

Sequential Intramolecular Epitope Spreading of Humoral Responses to Human BPAG2 in a Transgenic Model

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Bullous pemphigoid (BP) is a subepidermal autoimmune disease characterized by a humoral response to an epidermal basement membrane (BM) component, BP antigen 2 (BPAG2). BP patients have IgG autoantibodies against an immunodominant BPAG2 extracellular domain termed NC16A as well as additional epitopes located both in the intracellular and extracellular domains (ICD and ECD, respectively) of this autoantigen. To study the evolution of humoral responses to BPAG2, sequential serum samples obtained from C57BL/6Ncr mice grafted with otherwise syngeneic skin from transgenic mice expressing human BPAG2 (hBPAG2) in epidermal BM were studied for IgG reactivity to seven ECD and ICD hBPAG2 epitopes. All grafted mice developed specific IgG against hBPAG2 ECD and ICD epitopes. In seven of eight mice, anti-hBPAG2 IgG was initially directed against ECD epitopes; in six mice, humoral responses subsequently targeted additional ECD and ICD BPAG2 epitopes. In contrast to IgG specific for ECD epitopes, IgG against ICD epitopes was present at lower levels, detectable for shorter periods, and non-complement fixing. Interestingly, the appearance of IgG directed against ICD epitopes correlated with the development of graft loss in this experimental model. These studies provide a comprehensive and prospective characterization of the evolution of humoral immune responses to hBPAG2 *in vivo*.

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INTRODUCTION

Bullous pemphigoid (BP) is a subepidermal autoimmune bullous disease of the skin and mucosae characterized by a humoral response against structural components of the cutaneous basement membrane (BM): BP antigen 2 (BPAG2, BP180 or collagen XVII) and BP antigen 1 (BPAG1, BP230) (Labib *et al.*, 1986; Stanley *et al.*, 1988). BPAG2 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; Schäcke *et al.*, 1998). BPAG1 is a cytoplasmic plakin protein family member with a central rod flanked by globular end domains (Stanley *et al.*, 1988; Sawamura *et al.*, 1991).

Although the role of autoantibodies directed against BPAG1 remains to be clarified, the pathogenic relevance of anti-BPAG2 autoantibodies is supported by several experimental observations: (1) passive transfer of experimental IgG against the murine homolog of the immunodominant domain of human BPAG2 (hBPAG2) elicits subepidermal blisters in neonatal mice like those seen in patients with BP (Liu *et al.*, 1993); (2) dermal–epidermal separation is also observed in neonatal hamsters after passive transfer of rabbit anti-BPAG2 antibodies (Yamamoto *et al.*, 2002); (3) levels of circulating IgG anti-BPAG2 autoantibodies in patients with BP are directly related to the disease activity (Haase *et al.*, 1998; Schmidt *et al.*, 2000; Amo *et al.*, 2001; Hofmann *et al.*, 2002; Di Zenzo *et al.*, 2008b); and (4) injection of IgG from patients with BP into BPAG2-humanized mice produces clinical, histological, and immunopathological alterations like those seen in affected individuals (Nishie *et al.*, 2007). Most BP patients have IgG autoantibodies directed against an immunodominant extracellular domain (ECD) of BPAG2 termed NC16A (amino acid (AA) 490–562) (Zillikens *et al.*, 1997). However, additional epitopes located both in the intracellular domain (ICD) and the ECD of BPAG2 are also recognized by BP sera (Murakami *et al.*, 1998; Egan *et al.*, 1999; Nie and Hashimoto 1999; Perriard *et al.*, 1999; Di Zenzo *et al.*, 2004). The current model of chronic autoimmune disease involves loss of tolerance to “dominant” self-epitopes that induce inflammatory cascades and tissue

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Abbreviations: AA, amino acid; BM, basement membrane; BP, bullous pemphigoid; BPAG1, BP antigen 1; BPAG2, BP antigen 2; ECD, extracellular domain; ES, epitope spreading; GST, glutathione-S-transferase; hBPAG2, human BPAG2; ICD, intracellular domain; IF, immunofluorescence; Tg, transgenic; Wt, wild type

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damage in the target organ. In this model, disease progression is thought to be associated with recruitment and activation of autoreactive lymphocytes specific for “secondary” epitopes on the disease-inducing antigen or on different self-proteins (Chan *et al.*, 1998). This phenomenon of disease progression is called epitope spreading (ES). In line with this model, it has been hypothesized that autoimmune responses in patients with BP first target BPAG2 ECD epitopes then expand to include reactivity against ICD epitopes as well.

