyme B, may represent the main mediator of epidermal apoptosis in dermatomyositis

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We read with interest the paper by Grassi *et al.*<sup>1</sup> reporting an immunohistochemical study on cutaneous lesions of

lupus erythematosus (CLE) and dermatomyositis (DM). A particularly interesting finding was that the expression of granzyme B (GrB), a pro-apoptotic serine-protease, was higher in CLE than in DM lesions. As GrB is mainly expressed by CD8+ lymphocytes, we were puzzled to read that 'in DM, the CD8+ subpopulation represented > 50% of the lymphocytic population'.<sup>1</sup> This finding is in striking contrast to a study in which our research group found that the CD4/CD8 ratio is approximately 2.5 in DM skin lesions.<sup>2</sup> On the other hand, the number of infiltrating CD8+ cells was reported to be similar to that of the CD4+ cells in CLE in both papers.<sup>1,2</sup> As previous studies also reported a similar level of apoptotic phenomena in lesional epidermis in both CLE and DM,<sup>3,4</sup> it is evident that GrB is not likely to represent a major mechanism of cell death in DM lesions.

We report the results from an ongoing study on skin lesions of six patients with DM, five patients with subacute CLE (SCLE) and five patients with discoid CLE (DLE). The sun-protected, clinically healthy skin of the patients served as internal controls, and five healthy donors comprised the control group. Immunohistochemical examination, performed as described previously,<sup>2</sup> included antibodies to Bax, a pro-apoptotic molecule involved in the mitochondrial pathway of apoptosis and activated by GrB; Bcl-2, its antiapoptotic counterpart also belonging to the mitochondrial pathway; Fas and Fas ligand (Fas-L), two key molecules triggering the death receptor pathway of apoptosis; and caspase 3, the final effector of both pathways. The following monoclonal antibodies were used. Bax: 1 : 500 dilution, clone YTH-2D2 (R&D Systems, MN, USA); Bcl-2: 1 : 1000 dilution, clone 124 (Dako, Copenhagen, Denmark); Fas: 1 : 60 dilution, clone GM30 (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK); FasL: 1 : 50 dilution, clone 5D1 (Novocastra); caspase 3: 1 : 50 dilution, clone 5A1 (Cell Signaling, Danvers, MA, USA).

There was higher expression of Bax than of Bcl-2 in patient epidermis (Table 1). Fas was strongly expressed in epidermal sections from patients with DM (Fig. 1a) and CLE, and there was focal positivity in the healthy skin of these patients. Caspase 3 was expressed strongly in all lesional specimens and moderately in the healthy skin of patients.

**Table 1** Semiquantitative evaluation of apoptotic molecules in lesional sections and controls.

|           | DM  | DM-HS | SCLE | SCLE-HS | DLE | DLE-HS | HC |
|-----------|-----|-------|------|---------|-----|--------|----|
| Bax       | ++  | ++    | +++  | ++      | +++ | +      | +  |
| Bcl-2     | +   | +     | +    | +       | ++  | +      | +  |
| Fas       | +++ | +     | +++  | +       | +++ | +      | –  |
| FasL      | ++  | +     | +++  | +       | ++  | +      | –  |
| Caspase 3 | +++ | ++    | +++  | ++      | +++ | ++     | +  |

DM, lesional epidermis of dermatomyositis; DM-HS, epidermis of sunprotected healthy skin from patients with DM; SCLE, lesional epidermis of subacute cutaneous lupus erythematosus; SCLE-HS, epidermis of sunprotected healthy skin from patients with SCLE; DLE, lesional epidermis of discoid cutaneous lupus erythematosus; DLE-HS, epidermis of sunprotected healthy skin from patients with DLE; HC, epidermis of sunprotected healthy skin from healthy controls. Semiquantitative evaluation of epidermal positivity for Bax, Bcl-2, Fas and caspase 3: –, negative; +, focal; ++, continuous basal; +++, basal and suprabasal. Semiquantitative evaluation of dermal positivity for FasL: –, negative; +, 0–5 cells; ++, 6–10 cells and +++, > 10 cells).



