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Identification and Characterization of Epitopes Recognized by T Lymphocytes and Autoantibodies from Patients with Herpes Gestationis

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Autoantibodies associated with herpes gestationis (HG), a pregnancy-associated autoimmune skin disease, target the hemidesmosomal protein BP180. It was shown that the major noncollagenous stretch of the BP180 ectodomain (NC16A) harbors epitopes recognized by HG sera. Furthermore, Abs reactive with the homologous domain of murine BP180 are known to trigger a cutaneous blistering disease in mice by passive transfer experiments. The present study was aimed at characterizing the T cell responses and specificities of autoantibodies from two HG patients. Using immunoblotting and T cell proliferation assays, we have identified a 14-amino-acid stretch of the BP180 ectodomain (MCW-1; aa 507–520) that is recognized by both T cells and autoantibodies produced by the HG patients. The neonate born to one of these HG patients showed no signs of skin disease and had no detectable T cell response to the BP180 Ag, but did have a low titer of circulating anti-BP180 autoantibodies, presumably of maternal origin. BP180-specific T cell lines and clones developed from an HG patient specifically reacted with the MCW-1 epitope. The proliferative responses of these clones were restricted to HLA-DR, but not -DQ or -DP. These Ag-specific T cells expressed αβ TCRs and a CD4 memory T cell phenotype and secreted IFN-γ and IL-2, but not IL-4 or IL-6, suggesting that they are Th1-type lymphocytes. Further characterization of these Ag-specific T cells and autoantibodies will aid in elucidating the autoimmune mechanism(s) leading to the development of HG.

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3 Abbreviations used in this paper: HG, herpes gestationis; BMZ, basement membrane zone; BP, bullous pemphigoid; Dsg, desmoglein; GST, glutathione S-transferase; NC, noncollagenous; PF, pemphigus foliaceus; PV, pemphigus vulgaris; S.I., stimulation index.
The purpose of this study was to characterize the fine specificity of the anti-BP180 autoantibodies present in the sera of two HG patients and a neonate from one of these patients. We have also characterized the proliferative response of T cells from these three individuals and developed BP180-specific T cell clones. The surface markers and cytokine profiles of these clones indicated that they are CD4^+ memory T cells of the Th1 type. Our findings revealed that both autoantibodies and T cells from HG patients recognize a common site within the NC16A domain of the human BP180 Ag.

**Materials and Methods**

**HG patients and controls**

Sera and peripheral blood were obtained from two HG patients. Cord blood from a neonate born to one of these patients was also used. The HG patients exhibited subepidermal blistering and linear deposition of IgG and C3 at the BMZ of perilesional skin. Skin lesions were found in the neonate. Indirect immunofluorescence staining was performed using human foreskin as substrate to detect IgG anti-BMZ Abs (3). Serum and peripheral blood were obtained from two HG patients. Cord blood from a neonate born to one of these patients was also used. The HG patients and controls were then centrifuged as 10,000 g for 15 min at 4°C. The preadsorbed sera were subsequently tested for reactivity with various BP180 fusion proteins by immunoblotting. After a 15-h incubation with the primary Ab, the blots were washed, blocked for 30 min with PTX buffer and used in the immunoblotting assays. After a 15-h incubation with the primary Ab, the blots were washed, blocked for 30 min with PTX buffer and incubated for 2 h with ^3H-labeled Staphylococcus aureus protein A at a concentration of 10^5 cpm/ml. The bound Abs were visualized by autoradiography.

**Immunoblotting**

SDS-PAGE was performed as described (18). Briefly, fusion proteins were fractionated by 15% SDS-PAGE and transferred to nitrocellulose (Life Technologies, Gaithersburg, MD). Blots were blocked with PTX buffer (0.01 M phosphate, pH 7.5, 0.2% Triton X-100, 0.15 M NaCl, 1 mM EGTA, 4% BSA) for 45 min. HG and control sera were diluted 100-fold in PTX buffer and used in the immunoblotting assays. After a 15-h incubation with the primary Ab, the blots were washed, blocked for 30 min with PTX buffer, and incubated for 2 h with ^3H-labeled Staphylococcus aureus protein A at a concentration of 10^5 cpm/ml. The bound Abs were visualized by autoradiography.

