
IgG4 and IgE are the major immunoglobulins targeting the NC16A domain of BP180 in bullous pemphigoid: Serum levels of these immunoglobulins reflect disease activity

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Background: Bullous pemphigoid (BP) is an autoimmune blistering disease associated with autoantibodies against the hemidesmosomal glycoprotein BP180. The noncollagenous (NC)16A domain of BP180 has recently been shown to harbor major antigenic sites recognized by BP sera.

Objective: The purpose of this study was to characterize the subclass distribution and fine specificities of autoantibodies to BP180 NC16A present in the circulation of patients with BP before, and during the course of, therapy for this disease.

Methods: Eighteen BP sera were analyzed by immunoblotting and enzyme-linked immunosorbent assay for the presence of IgG1, IgG2, IgG3, IgG4, and IgE reactive with various sites on the BP180 NC16A domain. The sera were collected before treatment was started and at 4- and 8-week time points after initiation of treatment.

Results: We identified IgG4 and IgE as the major immunoglobulins that preferentially react with two distinct epitopes (MCW-1 and MCW-2) within BP180 NC16A. Levels of these autoantibodies correlated with disease activity in BP. During the course of disease, no change was observed with regard to the immunoglobulin subclass predominantly reacting with BP180 NC16A or the specific epitopes within this domain.

Conclusion: Our data demonstrate that remission of BP is paralleled by a decrease of serum levels of IgE and the different IgG subclasses reactive with BP180 NC16A. (*J Am Acad Dermatol* 2000;42:577-83.)

Bullous pemphigoid (BP) is an autoimmune subepidermal blistering disease characterized by the linear deposition of IgG and C3 at the cutaneous basement membrane zone (BMZ).¹ Autoantibodies in BP are directed against two hemidesmosomal proteins designated BP230 and BP180.²⁻⁵ BP230 localizes to the intracellular hemidesmosomal plaque.⁶ In contrast, BP180 is a

transmembrane glycoprotein with a large extracellular portion that spans the lamina lucida of the BMZ and consists of 15 interrupted collagen domains.⁷ Data from a passive transfer animal model strongly suggest that antibodies to BP180 are directly involved in the pathogenesis of BP.⁸ Epitope mapping studies have shown that the membrane-proximal noncollagenous (NC)16A domain of human BP180 harbors a cluster of antigenic sites recognized by the vast majority of BP sera.⁹ With the NC16A domain used as the antigenic target, a highly sensitive and specific enzyme-linked immunosorbent assay (ELISA) has been developed that detects anti-BP180 antibodies in more than 90% of BP sera.¹⁰ More recently, we have shown that serum levels of antibodies specific for BP180 NC16A, in contrast to immunofluorescence titers of anti-BMZ antibodies, correlate with disease activity in patients with BP.¹¹ In this study, 18 BP sera were investigated for the subclass distribution of antibodies to full-length BP180

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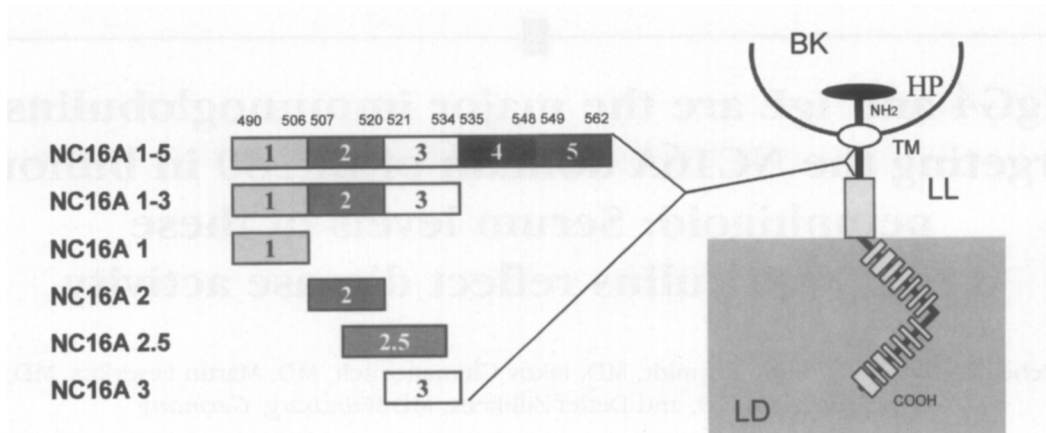


