

Demonstration of Epitope-Spreading Phenomena in Bullous Pemphigoid: Results of a Prospective Multicenter Study

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Bullous pemphigoid (BP), the most common autoimmune subepidermal bullous disease, is associated with an autoantibody response to BP180 and BP230, two components of junctional adhesion complexes in human skin promoting dermo-epidermal cohesion. Retrospective analyses demonstrated that these autoantigens harbor several epitopes targeted by autoaggressive B and T cells. The aim of this prospective multicenter study was to assess the evolution of IgG autoantibodies in 35 BP patients over a 12-month observation period. Epitope-spreading (ES) events were detected in 17 of 35 BP patients (49%). They preferentially occurred in an early stage of the disease and were significantly related to disease severity at diagnosis. Moreover, in three patients, spreading of IgG reactivity to intracellular epitopes of BP180 and BP230 was preceded by recognition of the BP180 ectodomain. Finally, IgG reactivity with extracellular epitopes of BP180 and intracellular epitopes of BP230 correlated with the severity of BP in disease course. These findings support the idea that IgG recognition of the BP180 ectodomain is an early and crucial event in BP disease, followed by variable intra- and intermolecular ES events, which likely shape the individual course of BP.

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INTRODUCTION

Bullous pemphigoid (BP) is the most common autoimmune bullous skin disease associated with a humoral immune response against components of the skin basement membrane zone, i.e., BP180 (collagen XVII, BP antigen 2) and BP230 (BP antigen 1-e; Labib *et al.*, 1986; Stanley *et al.*, 1988). BP180 is a type II transmembrane protein, which consists of a globular cytoplasmic domain and a large extracellular region containing 15 collagenous domains (Giudice *et al.*, 1992; Hirako *et al.*, 1996). BP230 is a cytoplasmic plakin protein family member with a central rod flanked by globular end domains (Stanley *et al.*, 1988; Green *et al.*, 1992). Most BP patients have IgG autoantibody and

T-cell response directed against an immunodominant extracellular domain of BP180, termed BP180-NC16A (Zillikens *et al.*, 1997; Nishie *et al.*, 2010). The pathogenic relevance of immunological recognition of BP180-NC16A is supported by the observation that experimental transfer of IgG against this domain into neonatal mice and hamsters elicits subepidermal blisters like those seen in patients with BP (Liu *et al.*, 1993; Yamamoto *et al.*, 2002). In addition, it has been shown that injection of IgG from BP patients' sera into BP180-humanized mice produces immunopathological alterations resembling those seen in BP (Nishie *et al.*, 2007). Moreover, inactivation of BP180-NC16A-reactive IgG with a recombinant NC16A decoy peptide blocks the pathogenic activity of IgG from the BP sera *in vivo*. Finally, an active BP mouse model has been recently established by transferring into Rag-2(–/–)/BP180-humanized mice splenocytes from wild-type mice grafted with human BP180-transgenic mouse skin (Ujiie *et al.*, 2010). Retrospective analyses in BP patients demonstrated that, in addition to the BP180-NC16A domain, there are several epitopes of BP180 and BP230 that are targeted by both autoaggressive B and T cells (Tanaka *et al.*, 1991; Murakami *et al.*, 1998; Perriard *et al.*, 1999; Skaria *et al.*, 2000; Di Zenzo *et al.*, 2004; Mariotti *et al.*, 2004; Thoma-Uszynski *et al.*, 2006).

The current model of chronic autoimmune diseases predicts that “dominant” self-epitopes induce the inflammatory cascade and tissue damage in the target organ. Recruitment

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Abbreviations: BP, bullous pemphigoid; ES, epitope spreading

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and activation of autoreactive T lymphocytes specific for “secondary” epitopes of the nominal autoantigen or on distinct self-proteins subsequently contribute to disease course (Chan *et al.*, 1998; Vanderlugt and Miller, 2002). This phenomenon is called epitope spreading (ES). There is growing evidence that inter- and intramolecular ES events are not simply an epiphenomenon but are critical in the evolution and progression of autoimmune diseases (Naserke *et al.*, 1998; Vader *et al.*, 2002; Arbuckle *et al.*, 2003; Di Lorenzo *et al.*, 2007; Griffiths, 2008).

The dynamics of IgG reactivity has been recently analyzed in a mouse model of BP, in which grafting of skin obtained from transgenic mice expressing human BP180 on wild-type mice resulted in triggering of an immune response to BP180. In this model, IgG reactivity with extracellular epitopes preceded IgG recognition of intracellular domain. Appearance of IgG autoantibodies directed against intracellular epitopes correlated with the development of graft loss (Di Zenzo *et al.*, 2010). A recent study that utilized a vast array of BP230 and BP180 peptides strongly suggested that BP patients have a specific IgG autoantibody profile, including IgG recognition of intracellular epitopes, which is detectable already at an early stage of the disease (Di Zenzo *et al.*, 2008). However, it is still not well understood whether IgG reactivity with BP180 and BP230 spreads toward other epitopes during the course of BP and whether recognition of different epitopes is related to disease severity.