Recent studies have shown that grafts of skin from transgenic (Tg) mice expressing hBPAG2 in epidermal BM elicit experimental humoral immune responses against hBPAG2 in wild-type (Wt) mice that show fidelity to those seen in patients with BP (Olasz *et al.*, 2007). To investigate the dynamics of humoral immune responses against hBPAG2 in this model as well as the potential sequence by which ES occurs *in vivo*, serum samples were obtained from Wt mice at various time points after placement of hBPAG2 Tg skin grafts and then tested for reactivity against seven BPAG2 epitopes known to be recognized by patients with BP and other pemphigoid forms (Di Zenzo *et al.*, 2004, 2007, 2008b; Mariotti *et al.*, 2004; Calabresi *et al.*, 2007). These studies show that mice grafted with hBPAG2 Tg skin develop circulating IgG reactive with the majority of BPAG2 epitopes tested and that reactivity against hBPAG2 ECD epitopes precedes that against ICD epitopes.

RESULTS

Sera from Wt mice grafted with Tg skin contain IgG that recognizes numerous ECD and ICD hBPAG2 epitopes

Wild-type C57BL/6Ncr mice grafted with hBPAG2 Tg (but not syngeneic Wt) skin (Supplementary Figure S1) develop IgG that binds hBPAG2, including its NC16A immunodominant domain (Olasz *et al.*, 2007). As adsorption of immune sera with NC16A did not completely remove its reactivity against full-length hBPAG2, it was evident that such sera contained IgG targeting other domains of this antigen (Olasz *et al.*, 2007). To characterize further humoral immune responses to hBPAG2 in this experimental model, the reactivity of IgG from eight Wt mice grafted with hBPAG2 Tg skin 40 or 100 days earlier was analyzed in indirect immunofluorescence (IF) microscopy studies of 1 M NaCl split skin and ELISAs using seven previously characterized ICD and ECD hBPAG2 epitopes as target antigens (Figure 1). These studies found that all sera displayed high titers of IgG reactive with the epidermal side of 1 M NaCl split human skin (titers ranging from 640 (mouse 8) to 5120 (mice 1, 2, 3, and 5)) (Table 1 and Figure S2). No grafted mouse sera analyzed (all blood drawn and mice reported in Table 1) develop IgA or IgE against hBPAG2 (data not shown). In addition, sera from mice bearing Tg skin grafts were found capable of fixing C3 to epidermal BM as assessed by indirect IF microscopy (data not shown). As shown previously (Olasz *et al.*, 2007), immune sera from Tg graft recipients displayed no reactivity to the epidermal BM of Wt C57BL/6Ncr mice. Moreover, sera from Wt mice grafted with gender-matched syngeneic Wt skin showed no reactivity to human epidermal BM by indirect IF microscopy or to numerous hBPAG2

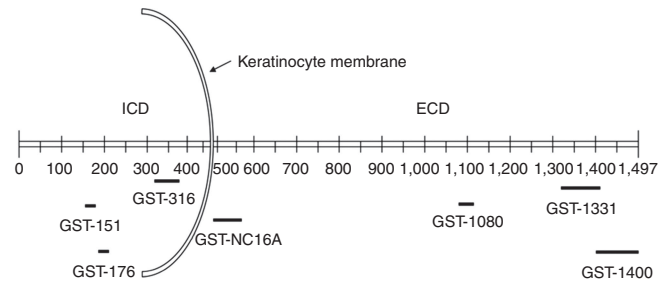


Figure 1. The seven human BPAG2 (hBPAG2) epitopes studied by ELISA.