Fig 1. Schematic diagram of BP180 and recombinant forms of the NC16A domain used in this study. The intracellular portion of BP180 associates with the hemidesmosomal plaque (HP) of the basal keratinocyte (BK). The transmembrane region is labeled TM. The extracellular domain of BP180, consisting of 15 interrupted collagen domains (shaded boxes), completely traverses the lamina lucida (LL) and projects into the lamina densa (LD).^{7,37} The NC16A domain has been subdivided into 5 regions, each of approximately 15 amino acids in length. Each of the fusion proteins contains an N-terminal GST-moiety (not shown). Amino acid residue numbers are shown above the boxes.

NC16A and recombinant fragments of this domain. In addition, we analyzed the change of this reactivity during the course of the disease.

MATERIAL AND METHODS

Human and rabbit sera

Sera from 18 patients with BP were included in this study. Eleven patients were men and 7 women. The ages of the patients ranged from 50 to 91 years (median, 72 years). By direct immunofluorescence (DIF), linear deposits of C3 and/or IgG at the BMZ of perilesional skin biopsy specimens were revealed. By indirect immunofluorescence (IIF) on 1 mol/L NaCl-split human skin, circulating autoantibodies labeled the epidermal side of the split in all patients. All BP sera contained autoantibodies against BP180 NC16A as determined by both ELISA and immunoblot analysis. Blood samples were obtained before treatment was started and at 4- and 8-week time points after initiation of treatment. Patients were treated with a combination of oral prednisolone plus dapsone or doxycycline plus nicotinamide. Treatment was tapered according to clinical improvement as described previously.^{11,12} Sera from 50 healthy volunteers were used as controls. Rabbit serum R58 was raised against recombinant glutathione S-transferase (GST).⁹

Preparation of human BP180 recombinant proteins

The following previously described GST fusion proteins containing full-length BP180 NC16A and

various fragments of this domain were expressed in *Escherichia coli* DH5 α : GST-NC16A1, GST-NC16A2, GST-NC16A2.5, GST-NC16A3, GST-NC16A1-3, and GST-NC16A1-5 (Fig 1). Fusion proteins were purified by glutathione agarose affinity chromatography.⁹

Immunoblotting

Protein extracts were fractionated by 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose.⁹ After blocking in 3% solution of skimmed milk powder in TBST (0.02 mol/L TRIS-[hydromethyl]-aminomethan, 0.14 mol/L NaCl, Tween-20 0.01%, pH 7.5) buffer for 45 minutes, blots were incubated for 4 hours with human (1:100 in 1% TBST bovine serum albumin [BSA]) or rabbit (1:500) serum. Bound antibodies were visualized enzymatically with the use of peroxidase-conjugated secondary antibodies at the following dilutions: rabbit anti-human IgG 1:15,000 (DAKO, Glostrup, Denmark), monoclonal mouse anti-human IgG1 1:1,000, anti-IgG2 1:500, anti-IgG3 1:200, anti-IgG4 1:4,000, and sheep anti-human IgE 1:100 (all Binding Site, Birmingham, UK). Sensitivities of secondary monoclonal antibodies used at their working dilutions were shown to be comparable by immunoblot analysis against equal amounts of human myeloma proteins.¹³

ELISA

The procedure for development of the optimal working conditions of the ELISAs to detect IgE, IgG1, IgG2, IgG3, and IgG4 autoantibodies to NC16A1-5

Table I. Immunoglobulin subclass distribution of autoantibodies to BP180 NC16A in sera from patients with bullous pemphigoid*