In this multicenter prospective study, we have assessed the profile of IgG autoantibody response to distinct BP180 and BP230 epitopes during the clinical course of 35 BP patients. We have been able to detect ES events in up to 50% of the patients. Specifically, we observed intramolecular ES events consisting of early IgG reactivity with extracellular epitopes, which was followed by IgG reactivity with intracellular epitopes of BP180. Moreover, we also detected intermolecular ES consisting of an initial IgG reactivity with BP180 epitopes, with subsequent recognition of BP230 epitopes. Finally, we found that IgG reactivity with extracellular epitopes of BP180 and intracellular epitopes of BP230 correlated with the severity of BP during disease course, whereas IgG recognition of intracellular epitopes of BP180 did not.

RESULTS

ES in the BP patients

This prospective study was designed to characterize and correlate clinical and immunological parameters of BP patients at the time of diagnosis and during disease course. Therefore, we tested IgG reactivity against BP180 and BP230 recombinants (Figure 1) by different approaches before treatment, as described elsewhere (Di Zenzo *et al.*, 2008), as well as during the course of the disease. Thirty-five BP patients gave consent to be included in the follow-up study, and the dynamics of the humoral response over a 12-month period was investigated (Supplementary Table S1 online).

On performing ELISA, 17 of 35 (49%) BP patients were found to have developed IgG reactivity with epitopes that were not recognized at the time of first diagnosis (Table 1). In contrast, 51% of the studied BP patients did not

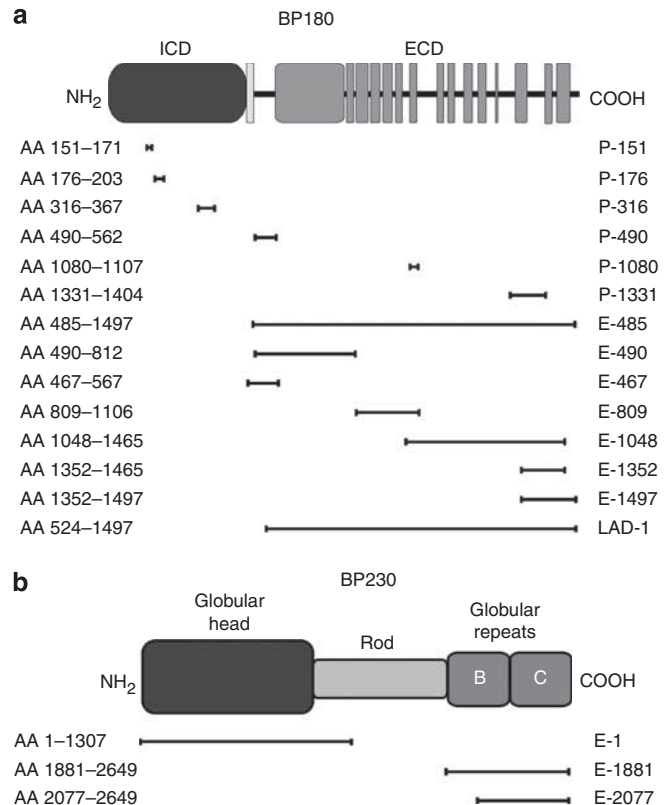


Figure 1. Bullous pemphigoid (BP)180 and BP230 recombinant proteins.

(a) Schematic representation of BP180, a type II transmembrane protein containing a globular intracellular domain and an extracellular domain with 15 interrupted collagenous regions. (b) Schematic representation of BP230, an intracellular hemidesmosomal component that consists of a central rod flanked by globular end domains. The nomenclature of the recombinant BP180 and BP230 fusion proteins, which are either expressed prokaryotically (P-) or eukaryotically (E-), follows the amino-acid residues (AA) of the reference protein. Linear IgA disease-1 (LAD-1) represents the shed ectodomain of BP180 that was prepared from conditioned keratinocyte medium and not a fragment produced as recombinant protein. ECD, extracellular domain; ICD, intracellular domain.

show a shifted recognition of BP180 and BP230 epitopes (Supplementary Table S2 online). All the 17 patients, in whom ES occurred, had IgG reactivity with one or more BP180 epitopes at the time of diagnosis, whereas 12 of 17 BP (71%) patients showed IgG reactivity with BP230 epitopes. In 17 of the studied patients, we observed 24 different ES events (considering as the same event the targeting of overlapping recombinants): in 12 of these cases, ES events involved extracellular epitopes and in the remaining 12 cases, intracellular ones (Table 1). In particular, ES events involved epitopes of: (1) the BP180 intracellular domain (5 of 24); (2) the mid-portion of the BP180 ectodomain (6/24); (3) the BP180 COOH-terminal region (4/24); and (4) the NH2 and COOH termini of BP230 (7/24). Noteworthy, ES events targeting epitopes of BP180-NC16A were observed in two patients. In contrast, patients who recognized BP180-NC16A at diagnosis showed ES events involving extracellular (BP180; *n*=8) and intracellular (BP180 and BP230; *n*=7) epitopes.

Table 1. Epitope spreading in 17 BP patients with IgG reactivity against BP180 and/or BP230

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Abbreviations: AA, amino acid; BP, bullous pemphigoid; E, eukaryotically; ES, epitope spreading; P, prokaryotically; R, relapse.