Schematic diagram representing hBPAG2 and the position of the seven fragments produced as glutathione-S-transferase (GST) fusion proteins for use in this study. Each epitope is termed as GST followed by the number of first amino acid residue. GST-151, represents a fragment of hBPAG2 encompassing amino acid (AA) residues from 151 to 170; GST-176, AA 176–203; GST-316, AA 316–367; GST-NC16A, AA 490–562; GST-1080, AA 1080–1107; GST-1331, AA 1331–1404; GST-1400, AA 1400–1497. ICD, intracellular domain; ECD, extracellular domain.

recombinant peptides by ELISA. Interestingly, all sera from hBPAG2 skin graft recipients contained IgG that bound ECD epitopes encompassing AA residues 490–562 (glutathione-S-transferase (GST)-NC16A), 1080–1107 (GST-1080), and 1331–1404 (GST-1331). However, only one mouse had IgG that bound a peptide corresponding to the carboxyl terminus of BPAG2 (that is, AA 1400–1497 (GST-1400)). Epitopes encompassing ICD residues AA 151–170 (GST-151) and AA 316–367 (GST-316) were recognized by IgG from five of eight and seven of eight hBPAG2 skin graft recipients, respectively; no serum samples in this series contained IgG that reacted with ICD epitope AA 176–203 (GST-176) (Table 1). The frequency of reactivity of sera from hBPAG2 graft recipients showed no obvious correlation with the degree of homology between murine and hBPAG2 AA sequences (Figure S3).

Anti-hBPAG2 IgG in Wt mice grafted with Tg skin develops first and foremost against ECD epitopes

Earlier studies have shown that Wt mice grafted with syngeneic hBPAG2 Tg skin develop high (that is, ≥ 640) and durable (that is, at least as long as 380 days) titers of anti-hBPAG2 IgG starting 16 ± 2 days after graft placement (Olasz *et al.*, 2007). The comprehensive ELISA studies described above show that IgG in grafted mice recognize a variety of ECD and ICD BPAG2 epitopes, including its NC16A immunodominant domain. To define in detail the dynamics of humoral immune responses against hBPAG2, serum samples were obtained from eight Wt mice at specific time points after placement of Tg skin grafts and tested for evidence of IgG reactivity against seven hBPAG2 epitopes by ELISA. Although no anti-hBPAG2 IgG was detectable in grafted mice at baseline (that is, the day of grafting), specific IgG appeared in all eight mice 15–20 days after graft placement and bound ECD epitopes GST-NC16A, GST-1080, and GST-1331 (Table 2). ES is defined as occurring when IgG seroreactivity against a given hBPAG2 epitope is followed by IgG reactivity against a different epitope. In seven of eight mice ES events occurred, in particular serologic reactivity to hBPAG2 was found to extend to ICD

Table 1. Sera from Wt mice grafted with Tg skin contain IgG that recognizes human epidermal BM and numerous intra- and extracellular hBPAG2 domains

Mouse serum	IIF titer	GST-151	GST-176	GST-316	GST-NC16A	GST-1080	GST-1331	GST-1400
1	5120	+	—	+	+	+	+	+
2	5120	—	—	+	+	+	+	—
3	5120	—	—	+	+	+	+	—
4	1280	+	—	+	+	+	+	—
5	5120	+	—	+	+	+	+	—
6	2560	—	ND	—	+	+	+	—
7	2560	+	ND	+	+	+	+	—
8	640	+	ND	+	+	+	+	—
C ¹	Neg	—	—	—	—	—	—	—
Positive sera		5/8	0/5	7/8	8/8	8/8	8/8	1/8

BM, basement membrane; GST, glutathione-S-transferase; hBPAG2, human BPAG2; IIF, indirect immunofluorescence; ND, not done; Neg, negative; Tg, transgenic; Wt, wild type.

¹Representative control serum from a Wt mouse grafted with Wt skin.