BP sera	IgG1	IgG2	IgG3	IgG4	IgE
BP-1	+	+	-	+	++
BP-2	+	-	+	++	+
BP-3	+	-	+	++	++
BP-4	+	+	-	-	+
BP-5	++	++	++	+	++
BP-6	-	-	+	-	-
BP-7	+	-	-	-	-
BP-8	-	++	-	+	-
BP-9	-	-	-	++	-
BP-10	++	++	+	++	+
BP-11	-	-	+	-	-
BP-12	++	+	+	++	+
BP-13	-	-	-	+	+
BP-14	-	+	-	-	++
BP-15	-	-	-	++	+
BP-16	+	-	++	-	-
BP-17	-	++	-	+	-
BP-18	-	-	-	+	-
Positive sera	50%	44%	44%	66%	55%

*BP sera (n = 18) were depleted of anti-GST immunoreactivity and assayed for immunoblot reactivity with recombinant GST-NC16A1-5.

followed a previous protocol.¹⁰ Briefly, analysis of the different plots generated by chessboard titrations of serial dilutions of antigen (4 ng-9 µg), primary antibody (25- to 1600-fold dilution of BP serum), and secondary antibody (50- to 16,000-fold dilutions) demonstrated that coating of each well with 400 ng of GST-NC16A1-5 and a 50-fold dilution of the primary antibody yielded the highest level of discrimination. The 6 different ELISAs to detect polyclonal IgG, IgE, and IgG subclasses were performed according to the following protocol: each well was coated with purified recombinant GST-NC16A1-5 for 2 hours. Wells were then washed with phosphate-buffered saline (PBS), pH 7.4, and blocked for 1 hour with PBS containing 1% BSA (Sigma, Deisenhofen, Germany). Subsequently, wells were incubated for 1 hour with serum (BP or control) diluted 50-fold in PBS containing 1% BSA and 0.05% Tween-20 (Sigma). After washing, wells were reacted for 1 hour with peroxidase-labeled rabbit anti-human IgG (1:10,000) and monoclonal mouse anti-human IgG1 (1:600), IgG2 (1:300), IgG3 (1:200), IgG4 (1:3000), or sheep anti-human IgE (1:100). Followed by another series of washes, o-phenylenediamine in 0.01% H₂O₂ was added to each well. After 10 minutes, the reaction was stopped and the optical density (OD) was read at 492 nm. Each serum was analyzed in trip-

Table II. NC16A2 and NC16A2.5 are the major antigenic sites targeted by IgG subclasses and IgE before treatment is initiated*

Antibody	BP 180 NC16A					
	1-5	1-3	3	2.5	2	1
IgG1	100%	100%	0%	60%	80%	80%
IgG2	100%	100%	40%	100%	60%	20%
IgG3	100%	100%	0%	33%	66%	0%
IgG4	100%	100%	40%	80%	60%	20%
IgE	100%	100%	0%	33%	100%	33%

*Eight BP sera that showed the strongest reactivity of immunoglobulin subclasses with full-length BP180 NC16A were assayed by immunoblotting, after depletion of anti-GST reactivity, against different fragments of this domain.

licate for reactivity with both GST-NC16A and recombinant GST alone. The mean OD reading obtained with GST was subtracted from the mean OD reading for GST-NC16A. To minimize plate-to-plate variability, each ELISA plate included the same 4 internal controls.¹⁰ The cut-off value was set at an OD reading corresponding to the mean value of 50 negative control sera + 2 SD.

RESULTS

IgG4 and IgE are the major immunoglobulin isotypes targeting BP180 NC16A before initiation of treatment in patients with BP

Serum samples obtained from 18 BP patients before initiation of treatment were analyzed by immunoblotting for reactivity to BP180 NC16A. Sixty-six percent of these BP sera contained IgG4, 50% IgG1, 44% IgG2, and 44% IgG3 antibodies that showed specific reactivity in this assay. In addition, 55% of these BP sera contained IgE autoantibodies that were reactive with the NC16A domain. The results of this analysis are summarized in Table I.

