Epitopes Targeted by Bullous Pemphigoid T Lymphocytes and Autoantibodies Map to the Same Sites on the Bullous Pemphigoid 180 Ectodomain

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Bullous pemphigoid is a blistering skin disease characterized by autoantibodies directed against the NC16A domain of bullous pemphigoid 180 (collagen XVII), a transmembrane protein of epidermal basal cells. Passive transfer studies in mice have shown that antibodies that bind to this immunodominant region of bullous pemphigoid 180 are capable of inducing a skin disease that closely mimics bullous pemphigoid, supporting the hypothesis that epitopes within NC16A are involved in the pathogenesis of bullous pemphigoid. In this study, we examined the autoimmune T cell response in bullous pemphigoid patients. T cells from eight of 12 bullous pemphigoid patients, all of whom had circulating anti-bullous pemphigoid 180 autoantibodies, showed a specific proliferative response to recombinant forms of NC16A. T cell lines and clones developed from four of these patients recognize the same NC16A peptides as those targeted by autoantibodies from the corresponding individuals. These NC16A-responding T lymphocytes express α/β T cell receptors and CD4 memory T cell surface markers and exhibited a Th1/Th2 mixed cytokine profile that may support the production of antibodies. This new information will aid in defining the key steps involved in the development of the autoimmune response in bullous pemphigoid. ***Key words: autoimmunity/basement membrane zone/collagen/hemidesmosome/skin. J Invest Dermatol 115:955–961, 2000***

Bullous pemphigoid (BP) is a cutaneous autoimmune disorder first described by Lever in 1953 (Lever, 1953). This disease, which mainly occurs in elderly patients without gender bias (Lin *et al*, 1998), is characterized by subepidermal blistering and deposition of IgG and complement in the basement membrane zone (BMZ) of the skin (Jordon *et al*, 1967; Provost and Tomasi, 1973). Two hemidesmosomal antigens are recognized by the great majority of BP sera, one associated with the intracellular plaque, BP230 (BPAg1) (Mueller *et al*, 1989) and the other the transmembrane glycoprotein known as BP180 (BPAg2 or collagen XVII) (Labib *et al*, 1986; Diaz *et al*, 1990; Giudice *et al*, 1992). Immunodominant epitopes, including one designated MCW-1, recognized by BP autoantibodies are located within the NC16A domain of the BP180 antigen (Giudice *et al*, 1993; Zillikens *et al*, 1997).

The major clinical, histologic, and immunologic features of BP are also exhibited by patients with herpes gestationis (HG), a pregnancy-associated nonherpetic blistering disease (Provost and Tomasi, 1973; Shornick *et al*, 1983). Over 95% of HG sera recognize the MCW-1 epitope of the BP180 ectodomain (Lin *et al*, 1999a). There are, however, some interesting differences, e.g., the majority of BP autoantibodies belong to the IgG4 subclass (Suzuki *et al*, 1994), whereas in HG, IgG1 is the predominant autoantibody isotype (Kelly *et al*, 1989). Furthermore, immunogenetic studies have shown that BP is associated with the HLA-DQB1*0301 allele (Delgado *et al*, 1996), whereas HG is linked to HLA-DRB1*0401/x or DRB1*0301 MHC II alleles (Shornick *et al*, 1995).

Several lines of evidence support the hypothesis that an autoimmune response against BP180 is a key step in the initiation and evolution of BP. For example, our research group has developed and characterized a passive transfer model of BP, in which neonatal BALB/C mice are injected with antibodies that recognize a segment of the murine BP180 protein that is homologous with the human NC16A domain (Liu *et al*, 1993, 1995a). The antibody-treated animals consistently develop subepidermal blisters that duplicate the features of the human disease, such as complement fixation at the BMZ (Liu *et al*, 1995b) and neutrophil recruitment (Liu *et al*, 1997). These findings support the idea that BP autoantibodies against the BP180 antigen are directly involved in the initiation of the subepidermal blistering in BP patients and, more specifically, implicate the NC16A domain in the initiation of the BP autoimmune response.

The humoral autoimmune response in BP and HG and the epidermal antigens recognized by BP autoantibodies have been the focus of numerous studies carried out over the past 25 y. Very little, however, is known about the immunoregulatory cellular mechanisms involved in the pathogenesis of BP or HG.

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Table I. The MHC class II phenotype and autoimmune response of BP patients

Patients	MHC II alleles		Autoantibody responses[a]			T cell response to NC16A1–5[b]	Sex	Age	Indirect immunofluorescence titer
	HLA-DRB1	HLA-DQB1	230 kDa antigen	180 kDa antigen	sec180e				
ED[c]	1402/0802	0301/0402	–	+	+	3.7	M	74	1:80
KL	1501/1501	ND	+	+	+	3.4	M	79	1:40
SC	1301/1501	0602/0603	+	+	+	3.5	M	30	–
BM	0701/1302	0201/0604	+	+	+	7.8	M	65	1:40
LT	1601/1201	0502/0301	–	+	+	6.2	F	68	1:160
IK	0102/1101	0301/0501	–	+	+	6.1	F	79	–
HP	0401/1101	0301/0302	–	+	+	9.5	M	67	–
AN	04011/0101	0501/0301	+	+	+	3.4	F	81	1:40
ES	0701/1101	0502/0301	+	–	–	–	F	82	1:20
AP	1201/1301	0301/0501	+	–	–	–	M	79	1:20
LH	0804/1503	0301/0602	+	–	–	–	M	69	1:40
NE	0701/1101	0201/0304	+	–	–	–	F	65	1:320

[a] Epidermal antigens used in the immunoblotting assays were extracted from human foreskin. ND, not done.
[b] The proliferation of BP T cells to NC16A1–5 was performed as described in the *Materials and Methods*. The results were shown as SI. SI ≥ 3.0 was considered a positive response. SI < 3.0 was marked as –.
[c] Patient ED deceased at age of 74.

