Accuracy of indirect immunofluorescence testing in the diagnosis of paraneoplastic pemphigus

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Background: Paraneoplastic pemphigus (PNP) is an autoimmune disease defined in part by autoantibodies with unique specificity. Initial reports suggested that indirect immunofluorescence with rodent bladder epithelium was highly reliable in detecting these autoantibodies.

Objective: We compared the sensitivity and the specificity of indirect immunofluorescence in the diagnosis of PNP in a large number of cases.

Methods: Indirect immunofluorescence was performed on stratified squamous epithelium of monkey esophagus and mouse tongue, bladder, liver, and myocardium. Sera were obtained from 28 patients with PNP and from 29 control subjects with autoimmune blistering diseases.

Results: The sensitivity of murine bladder as a substrate was 75%, with a specificity of 83%. Indirect immunofluorescence on liver was specific (96.5%) but insensitive (43%). Sensitivity and specificity with myocardium were intermediate.

Conclusion: Indirect immunofluorescence on murine bladder epithelium is an adequate screening test for PNP but is negative or indeterminate in as many as one fourth of patients. Negative indirect immunofluorescence does not exclude the diagnosis of PNP, and immunochemical techniques such as immunoprecipitation must be performed.

Paraneoplastic pemphigus (PNP) is an autoimmune blistering disease first described by Anhalt et al.1 in 1990. It was originally defined by the following criteria: (1) clinically, by painful erosive mucositis and a polymorphous skin eruption in patients with an associated neoplasm; (2) histologically, by suprabasilar acantholysis, dyskeratotic keratinocytes, and vacuolar interface changes; (3) by direct immunofluorescence showing deposition of IgG and C3 in the epidermal intercellular space (ICS) and often along the basement membrane zone (BMZ); (4) by serologic detection of autoantibodies reactive with simple, columnar, and transitional epithelia, and nonepithelial tissue; and (5) by immunoprecipitation of a distinctive complex of proteins with carbon 14–labeled keratinocyte extracts. The original report of the syndrome was based on five patients. To date our laboratory has identified a total of 45 cases, some of which have been reported by us and by others.2–6

With additional experience we have come to appreciate that the syndrome is reproducible and distinctive, and the original clinical histologic and immunopathologic features are shared by all patients. However, some variability exists in the autoantibody profile in individual cases. The original report and subsequent studies have shown that the antigens recognized in this syndrome include polypeptides at
250, 230, 210, 190, and 170 kd. It is now known that the bands at 250 and 210 kd represent desmoplakins I and II, respectively; the 230 kd band is the BP 230 antigen, and the 190 and 170 kd antigens have not been identified. Immunochemical studies have shown that a subset of patients have weak or undetectable reactivity with desmoplakin I and the BP 230 antigen but have consistent autoantibody reactivity against the remaining protein bands, desmoplakin II and the 190 and 170 kd antigens. This weak reactivity with desmoplakin I seemed to be associated with a failure of these sera to be easily identified by indirect immunofluorescence (IIF) screening. Therefore it seemed important to review our experience with immunofluorescence evaluation of these cases, to correlate it with the antigenic specificity of the patients' autoantibodies, and to gain more insight into the reliability of laboratory tests in the diagnosis of this syndrome.

MATERIAL AND METHODS

Patient selection

Patients were considered to have a confirmed case of PNP if they displayed at minimum the following findings:

1. Persistent and refractory stomatitis and polymorphous or blistering skin eruption were present in the context of an underlying neoplasm. Of the 28 patients included in the current study, associated neoplasms included non-Hodgkin’s lymphoma (n = 12), chronic lymphocytic leukemia (n = 5), Castleman’s tumor (n = 4), thymoma (n = 3), Waldenström’s macroglobulinemia (n = 2), and poorly differentiated spindle cell neoplasm (n = 2).

2. All patients at some point in their examination had a biopsy of mucous membrane or skin that showed suprabasilar acantholysis. Additional findings of keratinocyte necrosis and vascular interface change were sporadically seen.

3. Biopsy of skin or mucous membrane showed in vivo bound IgG and complement components on the cell surface of affected epithelium. The coexistent finding of BMZ staining was seen frequently but was not an absolute requirement for diagnosis.

4. Circulating autoantibodies bound the cell surface of stratified squamous epithelium in a pattern consistent with pemphigus.

5. Immunoprecipitation studies with carbon 14-labeled keratinocyte extracts demonstrated (1) lack of detection of pemphigus vulgaris or foliaceus antigen and (2) identification of at least three of the five antigen bands identified by patients with PNP. Of the five antigen bands that are detectable by immunoprecipitation, desmoplakin I (at 250 kd) and the BP 230 antigen were variably absent. All patients studied had at minimum reactivity by immunoprecipitation with desmoplakin II (at 210 kd) and the uncharacterized antigen bands at 190 and 170 kd.