NC16A2 and NC16A2.5 are the major antigenic sites targeted by IgG subclasses and IgE autoantibodies before initiation of treatment

Eight BP sera obtained before treatment was initiated were preadsorbed against recombinant GST and assayed by immunoblotting for reactivity with 6 recombinant forms of the human BP180 NC16A domain (GST-NC16A1, GST-NC16A2, GST-NC16A2.5, GST-NC16A3, GST-NC16A1-3, and GST-NC16A1-5). Equal amounts of the various recombinant proteins were used for this analysis as confirmed by immunoblotting with R58 serum. IgE and the 4 different IgG subclasses targeted 4 distinct antigenic sites within BP180 NC16A. GST-NC16A2 (MCW-1)

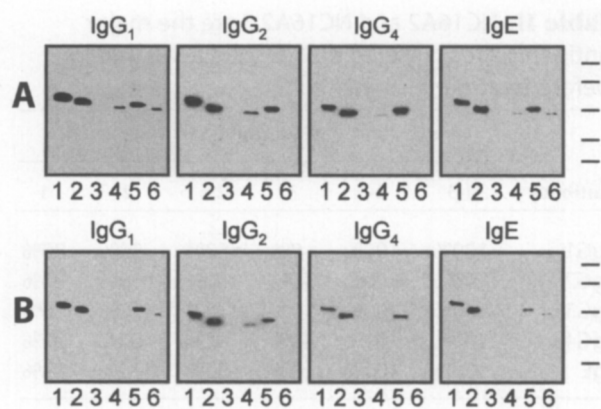


Fig 2. The fine specificity of immunoglobulins targeting epitopes within BP180 NC16A does not change during the course of the disease. Results of a representative patient (BP-1; see Table I) are shown. Blots containing equal amounts of fusion proteins GST-NC16A1-5, GST-NC16A1-3, GST-NC16A3, GST-NC16A2.5, GST-NC16A2, GST-NC16A1 (lanes 1-6, respectively) were labeled with BP serum (1:100) before treatment (A) and after 4 weeks of therapy and marked clinical improvement (B). Autoantibodies were visualized using peroxidase-conjugated monoclonal antibodies against anti-human IgG₁, IgG₂, IgG₄, and IgE. Molecular weight markers corresponding to 66, 45, 31, 21, and 14 kD are indicated at right.

and GST-NC16A2.5 (MCW-2) were the two epitopes most consistently recognized. GST-NC16A1 (MCW-0) and GST-NC16A3 (MCW-3) were targeted less frequently. Taking together the reactivity of all isotypes, GST-NC16A2 was recognized by 71%, GST-NC16A2.5 by 66%, GST-NC16A1 by 33%, and GST-NC16A3 by 19% of BP sera. Results of this analysis are summarized in Table II, and representative patterns of reactivity are shown in Fig 2, A.

Serum levels of IgG subclasses and IgE antibodies to BP180 NC16A parallel disease activity during the course of the disease

In 10 BP patients, immunoglobulin subclass reactivity against NC16A was assayed by ELISA before treatment was initiated as well as 2, 4, 6, and 8 weeks later. In all BP sera, levels of antibodies against BP180 NC16A decreased during the observation period. This decrease included all IgG subclasses and IgE autoantibodies. A representative example is shown in Fig 3.

The fine specificity of autoantibodies to epitopes within BP180 NC16A does not change during the course of the disease

In 8 BP patients, the fine specificity of autoantibodies to the various fragments of NC16A was

monitored by immunoblotting before treatment was initiated and 4 weeks later. After 4 weeks of treatment, at which time there was significant clinical improvement, all BP sera showed a marked decrease in levels of IgG subclasses and IgE autoantibodies to the same antigenic sites that were targeted before therapy. No change in the pattern of reactivity was observed (Fig 2).