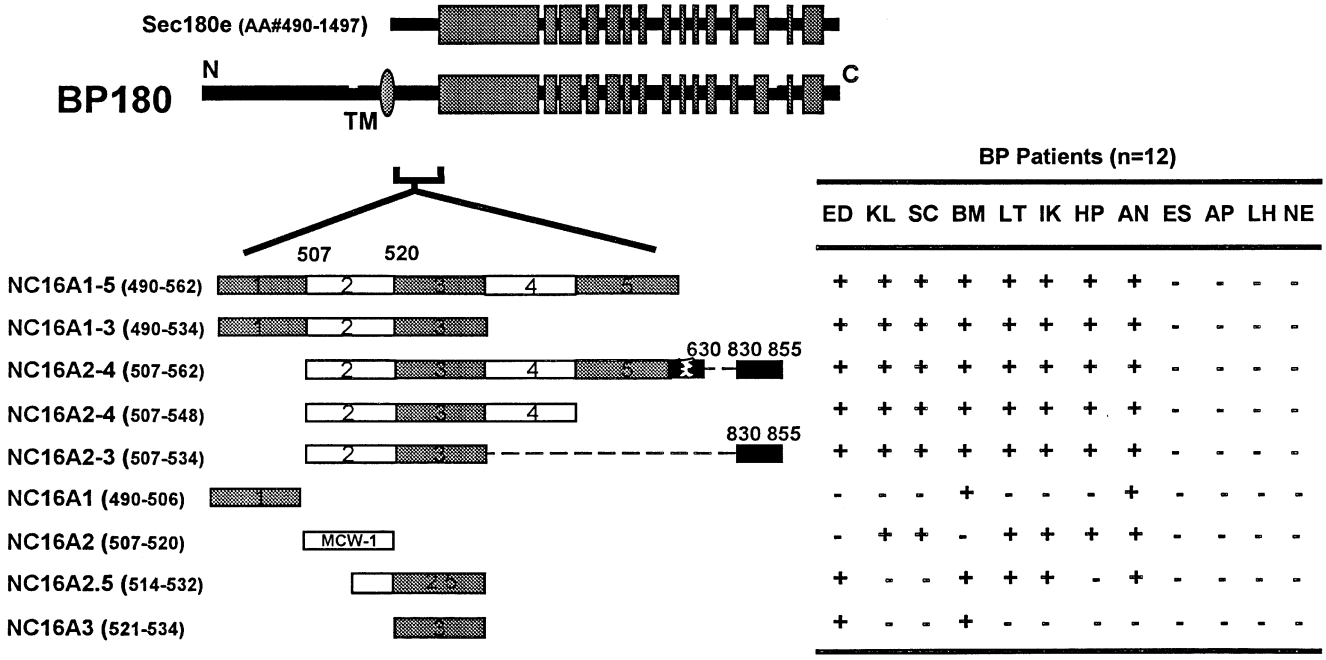

Figure 1. Recombinant BP180 GST fusion proteins encompassing the NC16A region. BP180 contains a single transmembrane domain ("TM"). The cytoplasmic region is shown to the left and the carboxy-terminal ectodomain, consisting of 15 interrupted collagen domains, is shown to the right. The NC16A region has been arbitrarily divided into five subregions, each of approximately 15 amino acids in length. Every BP180 fusion protein shown in this diagram possesses an N-terminal GST tail, and the dotted lines represent deleted peptide stretches. The immunoblotting results of BP sera included in this study are shown in the table to the right. "+" and "–" indicate positive and negative immunoblotting results, respectively.

Recently, it was demonstrated that T cells from normal individuals or BP patients expressing the HLA-DQB1*0301 allele respond to recombinant proteins encompassing the entire extracellular domain of BP180, providing the first evidence that T lymphocytes participate in the autoimmune response in this disease (Büdinger *et al*, 1998). As it was shown that the NC16A domain of the BP180 bear autoantibody epitopes that may be involved in the pathogenesis of BP, in this study, we concentrated our effort in characterizing the T cell autoimmune response in BP patients and defining the responsive T cell epitopes within the NC16A domain. Here, we demonstrate unequivocally that the NC16A region of BP180 harbors epitopes recognized by both T cells and autoantibodies from BP patients. Furthermore, BP180-specific T cell lines and clones generated from BP patients express the $CD4^+$ memory T cell phenotype and secrete Th1/Th2 mixed cytokines.

MATERIALS AND METHODS

BP patients and controls Sera and peripheral blood were obtained from 12 BP patients. All samples were collected from patients when their diseases are in an active stage. Clinically, these patients showed linear IgG and C3 deposits in the BMZ of lesional skin. Indirect immunofluorescence staining (**Table I**) was performed using human foreskin as substrate to detect anti-BMZ IgG (Jordon *et al*, 1976; Katz *et al*, 1976). Patients with

other cutaneous autoimmune diseases, such as pemphigus vulgaris (n = 11), pemphigus foliaceus (n = 4), systemic lupus erythematosus (SLE) (n = 2), epidermolysis bullosa acquisita (n = 1), and psoriasis (n = 2), were included along with normal volunteers (n = 7) as controls. Other information about the BP patients recruited in this study, including age, sex, indirect IF serum titers, and the expression of HLA-DRB1 and -DQB1 alleles, are presented in **Table I**.