Control sera for IIF studies were obtained from 29 patients with other bullous disorders such as pemphigus vulgaris, pemphigus foliaceus, bullous pemphigoid, and cicatricial pemphigoid. Seven of these control subjects had pemphigus vulgaris or pemphigus foliaceus associated with nonlymphoid neoplasms such as squamous cell carcinoma of the cervix, lung, or skin, and adenocarcinoma of the bowel.

Results were recorded as positive or negative in a blinded reading. The sensitivity of each tissue tested was calculated as the ratio of true positives to the sum of the true positives and false negatives, multiplied by 100. The specificity is the ratio of true negatives to the sum of the true negatives and false positives, multiplied by 100.

Immunofluorescence techniques

IIF was performed according to established methods with the following tissues: monkey esophagus, and murine tongue, liver, heart, and urinary bladder. Fresh tissues were embedded in OCT medium (Tissue Tek, Miles Laboratories, Elkhart, Ind.) and snap-frozen in liquid nitrogen. Four-micrometer sections were incubated for 30 minutes with a 1:20 dilution of patient or control serum, washed, and incubated with fluorescein isothiocyanate-labeled anti-human IgG (Cappel, Organon Teknika, Durham, N.C.). The sections were mounted and examined with an epifluorescence microscope. Patients who appeared to have PNP by other criteria, including immunoprecipitation, but who had negative IIF findings on mouse bladder were tested again on bladder epithelium by complement-fixation IIF. For this purpose 4 μm frozen sections of mouse urinary bladder was exposed sequentially to a 1:10 dilution of the patient’s serum, to a 1:5 dilution of fresh normal human serum as a complement source, and then to fluorescein-conjugated anti-C3 (Cappel).

RESULTS

Use of stratified squamous epithelium

PNP sera that had a high titer of autoantibody (>640) could often be distinguished from pemphigus vulgaris or foliaceus sera morphologically with monkey esophagus. These PNP sera bound uniformly throughout the epithelium of monkey esophagus and also stained all surfaces of the basal epithelial cells, including the basilar surface, producing combined ICS and BMZ staining. Pemphi-
Fig. 1. Usefulness of IIF in discriminating between pemphigus vulgaris and PNP with stratified squamous epithelium of monkey esophagus and murine urinary bladder epithelium, is shown. A and B, IIF with pemphigus vulgaris sera on monkey esophagus and mouse urinary bladder. A, Binding of IgG to cell surface of suprabasilar epithelial cells in monkey esophagus is clearly seen. BMZ of esophagus is demonstrated by the white arrows, and there is no reactivity with basal cells or with BMZ. B, No reactivity is shown with urinary bladder epithelium (outline of bladder epithelium is detailed by square white dots.) C and D, IIF with PNP sera. Binding is diffuse on cell surface of all layers of epithelium of monkey esophagus and binding to basilar surface of basal epithelial cells is evident as well (white arrows). D, Characteristic cytoplasmic and cell surface staining of urinary bladder epithelium from antibodies in sera of patient with PNP. This illustration shows an optimal result in which it is easy to discriminate between these two diseases with IIF. (A and C, ×400; B and D, ×200.)

gus vulgaris sera do not stain the basilar surface of basal cells and bind most intensely in suprabasilar cell layers (Fig. 1). This finding was not as apparent in low-titer sera (IIF titers, <40).

When IIF was performed on stratified squamous epithelium of monkey esophagus, 24 of 28 PNP sera had readily observable pemphigus-like autoantibodies, staining the ICS of the epithelial cells in a pattern that was often indistinguishable from that seen with pemphigus vulgaris or foliaceus. Four patients with PNP demonstrated strong cytoplasmic background fluorescence that obscured the ICS staining on monkey esophagus, but on mouse tongue the cytoplasmic background was less intense and ICS staining could be observed. This background cytoplasmic fluorescence could not be eliminated
Fig. 2. Problematic case of PNP in which IIF could not reliably detect circulating antibodies. A, IIF with patent serum at dilution of 1:10 on monkey esophagus. Heavy cytoplasmic staining of epithelial cells obscures any discernable cell surface binding. Use of higher dilutions of serum did not produce any clearer resolution of these findings. B, IIF of same serum on mouse tongue epithelium. Very strong cytoplasmic staining persists, but there is some detection of cell surface staining in suprabasilar epithelium. C, IIF with mouse urinary bladder epithelium. Characteristic cytoplasmic and cell surface staining of bladder epithelium is evident on this tissue. This is representative of case in which IIF with monkey esophagus alone was considered negative for circulating antibodies but IIF on bladder epithelium suggested that PNP autoantibodies were present in serum. (A, ×400; B, ×200; C, ×100.)

