Usefulness of BP180 NC16a Enzyme-Linked Immunosorbent Assay in the Serodiagnosis of Pemphigoid Gestationis and in Differentiating Between Pemphigoid Gestationis and Pruritic Urticarial Papules and Plaques of Pregnancy

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Background: Pemphigoid gestationis (PG) is a rare pregnancy-associated subepidermal immunobullous disease that targets hemidesmosomal proteins, particularly BP180. Clinically, PG can resemble the eruption known as polymorphic urticarial papules and plaques of pregnancy (PUPPP), and accurate differentiation between these 2 pruritic pregnancy dermatoses has important implications for fetal and maternal prognoses. Results of epitope mapping studies show that IgG autoantibodies in up to 90% of PG serum samples target the well-defined membrane-proximal NC16a domain of BP180.

Objective: To examine the usefulness of a commercially available NC16a domain enzyme-linked immunosorbent assay in the serodiagnosis of PG and in the differentiation of PG from PUPPP.

Participants: A total of 412 women consisting of pretreatment patients with PG (n=82), patients with PUPPP (n=164), and age- and sex-matched controls (n=166).

Methods: All serum samples were assayed in duplicate. Receiver operating characteristic analyses were performed to determine a cutoff value for the diagnosis of PG and for differentiation from PUPPP and controls.

Results: A cutoff value of 10 enzyme-linked immunosorbent assay units was associated with specificity and sensitivity of 96%.

Conclusions: The NC16a enzyme-linked immunosorbent assay is highly sensitive and highly specific in differentiating PG from PUPPP, and it is potentially a valuable tool in the serodiagnosis of PG.

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Pemphigoid gestationis (PG) is a pregnancy-associated subepidermal immunobullous disease. It manifests most commonly during the second or third trimester, but it may present at any stage during pregnancy or puerperium. Pruritus is typically a feature of PG, with urticated papules and plaques often preceding actual blister formation. Clinically and immunopathologically, PG is related to the pemphigoid group of disorders. It is characterized by linear deposition of C3 and, in 30% of cases, IgG along the basement membrane zone (BMZ) by direct immunofluorescence (IF) of perilameloid skin. Using an indirect complement-fixing IF technique, most patients’ serum samples contain complement-fixing IgG directed against an antigen of the cutaneous BMZ. Immunoblotting studies have demonstrated that 90% of PG serum samples recognize the hemidesmosomal protein BP180.

One of the most important differential diagnoses of PG is polymorphic urticarial papules and plaques of pregnancy (PUPPP), a pruritic eruption of unknown etiology that is also synonymous with polymorphic eruption of pregnancy. Clinically and histologically, PUPPP resembles nonbullous PG (Figure 1). Differentiation of PG from PUPPP depends on the demonstration of anti-BMZ autoantibodies by IF, immunoblotting, or enzyme-linked immunosorbent assay (ELISA). The accurate differentiation of these 2 conditions is important. Polymorphic urticarial papules and plaques of pregnancy can usually be managed with emollients, sedating antihistamines, and moderately potent topical corticosteroids, and it is not associated with an adverse fetal outcome. On the other hand, PG requires potent topical corticosteroids and of...
ten systemic corticosteroid therapy. In 5% to 10% of patients, PG is associated with blistering of the neonate due to passive transfer of circulating autoantibodies,2,7 and there is a potential risk of small-for-dates and low-birth-weight babies, probably due to placental failure induced by PG autoantibodies (BP180 is present in placental amnion).8-10 In addition, a diagnosis of PG has important prognostic implications for future pregnancies; PG generally recurs in subsequent pregnancies, and when it does, it has an earlier onset and often a more aggressive disease course.7,11

The purpose of this study is to assess the usefulness of a commercially available NC16a domain ELISA in the serodiagnosis of PG. In contrast to other studies of BP180 ELISA, we address the specificity and sensitivity of NC16a ELISA in differentiating PG from PUPPP.

**METHODS**

**PATIENTS AND CONTROLS**

Serum samples were obtained from 164 patients with PUPPP (mean age, 31 years), 166 controls aged 18 to 50 years (mean age, 33 years), and 82 patients with PG (mean age, 31 years) before any treatment was initiated. The PG and PUPPP groups were characterized by a polymorphic pruritic eruption during pregnancy. All patients with PG showed linear deposition of C3 or IgG at the cutaneous BMZ by direct IF; PUPPP was diagnosed in the absence of blister formation and by negative direct and indirect IF.