**Immunoadsorption**

Immunoadsorption procedures were conducted as described previously (36). The sera were diluted in PTX and incubated overnight with rGST or with GST fusion proteins containing segments of NC16A. The mixtures were then centrifuged as 10,000 x g for 15 min at 4°C. The preadsorbed sera were subsequently tested for reactivity with various BP180 fusion proteins by immunoblotting.

**MHC class II analysis**

The analysis of HLA-DRB1 and DQB1 expression in HG patients was conducted by the use of the sequence-specific oligonucleotide hybridization technique of PCR-amplified DNA (37, 38). Patient RM expressed HLA-DRB1*0401/1301 and DQB1*0302/0604, while patient RW expressed HLA-DRB1*1001/0101 and DQB1*0501.
Purification of PBMC and isolation of T lymphocytes

PBMC were isolated by Ficoll-Hypaque (Pharmacia) density-gradient separation (39). T cells were then purified by E-rosetting (39) by treating 2-aminoethylisothiourea bromide (AET; Sigma, St. Louis, MO)-treated SRBC (Colorado Serum, Denver, CO) (40). The purified T cells were washed three times with medium, and resuspended in RPMI 1640 medium supplemented with 10% human AB serum (NABI, Miami, FL) for the T cell proliferation assays.

T cell proliferation assays

T cell responses to BP180 fusion proteins were determined by proliferation assays, as described (39). Briefly, T cells at a density of 10^5/ml were cultured for 7 days with 10^7/ml of irradiated autologous PBMC as APCs along with fusion proteins in wells of 96-well U-bottom plates. Cells treated with PHA (Sigma) at 0.25 μg/ml or IL-2 (Collaborative Research, Bedford, MA) at 10 U/ml served as positive controls in the T cell proliferation experiments, while cells treated with GST, GST-Dsg1, and GST-Dsg3 fusion proteins were included as negative controls. Cells in individual wells were pulsed with 1 μCi of [3H]thymidine (ICN, Costa Mesa, CA) during the last 18 h of incubation and then harvested using an automated cell harvester (Inotech Biosystems, Lansing, MI). The proliferation of T cells was determined by measuring the [3H]thymidine uptake using a liquid scintillation counter (Wallac, Gaithersburg, MD). Data were presented as average cpm ± SD or as a stimulation index (S.I.) (average cpm of cells treated with fusion protein = average cpm of cells treated with PBS). An S.I. equal to or greater than three was considered a positive response.

Development and characterization of BP180-specific T cell lines and clones

The BP180-specific T cell lines and clones were developed from an HG patient using the in vitro repeat stimulation protocol described previously (39). The cell surface expression of CD3, CD4, CD8, CD19, CD45RA, and the T cell responses to BP180 fusion proteins were determined by proliferation assays. Mouse IgG1 was used as a control in these assays. Abs were conjugated F(ab)2 anti-mouse Ig was used as the secondary Ab. Cytometric analysis using a FACScan flow cytometer (Becton Dickinson) was used to analyze the expression of accessory molecules (HLA-DR, HLA-DQ, HLA-DP) in the T cell lines. The Ag specificity of the T cell lines and clones was examined by culturing 5 × 10^4/ml T cells with 5 × 10^5/ml of irradiated autologous PBMC in the presence of 20 μg/ml of Ags or with Ag-pulsed EBV-transformed B cells at a ratio of 5:1. T cells in each well were pulsed with 1 μCi of [3H]thymidine (ICN) during the last 18 h of incubation. An S.I. equal to or greater than 3 was considered a positive response.