Epstein–Barr virus-transformed B cell lines and antibodies Epstein–Barr virus-transformed B cell lines from BP patients were developed as previously described (Ohlin *et al*, 1989). These cell lines were cultured in RPMI 1640 medium containing 10% fetal bovine serum (Hyclone, Salt Lake City, UT) and were used as antigen-presenting cells in the maintenance of T cell clones and in T cell proliferation assays.

Anti-CD3, -CD4, -CD8, CD19, -HLA-DR (clone B-F1), -CD45RA, and fluorescein-labeled goat $F(ab')_2$ anti-mouse immunoglobulin were purchased from Biosource International (Camarillo, CA). Anti-HLA-DQ and anti-HLA-DP and anti-T cell receptor-α/β antibodies were obtained from Becton-Dickinson (San Jose, CA). Anti-CD45RO was purchased from Immunotech (Westbrook, ME). Murine IgG2a and IgG1 were used as negative controls and were obtained from DAKO (Carpinteria, CA).

Preparation of recombinant BP180 fusion proteins and sec180e The following segments of the human BP180 antigen were expressed in *Escherichia coli* as glutathione S-transferase (GST) fusion proteins: NC16A1–5 (aa 490–562) (Giudice *et al*, 1993); NC16A2–5 (aa 507–562); NC16A2–4 (aa 507–548); NC16A1–3 (aa 490–534); NC16A2–3 (aa 507–534); NC16A1 (aa 490–506); NC16A3 (aa 521–534); NC16A2.5 (aa 514–532); and NC16A2 (aa 507–522) (see **Fig 1**; Giudice *et al*, 1993; Zillikens *et al*, 1997). To generate the above BP180 segments, the BP180 cDNAs encoding the respective regions were amplified by polymerase chain reaction (PCR) and subcloned into the bacterial expression vector pGEX2T (Pharmacia, Piscataway, NJ) as described (Liu *et al*, 1995a). These BP180-GST fusion proteins were expressed in *E. coli* strain DH5α and purified by glutathione agarose affinity chromatography (Liu *et al*, 1992). The purified fusion proteins were dialyzed against phosphate-buffered saline, concentrated by ultrafiltration and filter-sterilized. The protein concentration was determined by the Bradford protein assay (Bio-Rad, Hercules, CA). Sec180e, a recombinant protein encompassing the entire extracellular domain of BP180 was constructed and expressed in a mammalian expression system as described (Balding *et al*, 1997). Other unrelated antigens, such as recombinant GST and fragments of desmoglein-1 (Dsg1-K; aa 424–510) and desmoglein-3 [Dsg3-A (aa 145–192), -B (aa 240–303), and -C (aa 570–614)] (Lin *et al*, 1997b), were used as controls in this study.

Immunoblotting assays Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed as described (Zillikens *et al*, 1997). Briefly, sec180e, human foreskin epidermal extracts, and NC16A fusion proteins were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred on to nitrocellulose (Gibco BRL, Gaithersburg, MD). Blots were blocked with PTX buffer (0.01 M phosphate, pH 7.5, 0.2% Triton X-100, 0.15 M NaCl, 1 mM ethylenglycol-bis-(β-amino-ethylether)-*N,N,N′,N′*-tetraacetic acid, 4% bovine serum albumin) for 45 min. Sera from BP patients and controls were diluted in PTX buffer at 1:100 or 1:50 and incubated with the blots overnight at 4°C. The blots were then washed, blocked for 30 min with PTX buffer, and incubated for 2 h with ^{125}I-labeled *Staphylococcus aureus* protein A (Sigma, St Louis, MO) at a concentration of 10^5 cpm per ml. The immunoreactive bands were visualized by autoradiography.

Liquid phase immunoadsorption Immunoadsorption procedures were conducted as described previously (Zillikens *et al*, 1997). The sera were diluted in PTX and incubated overnight with recombinant GST or with GST fusion proteins containing segments of the BP180 NC16A domain. The mixtures were then centrifuged at $10{,}000 \times g$ for 15 min at 4°C. The preadsorbed sera were subsequently used in the immunoblotting assays against various NC16A fusion proteins and sec180e.

Major histocompatibility complex (MHC) class II analysis The analysis of HLA-DRB1 and DQB1 of BP patients was carried out using the sequence-specific oligonucleotide hybridization technique of PCR amplified DNA (Fernandez-Vina *et al*, 1994; Moraes *et al*, 1997). The results are shown in **Table I**.

Purification of peripheral blood mononuclear cells (PBMC) and isolation of T lymphocytes PBMC were isolated by Ficoll-Hypaque (Pharmacia) density gradient separation (Lin *et al*, 1997a). T cells were subsequently purified by E-rosetting using 2-amino-ethylisothiouronium bromide (Sigma)-treated sheep red blood cells (Colorado Serum, Denver, CO) (Indiveri *et al*, 1980). The purified T cells were washed with medium three times, and resuspended in RPMI 1640 medium supplemented with 10% human AB serum (NABI, Miami, FL) for the T cell proliferation assays. In some experiments, CD4- or CD8-positive T cells were depleted from purified T cells using specific antibodies and a magnetic cell sorter (Miltenyi *et al*, 1989). The CD4- or CD8-depleted T cells were used in the proliferation assays as described below.

