molecules. Our experiments focused on the injection of the soluble form of SCF and inhibition of the KIT pathway with monoclonal antibody K44.2 (Grichnik *et al* 1998). Because both forms of SCF stimulate the KIT receptor, we believe the results of our experiments are applicable for both the soluble and the membrane bound forms of SCF. K44.2 inhibits the binding of soluble SCF to KIT (Blechman *et al*, 1995) but we have no reason to suspect that it does not also inhibit the binding of membrane bound SCF. Our findings support the importance of the SCF/KIT interaction to human melanocytes and do not exclude bound SCF.

While both forms of SCF activate KIT, we suspect that the duration of activation and potential for receptor degradation is different for each form. Keratinocytic bound SCF may lock on to the melanocyte's KIT receptor resulting in persistent KIT activation (without KIT receptor internalization and degradation), while soluble SCF may transiently activate the KIT receptor followed by internalization and degradation.

The role of KIT on the variety of melanocytic differentiation states remains for the most part unknown. It is possible that KIT expression on melanocytes is regulated through at least two mechanisms, one central (transcriptional) and one peripheral (feedback through receptor). If the central expression is low the melanocytic cells may function independently from the KIT pathway. If central expression is high, then cells become susceptible to KIT activation or inhibition. Peripheral KIT activation may also result in apparent KIT downregulation through negative feedback. Following this logic the KIT + TRP-1(–) cells (normal skin, Grichnik *et al*, 1996; nevus depigmentous, Dippel *et al*, 1995) should be particularly susceptible to KIT inhibition; however, we have no specific data to support or refute this hypothesis.

There are still quite a number of challenges ahead. How is SCF normally regulated on human keratinocytes? How is the KIT receptor regulated on the melanocyte? Is there a functional autocrine pathway for SCF/KIT in melanocytic disease states? What are the different differentiation states of melanocytes? Which differentiation states are dependent or independent of SCF? Can these states be reversed in normal melanocytes, or in melanoma?

The myriad of different pigmentation states, nevi, and melanoma types suggests that we have a relatively complex biologic puzzle to work out. We believe the SCF/KIT pathway will factor into many of these processes at some capacity. We look forward to the next millennium and to the answers for many of these questions.

James M. Grichnik
Duke University Medical Center, Division of Dermatology,
Durham, North Carolina, USA

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# $IgG_1$ and $IgG_3$ are the Major Immunoglobulin Subclasses Targeting Epitopes within the NC16A Domain of BP180 in Pemphigoid Gestationis

To the Editor:

Pemphigoid gestationis (PG) is a subepidermal blistering disease characterized by the linear depositon of C3 and, to a lesser extent, IgG at the cutaneous basement membrane zone (BMZ) as detected by direct immunofluorescence (IF) (Shornick, 1987). Indirect IF studies demonstrated that circulating autoantibodies of PG sera are predominantly of the $IgG_1$ subclass (Kelly *et al*, 1989). The autoimmune response in PG is directed against two hemidesmosomal proteins, BP180 and, less frequently, BP230 (Morrison *et al*, 1988). The pathogenic relevance of autoantibodies against BP180 in PG and bullous pemphigoid (BP) was established using a passive transfer animal model (Liu *et al*, 1993). BP180 is a transmembrane glycoprotein with a large extracellular C-terminal ectodomain containing 15 interrupted collageneous domains (Giudice *et al*, 1992; Hopkinson *et al*, 1992; Li *et al*, 1993). Immunoelectron microscopy studies have shown that the BP180 ectodomain tranverses the lamina lucida and projects into the lamina densa (Bedane *et al*, 1997; Masunaga *et al*, 1997). It might interact with one or more proteins of the epidermal BMZ thus promoting the adhesion of basal keratinocytes. Recently, it was demonstrated that anti-BP180 antibodies in BP recognize four major epitopes on its membrane-proximal NC16A domain (Zillikens *et al*, 1997a), and that one of these antigenic sites (designated NC16A2 or MCW-1) is also recognized by PG sera (Giudice *et al*, 1993). The purpose of this study was to characterize the epitopes within BP180 NC16A targeted by PG autoantibodies and to determine their subclass distribution.