¹IgG reactivity against defined BP180 and/or BP230 recombinants is shown as gray boxes. ES is indicated in black. AA sequences of the recombinant proteins are shown in Figure 1.

Interestingly, most of the BP patients showed IgG reactivity with BP180-NC16A and additional epitopes, whereas the sera of three BP180-NC16A-negative BP patients (A12, B04, and E17) recognized other BP180 and BP230 epitopes at diagnosis (Supplementary Table S2 online).

Noteworthy, several patients showed IgA reactivity with linear IgA disease-1 (LAD-1) at baseline as detected by immunoblotting (11/35). In four BP patients (A01, C11, D03, and E17), IgG reactivity with BP180 and BP230 at time of diagnosis further spread to include IgA reactivity against LAD-1 within 1 to 12 months after diagnosis (Supplementary Table S2 online).

In three patients, IgG reactivity against the BP180 ectodomain precedes recognition of intracellular epitopes

In three BP patients (A11, B01, and C19), IgG reactivity was first specifically directed against the BP180 ectodomain, which was followed by the development of a reactivity with intracellular epitopes of BP180 and BP230 (Figure 2,

Supplementary Table S3 online). Initial IgG recognition of intracellular epitopes with subsequent appearance of reactivity with extracellular epitopes was never observed in the course of the disease.

Intermolecular IgG reactivity spreading from BP180 to BP230 epitopes

Most ES events during the course of BP occurred intramolecularly, i.e., IgG autoantibodies of BP180, and in four cases of BP230 epitopes (B06, C09, C12, and C15; Table 1). Intermolecular ES was detected in 3 of 17 BP cases (A11, C09, and C19; Table 1). Noteworthy, the IgG response was first directed against BP180 and subsequently targeted BP230 epitopes, whereas IgG recognition of BP230 epitopes never preceded that of BP180 epitopes (Table 1).

T-cell recognition of BP180 and BP230

We also investigated the dynamics of T-cell responses against BP180 and BP230 recombinants during the course of

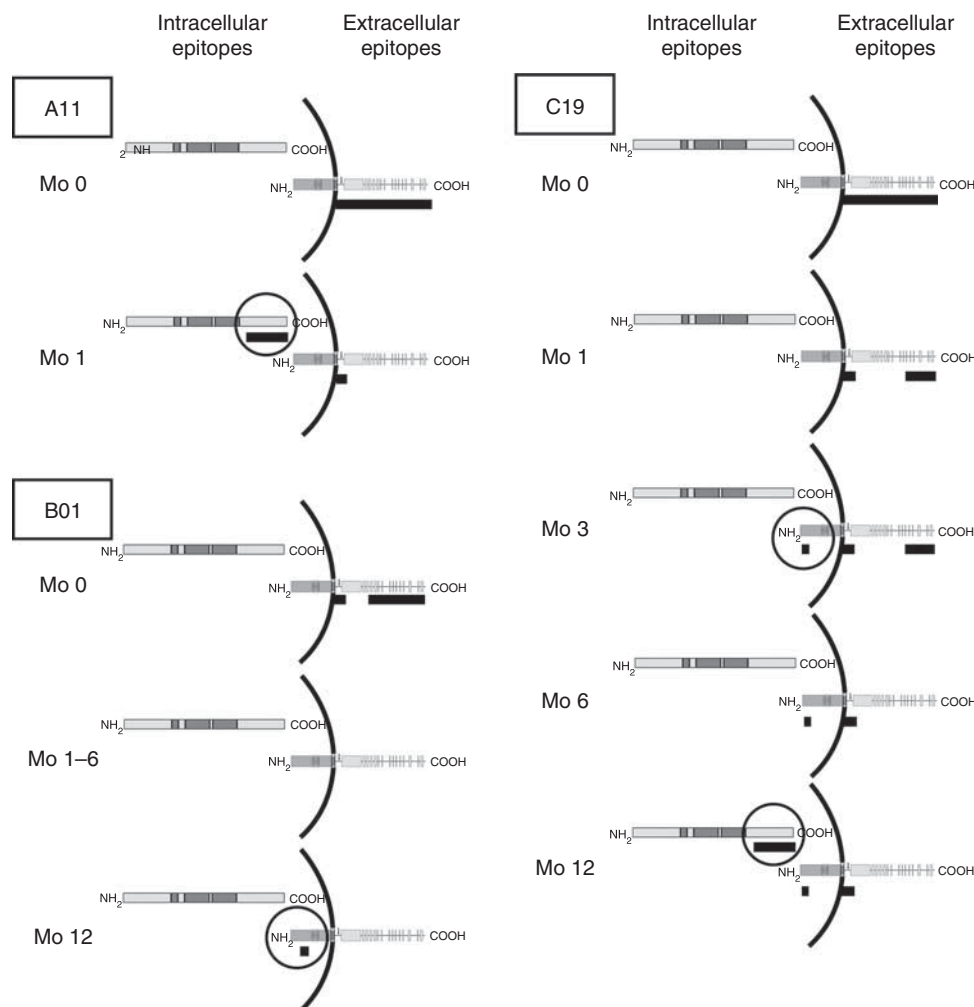


Figure 2. IgG recognition of the bullous pemphigoid (BP)180 ectodomain precedes reactivity against intracellular BP180 epitopes. Schematic representation of the IgG autoantibody reactivity of three bullous pemphigoid (BP) patients (A11, B01, and C19) against intracellular and extracellular domains of BP180 and intracellular epitopes of BP230 during the course of the disease. The IgG autoantibody profile at different time points (expressed in months) is shown. The targeted regions are highlighted as black bars. Secondary epitopes that arose as a consequence of epitope spreading are circled.