The MHC class II restriction of Ag responses of HG T cell lines and clones was determined by using anti-HLA-DR (BioSource International), -DQ (B-D) and -DP (B-D) Abs at concentrations of 1 μg/ml in proliferation assays. Mouse IgG1 was used as a control in these assays. T cells were dialyzed against PBS before use in cell cultures. It was determined that the Ab concentrations used in the proliferation assays were sufficient to saturate binding on at least 10^6 APCs. For cytokine profile analysis, 10^5 T cells/well were cultured in a 24-well plate in the presence of 10 ng/ml of PMA (Sigma) and 100 ng/ml of anti-CD3 Abs (41). Cell culture supernatants were collected after 30 h of stimulation, and were subjected to lymphokine bioassays. The activity of IL-2, IL-4, and IFN-γ was determined by the cytokine-specific ELISA kits following the protocols provided by the manufacturer (Genzyme, Cambridge, MA). The presence of IL-6 was determined by the bioassay using B9 as the indicator cell line (42). rIL-6 was purchased from Biosource International and used as the standard in the IL-6 bioassay.

Results

Autoantibodies of HG patients react with fusion proteins encompassing the NC16A domain of the BP180 molecule

To define the epitope(s) recognized by HG autoantibodies on the BP180 Ag, immunoblotting assays were performed using GST-BP180 fusion proteins as substrates. We found that sera from the two HG patients, RM and RW, and the newborn baby (MW) recognize various peptide fragments encompassing the NC16A domain of BP180, including NC16A1–3, NC16A2–5, NC16A2–4, and NC16A2–3 (not shown). Of the individual NC16A subregions that were tested, only subregion 2 (NC16A2) was recognized by the patients’ sera (Fig. 2).

To determine whether NC16A2 is the only antigenic site recognized by these HG sera, samples were first depleted of NC16A2 reactivity and then tested for reactivity to NC16A1–5 by immunoblotting. As shown in Fig. 3, the GST-preadsorbed HG sera reacted with the NC16A1–5 (lanes 1, 3, and 5), as expected. Reactivity with this BP180 fusion protein was abolished, or greatly diminished, when these HG sera were preadsorbed with NC16A2 (lanes 2, 4, and 6). Moreover, after depleting reactivity with subregion NC16A2, the HG sera no longer reacted with a recombinant protein encompassing the entire extracellular domain of BP180 by immunoblotting and did not label the BMZ of human skin by indirect immunofluorescence (not shown), supporting the conclusion that the NC16A2 segment contains the major autoantibody-reactive epitope of these HG patients.

T lymphocytes from HG patients specifically respond to NC16A fusion proteins

To examine whether T lymphocytes from HG patients respond to the BP180 Ag, T cells purified from PBMC of two HG patients and

FIGURE 2. Sera of HG patients recognize fusion proteins encompassing the NC16A region. The sera of two HG patients (RW and RM) and a neonate (MW) of an HG patient were tested for reactivity with various BP180 fusion proteins by immunoblotting. The sera were preadsorbed with GST to eliminate reactivity against the GST moiety of the fusion protein. Lane 1, GST; lane 2, NC16A1–5; lane 3, NC16A1; lane 4, NC16A2; and lane 5, NC16A3.

FIGURE 3. NC16A2 (MCW-1) is the only NC16A autoantibody epitope recognized by HG sera. Immunoblots containing the NC16A fusion protein were labeled with the sera of two HG patients (RW and RM) and the neonate (MW) preadsorbed with either GST (lanes 1, 3, and 5) or NC16A2 (lanes 2, 4, and 6). Lanes 1 and 2, patient RW; lanes 3 and 4, neonate MW; and lanes 5 and 6, patient RM.
a neonate of one of these patients were characterized using a standard cell proliferation assay. As shown in Fig. 4A, T cells from HG patient RW responded to BP180 fusion proteins encompassing the NC16A region in a dose-dependent fashion. Furthermore, using smaller fusion proteins in the proliferation assay, we identified NC16A2 as a major epitope that elicits the anti-BP180 T cell response in this HG patient (Fig. 4C). T cells from HG patient RM, a woman with recurrent subepidermal blistering associated with her menstrual cycle, also exhibited a proliferative response only to fusion proteins containing the NC16A2 peptide segment (Fig. 4D).