DISCUSSION

By DIF and IIF microscopy or Western blot analysis of epidermal extracts, IgG₄ and IgG₁ have been identified as the major IgG subclasses of autoantibodies in BP.¹⁴⁻¹⁸ Immunofluorescence analysis, however, may detect antibodies to different components of the BMZ, including both BP180 and BP230. On the other hand, Western blotting of epidermal extracts is capable of identifying antibodies specific to BP180, but cannot differentiate between reactivity to different antigenic sites within BP180. Antibodies to various extracellular and intracellular epitopes of BP180 have recently been described in BP sera.^{9,19-21} However, data from a passive transfer mouse model of BP suggest that autoantibodies to the NC16A domain of BP180 are of pathogenic relevance for blister formation in BP.⁸ The aim of the present study was to characterize reactivity of IgG subclasses and IgE autoantibodies to full-length NC16A and recombinant fragments of this domain before and during treatment.

We found that IgG₄ (66%) and IgG₁ (50%) antibodies were the major IgG subclasses targeting BP180 NC16A before treatment was initiated; this is similar to data of immunofluorescence microscopy and Western blot analysis of epidermal extracts.¹⁴⁻¹⁸ In 44% of the BP sera, we detected IgG₂ and IgG₃ antibodies to BP180 NC16A, which is in contrast to immunofluorescence and immunoblotting studies that found IgG₂ and IgG₃ autoantibodies in only a small minority of BP sera. IgG₁ and IgG₃ are the IgG subclasses with the strongest complement fixing properties²² and may account for complement activation by the classical pathway in the skin of BP patients. In fact, complement activation has been shown to be a prerequisite for blister formation in an in vitro model and the passive transfer mouse model of BP.^{23,24} Interestingly, in 44% of our BP sera, no IgG₁ or IgG₃ antibodies to BP180 NC16A were detected. In this subset of BP patients, complement may be activated by the alternative pathway. Components of the alternative pathway have previously been identified in blister fluid and lesional skin of BP patients.^{25,26}

We have recently demonstrated that reactivity of BP sera to BP180 NC16A is mainly restricted to 4 dif-

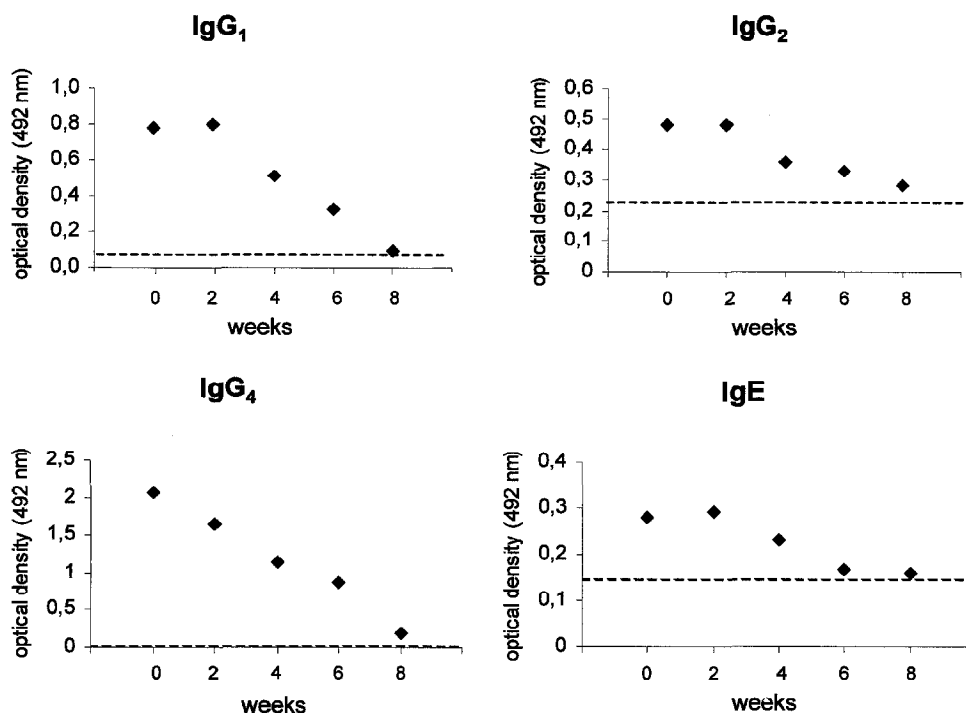


Fig 3. Levels of IgG subclasses and IgE antibodies to BP180 NC16A decrease with clinical improvement of BP. Data are shown for one representative patient (BP-1; see Table I). Serum reactivity to BP180 NC16A (diamonds) was measured by ELISA. Samples were analyzed in triplicates and results are expressed as mean of OD₄₉₂ readings. The cut-offs (mean of controls + 2 SD) of the different assays are shown as dotted lines.