Table 2. Shifted T-cell recognition of BP180 and BP230 in five of nine patients with BP

Patient	Months	BP180 ¹						BP230 ¹		
		E 467	E 490	E 485	E 809	E 1048	E 1352	E 1	E 1881	E 2077
A08	0									
	1									
	6									
	12									
A11	0									
	1									
A12	0									
	1									
	6									
	12									
D02	0									
	1									
	6									
	12									
D11	0									
	1									
	6									

Abbreviations: AA, amino acid; BP, bullous pemphigoid; E-, eukaryotically; ES, epitope spreading; P-, prokaryotically; SI, stimulation index. ¹Significant T-cell reactivity against defined BP180 and/or BP230 recombinants is shown as gray boxes. ES is indicated in black. AA sequences of the recombinant proteins are shown in Figure 1. Proliferative T-cell responses were defined by an SI (ratio of [3H]thymidine uptake in T-cell cultures with/without Ag) ≥ 2.0 .

the disease in nine BP patients. Five of nine BP patients (A08, A11, A12, D02, and D11) showed a proliferative T-cell response against BP180 and/or BP230 before treatment (Table 2). Two of the five BP patients (A11 and D11) showed ES events. In particular, in one patient (A11), T cell-reactivity was initially directed against epitopes of the BP180 ectodomain and later on also involved the COOH-terminal region of BP230 (Table 2). The second BP patient (D11) first showed T-cell reactivity against BP180-NC16A, which shifted later on toward T-cell recognition of the mid-portion and COOH-terminal region of BP180 (Table 2). In the majority of the BP patients, autoreactive B and T cells showed a similar epitope profile during the disease course (Supplementary Table S4 online). In some patients, T-cell reactivity against the BP recombinants was strongly impaired by treatment with systemic glucocorticoids (Table 2).

ES events preferentially occur at an early stage of BP and are related to clinical activity and severity

Incidence of ES events was more frequent during the first 3 months after diagnosis of BP (incidence=0.26 person-month) than at later observation periods (i.e., 6–12 months; incidence=0.07 person-month; $P=0.0013$; (Figure 3a), strongly suggesting that ES events are an early event in BP. An association study aimed at determining whether specific clinical and immunological features of BP at the time of first diagnosis were related to ES events did not reveal a significant association between ES and age, sex, mucosal involvement, pruritus intensity, duration and extent of BP. There was also no significant association between ES events and IgG reactivity by ELISA and immunoblotting against distinct single or grouped BP180 and/or BP230 epitopes. Of note, the

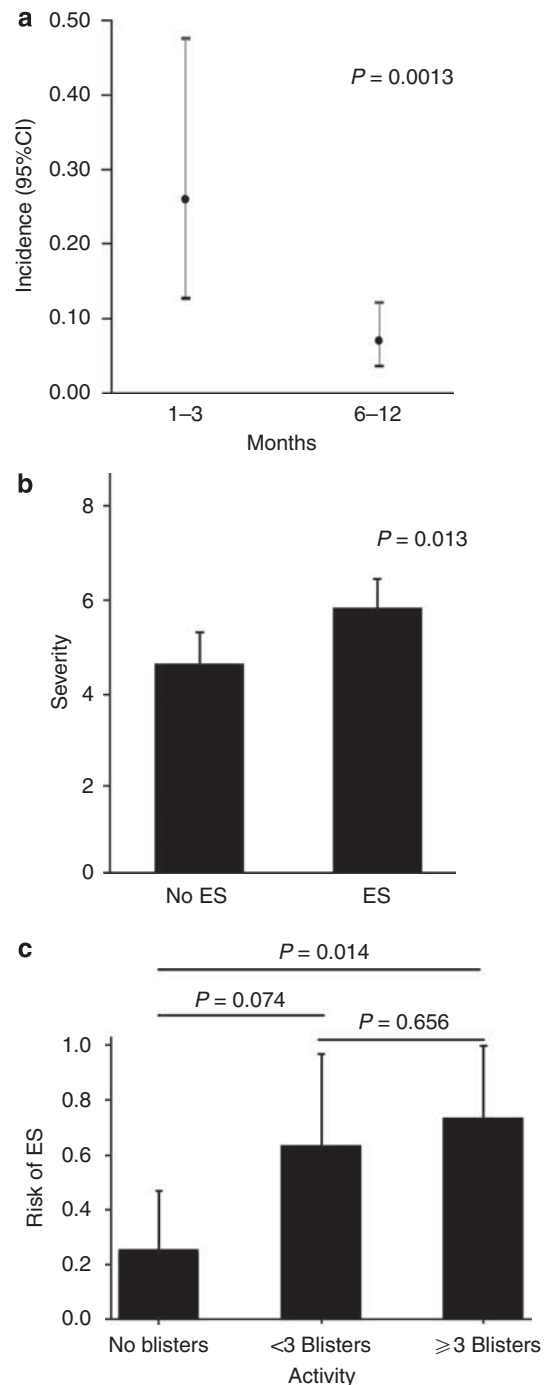


Figure 3. Epitope-spreading (ES) events preferentially occur early on and are correlated with the clinical activity and severity of bullous pemphigoid.