**INDIRECT IF**

Basement membrane zone autoantibody titers were measured by indirect IF using normal human skin substrate.12 Indirect complement IF was performed as described previously.12 Briefly, this 3-stage procedure involves incubating 4-µm cryosections of normal human skin substrate with the test serum (1:5 and 1:10 dilutions in phosphate-buffered saline solution) and then washing and incubating the cryosections with fresh normal human serum as a source of complement at 37°C for 60 minutes in a wet chamber. Serial dilutions of anti-C3 are used to assess the intensity of binding. This technique demonstrates whether skin-specific circulating antibodies in test serum are capable of binding C3, and it is used particularly in the diagnosis of PG. By indirect IF, linear binding of BMZ antibodies at the epidermal side of salt-split skin was detected in 71 (87%) and 67 (82%) of 82 PG serum samples using the C3 and IgG techniques, respectively (Table 1). One patient with PG had no evidence of circulating anti-BMZ antibodies by indirect IF when first seen (this patient is further described in the “Results” section). Indirect IF was subsequently performed on all control serum samples, and the results were found to be negative in all cases tested.
ELISA QUANTIFICATION

To allow comparison of results from different plates, test sample ODs were adjusted relative to positive and negative control samples supplied in each kit, which were assigned arbitrary values of 100 and 0, respectively. The index value was defined using the following formula:

\[
\text{Index} = \frac{(\text{Mean OD of Tested Serum Samples} - \text{Mean OD of Negative Control Samples})}{(\text{Mean OD of Positive Control Samples} - \text{Mean OD of Negative Control Samples})} \times 100.
\]

STATISTICAL ANALYSIS

Statistical analysis was performed using a software program (SPSS 11.5; SPSS Inc, Chicago, Ill). Receiver operating characteristic curves were used (1) to evaluate the ability of the ELISA to detect autoantibodies against the NC16a domain to diagnose PG, (2) to specifically differentiate between PG and PUPPP, and (3) to determine a cutoff value for the ELISA in the diagnosis of PG in our population. The areas under the curve were used (1) to evaluate the ability of the ELISA to distinguish between PG and PUPPP, and (2) to determine a cutoff value for the ELISA in the diagnosis of PG in our population. The Youden index \((J = \text{sensitivity} + \text{specificity} - 1)\) was used to select the best cutoff values. Sensitivity and specificity were plotted when different scores (possible range, 0-223) were used for cutoff values. The cutoff value was determined to be the value that gives a maximum sum of sensitivity and specificity.

To assess the reproducibility of the ELISA results, coefficients of variance were calculated for 2 PG reference serum samples with intermediate OD readings. The intraplate and interassay coefficients of variation were 3% and 3%, respectively. All samples were assayed in duplicate, and the mean overall coefficient of variation was 4%.

RESULTS

PARTICIPANTS

Serum samples from 412 women were studied: 82 with PG, 164 with PUPPP, and 166 controls (women aged 18-50 years) by NC16a ELISA. The mean (SD) age of patients with PG was 31.2 (7.8) years, of patients with PUPPP was 30.9 (5.9) years, and of controls was 33.2 (8.5) years (Table 2).

DETERMINATION OF CUTOFF VALUE AND GRAY ZONE

The mean (SD) ELISA value of the control serum samples \((n = 166)\) was 2.5 (2.8) \((SE=0.2)\) and of the PUPPP serum samples \((n = 164)\) was 2.6 (3.7) \((SE=0.3)\) (Figure 2 and Table 2). There was no significant difference between the mean ELISA values of the 2 control cohorts \((P=.7)\). In the absence of PG, pregnancy is not associated with an increased incidence of anti-NC16a antibodies. We, therefore, pooled the data from both control groups for receiver operating characteristic analysis.

When ELISA values from PG serum samples were compared with those from the pooled cohort of control and PUPPP serum samples (defined as negative), there were 3 different values associated with the maximum Youden index.

Table 1. Sensitivities of Indirect Immunofluorescence Using the IgG or C3 Technique Alone*

<table>
<thead>
<tr>
<th>ELISA</th>
<th>Complement Technique, No.</th>
<th>IgG Technique, No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>69</td>
<td>10</td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>71</td>
<td>11</td>
</tr>
</tbody>
</table>

Abbreviation: ELISA, enzyme-linked immunosorbent assay.

*Traditionally, we combine the results of indirect immunofluorescence using the IgG and C3 techniques, which increases sensitivity to 99%, higher than that of ELISA.

Table 2. Age and ELISA Values of Patients With PG, Patients With PUPPP, and Controls*

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Age, Mean (SD), y</th>
<th>ELISA Value, Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG ((n = 82))</td>
<td>31.2 (7.8)</td>
<td>70.0 (37.2)</td>
</tr>
<tr>
<td>PUPPP ((n = 164))</td>
<td>30.9 (5.9)</td>
<td>2.6 (3.7)</td>
</tr>
<tr>
<td>Controls ((n = 166))</td>
<td>33.2 (8.5)</td>
<td>2.5 (2.8)</td>
</tr>
</tbody>
</table>

Abbreviations: ELISA, enzyme-linked immunosorbent assay; PG, pemphigoid gestationis; PUPPP, polymorphic urticarial papules and plaques of pregnancy.