Each serum was assayed in duplicate and the mean OD at 450 nm, obtained with GST protein alone (with the correlation wavelength set at 620 nm), was subtracted from the mean reading obtained with GST epitope. Sera from grafted mice were collected 100 (mice 1, 2, 3, 5, 7, and 8) or 40 (mice 4 and 6) days after grafting.

and/or different ECD epitopes between 20 and 60 days after graft placement (Table 2). More specifically, in six of eight mice, anti-hBPAG2 IgG spread toward an ICD epitope (that is, mice 1, 2, 4, 5, 7, and 8) within 40–100 days of grafting or a different ECD epitope (that is, mice 1, 4, 5, 6, 7, and 8) within 20–60 days of grafting (Table 2). Interestingly, ES of serologic reactivity from one ICD epitope to another was never detected. These longitudinal epitope mapping studies also found that the time of appearance of anti-hBPAG2-ICD IgG closely approximated the date when Tg skin grafts were deemed lost (range, 13 days before to 4 days after graft loss in four of six mice (mice 1, 2, 4, and 5)) (Table 2). It was not possible to assess the correlation between the development of anti-hBPAG2-ICD IgG and graft loss in two mice (mice 7 and 8) due to the lack of blood samples from relevant time points.

In contrast to anti-hBPAG2-ECD IgG in Tg graft recipients, IgG against ICD epitopes were present at lower levels, detectable for shorter periods, and non-complement fixing

The consistency of humoral immune responses in this grafting model coupled with the ability to use low volumes of serum in hBPAG2-specific ELISAs facilitated longitudinal studies of humoral immune responses in Wt mice grafted with Tg skin. Studies of this sort found that levels of IgG directed against hBPAG2 ICD epitopes were lower and less durable than those against ECD epitopes. More specifically, IgG reactivity against ICD epitope GST-316 decreased and then disappeared in three mice (mice 3, 7, and 8) 130–370 days after grafting, whereas IgG directed against ECD epitopes GST-NC16A, GST-1080, and GST-1331 remained high (Figure 2 and data not shown). Similarly, the reactivity against the ICD epitope GST-151 disappeared in three mice (mice 4, 7, and 8) between days 100 and 570, when reactivities against ECD epitopes (GST-NC16A, GST-1080, and GST-1331) remained high (Figure 2 and data not shown). Moreover, in five mice

(mice 1, 3, 5, 7, and 8), relative levels of IgG against ICD epitopes were lower than those against ECD ones (Figure 2 and data not shown). Finally, ELISA studies of sera from three representative graft recipients (mice 1, 2, and 5) found that IgG against hBPAG2 ECD epitopes (GST-NC16A and GST-1331) was able to fix complement, whereas IgG against ICD epitopes (GST-151 and GST-316) was not (Figure 3). The ability of mouse sera to fix complement was also confirmed by complement-fixing IF assay (data not shown). Interestingly, the analysis of IgG subclasses showed that the complement-fixing subclasses (IgG2a and 2b) reacted both with ICD (GST-151) and ECD (GST-NC16A) epitopes in mouse 5, whereas recognized only ECD epitopes (GST-1331) in mouse 2 and ECD (GST-NC16A) and only one of two ICD epitopes (GST-151) in mouse 1 (see inserts of Figure 3). To extend this study, we then performed a sequential analysis of IgG subclasses in two grafted mice (mouse 1 and 4) during the evolution of humoral immune response against four representative epitopes (GST-151, GST-316, GST-NC16A, and GST-1331) of hBPAG2. At all the time points positive for IgG reactivity against hBPAG2, complement-fixing IgG subclasses bound to ECD epitopes (GST-NC16A and GST-1331), whereas did not recognize one out of two ICD epitopes studied (GST-316 in mouse 1 and GST-151 in mouse 4) (Supplementary Table 1). These data suggest that mouse sera prevalently contain complement-fixing IgG subclasses reacting with ECD epitopes and that hBPAG2 epitopes may possess different biological capabilities.

DISCUSSION

This study characterizes in detail the specific evolution of humoral immune responses to hBPAG2 in an *in vivo* experimental model in which the immunizing antigen is located in its natural context (that is, within epidermal BM). These studies found that (1) Wt mice grafted with hBPAG2 Tg

Table 2. Sequential studies of sera from Wt mice grafted with Tg skin show evolution of humoral immune responses against hBPAG2