(a) The incidence of ES events was significantly ($P=0.0013$) different between months 1–3 (incidence=0.26 person-month, 95% confidence interval (CI): 0.12; 0.48) versus months 6–12 (incidence=0.07 person-month, 95% CI (0.03; 0.12)). (b) ES events assessed by ELISA correlated with disease severity ($P=0.013$). Error bars show mean and $\pm$ SE of disease severity in patients with and without ES. (c) ES phenomena assessed by ELISA appeared to be associated with disease activity, which was evaluated as the number of new blisters during two consecutive days ($P=0.034$). A bar plot shows the ES risk ($\pm$ SE) according to disease activity. When pairwise comparisons were considered, a difference for ES risk was observed between absence of blisters and ≥ 3 blisters ($P=0.014$). The association was statistically significant after taking into account Bonferroni correction for multiple comparisons.

occurrence of ES events appeared to be significantly related to the severity ($P=0.013$) and clinical activity ($P=0.014$) of BP at the time of diagnosis (Figure 3b and c). Finally, during disease course in a given BP patient, the level of IgG reactivity against epitopes targeted by ES decreased more rapidly than that against epitopes already recognized at diagnosis (Supplementary Table S5 online).

IgG reactivity with the BP180 ectodomain but not with intracellular epitopes of BP180 is related to the severity of BP

The present prospective study demonstrates a significant relationship between the extent of IgG autoantibody reactivity and the clinical severity of BP. On the basis of multiple comparison analyses, there was a significant correlation of disease severity with IgG reactivity against (1) BP180-NC16A (recombinants E-467, E-490, and P-490); (2) the entire BP180 ectodomain (E-485); (3) COOH-terminal regions of the BP180 ectodomain (E-1048 and E-1352); and (4) distinct BP230 domains (E-1 and E-1881; Figure 4). In contrast, IgG reactivity against intracellular BP180 epitopes (P-151, P-176, and P-316) and the mid-portion (E-809 and P-1080) of the BP180 ectodomain was not significantly related to disease severity (Figure 4).

DISCUSSION

The present prospective multicenter study shows for the first time that ES occurs early on in BP and is associated with a more severe disease at diagnosis. Moreover, in three patients, spreading of IgG reactivity to intracellular epitopes followed IgG recognition directed against the BP180 ectodomain. Moreover, this study shows that IgG reactivity with BP230 and extracellular, but not intracellular, epitopes of BP180 correlates with the severity of BP.

There is ample evidence obtained from studies in distinct autoimmune diseases indicating a crucial role of ES during the early stage of disease development (Brooks-Worrell *et al.*, 2001; Matsumoto *et al.*, 2007; Poole *et al.*, 2009). The current model of autoimmune disease predicts that the initial reactivity with the BP180 ectodomain induces tissue damage that subsequently leads to disease progression associated with further IgG recognition of intracellular epitopes. In particular, we have previously demonstrated that in BP, IgG binding to intracellular epitopes of BP180 and/or BP230 is detectable early in disease course (Thoma-Uszynski *et al.*, 2004; Di Zenzo *et al.*, 2004, 2007, 2008). In line with these data, the present study shows that (1) at diagnosis, almost all BP patients (34/35; 97%) presented IgG reactivity with the BP180 ectodomain, whereas 27 of 35 patients (69%) had IgG against intracellular epitope(s) (BP180 intracellular domain and/or BP230); (2) whereas only one BP patient serum (B04) exclusively bound to intracellular epitopes, eight patients (23%; A11, A19, B01, C02, C19, E09, E11, and E17) presented exclusive recognition of BP180 ectodomain at diagnosis; (3) in 18/35 BP patients, IgG recognition of multiple extracellular and intracellular epitopes was already detected at diagnosis and did not evolve with ES; and (4) when ES occurred (17/35 patients), it was preferentially an early event in the disease course. Altogether,