T cell proliferation assays T cell responses to BP180 fusion proteins were determined by proliferation assays as described (Lin *et al*, 1997a). Briefly, T cells at a density of 10^5 per ml were cultured with 10^5 per ml of irradiated autologous PBMC as antigen-presenting cells along with fusion proteins in wells of 96-well U-bottom plates for 7 d. Phytohemagglutinin (Sigma) at 0.25 μg per ml or interleukin (IL) –2 at 10 U per ml (Collaborative Research, Bedford, MA) served as positive controls in T cell proliferation experiments. The GST, GST-Dsg1, and GST-Dsg3 fusion proteins were included in these experiments as negative controls. Cells in individual wells were pulsed with 1 μCi of ^{3}H-thymidine (ICN, Costa Mesa, CA) during the last 18 h of incubation and then harvested using an automated cell harvester (Inotech Biosystems, Lansing, MI). The proliferation of T cells was determined by measuring the ^{3}H-thymidine uptake by liquid scintillation counting. Data were presented as average cpm ± SD or stimulation index (SI) (average cpm of cells treated with fusion proteins ÷ average cpm of cells treated with GST at the same concentration). An SI equal to or greater than 3 was considered a positive response.

Development and characterization of BP180-specific T cell lines and clones The NC16A-specific T cell lines and clones were developed from four BP patients using NC16A fusion protein (aa 490–560) following the *in vitro* repeat stimulation protocol as described previously (Lin *et al*, 1997a). The cell surface expression of CD3, CD4, CD8, CD19, CD45RA, CD45RO, and T cell receptor-α/β on BP180-specific T cells was examined by flow cytometric analysis using a FACScan flow cytometer (Becton Dickinson) and specific monoclonal antibodies. Mouse IgG was used as a negative control. Fluorescein-conjugated $F(ab')_2$ anti-mouse immunoglobulin was used as the secondary antibody.

The antigen specificity of derived T cell lines and clones was examined by culturing 5×10^4 per ml T cells with 5×10^4 per ml of irradiated autologous PBMC or Epstein–Barr virus-transformed B cells pulsed with 20 μg per ml of antigen for 5 d at 37°C in 96 well plates (Fisher Scientific, Pittsburgh, PA) at a final volume of 200 μl. T cells in each well were pulsed with 1 μCi of ^{3}H-thymidine during the last 18 h of incubation. An SI equal to or greater than 3 was considered a positive response. Phytohemagglutinin at 0.25 μg per ml or IL-2 (Biosource) at 10 U per ml were used as positive controls in these proliferation assays.

Cytokine profile analysis of NC16A-specific T cells T cells from patients and controls were cultured in a 24-well plate (10^6 cells per well) in the presence of 10 ng per ml of phorbol myristate acetate (Sigma) and 100 ng per ml of anti-CD3 antibodies (Del Prete *et al*, 1995). Stimulated cells were collected and washed before total RNA was extracted by Trisol reagent (Gibco BRL). The first-strand cDNA was synthesized using Superscript (Gibco BRL) following the protocol provided by the manufacturer. The mRNA of cytokines expressed by NC16A-specific T lymphocytes were PCR-amplified using specific primer sets (Brenner *et al*, 1989) and Taq polymerase (Gibco BRL). The PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. The following are the sequences of the PCR primer sets: β-actin: 5′-TCCTGTGGCATCCACGAAACT-3′ and 5′-GAAGCATTTGCG-GTGGACGAT-3′; IL-2: 5′-AACTCCTGTCTTGCATTGCA-3′ and 5′-GTGTTGAGATGATGCTTTGAC-3′; IL-4: 5′-CAACTTTGCTG-GACTTCAACA-3′ and 5′-TCCAACGTACTCTGGTTGG-3′; IL-5: 5′-AGGATGCTTCTGCATTTGAG-3′ and 5′-CTATTATCCATC-TCGGTGTTC-3′; IL-6: 5′-AACTCCTTCTCCATAAGCG-3′ and 5′-TGGACTGCAGGAACTCCTT-3′; transforming growth factor (TGF) -β: 5′-AACA-TGATCGTGCGCTCCTGCAAGTGCAGC-3′ and 5′-AAGGAATAGTGCAGACAGGC-AGGA-3′; and interferon (IFN) -γ: 5′-ATGAAATATACAAGTTATATC-3′ and 5′-TTACTGGGATGCT-CTTCGACCTCGAAACAGCAT-3′. The expected sizes of PCR products are as follows: β-actin, 314 bp; IL-2, 441 bp; IL-4, 345 bp; IL-5, 394 bp; IL-6, 610 bp; TGF-β, 200 bp; and IFN-γ, 501 bp. Commercial enzyme-linked immunosorbent assay kits were used to verify the cytokine profiles of these BP180-specific T cells (Cytimmune Sciences, College Park, MD).