Mouse number	T ¹	GST-151 ²	GST-176	GST-316	GST-NC16A	GST-1080	GST-1331	GST-1400
1 (d 73 ³)	0	—	—	—	—	—	—	—
	20	—	—	—	+	+	+	—
	40	—	—	—	+	+	+	—
	60	±	—	±	+	+	+	±
	100	+	—	+	+	+	+	+
	130	+	—	+	+	+	+	+
	145	+	—	+	+	+	+	+
2 (d 36)	0	—	—	—	—	—	—	—
	20	—	—	—	+	+	+	—
	40	—	—	±	+	+	+	—
	60	—	—	+	+	+	+	—
	100	—	—	+	+	+	+	—
	130	—	—	+	+	+	+	—
	145	—	—	+	+	+	+	—
3 (d 50)	0	—	—	—	—	—	—	—
	20	—	—	+	+	+	+	—
	40	—	—	+	+	+	+	—
	60	—	—	+	+	+	+	—
	100	—	—	+	+	+	+	—
	130	—	—	—	+	+	+	—
	145	—	—	—	+	+	+	—
4 (d 50)	0	—	—	—	—	—	—	—
	20	—	—	—	+	—	+	—
	40	±	—	±	+	±	±	—
	60	+	—	+	+	+	+	—
	100	—	—	+	+	+	+	—
	130	—	—	+	+	+	+	—
	145	—	—	+	+	+	+	—
5 (d 36)	0	—	—	—	—	—	—	—
	20	—	—	—	—	—	+	—
	40	±	—	±	±	±	+	—
	60	+	—	+	+	+	+	—
	100	+	—	+	+	+	+	—
	130	+	—	+	+	+	+	—
	145	+	—	+	+	+	+	—
6 (d 29)	0	—	ND	—	—	—	—	—
	15	—	ND	—	—	+	—	—
	20	—	ND	—	±	+	±	—
	40	—	ND	—	+	+	+	—
7 (d 21)	0	—	ND	—	—	—	—	—
	15	—	ND	—	—	+	+	—
	20	—	ND	—	±	+	+	—
	100	±	ND	±	+	+	+	—

Table 2 continued on the following page

Table 2. Continued

Mouse number	T ¹	GST-151 ²	GST-176	GST-316	GST-NC16A	GST-1080	GST-1331	GST-1400
	235	+	ND	+	+	+	+	–
	370	–	ND	+	+	+	+	–
	570	–	ND	–	+	+	+	–
8 (d 27)	0	–	ND	–	–	–	–	–
	15	–	ND	–	+	–	–	–
	20	ND	ND	–	+	±	±	ND
	100	±	ND	±	+	+	+	–
	235	+	ND	+	+	+	+	–
	370	+	ND	–	+	+	+	ND
	570	–	ND	–	+	+	+	–

d, day; GST, glutathione-S-transferase; hBPAG2, human BPAG2; ND, not done; Tg, transgenic; Wt, wild type.

¹Time points after grafting.

²Epitope encompassing amino acid (AA) residues 151–170 (GST-151) produced as GST fusion (see the Result section for the others). Epitope spreading phenomena are underlined.

³The time of graft loss in recipients is indicated in parenthesis (d, days between graft placement and loss).

Each serum was assayed in duplicate and the mean OD at 450 nm, obtained with GST protein alone (with the correlation wavelength set at 620 nm), was subtracted from the mean reading obtained with GST epitope.