Sera were obtained from 21 PG patients before treatment was initiated. All patients showed deposits of C3 and/or IgG at the BMZ by direct and/or indirect IF and demonstrated autoantibodies against BP180 NC16A by both ELISA and immunoblot analysis (Zillikens *et al*, 1997b). Twelve well-characterized BP sera and 10 normal human sera (NHS) were used as controls. Rabbit serum R58 was raised against recombinant glutathione S-transferase (GST). Reactivity of PG sera with different segments of BP180 NC16A was assayed by immunoblotting using the following recombinant GST fusion proteins: GST-NC16A1, GST-NC16A2, GST-NC16A2.5, GST-NC16A3, GST-NC16A1–3, GST-NC16A2–4, GST-NC16A2–5, and GST-NC16A1–5. Preparation of these proteins, and immunoblotting and immunoadsorption procedures were performed as described (Giudice *et al*, 1993; Zillikens *et al*, 1997a). The secondary peroxidase-conjugated antibodies were used at the following dilutions: goat antirabbit polyclonal IgG 1:5000; rabbit antihuman polyclonal IgG 1:15 000; antihuman IgM 1:5000 (all DAKO, Glostrup, Denmark); antihuman IgA 1:20 000 (Jackson Immunoresearch Laboratories, West Grove, PA); sheep antihuman IgE 1:100; mouse antihuman $IgG_1$ (clone 8c/6–39) 1:1000; antihuman $IgG_2$ (clone HP6014) 1:500; antihuman $IgG_3$ (clone HP6050) 1:200; antihuman $IgG_4$ (clone HP6023) 1:4000 (all Binding Site, Birmingham, UK). Monoclonal antibodies were demonstrated to be specific by direct hemagglutination and hemagglutination inhibition assays (Jefferis *et al*, 1985), and by immunoblot analysis using human $IgG_1$, $IgG_2$, $IgG_3$, $IgG_4$ myeloma proteins (Sigma, St. Louis, MO). Sensitivities of secondary monoclonal

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Correspondence: Dr. Detlef Zillikens, Department of Dermatology, University of Würzburg, Josef-Schneider-Str. 2, 97080 Würzburg, Germany.

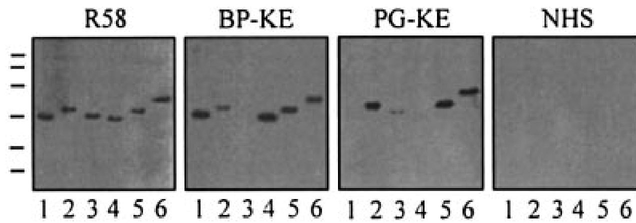


**Figure 1. PG, like BP sera, react with distinct antigenic sites within BP180 NC16A.** Recombinant proteins were fractionated by 15% SDS-PAGE, transferred to nitrocellulose, and labeled with a PG serum (PG-KE), a reference control BP serum (BP-KE), and a normal human serum (NHS). Sera were diluted 1:100 and completely preadsorbed for reactivity against recombinant GST. Equal amounts of recombinant fusion proteins NC16A1, NC16A2, NC16A2.5, NC16A3, NC16A1–3, and NC16A1–5 were loaded in *lanes* 1–6, respectively, as verified by immunoblotting analysis with rabbit serum R58 raised against recombinant GST. The migration positions of molecular weight markers of 97, 66, 45, 31, 21, and 14 kDa are indicated at the left.

**Table I. IgG1 and IgG3 are the two major immunoglobulin subclasses reactive with BP180 NC16A in PG sera[a]**

| IgG1 | IgG2 | IgG3 | IgG4 | IgE | IgM | IgA |
|---|---|---|---|---|---|---|
| 17/21[b] | 6/21 | 14/21 | 7/21 | 5/21 | 2/21 | 1/21 |

[a]Immunoglobulin subclass distribution of autoantibodies in PG sera was analyzed by immunoblotting using recombinant GST-NC16A1–5 and monoclonal peroxidase-labeled secondary antibodies.
[b]Number of positive sera/number of sera tested.

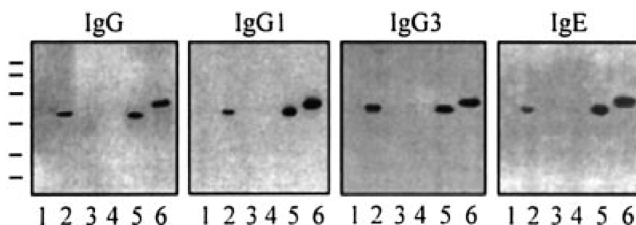


**Figure 2. IgG subclasses and IgE antibodies reveal a similar pattern of reactivity compared with polyclonal IgG antibodies.** Blots containing equal amounts of fusion proteins NC16A1, NC16A2, NC16A2.5, NC16A3, NC16A1–3, NC16A1–5 (*lanes* 1–6, respectively) were labeled with PG-SP serum (1:100). Bound antibodies were visualized using peroxidase conjugated antihuman IgG and monoclonal antibodies against human $IgG_1$, $IgG_3$, and IgE. Molecular weight markers corresponding to 97, 66, 45, 31, 21, and 14 kDa are indicated at the left.

antibodies used at their working dilutions were shown to be comparable by immunoblot analysis against equal amounts of human myeloma proteins.