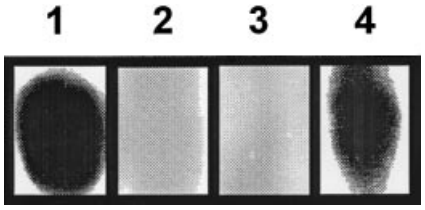

Figure 2. A long-term BP patient expresses anti-BP180 antibodies during the early, but not the later, stages of disease. Serum samples of patient NE [collection dates 1992 (shortly after disease onset) and 1998 (late phase of disease)] were assayed by immunoblotting for reactivity to sec180e. Human sera were used at a dilution of 1:100, and the rabbit anti-BP180 at a dilution of 1:1000. *Lane 1*, positive control (rabbit anti-BP180 antibodies); *lane 2*, negative control (serum from a normal individual); *lane 3*, serum from NE (1998 bleeding); *lane 4*, serum from NE (1992 bleeding).

RESULTS

Most, but not all, BP patients express autoantibodies directed against the BP180 extracellular domain Serum samples from the BP patients were assayed by immunoblotting for reactivity with antigens from a human epidermal extract and with a recombinant protein, sec180e, corresponding to the entire BP180 ectodomain. A summary of the results is presented in **Table I**. Eight of the 12 BP sera (SC, BM, KL, AN, ED, IK, HP, and LT) recognized BP180 extracted from human epidermis as well as the sec180e protein, indicating that these patients were producing autoantibodies that react with the extracellular domain of BP180. Four of these BP180-positive sera (SC, BM, KL, AN) and the four BP180-negative BP sera (LH, ES, AP, and NE) contained immunoreactivity with the BP230 antigen.

Interestingly, although serum NE (obtained in 1998) reacted with BP230, but not BP180, a serum sample that had been obtained from this same patient 6 y earlier (1992) was also tested and was shown to contain IgG reactivity against human epidermal BP180 and sec180e, but not BP230, when tested by immunoblotting at a dilution of 1:100 (**Fig 2**). Epitope mapping analysis using recombinant BP180 proteins revealed that the serum sample collected in 1992 recognized NC16A3 (aa 521–534) and NC16A2.5 (aa 514–532) segments of the NC16A domain. Similar to the results obtained by serum NE reported in **Table I**, two other serum samples from this patient obtained more recently (in 1998) failed to recognize BP180 (data not shown).

Sec180e positive BP sera recognize NC16A fusion proteins To define the epitope(s) recognized by the BP sera in this study, immunoblotting assays were performed using GST-BP180 fusion proteins as the antigen source (**Fig 1**). As reported in **Fig 1**, all eight of the sec180e-positive BP sera reacted with one or more sites within the NC16A domain: six sera reacted with the NC16A2 (aa 507–520) epitope, five with NC16A3 (aa521–534), five with NC16A2.5 (aa 514–532), and two with NC16A1 (aa 490–506). The four BP sera that reacted with BP230, but not BP180, isolated from human epidermis also failed to react with any of these BP180 fusion proteins even at a low serum dilution (1:25).

In order to determine whether these BP sera reacted with sites on the BP180 ectodomain outside of the NC16A domain, the sec180e-positive BP sera were depleted of reactivity with NC16A1–3 and subsequently assayed for reactivity with sec180e. It was clear that after preadsorption against NC16A1–3, all eight BP sera lost their ability to react with sec180e (not shown). Therefore, within the limits of the sensitivity of this immunoblotting assay, we were unable to detect any extracellular BP autoantibody-reactive epitopes outside the BP180 NC16A domain.

T lymphocytes from BP patients specifically respond to NC16A To examine whether T lymphocytes respond to BP180, T cells were purified from PBMC of BP patients and analyzed using a standard T cell proliferation protocol. As reported in **Table I**, T cells from the eight BP patients whose sera recognized sec180e and NC16A proliferated in response to NC16A1–5 (aa 492–560), but showed no response to GST or unrelated epidermal antigens, such as Dsg-1 and Dsg-3. These findings indicated that BP T cells specifically proliferate in response to BP180. Furthermore, T cells from other patient groups, such as pemphigus vulgaris (n = 11), pemphigus foliaceus (n = 4), systemic lupus erythematosus (n = 2), epidermolysis bullosa acquisita (n = 1), and psoriasis (n = 2), and normal controls (n = 7) did not respond to NC16A1–5 (not shown), indicating that the NC16A-induced T cell proliferation response is specific to BP. Interestingly, T cells isolated from BP patients expressing only anti-BP230 autoantibodies failed to respond to the NC16A fusion protein (**Table I**).

NC16A responsive BP T lymphocytes are CD4-positive cells It is known that CD4 Th cells are capable of promoting antibody production in B cells in an antigen-dependent response. As BP is an antibody-mediated autoimmune disease, it is postulated that the BP180-specific T cells are of the CD4 lineage. To examine further the phenotype of the NC16A-responsive T cells, the T cell preparations were selectively depleted of $CD4^+$ or $CD8^+$ T cells by magnetic sorting using anti-CD4 or anti-CD8 antibodies, respectively, and subsequently examined in the proliferation assay. As shown in **Fig 3(*A*)**, T cells from a representative patient proliferated when incubated with NC16A fusion proteins in a concentration-dependent manner. These observed T cell responses were abolished when $CD4^+$ T cells were depleted (**Fig 3*C***), but not when the $CD8^+$ T cells were depleted (**Fig 3*B***), suggesting that the NC16A-responsive T cells are of the CD4 T cell lineage.