*There was no statistically significant difference between the mean ELISA values of controls and patients with PUPPP, suggesting that in the absence of PG, pregnancy is not associated with an increased incidence of anti-NC16a antibodies.

BP180 ELISA

Ninety-six–well ELISA plates were precoated with recombinant proteins encompassing the NC16a portion of the extracellular domain of BP180 (Medical and Biological Laboratories Co Ltd, Nagoya, Japan). Following the manufacturer’s instructions, serum samples were diluted 1:101. NC16a autoantibodies were detected by a standard indirect ELISA using horseradish peroxidase–conjugated anti-human IgG, followed by tetra-methyl-benzidine as the horseradish peroxidase substrate. Tetra-methyl-benzidine is oxidized in the presence of horseradish peroxidase, and this results in a detectable color change related to the amount of horseradish peroxidase and, thus, indirectly to the quantity of anti-NC16a antibodies. The absorbance or optical density (OD) of each well was read at 450 nm using an automated plate reader (Dynex Technologies, Chantilly, Va). Incubation steps were performed at room temperature and totaled 2½ hours. All samples were assayed in duplicate, the average OD was taken for each, and a coefficient of variation for each sample assayed was determined.
index ($j=0.92$) (Table 3). Setting the cutoff value at 8, 10, or 14 maximizes the sum of the sensitivities and specificities at 192 (specificities and sensitivities of 94% [310/330] and 98% [80/82], 96% [317/330] and 96% [79/82], and 98% [323/330] and 94% [77/82], respectively). The cutoff value was set at 10 because it maximizes sensitivity and specificity (Table 3); however, there is a gray zone from 8 to 14 (Figure 2). An ELISA value of 14 is equal to the average OD of PUPPP serum samples plus 3 SD (2.64 + [3 × 3.65]), which is an alternative method of calculating a cutoff value by setting the specificity at 98%. The diagnostic accuracy of the ELISA, as determined by receiver operating characteristic curves, is represented by the area under the curve, which was 0.99 (95% confidence interval, 0.982-1.002) (Figure 3). No correlation was observed between titers of anti-BMZ IgG autoantibodies as detected by indirect IF and NC16a ELISA values.

Only 3 patients with PG (4%) had NC16a ELISA values less than 10; of these, 2 had no evidence of circulating anti-BMZ IgG autoantibodies on indirect IF but had positive indirect IF findings using the complement technique. There were 13 positive results among patients with PUPPP and controls; none had ELISA values greater than 20. There was 1 patient with PG, diagnosed on the basis of positive direct IF findings and clinical and histologic features consistent with PG, who had negative indirect IF findings (using the IgG and C3 techniques) but a positive ELISA value of 71 when first seen. Essentially, she was seen 4 days post partum with an acute blistering eruption that resolved within 2 weeks with application of a moderately potent topical corticosteroid; her indirect IF result subsequently became positive.

By ELISA, we studied the levels of BP180-NC16a IgG autoantibodies in a relatively large cohort of untreated patients with PG compared with nonpregnant women of childbearing age and women with PUPPP. The goal of this study was to analyze the sensitivity and specificity of a BP180-NC16a ELISA in the diagnosis of PG and particularly in differentiating PG from PUPPP. By setting the cutoff value at 10 ELISA units, this assay is highly specific (96%) and sensitive (96%).

The results of this study are consistent with those of previous studies using recombinant portions of the BP180 extracellular domain, which have shown that most PG serum samples recognize an epitope in a common immunodominant segment of the extracellular domain of BP180 (NC16a). In 1 study, 34 (92%) of 37 PG serum samples reacted to 1 or all 4 epitopes clustered in a 22–amino acid region of the NC16a domain of BP180, although an earlier study reported a lower incidence (34 [79%] of 43) of reactivity to the NC16a domain. A study of T-cell autoreactivity to BP180 of 2 patients with PG demonstrated a T$_\alpha$1-type, HLA-DR-restricted, T-cell-proliferative response to a 14–amino acid (507-520) peptide located in the NC16a domain of BP180, providing additional evidence that the major epitope of PG lies in the NC16a domain.