Like in BP, PG sera revealed major polyclonal IgG reactivity with NC16A regions 2 (MCW-1) (71%) and 2.5 (MCW-2) (57%) and showed a reduced level of reactivity with NC16A region 3 (MCW-3) (29%) (**Fig 1**). In contrast to BP, only 30% of PG sera reacted with NC16A region 1 (MCW-0) (Zillikens *et al*, 1997a). Interestingly, 2 PG sera recognized NC16A region 5 (which we designate MCW-5) that has not been identified as a target of autoantibodies so far. Neither PG nor 12 control BP sera reacted with NC16A region 4 (MCW-4) that is targeted by autoantibodies in lichen planus pemphigoides (Zillikens *et al*, 1999).

We identified $IgG_1$ (81%) and $IgG_3$ (67%) as the major subclasses of PG autoantibodies recognizing BP180 NC16A (**Table I**). $IgG_2$ and $IgG_4$ reactivity was detected less frequently (33% and 29%, respectively). In contrast, circulating anti-BMZ antibodies in PG, as detected by indirect IF, belong almost exclusively to the $IgG_1$ subclass (Kelly *et al*, 1989). It is, however, important to keep in mind that the reactivity revealed by indirect IF reflects the binding properties of autoantibodies to different BMZ proteins, including BP180 and BP230. The IgG subclass distribution in PG differs from that in BP, where $IgG_4$ is the major subclass of anti-BP180 NC16A antibodies.[1]

It is known that some BP sera contain IgE antibodies against BP230 (Delaporte *et al*, 1996). In PG, no IgE reactivity has been reported. We now demonstrate that 24% of PG sera, but none of 10 NHS, show circulating IgE antibodies to BP180 NC16A that are mainly directed against the MCW-1 epitope (**Fig 2**). In addition, we detected IgM and IgA antibodies recognizing BP180 NC16A in two and one of our 21 PG sera, respectively, but not in NHS. IgM and IgA anti-BMZ antibodies have been occasionally found in the skin of PG patients (Wever *et al*, 1995). In addition, in 13% of patients with polymorphic eruption of pregnancy, circulating IgM antibodies to 180 and/or 230 kDa epidermal proteins were detected (Borradori *et al*, 1995). No circulating IgM and IgA antibodies, however, had yet been reported in PG patients. The pathogenic relevance of IgE, IgM, or IgA isotypes reactive with BP180 NC16A in PG remains to be elucidated.

We also addressed the question of whether certain subclasses of PG autoantibodies preferentially react with specific epitopes on BP180 NC16A. PG sera (n = 7) revealing the strongest immunoblot reactivity of the different immunoglobulin subclasses with full-length BP180 NC16A were assayed against fusion proteins containing different subregions of NC16A. MCW-1 was the epitope most commonly recognized by autoantibodies of all IgG subclasses and IgE. In addition, $IgG_2$, $IgG_3$, and $IgG_4$ targeted MCW-2 in 40%–50% of PG sera studied. As we found $IgG_1$ and $IgG_3$ to be the most common subclasses directed against BP180 NC16A in PG, the observed preferential reactivity of polyclonal IgG with MCW-1 and MCW-2 appears to mainly reflect the strong preferential reactivity of $IgG_1$ and $IgG_3$ with these epitopes (**Fig 2**).

In conclusion, we demonstrate that PG sera recognize five distinct epitopes within BP180 NC16A, four of which have been reported as major antigenic sites targeted by BP autoantibodies (Zillikens *et al*, 1997a). In contrast to BP, we found $IgG_1$ and $IgG_3$ to be the major subclasses targeting MCW-1 and MCW-2 within BP180 NC16A. As $IgG_3$ and $IgG_1$ are the subclasses with the strongest complement fixing properties, our findings may well explain complement deposition at the BMZ, which is the most consistent immunopathological feature in PG and a prerequisite for the induction of blisters by anti-BP180 antibodies in the mouse model of PG and BP (Liu *et al*, 1995).

Iakov Chimanovitch, Enno Schmidt, Gerald Messer,*
Rebekka Döpp, Karin Partscht,* Eva-Bettina Bröcker,
George J. Giudice,† and Detlef Zillikens
Department of Dermatology, University of Würzburg, Germany
*Department of Dermatology, Ludwig–Maximilians–University of Munich, Germany
†Department of Dermatology, Medical College of Wisconsin, Milwaukee, Wisconsin, USA

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