ferent epitopes clustered within the N-terminal portion of NC16A (MCW-0 through MCW-3).⁹ In the present study, we also addressed the question of whether certain subclasses of BP autoantibodies preferentially react with specific epitopes within NC16A. The antigenic sites most consistently recognized by all IgG subclasses were NC16A2 (MCW-1) and NC16A2.5 (MCW-2). Less frequent reactivity was seen against NC16A1 (MCW-0) and NC16A3 (MCW-3). Consistent with previous observations,⁹ no reactivity was detected with NC16A4 (MCW-4) and NC16A5 (MCW-5). Interestingly, sera from patients with lichen planus pemphigoides were recently found to react with MCW-4.²⁷ In the present study, we did not detect a preferential binding of certain IgG subclasses in BP sera with specific epitopes within NC16A.

Antibodies to BP180 NC16A, in contrast to IIF titers of anti-BMZ antibodies, parallel disease activity in BP patients.¹¹ In the present study, we addressed the question of whether a certain IgG subclass or reactivity with a certain antigenic site within BP180 NC16A is preferentially linked with disease activity. Using the BP180 NC16A ELISA, we investigated 10 BP sera for their levels of IgG subclasses before and dur-

ing treatment. All IgG subclasses of anti-BP180 antibodies decreased in parallel according to a decrease of disease activity. After 8 weeks of therapy and a marked clinical improvement in all patients, levels of all 4 IgG subclasses were close to, or below, the cut-off of the ELISA. When the reactivity of IgG subclasses with various recombinant fragments of BP180 NC16A was monitored over time, we found that the different IgG subclasses targeted the same epitopes at the pretreatment and the 4-week posttreatment time points.

Elevated IgE levels in sera and blister fluid of BP patients have been reported, and binding of circulating IgE antibodies along the BMZ is also found in certain BP patients.^{28,29} Before this study, IgE reactivity was only reported to BP230.^{30,31} Using our immunoblot system, we detected IgE reactivity to BP180 NC16A in 55% of BP sera. This discrepancy is likely due to the lower sensitivity of immunoblotting of epidermal or keratinocyte extracts to detect anti-BP180 antibodies compared with the use of recombinant BP180 proteins and ELISA, as was described previously.¹⁰ When the fine specificities of IgE antibodies to BP180 were studied, MCW-1 and MCW-2 were identified as the major antigenic sites within

NC16A targeted by IgE. As was the case with the IgG subclasses, serum concentrations of IgE anti-BP180 antibodies paralleled disease activity, and no change of the specific target epitopes was noticed during the course of the disease. Interestingly, we found the highest reactivity with BP180 NC16A for IgG4 and IgE autoantibodies. IgG4 and IgE production is regulated concordantly and requires the stimulation of T helper 2 (T_H2) cells. Interleukin (IL) 4, IL-13, and sCD23 have been shown to be involved in this process.^{32,33} In sera or blister fluid of BP patients, elevated levels of IL-4, IL-13, and sCD23 have been detected,^{31,34,35} and autoreactive T_H2 cells in the peripheral blood of BP patients have been identified.³⁶ Taken together, these data support the concept that T_H2-mediated immune mechanisms may contribute to the pathogenesis of BP.

In conclusion, we demonstrate that circulating IgG4 and IgE antibodies are the major immunoglobulins preferentially targeting two distinct epitopes on BP180 NC16A (MCW-1 and MCW-2). In addition, we show that levels of IgG subclasses and IgE antibodies to BP180 NC16A parallel disease activity. In contrast, the fine specificity of autoantibodies to the different epitopes within BP180 NC16A does not change during the course of the disease.

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