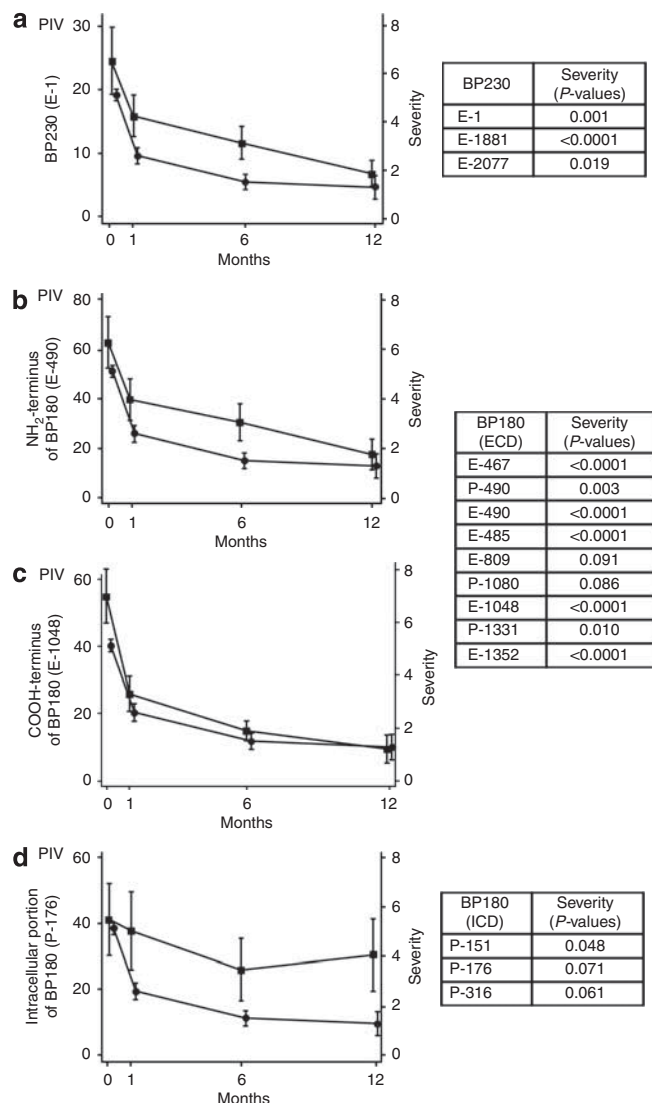


Figure 4. IgG reactivity against bullous pemphigoid (BP)230 and BP180 extracellular, but not intracellular, epitopes relates to the clinical severity of BP. Levels of IgG against: (a) BP230 (E-1); (b) the NH₂-terminal (E-490); and (c) the COOH-terminal region of the BP180 ectodomain (E-1048) paralleled the mean decrease of disease severity, whereas mean IgG levels against (d) the intracellular domain of BP180 (P-176) did not. Disease severity and serum IgG reactivity, expressed as pemphigoid index value (PIV), are shown as filled circles and squares, respectively. Error bars indicate mean $\pm$ SE. A longitudinal analysis (subject as fixed effect) conducted considering time points at months 0, 1, 6, and 12 is shown in tables on the right side of the figure. The associations that proved statistically significant after multiple comparison analyses (in bold) had a $P \leq 0.003$. ECD, extracellular domain; ICD, intracellular domain.

these findings indicate the idea that ES can occur also before the development of overt clinical manifestations of BP.

The dynamics of T-cell responses against BP180 and BP230 during the course of the disease were also investigated in selected BP patients. The data show that in two of five BP patients the proliferative T-cell response, which was initially directed against epitopes of the BP180 ectodomain, subsequently involved epitopes within BP230 or BP180

ectodomain. Noteworthy, in one of these two patients (A11), both B- and T-cell reactivity with the BP180 ectodomain was initially found, which subsequently spread involving BP230 as well. In addition, the comparison of B- with T-cell reactivity during disease course showed a similar epitope profile. These preliminary data support the relevance of T-B cooperation and possible interplay in the ES process.

A technical limitation in interpreting the present *ex vivo* T-cell proliferative responses to BP180 and BP230 was the significant impairment of this assay by systemically applied glucocorticoids, which are therapeutic mainstay in BP. In contrast, systemic glucocorticoid treatment had a more delayed inhibitory effect on serum IgG autoantibodies in the studied BP patients.

The different patterns of epitope recognition/spreading presumably shape the individual course of BP. Along this line, we have investigated a possible relationship between ES events and the clinical severity of BP. Interestingly, there was a significant relation between the occurrence of ES and both severity and clinical activity of BP at the time of first diagnosis. On the other hand, with the exception of two BP patients who showed an ES event during a relapse, we could not observe a significant association between ES and severity or remission during disease course. Evidences from animal models of autoimmune diseases indicate that ES may be due to the activation and recruitment of autoreactive T lymphocytes specific for epitopes that are distinct from the disease-inducing epitope. In this context, it is possible that during the disease course under immunosuppressive therapy, activation and recruitment of T and B cells are impaired, leading to a reduction of further ES events.

Several retrospective studies reported a direct correlation between the levels of anti-BP180-NC16A IgG autoantibodies and BP severity (Haase *et al.*, 1998; Schmidt *et al.*, 2000; Hofmann *et al.*, 2002). We have previously found a significant relation between clinical severity of BP at diagnosis and the levels of IgG reactivity with the NH₂- and COOH-terminal regions of the BP180 ectodomain (Di Zenzo *et al.*, 2008). In the present prospective study, IgG levels against the extracellular but not the intracellular domain of BP180 were related to the severity of BP. Specifically, a significant relation between clinical activity in disease course and reactivity to the NC16A domain and COOH-terminal region of the BP180 ectodomain was found. Furthermore, in line with a recent study (Charneux *et al.*, 2011), we have found that IgG levels against all the fragments comprising the BP180-NC16A domain were much higher than those measured for other epitopes of BP180 and BP230 (not shown). Finally, two of three BP180-NC16A-negative BP patients (A12 and E17) at diagnosis showed IgG reactivity with the COOH-terminal region of the BP180. Altogether, these findings extend previous observations and provide additional support to the idea that IgG autoantibodies against the BP180-NC16A domain and the COOH-terminal region of BP180 have a central role in the pathogenesis of BP.