Antigen specificity of BP180-specific T cells developed from BP patients To facilitate the characterization of BP-specific T cells, T cell lines were developed from BP patients as described (Lin *et al*, 1997a), and T cell clones were subsequently derived from the cell lines by limiting dilution (Taswell, 1981). The antigen specificity of these T cells was determined by proliferation assays using BP180 fusion proteins as the presenting antigens. As shown in **Fig 4**, T cells developed from BP patients specifically respond to NC16A fusion proteins in a concentration-dependent manner. These BP T cell lines and clones do not proliferate to fusion proteins derived from unrelated skin antigens, such as Dsg1 and Dsg3 (not shown), establishing the specificity of the BP180 response exhibited by these cells. T cell clones (n = 6) from patient BM, whose serum recognized the NC16A3 peptide, proliferated in response to NC16A3, but did not exhibit a response to other fragments of NC16A, such as NC16A2 and NC16A1 (**Fig 4*A***, ***B***). On the other hand, T cell lines and clones developed from two other BP patients with antibodies recognizing the NC16A2 site showed an exclusive proliferation response to the NC16A2 peptide (aa 507–520) (**Fig 4*C***, ***D***). These results indicate that both autoimmune T and B cells from a given patient recognize extracellular BP180 epitopes located in close proximity to one another (within a 15 amino acid stretch).

Cell surface phenotypes and cytokine profiles of NC16A-specific T cells Flow cytometric analysis was used to determine the cell surface phenotype of the BP180-specific T cells developed from BP patients. We found that all cell lines and clones expressed CD3, CD4, CD45RO, and T cell receptor-α/β, but was negative for CD8, CD45RA, and the B cell marker CD19, indicating that BP180-specific T lymphocytes from BP patients exhibit a CD4 memory T cell phenotype (data not shown).

The cytokine profile of the BP T cells was determined as followed. T cells were activated by anti-CD3 antibodies in the presence of phorbol myristate acetate for 30 h. The expression of IL-2, IL-4, IL-5, IL-6, TGF-β, and IFN-γ in these T cells was analyzed by reverse transcription–PCR using specific sets of cytokine primers as described (Brenner *et al*, 1989) and commercial human cytokine enzyme-linked immunosorbent assay kits. As shown in **Fig 5**, a representative T cell clone predominantly expressed IL-2, IL-5, and IFN-γ mRNA, and also relatively smaller

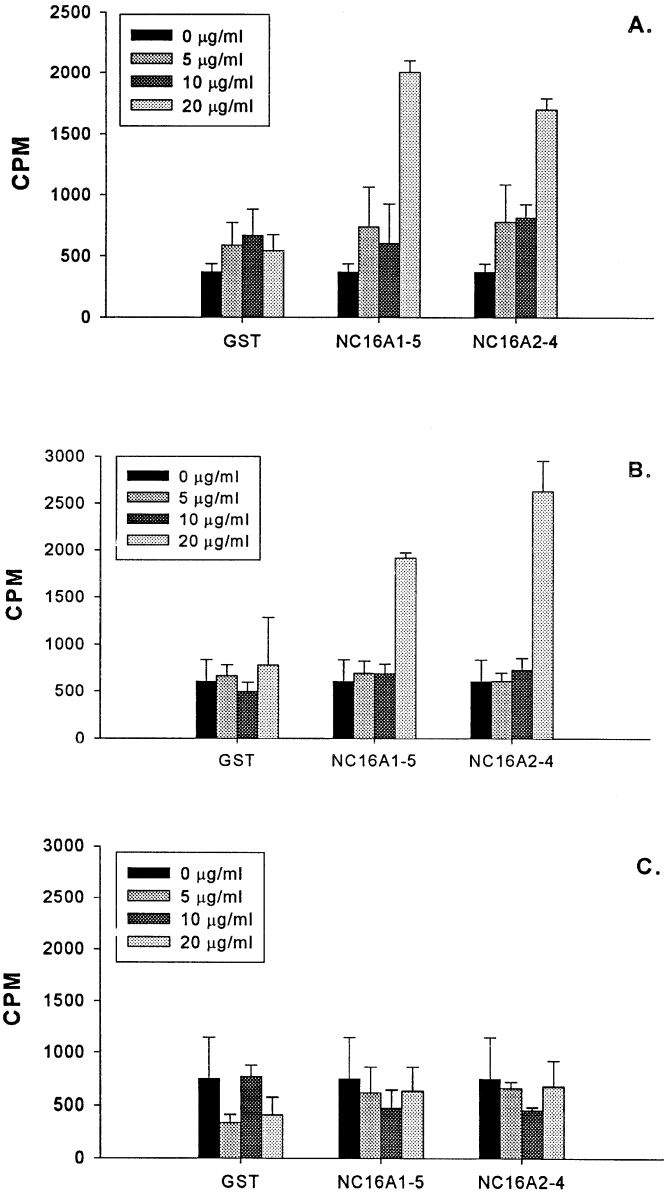

Figure 3. T cells from BP patients respond to NC16A fusion proteins. The response of BP T cells to NC16A fusion proteins was examined using the proliferation assay as described. T cells at a density of 10^5 per ml were cultured with antigen-pulsed autologous antigen-presenting cells in a 96-well plate for 7 d. Cells were pulsed with ^{3}H-thymidine during the last 18 h of incubation and subsequently analyzed for radioactivity uptake by liquid scintillation counting. Data of a representative patient, ED, are displayed here. Total T cells (*A*), CD8-depleted T cells (*B*), and CD4-depleted T cells (*C*).

amounts of IL-4 and IL-6, but was negative for TGF-β mRNA. Similar results were obtained from other T cell lines and clones recognizing either NC16A2 or NC16A3. These data were confirmed by enzyme-linked immunosorbent assay methods (not shown). These results indicated that NC16A-specific T cells from BP patients produce a Th1/Th2 mixed cytokine profile.