Interestingly, although the serum of the neonate MW contains IgG1 Abs that label the BMZ by indirect immunofluorescence and recognizes NC16A fusion proteins by immunoblotting, the T cells from MW did not proliferate when cultured with BP180 fusion proteins (Fig. 4B). T cells from healthy controls and from patients with PV, PF, psoriasis, and lupus did not respond to any of the BP180 fusion proteins. We also showed that the T lymphocytes from the HG patients responded only to BP180 peptides and not to unrelated Ags such as GST, Dsg-1, and Dsg-3 (not shown), indicating that HG T cells specifically proliferate in response to BP180.

Ag specificity of BP180-specific T cells from HG patient

To further study the properties of HG-specific T cells and characterize the antigenic BP180 epitope(s) involved in the autoimmune responses, long-term T cells were developed from an HG patient using a previously described protocol (39). T cell clones were subsequently derived from cell lines by limiting dilution. The Ag specificity of these T cells to BP180 fusion proteins was confirmed by the results from proliferation assays. As shown in Fig. 5A, RW21 and RW67, two cell clones developed from patient RW, specifically respond to various BP180 fusion proteins containing the NC16A2 epitope, but not to peptides without this particular region. This result indicated that NC16A2 is the T cell epitope recognized by HG-specific T cells.

To investigate the MHC class II restriction of the BP180-specific responses of T cell clones developed from the HG patient, anti-HLA-DR, -DQ, and -DP Abs were applied to the T cell proliferation assays. Nonspecific mouse IgG1 was used as an isotype control. As demonstrated in Fig. 5B, only the anti-HLA-DR Ab, but not the anti-HLA-DQ or -DP Abs, inhibited the proliferation responses of the BP180-specific T lymphocytes to the NC16A2 peptide. This result clearly demonstrates that the recognition of BP180 by HG T cells is restricted to HLA-DR molecules.

Cell surface phenotype and cytokine profile of HG-specific T cells

Flow-cytometric analysis was used to determine the cell surface phenotype of the BP180-specific T cell clones developed from the HG patient RW. As shown in Fig. 6, RW67, a representative NC16A2-specific T cell clone, expressed CD3, CD4, CD45RO, and TCRαβ, but was negative for CD8, CD45RA, and the B cell marker CD19. All other T cell clones and lines tested expressed this same set of markers. These results showed that NC16A2-specific T lymphocytes in this HG patient exhibit a CD4 memory T cell phenotype.

The cytokine profiles of the T cells from patient RW were also determined as follows. T cells were activated by anti-CD3 Abs in the presence of PMA for 30 h. The presence of IL-2, IL-4, and IFN-γ in the T cell culture supernatants was then examined by the ELISA method using commercial kits, as described. The activity of IL-6 was verified using a standard bioassay. As summarized in Table I, the Ag-specific T cell clones derived from patient RW
secrete IL-2 and IFN-γ, but not IL-4 nor IL-6, indicating that these T cells express a Th1-type cytokine profile.

Discussion

In this study, we report that sera from two active HG patients and a neonate of one patient specifically react with the NC16A domain of the BP180 Ag. Furthermore, these three sera only recognized the NC16A2 subregion (a 14-aa segment of NC16A that was previously designated MCW-1). Interestingly, T lymphocytes from these two HG patients also showed a specific response to the MCW-1 peptide. Based on cell surface markers and cytokine profiles, MCW-1-responsive T cell clones generated from one of the HG patients were identified as Th1-type cells. Thus, the MCW-1 epitope on the BP180 ectodomain appears to be a major target for both T cells and autoantibodies in the HG patients studied. Our findings suggest that this particular BP180 epitope may play a key role in the initiation of the anti-BP180 autoimmune response in HG.