It is still unclear as to whether IgG autoantibodies to BP230 are critical in the pathogenesis of BP. In the present

study, IgG reactivity against the cytoplasmic BP230 appeared to be related to the severity of BP. A possible pathogenic role of anti-BP230 IgG has been strongly suggested by the following observations: (1) immunization of rabbits with BP230-derived peptides appeared to increase the local inflammatory response following epidermal damage (Hall *et al.*, 1993) and (2) IgG antibodies against specific portions of BP230 were able to induce subepidermal blister formation (Kiss *et al.*, 2005). Additional support for a possible pathogenic role of anti-BP230 IgG is provided by the reported direct relationship between clinical severity of BP and IgG reactivity against BP230 (Yoshida *et al.*, 2006). In this context, it should be noted that in the present study, two of three BP180-NC16A-negative sera (A12 and B04) bound to BP230 epitopes at time of initial diagnosis. Hence, it is conceivable that, at least in a subset of patients, an autoantibody response to BP230 contributes to the pathogenesis of BP, e.g., by interfering with BP230 binding to keratin intermediate filaments, and thus leading to a loss of adhesion at the dermal-epidermal junction (Skaria *et al.*, 2000). In fact, several experimental data suggest an involvement of intracellular antigens in the development of autoimmune diseases. In particular, it has been reported that penetration of mature autoantibodies into living cells might participate in disease pathogenesis (Ruiz-Argüelles *et al.*, 2003).

In the majority of the patients, ES events occurred intramolecularly mainly involving IgG responses to BP180. Intermolecular ES was detected in three BP patients in whom IgG autoantibodies first targeted the BP180 ectodomain and only subsequently recognized intracellular epitopes of BP230 and in one case also of BP180. On the other hand, IgG recognition of the cytoplasmic BP230 autoantigen never preceded reactivity against BP180. IgG spreading from BP180 to BP230 might be attributed to the physical interaction between the NH₂-terminal portions of BP230 and BP180 (Borradori *et al.*, 1998). These findings are in line with a previous study aimed at investigating the dynamics of the IgG response against human BP180 in a mouse model, in which transgenic mouse skin expressing human BP180 was grafted on wild-type animals (Di Zenzo *et al.*, 2010). These mice developed first IgG against extracellular BP180 epitopes and thereafter IgG binding to intracellular epitopes. These findings are also in keeping with the current model of autoimmune disease that predicts an initial involvement of an extracellular immunodominant epitope (i.e. BP180 ectodomain), inducing tissue damage that eventually leads to disease progression associated with further IgG recognition of intracellular epitopes.

As for IgA response, in all four BP patients in whom IgG reactivity with BP180 and BP230 spread toward an IgA reactivity with LAD-1, the disease went concomitantly into remission. In addition, no significant association was detected between IgA reactivity and any clinical and immunological features, including disease severity and duration, mucosal lesions, and ES events (data not shown). These preliminary observations suggest a rather limited pathogenic role of IgA autoantibodies, which, however, remains to be further elucidated.

The sequential development of IgG reactivity with different epitopes and antigens during the course of the disease could represent an obstacle to the application of therapeutic approaches based on decoy peptides, tolerance inducing peptides, or antibody-specific immunoadsorption. In this context, the observation that the levels of IgG against epitopes targeted by ES decreased more rapidly than those against epitopes recognized at diagnosis could be of interest.

In conclusion, our study provides for the first time a comprehensive and prospective characterization of the evolution of humoral immune responses in BP patients. Altogether, our findings lend support to the concept that IgG recognition of the BP180 ectodomain is an early and crucial event in BP.

MATERIALS AND METHODS

Prospective multicenter observational study in BP

Thirty-five BP patients were recruited in a prospective multicenter study involving five European centers (Departments of Dermatology in Rome, Geneva, Freiburg, Erlangen, and Nice). Before inclusion, none of the patients had received any systemic immunosuppressive treatment. Controls ($n=50$) included patients with other bullous diseases and healthy volunteers. Time points for clinical and immunological assessment were months 0, 1, 3, 6, and 12, and in case of relapse. Diagnosis of BP was established in each center based on previously described criteria, and assessment of disease severity was based on disease activity, disease extent, and pruritus intensity, which was calculated on a scale ranging from 0 to 8, as previously described (Di Zenzo *et al.*, 2008). Clinical remission corresponded to the absence of signs of disease lasting for at least 2 weeks. Complete remission was defined as remission lasting for more than 3 months without immunosuppressive treatment. Clinical relapses were defined as the reappearance of clinical symptoms after the patient had attained remission, adapted from a previously introduced definition of clinical outcome parameters in pemphigus (Murrell *et al.*, 2008).