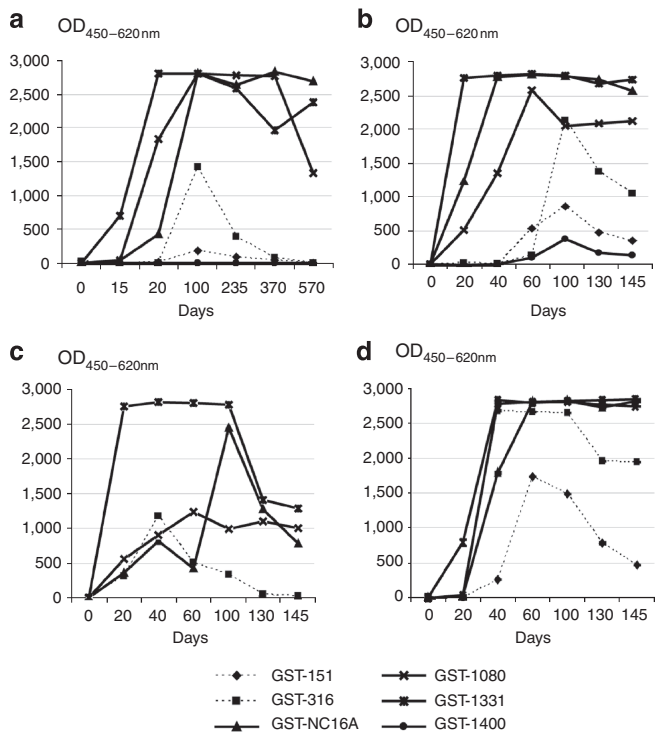


Figure 2. Analysis of sera from grafted mice showed that levels of IgG against ICD epitopes were lower and less durable than those against ECD epitopes. IgG against specific human BPAG2 (hBPAG2) epitopes at various time points after grafting was monitored by ELISA. Longitudinal studies of four representative mice (a, mouse 7; b, mouse 1; c, mouse 3; d, mouse 5) are shown. Summary data show evolution of serologic reactivity against ECD (black line) and ICD (dotted line) epitopes. The y axis corresponds to optical density at 450 nm with the correlation wavelength set at 620 nm.

skin developed circulating IgG reactive with the epidermal side of 1 M NaCl split human skin as well as the majority of hBPAG2 epitopes tested; (2) intramolecular ES of humoral

responses to hBPAG2 occurred in six of eight grafted mice; (3) during ES, reactivity against hBPAG2 ECD epitopes preceded those against ICD epitopes; (4) when grafted mice developed humoral responses against hBPAG2, IgG against ICD epitopes (in comparison with that against ECD epitopes) was of lower titer, lesser duration, and non-complement fixing; and (5) the appearance of IgG directed against hBPAG2 ICD epitopes correlated with the development of graft loss. Moreover, five of seven hBPAG2 epitopes were recognized by sera from the majority of mice grafted with hBPAG2 Tg skin, showing that the reactivity developed against hBPAG2 in this model was not restricted to its NC16A immunodominant domain—findings that again highlight the similarity between immune responses in this experimental model and those seen in patients with BP.

The grafting model used in this study allowed the development of experimental immune responses to BPAG2 in epidermal BM without the influence of adjuvants or potentially biasing immunization strategies. This approach allowed the dynamics of humoral immune responses to this target antigen to be defined prospectively over time and compared with observations made in patients with BP. Indeed, the finding that IgG reactivity against hBPAG2 ECD epitopes in grafted mice preceded (and predominated over) that against ICD epitopes is in line with recent observations in BP patients, namely, that humoral immune responses against BPAG2 initially target ECD epitopes such as NC16A, and later are directed against ICD epitopes of BPAG2 and BPAG1 (Di Zenzo *et al.*, 2008a). The observed sequence of ES might have been due to delayed exposure of hBPAG2 ICD epitopes to the immune system. Delayed exposure of ICD epitopes may also explain the lower and less durable levels of specific IgG against this portion of the molecule (in contrast to IgG against ECD epitopes). Of note, the spread of humoral immune responses in this graft model was exclusively