DISCUSSION

The data presented in this paper demonstrate that the NC16A stretch of the BP180 ectodomain contains epitopes recognized by both autoantibodies and T cells from the majority of BP patients. T lymphocytes from the control groups did not respond to the BP180 peptides and T cells from BP patients did not respond to control epidermal antigens, findings which established the antigen specificity and disease association of this T cell response. T lymphocytes exhibiting a proliferative response to BP180 NC16A peptides were found only in those BP patients who had detectable levels of circulating autoantibodies directed against this same antigen. Of particular interest were the epitope mapping results that revealed that T cell lines and clones generated from four BP patients recognize the same narrowly defined antigenic sites as those of the autoantibodies from the corresponding patients. These results provide evidence that antigenic sites located on the BP180 NC16A domain may well be involved in triggering the autoimmune response in BP.

It remains unclear, however, which, if any, of the BP180 epitopes are involved in dermal–epidermal detachment in BP. Passive transfer mouse model studies have clearly shown that the pathogenically active antibodies recognize a site on murine BP180 homologous with the human NC16A domain. These findings formed the basis for our in-depth analysis of the T and B cell responses directed to the NC16A domain of human BP180. Nevertheless, it must be kept in mind that BP-associated epitopes on the BP180 antigen are not solely restricted to NC16A. For example, Büdinger *et al* (1998) showed that T cells from some BP patients recognize BP180 fragments outside the NC16A domain, although these epitopes were not precisely defined. In another study (Perriard *et al*, 1999), it was shown that autoantibodies from some BP sera react with sites on the BP180 intracellular domain.

In order to determine whether the NC16A-responsive T cells belong to the CD4 or CD8 lineage, BP T cells were negatively selected using antibodies to these surface markers and were assayed by the cell proliferation protocol. The results of this analysis clearly demonstrated that the NC16A-specific BP T cells are of the CD4-positive cell lineage, a T cell population known to support B lymphocytes in the production of antibodies. This conclusion was further supported by the findings that all NC16A-specific T cell lines and clones derived from four BP patients express the CD4, but not the CD8, T cell marker.

Of the 12 BP patients tested, four (AP, LH, ES, and NE) did not exhibit a T cell response to BP180 peptides. Significantly, these were the same four patients whose sera exhibited no immunoreactivity to the BP180 antigen. In at least one of these cases (NE), the serum sample (from which the T cells and circulating antibodies were isolated) was obtained from the patient over 6 y after disease onset. Interestingly, a serum sample that had been collected from this same patient during the early phase of disease did contain anti-BP180 autoantibodies, but, due to lack of cell samples, whether T cells respond to BP180 at that time could not be assessed. Thus, whereas it appears that anti-BP180 autoantibodies may be crucial in triggering subepidermal blister formation during the initial stages of BP, other mechanisms (possibly involving the action of anti-BP230 autoantibodies) may predominate in the later phases of this disease. Further, in a subset of BP cases (possibly represented by patients AP, LH and ES) it appears that even the initiatory autoimmune response may be directed to structural molecules of the dermo–epidermal junction besides the BP180.

In this study, we also report detailed autoantibody epitope mapping using an immunoblotting assay. We found that the NC16A region of BP180 contains the major epitopes recognized by BP autoantibodies, with NC16A1–3 (aa 492–534) as the dominant target area. After depletion of reactivity to NC16A1–3, the BP sera lost their ability to react with sec180e, indicating that, within the limits of sensitivity of the immunoblotting assay, no autoantibody-reactive sites on the BP180 ectodomain outside NC16A1–3 could be uncovered. A similar conclusion was reached in a previous study by our research group that focused on defining the BP-associated epitopes on the BP180 ectodomain (Zillikens *et al*, 1997). Interestingly enough, T cell lines and clones developed from four BP patients recognized the same peptide stretches within the NC16A region as those bound by the autoantibodies, indicating that the BP180 epitopes targeted by autoimmune T and B cells from BP patients are located in close proximity to one another. In a similar vein, we have recently shown that T cells and auto-

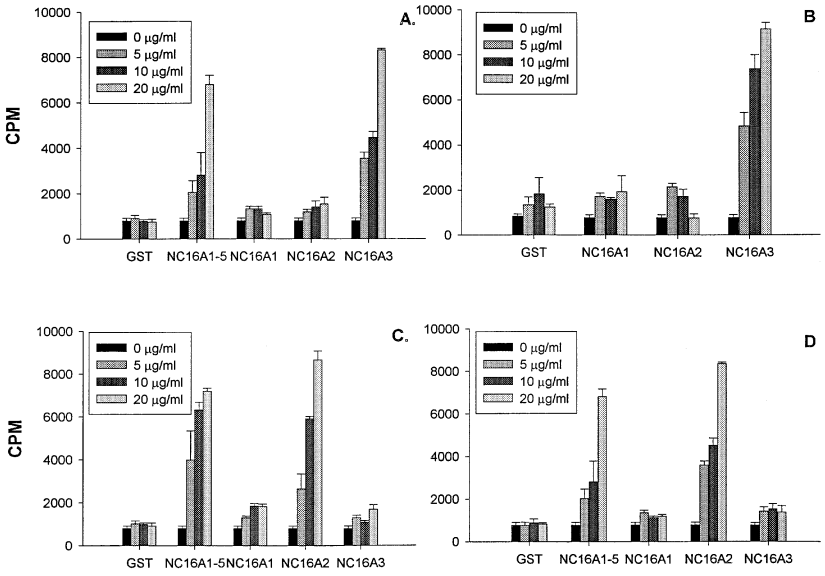

Figure 4. T cell lines and clones developed from BP patients respond to NC16A fusion proteins. T cell lines and clones were developed from BP patients as described. The specificity of these T cells to NC16A fragments was examined using the proliferation assay. Data of representative T cell lines and clones are shown here. T cell clone ED10 from patient ED (*A*), T cell clone BM78 from patient BM (*B*), T cell line LT1 from patient LT (*C*), and SC1 from patient SC (*D*).