Some patients were followed for <12 months because of loss to follow-up, withdrawal of consent, or death (Supplementary Table S1 online). However, no differences between subjects with complete or partial follow-up with respect to age, sex, disease duration, severity, activity, and itch were observed. Therapeutic regimens were not defined by the study protocol and the choice of therapy, and dosage was dependent on disease severity, patient's conditions, drug side-effect profile, and the physician's evaluation. Twenty-four (69%) BP patients underwent clinical remission during the 1-year follow-up study. Two BP patients relapsed during the follow-up period (Supplementary Table S1 online). The study was conducted in accordance to the Declaration of Helsinki Principles, approved by the local Ethics Committees of the participating institutions, and all patients gave written informed consent.

Expression and purification of BP180 and BP230 recombinant proteins

Previously published recombinants of BP180 and BP230 were produced and expressed in prokaryotic (*E. coli*, DH5 α) and eukaryotic (baculovirus-infected Sf21 cells) systems as previously described (Thoma-Uszynski *et al.*, 2004, 2006; Di Zenzo *et al.*, 2008). For the production of the shedded BP180 ectodomain,

normal human keratinocytes were grown to subconfluence in a serum-free medium that was subjected to methanol-chloroform precipitation. After centrifugation, the pellets were dissolved in sample buffer for immunoblotting (Di Zenzo *et al.*, 2008).

ELISA and immunoblotting analyses with BP180 and BP230 recombinant proteins

ELISA and immunoblotting analyses were performed as previously described (Di Zenzo *et al.*, 2008). For detection of IgA reactivity against LAD-1 protein, extracts prepared as described above were separated on SDS-PAGE and transferred onto nitrocellulose. The filter was incubated at first overnight at 4 °C with sera of patients and controls diluted at 1:20 and then for 90 min with an alkaline phosphatase-linked goat antihuman IgA antibody (Sigma, St Louis, MO; Di Zenzo *et al.*, 2008). To reduce unspecific variability of IgG reactivity between different samples, all the sera from a given patient were run on the same ELISA plate. All extinction data were standardized based on the positive and negative internal standards, which were set to 100 and 0 optical density units, respectively, and termed pemphigoid index value. In addition, to show that ES events are not due to a random variation of total IgG, the IgG levels of three representative BP patients were measured (Supplementary Table S3 online) using a human IgG-specific ELISA as previously reported (Pinna *et al.*, 2009). Certified Reference Material 470 (ERMs-DA470, Institute for Reference Materials and Measurements, Geel, Belgium) was used as standard material for human IgG. Probably because of corticosteroid therapy, IgG levels showed a trend to decrease and this variation had no effect on the ES data (Supplementary Table S3 online). Of note, the 24 ES events detected in our 35 BP patients corresponded to an increase of IgG reactivity levels against a given epitope that was 1-fold, 2-fold, and more than 5-fold in 2, 3, and 19 cases, respectively.

T-cell proliferative assays with BP180 and BP230 recombinants

T-cell proliferative assays were performed as previously described (Thoma-Uszynski *et al.*, 2006). Briefly, peripheral blood mononuclear cell from BP patients were isolated and plated in triplicate at 10^5 cells per well in microtiter plates. BP180 and BP230 recombinants were added ($10 \mu\text{g ml}^{-1}$), phytohaemagglutinin (1%) and recombinant glutathione-S-transferase protein ($10 \mu\text{g ml}^{-1}$) were used as controls. After 6 days, each well was pulsed with 0.5 μCi [^3H]thymidine for 12 h, and incorporation was measured. BP180- or BP230-specific T-cell proliferation was expressed as a stimulation index, which is the ratio of [^3H]thymidine uptake in cultures with antigen and cultures with glutathione-S-transferase. Stimulation index values ≥ 2.0 were considered positive.

Statistical analyses

The incidence of ES events was calculated as the ratio of the number of ES events and the person-months, with the latter calculated under the hypothesis of uniform occurrence of event and censoring. A 95% confidence interval has been estimated under Poisson model. The association of ES (categorized as absent/present) with sex, age of the studied BP patients (three classes: 60–75, 76–85, and 86+ years), disease extent, activity, itch, clinical relapse, and mucosal involvement was assessed with χ^2 -test (or Fisher exact test when expected counts were <5). The association between ES and disease severity and activity was investigated using non-parametric methods

(Mann–Whitney) and χ^2 -test, respectively. As for disease activity, BP patients were grouped into three classes: 0 new blisters ($n=16$), <3 new blisters ($n=8$), and ≥ 3 new blisters ($n=11$), and for disease severity into two classes: 21 patients with disease severity scores ranging from 2 to 5 and 14 patients with disease severity scores ranging from 6 to 8. The within-subject relationship between disease severity in BP patients and level of IgG reactivity during the course of the disease has been assessed by the Wald test of the coefficient of fixed-effect regression models, each considering disease severity as outcome variable and IgG reactivity as independent variable. The Bonferroni approach was used to take into account multiple comparison problems. All analyses were performed using Stata statistical package (StataCorp, 2005).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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