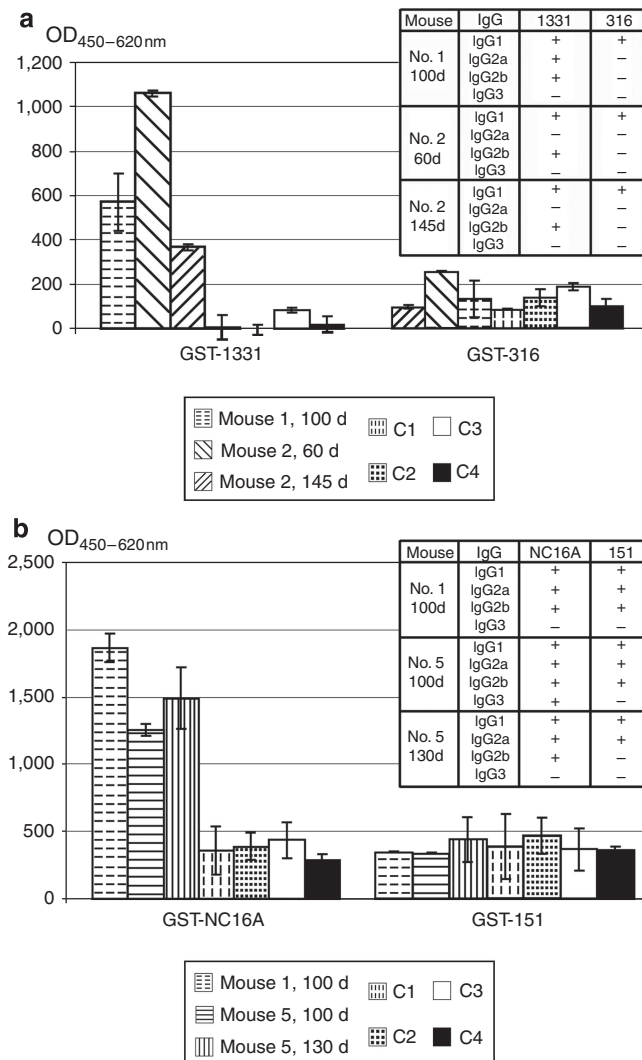


Figure 3. Analysis of sera from grafted mice showed that IgG against ECD epitopes was complement fixing in contrast to IgG against ICD epitopes.

Sera from three representative mice (mice 1, 2, and 5) were analyzed at different time points after grafting for their ability to fix complement on BPAG2 ECD (GST-1331 (a) and GST-NC16A (b)) and ICD (GST-316 (a) and GST-151 (b)) epitopes. The sera OD readings from mouse 1 at day 100, 2 at day 60 and 145, and 5 at day 100 and 130 were higher than cutoff values calculated for GST-1331 and GST-NC16A ELISAs and lower than cutoff values calculated for GST-151 and GST-316 ELISAs. In total, 4 representative normal mice sera out of 10 used as controls are shown. The results are the mean of data from two or four independent experiments. In the inserts are shown the IgG subclass distribution of the mice used in complement fixation studies. d, day; GST, glutathione-S-transferase.

target-specific (that is, hBPAG2); in fact, there is no loss of tolerance against mouse BM epitopes in this model (for example, mouse BPAG1, mouse BPAG2, and others, data not shown). In addition, the kinetics of graft loss in this model is completely different from alloantigen-related graft rejection, which is extensively shown in a previous paper (Olasz *et al.*, 2007). The histology of the graft loss in this model is nothing like GVHD-related graft loss (for example, no interface

dermatitis, no necrotic keratinocytes in the epidermis, and no satellite cell necrosis). Furthermore, we have shown that the kinetics of Tg skin graft loss on MHC I^{-/-} recipients (that is, mice with no CD8 cells) are the same as that noted in Wt recipients, and that graft loss in CD4^{-/-} mice was much delayed and associated with the appearance of anti-BM IgG.

Altogether, these findings have potential implications for the understanding of antibody-mediated tissue injury in this and other animal models, as well as in patients with immune-mediated subepidermal blistering diseases. In addition, the correlation between the appearance of anti-ICD IgG and graft loss may signify that antibodies directed against hBPAG2 ECD epitopes (in conjunction with complement) may have injured basal keratinocytes, and “exposed” hBPAG2 ICD epitopes. These findings, together with recent observations in BP patients showing that IgG against BPAG2 ICD epitopes are detectable early in disease (Di Zenzo *et al.*, 2004, 2008a, 2008b), raise the possibility that the development of IgG against ICD epitopes may correlate with the initial phase of the disease when the tissue damage starts occurring.

The demonstration that Wt mice grafted with Tg skin sequentially and rapidly develop different populations of IgG reactive with BPAG2 epitopes suggests that the therapeutic use of decoy peptides to block autoantibody binding *in vivo* may be difficult (Nishie *et al.*, 2007). Indeed, such interventions may require early and aggressive administration of several different peptide decoys other than those that correspond to the NC16A immunodominant portion of BPAG2. Relatedly, it is possible that the administration of decoy peptides may inadvertently enhance unwanted immune responses to hBPAG2. The availability of experimental animal models and sensitive screening assays for the detection of specific IgG will allow future studies to explore these questions directly.