Our research group previously reported that the fine specificities of anti-BP180 autoantibodies from BP patients are highly restricted (18). Only four major antigenic sites recognized by BP autoantibodies were detected on the BP180 ectodomain, and all four sites, one being MCW-1, are clustered within a 45-aa segment of NC16A. In the present study, HG sera were subjected to a similar type of analysis, i.e., liquid-phase immunoadsorption followed by immunoblotting. These results revealed that the MCW-1 peptide (aa 507–521) was the only portion of the BP180 NC16A domain that contained a detectable HG-associated epitope. One limitation of this type of assay is that conformational epitopes are not detectable.

Table 1. HGT cell clones secrete a Th1-like cytokine profile

<table>
<thead>
<tr>
<th>T Cell Clones</th>
<th>IL-2a (pg/ml)</th>
<th>IL-4b (pg/ml)</th>
<th>IL-6b (ng/ml)</th>
<th>IFN-γa (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RW5</td>
<td>1.04</td>
<td>&lt;16</td>
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<td>RW17</td>
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<td>&lt;16</td>
<td>Negative</td>
<td>2.04</td>
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<tr>
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<td>1.50</td>
<td>&lt;16</td>
<td>Negative</td>
<td>1.90</td>
</tr>
<tr>
<td>RW67</td>
<td>0.82</td>
<td>&lt;16</td>
<td>Negative</td>
<td>1.94</td>
</tr>
</tbody>
</table>

a The concentrations of IL-2, IL-4, and IFN-γ in the culture supernatants of activated T cell clones were analyzed using a commercial ELISA kit (ng/ml).

b The presence of IL-6 in the supernatants of activated T cell clones was determined by the B9 cell proliferation bioassay as described in Materials and Methods.

FIGURE 5. Ag response and the MHC II restriction of BP180-specific T cell clones from an HG patient. The Ag specificity of T cell clones RW21 and RW67, which were derived from HG patient RW, was examined by proliferation assays. In this experiment, autologous APCs were pulsed with 20 μg/ml of Ag for 8 h, then irradiated and used in the proliferation assays (A). The MHC II restriction of response to MCW-1 in the T cell clones was investigated by T cell proliferation assays in the presence of isotype control Ab mIgG1, anti-HLA-DR, anti-HLA-DQ, and anti-HLA-DP Abs. Irradiated autologous EBV-transformed B cells were used as APCs in this experiment. APCs were first pulsed with 10% human AB serum medium or 20 μg/ml of either GST or MCW-1 before the irradiation (B). Data were expressed as average cpm ± SD.

FIGURE 6. Cell surface phenotype of a MCW-1-specific T cell clone from an HG patient. The expression of cell markers on the surface of MCW-1-specific T cells was characterized by flow-cytometric analysis using primary monoclonal specific mouse anti-human Abs, as shown. Mouse IgG was used as a control in this experiment. Fluorescein-conjugated goat anti-mouse Ig was used as the secondary Ab. In each histogram, region M1 depicts the cell population that is labeled with the primary Abs. The data from one representative clone, RW67, are shown here.
likely to be destroyed under the harsh conditions of SDS-PAGE. To address this problem, we assayed the MCW-1-preabsorbed HG sera for reactivity with the basement membrane zone by indirect immunofluorescence on human skin. Interestingly, these preadsorbed sera failed to label the BMZ. Furthermore, depletion of autoantibodies that react with MCW-1 from the HG sera abolished immunoreactivity with a recombinant protein (sec180e) encompassing the entire BP180 ectodomain (Lin et al., unpublished data). Structural analyses have indicated that sec180e exists in a conformation that closely mimics endogenous BP180 (20, 21). Taken together, these findings indicate that, at least for the two HG cases studied, the MCW-1 epitope is the predominant extracellular BP180 site that is recognized by HG autoantibodies. We are currently testing a large number of HG sera to determine whether other epitopes within, or outside of, the NC16A domain are recognized by HG autoantibodies.