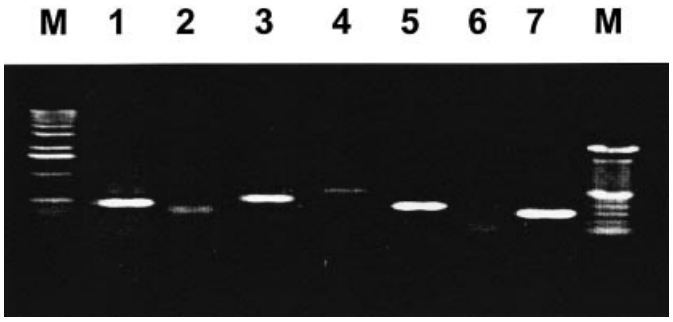

Figure 5. BP180-specific T cells express a Th1/Th2 mixed cytokine profile. The expression of cytokine mRNA in T cell lines or clones developed from BP patients was examined by reverse transcription–PCR as described (Brenner *et al*, 1989). The PCR products were subsequently fractionated on a 1.5% agarose gel and visualized with ethidium bromide. The data of a representative T cell clone BM77 are shown here. *Lane M*, molecular weight marker; *lane 1*, IL-2; *lane 2*, IL-4; *lane 3*, IL-5; *lane 4*, IL-6; *lane 5*, IFN-γ; *lane 6*, TGF-β; *lane 7*, β-actin.

antibodies from two HG patients react with the same NC16A peptide (Lin *et al*, 1999b). The colocalization of T and B cells epitopes in the same area of an antigen has been identified in other autoimmune diseases such as multiple sclerosis (Wucherpfennig *et al*, 1997) and myasthenia gravis (Atassi and Oshima, 1997). In our study, however, T cells from patients BM and AN expressing the anti-BP180 against NC16A1 epitope, failed to proliferate to this peptide. Currently, it is not clear why this peptide did not stimulate autoimmune BP T cells. It is possible that the frequency of NC16A1-reactive T cells is too low to be detectable in these two patients. These results further indicate the complexity of the BP autoimmune responses.

It is well established that the antibody isotype produced by a given B cell is dependent on the type of helper T lymphocytes that it encounters during activation (Lin and Chen, 1993). For example, T cells that secrete Th1 cytokines are capable of stimulating B cells to produce IgG1, whereas Th2 type cytokines can induce B cells to secrete IgG4 (Armitage *et al*, 1993). In HG, it was reported that NC16A2-specific T cells secrete a Th1 cytokine profile (Lin *et al*, 1999b). In this study, using reverse transcription–PCR and specific cytokine primers, we found that all NC16A-specific T cell lines and clones (n = 12) developed from four BP patients (ED, SC, LT, and BM) produce IL-2, IL-4, IL-5, IL-6, and IFN-γ at the mRNA and the protein level, indicating that these T cells express both Th1-type and Th2-type cytokines. This conclusion is similar to that drawn by Büdinger *et al* (1998), who showed that BP180-specific T cells secrete a Th1/Th2 cytokine profile. Whether these cytokines modulate the production of anti-BMZ antibodies in BP patients is currently under investigation.

Previously, Delgado *et al* (1996) reported that there is a strong association of HLA-DQB1*0301 with the development of BP. It remains unclear, however, as to whether the production of anti-BP180 and/or anti-BP230 autoantibodies is associated with the expression of this MHC II allele. It was shown that the T cell response from normal individuals and BP patients to the BP180 extracellular domain is restricted to the expression of DQB1*0301 (Büdinger *et al*, 1998). As almost all BP patients recruited for their study expressed this particular MHC II allele, this observation reflects the autoimmune reaction in a particular subset of BP patients. Although the majority of BP patients in our study express HLA-DQB1*0301, it is not clear whether the expression of this MHC II molecule is strongly associated with the development of both the humoral and cellular immune responses against BP180 in these patients. A preliminary study, however, demonstrated that anti-HLA-DQ antibodies inhibit the proliferative responses of patient ED T cells to NC16A3, suggesting that the T cell immune response to BP180 may be restricted to HLA-DQ (not shown).

In conclusion, the data presented in our study provide evidence that the NC16A region of the BP180 molecule harbors epitopes recognized by both autoimmune T lymphocytes and autoantibodies from BP patients, suggesting that this region may modulate the autoimmune reaction in BP. This information may help us to understand the underlying autoimmune mechanisms in the development of BP. Now that the epitopes recognized by BP autoimmune T lymphocytes have been identified, we can use this information to design inhibitory peptides or altered peptide ligands to downregulate the disease-associated T cell response (Sloan-Lancaster *et al*, 1994; Sloan-Lancaster and Allen, 1996).

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