MATERIALS AND METHODS

Skin grafting studies and sera sampling

Tail skin from hBPAG2 Tg (Olasz *et al.*, 2007) and Wt (control) C57BL/6Ncr mice was grafted onto the backs of age- and gender-matched Wt recipients (Rosenberg *et al.*, 1994). Bandages were removed at day 7 and grafts were inspected daily until day 30, then weekly, for viability and size. Skin grafts were graded as “lost” if their area became 20% or less of their original size (McFarland *et al.*, 2003). Longitudinal studies were conducted on 53 serum samples obtained at different time points from eight grafted mice (Table 2). All experimental studies in animals were approved by the Animal Care and Use Committee at the Medical College of Wisconsin.

IF microscopy

Direct IF microscopy studies of murine skin cryosections were performed as previously described (Olasz *et al.*, 2007). Serial dilutions of murine sera were tested against cryosections of intact or 1 M NaCl split human skin by indirect and complement-binding IF microscopy as described previously (Hodge *et al.*, 1978). Fluorescein-conjugated secondary antibodies diluted 1:20 (rat anti-mouse IgE (γ chain specific) and goat anti-mouse IgA (α chain specific) (SouthernBiotech, Chicago, IL)) were used.

Production of hBPAG2 peptides

Recombinant peptides corresponding to different portions of hBPAG2 ICDs and ECDs (Figure 1) were produced as previously described (Mariotti *et al.*, 2004; Calabresi *et al.*, 2007; Di Zenzo *et al.*, 2008b).

ELISAs

Immunomicrotiter plates (Maxisorb Immunoplate, Nunc, Wiesbaden, Germany) were coated with GST recombinant proteins (400 ng of GST-NC16A or a molar equivalent of other proteins) and a molar equivalent of GST in 100 μ l of coating buffer (50 mM NaHCO₃, pH 9.6) overnight at 4°C. Wells were then washed (phosphate-buffered saline containing 0.05% Tween 20) and blocked (5% dry no-fat milk in phosphate-buffered saline containing 0.05% Tween 20). Sera from grafted mice (diluted 1:100 and supplemented with bacterial extracts) were added to each well and incubated for 1 h at 37°C. After washing, horseradish peroxidase-conjugated anti-mouse IgG (Sigma, St Louis, MO), diluted 1:30,000, was added for 1 h at 37°C. Wells were washed and developed with 3,3',5,5' tetramethylbenzidine (Sigma). The reaction was stopped after 20 minutes, and OD at 450 nm, with the correlation wavelength set at 620 nm, was measured using a microplate reader (Bio-Rad Laboratories, Hercules, CA). For each serum assayed in duplicate, the mean OD reading obtained with GST was subtracted from the mean reading obtained with each GST-fusion protein. To measure the complement-fixing ability of IgG from grafted mice, after serum incubation and washing, a fresh source of mouse complement (control mouse serum diluted 1:20) was added to each well for 1 h at 37°C. After washing, peroxidase-conjugated anti-mouse C3 (Cappel Lab, Cochranville, PA), diluted 1:10,000, was added for 1 h at 37°C, washed and developed as above. The cutoff values were determined as the mean OD readings obtained with 28 and 10 control mouse sera (Wt and Wt grafted mice) + 3 standard deviations for ELISA based on hBPAG2 epitopes and complement-fixing ELISA, respectively. Finally, the subclass distribution of the IgG response to BPAG2 was assessed by ELISA with grafted mouse sera diluted 1:100. After serum incubation (for 1 h at 37°C) and washing, the antibodies against IgG subclasses (goat anti-mouse IgG1 (γ 1 chain specific) diluted 1:5000; goat anti-mouse IgG2a (γ 2a chain specific) diluted 1:2000; goat anti-mouse IgG2b (γ 2b chain specific) diluted 1:2000; goat anti-mouse IgG3 (γ 3 chain specific) diluted 1:2000, all HRP conjugated (SouthernBiotech)) were added to each well. The plates were washed and developed as above. The sensitivity and specificity of these antibodies at their working dilution was assessed by using as control substrate-purified mouse monoclonal IgG subclasses (IgG1, 2a, 2b, 3). The cutoff values were determined as the mean OD readings obtained with 10 control mouse sera + 5 standard deviations.

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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