Our study also demonstrates that T cells from HG patients proliferate when incubated with antigenic peptides derived from the NC16A domain presented by autologous APCs. It is worth noting that T cells from BP (n = 6) and linear IgA disease (n = 3) also react with peptides located within the NC16A region (Lin et al., unpublished data) (43, 44), suggesting that this BP180 stretch contains epitope(s) relevant to the development of these three diseases. The autoimmune mechanisms that precipitate these three different diseases are currently unknown. It is possible that the cytokine patterns of BP180-specific T lymphocytes or the expression of specific HLA alleles may predispose the development of specific diseases.

It was reported previously that some neonates born to HG patients exhibit high titers of anti-BMZ Abs and subepidermal blistering (1). Based on documented evidence for transplacental passage of IgG1 Abs, but not T cells, it was hypothesized that the cutaneous lesions in the babies were caused by the passively transferred maternal autoantibodies. The neonate of one of our HG patients possessed circulating anti-BP180 Abs; however, the T cells from this baby remained unresponsive when incubated with the BP180 antigenic peptides. This result supports the hypothesis that the anti-BP180 autoantibodies in the neonate were derived from the mother rather than resulting from an autoimmune response of the newborn. Furthermore, based on the above information, the absence of skin lesions in the neonate in our study is most likely due to the low autoantibody titer (1:10).

The proliferative response of T cells from HG patients to NC16A peptides was specific and dose dependent. T cells from normal individuals and other patient groups, such as PV, PF, systemic lupus erythematosus, and psoriasis, did not respond to these fusion proteins. Since the NC16A peptides were prepared as baculovirus GST fusion proteins, we also tested GST in the T cell proliferation assay. T lymphocytes from HG patients were unresponsive to GST or GST conjugated to other unrelated Ags, such as the th2-type cytokines induce B cells to secrete IgG4 (46, 47). Since IgG1 is the predominant isotype of the anti-BMZ Abs of HG, it is thought that T cells of the Th1 lineage may be relevant in this autoimmune disease. Our study supports this hypothesis, since T cell clones derived from our HG patient (RW) secrete IL-2 and IFN-γ, but not IL-4 and IL-6. The anti-BMZ autoantibodies in this HG patient were of the IgG1 isotype (not shown). Therefore, the Th1-type cytokine profile expressed by autoimmune T cells may modulate the production of anti-BP180 autoantibodies of the IgG1 subclass in HG.

In conclusion, the data presented in this study provide evidence that the MCW-1 site on the BP180 molecule is a major epitope that may modulate the autoimmune response in HG. This site is targeted by both T cells and autoantibodies produced by the two HG patients studied. Further efforts to characterize the immune cells responding to anti-BP180 will help to further elucidate the pathogenic mechanism in HG.

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3. Jordon, R. E., K. G. Heine, G. Tappeiner, L. L. Bushkell, and T. T. Provost. 1976. The isotype of Abs produced by a given B cell is dependent on the type of Th lymphocytes that it encounters during the T-B cell interaction (32). For example, T cells that secrete Th1 cytokines are capable of stimulating B cells to produce IgG1, while Th2-type cytokines induce B cells to secrete IgG4 (46, 47). Since IgG1 is the predominant isotype of the anti-BMZ Abs of HG, it is thought that T cells of the Th1 lineage may be relevant in this autoimmune disease. Our study supports this hypothesis, since T cell clones derived from our HG patient (RW) secrete IL-2 and IFN-γ, but not IL-4 and IL-6. The anti-BMZ autoantibodies in this HG patient were of the IgG1 isotype (not shown). Therefore, the Th1-type cytokine profile expressed by autoimmune T cells may modulate the production of anti-BP180 autoantibodies of the IgG1 subclass in HG.

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