Autoimmunity to the BP180 antigen characterizes several subepidermal immunobullous disorders, which include, in addition to PG, bullous pemphigoid (BP), mucous membrane pemphigoid, and linear IgA disease. Differences at fine specificity of target epitopes in the BP180 extracellular domain may account for differences observed at the clinical level within these conditions. The pathogenic relevance of antibodies to the NC16a portion of the ectodomain of BP180 is suggested by several lines of evidence from in vitro and in vivo experiments: (1) in an experimental murine BP model, antibodies to the murine homolog of human NC16a induced blisters in neonatal mice, findings that have been extended to a hamster BP model; and (2) antibodies to the NC16a domain were identified as triggering the expression and secretion of inflammatory mediators when incubated with cultured normal human keratinocytes and cytoskeletons of human skin. Various techniques have been used to detect and characterize anti-BP180 antibodies in the diagnosis of subepidermal immunobullous disease, including Western immunoblotting and ELISA using particular fragments of recombinant BP180 protein and, most recently, the use of eukaryotic cells genetically modified to express BP180 extracellular domain as IF substrate.

Other studies have explored the use of BP180 ELISA in the diagnosis of BP and PG. Most published studies on the use of BP180-based ELISAs have emphasized their use in the diagnosis of BP. Using various portions of the NC16a domain of BP180, specificities and sensitivities ranged from 98% to 100% and from 53% to 91%, respectively, in the diagnosis of PG or BP. Larger antigens can be synthesized using baculovirus-insect cell culture systems, and recently, an ELISA that uses almost the entire extracellular domain of BP180 (devoid of 68 amino acids at the C-terminus) has been developed. It was found to be 92% sensitive and 97% specific at differentiating BP (n=39) and PG (n=10) serum samples from pemphigus (n=15) and control (n=18) serum samples. Only 1 previous study has specifically explored the use of NC16a ELISA in the diagnosis and management of PG, and it demonstrated sensitivity and specificity of 88% and...
100%, respectively, comparing 44 patients with PG with controls. They also found that serial ELISA values showed a correlation with the clinical course of the disease activity. The variability of sensitivity and specificity among BP180 ELISA systems developed so far is probably due to a combination of variables, such as the antigens used, the populations studied, and variations in techniques of calculating a cutoff value. This study is only the second to focus on the use of this ELISA in the diagnosis of PG, and it involves the largest series of PG serum samples studied; it specifically includes controls restricted to women younger than 50 years and compares the reactivity to the NC16a domain between PG and PUPPP serum samples. This ELISA demonstrates the usefulness of a reliable and well-standardized commercial assay in the serodiagnosis of PG.

Table 1 summarizes the false-negative rates as observed by NC16a ELISA or indirect IF using either the IgG or the C3 technique alone. In fact, NC16a ELISA is more sensitive than IF using either the IgG or the C3 technique alone, but it is marginally less sensitive than the traditional combination of both indirect IF techniques (sensitivity, 99%). We observed no correlation between NC16a ELISA values and indirect IF titers as determined using either the conventional IgG or the C3 technique. It is difficult to directly compare the 2 techniques because they differ in the antigen substrate and the secondary antibody used; however, because anti-BMZ antibodies in PG serum samples have been demonstrated to target predominantly the NC16a domain of BP180, we initially expected to observe a correlation between the NC16a ELISA values and antibodies as detected by indirect IF. The PG situation seems to contrast with the pemphigus experience, where indirect IF titers have been shown to correlate with disease activity and desmoglein specific antibodies as measured by ELISA. However, in BP, no correlation is found between antibody titers as measured by indirect IF and clinical disease activity. This observation may be partly explained by the finding that indirect IF staining of BP autoantibodies mainly reflects reactivity to BP230 and, to a lesser degree, BP180. Therefore, in BP, no correlation has been observed between indirect IF titers and ELISA values. Recent observations suggest that serum levels of anti–BP180 antibodies as detected by ELISA may be related to disease severity in BP. The false-negative ELISA results and the lack of correlation between ELISA values and indirect IF titers may reflect technical or conformational differences between the 2 assays or reactivity to other BMZ antigens or epitopes, outside the NC16a domain.

For rapid routine diagnosis, NC16a ELISA is a powerful tool in differentiating between PG and PUPPP because (1) it is easy and rapid to perform (the whole assay can be performed in 3½ hours), (2) it is suitable for simultaneous testing of multiple serum samples, (3) it allows for the objective and semiquantitative analysis of NC16a specific antibodies, and (4) it seems to be highly specific and sensitive. Our results provide further evidence that the NC16a domain of BP180 contains the primary target epitopes of PG autoantibodies and that antibodies to NC16a are not present in PUPPP. Our results demonstrate that the BP180-NC16a ELISA is useful not only in the diagnosis of PG but also in making a prognostically important but often clinically difficult differentiation between PG and PUPPP.

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