simply by the use of higher dilutions of the patients’ sera (on monkey esophagus). Sera of these four patients did react with urinary bladder epithelium and immunoprecipitated the characteristic PNP antigen complex. An example of this finding is shown in Figs. 2 and 3. The patient used as an illustration had an associated non-Hodgkin’s lymphoma, severe mucositis and conjunctivitis, and flexural blisters that had histologic and direct immunofluorescence features of pemphigus, but initial IIF screen for pemphigus antibodies on monkey esophagus was interpreted as negative. The patient had a characteristic clinical course and died within 1 month of definitive diagnosis.

Use of nonstratified squamous epithelium: Mouse urinary bladder

Of the 28 patients with PNP, sera from 21 had unequivocal binding to urinary bladder epithelium. The staining consisted of both cell surface and cytoplasmic fluorescence in a pattern and intensity unique to these patients. Therefore the sensitivity for this substrate is 75% (Table I). In seven patients the intensity of staining of the bladder epithelium was faint and indistinct and it was not possible to identify these sera consistently with this substrate. All seven of these apparent false-negative sera did immunoprecipitate the PNP complex, but the reactivity of these sera with the desmoplakin I band was characteristically faint or undetectable. These sera, however, did reliably and reproducibly identify desmoplakin II and the 190 and 170 kd PNP antigens, and did not recognize either the pemphigus foliaceus or pemphigus vulgaris antigens.

Amplification of the immunofluorescence signal by indirect complement fixation allowed identification of four of the seven false-negative sera. These had in vitro C3 fixation to the epithelium of the bladder that was greater than that observed in any control sera. With complement immunofixation the
sensitivity of the technique was increased to 89%. Control sera in the C3 amplification included sera from 10 patients with bullous diseases such as pemphigus, bullous pemphigoid, and cicatricial pemphigoid. None of these 10 control sera produced a false-positive result.

Use of nonepithelial tissues: Liver and myocardium

Of the 28 PNP sera tested on mouse liver, only 12 stained the hepatocyte desmosomes in an unequivocal pattern. Only one control serum produced a false positive, giving this substrate the highest specificity but the lowest sensitivity. Mouse myocardium was used to test the reactivity against desmoplakins in the intercalated disks of the heart. Twenty-two patients with PNP had positive findings, but it was common to find low-intensity binding to this structure in control sera that could not be reliably differentiated in a blinded reading (8/29 controls).

DISCUSSION

During the initial investigation of patients with PNP, the unique autoantibodies associated with this syndrome were characterized by an immunochemical technique, specifically immunoprecipitation. We showed that patients with this syndrome did not identify the pemphigus vulgaris or foliaceus antigens but recognized a group of high-molecular-weight proteins not identified in patients with any other autoimmune disorder. It then became apparent that the 250 and 210 kd polypeptides represented desmoplakin I and II, the major plaque proteins of desmosomes and that the 230 kd protein was the BP 230 antigen, which is known to be the major plaque protein of hemidesmosomes. Although immunoprecipitation defines autoantibody specificity with precision, the technique is time consuming, expensive, technically demanding, and of extremely limited availability. With knowledge gained from immunoprecipitation studies, we believed that it may be possible to detect these autoantibodies with simple IIF screening. The distribution of these protein antigens in various tissues has been well studied. It is known that stratified squamous epithelia such as monkey esophagus and murine tongue express pemphigus vulgaris and foliaceus antigens as well as desmoplakins I and II. Other epithelia have desmosomes that contain desmoplakin I and/or desmoplakin II but do not express pemphigus vulgaris or foliaceus antigens. Examples would be respiratory, intestinal, and urinary bladder epithelium. Nonepithelial tissues that contain desmoplakins include the intercalated disks of myocardium and desmosomes on the cell surface of hepatocytes and lining bile canaliculi in the liver. Therefore it was originally proposed that patients with PNP could be identified and distinguish from those with pemphigus vulgaris by IIF performed on stratified squamous epithelia such as monkey esophagus and nonstratified squamous epithelium such as rodent urinary bladder.
Table I. Summary of results

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<th>Tissue</th>
<th>Tongue</th>
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<th>Liver</th>
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<td>21</td>
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<tr>
<td>Total No.</td>
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<td>28</td>
<td>28</td>
<td>28</td>
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<td>Sensitivity (%)</td>
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<td>78.5</td>
<td>43</td>
<td>75</td>
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- Sensitivity:* of PNP sera binding to substrates
- Specificity:* of substrates with control sera

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<tr>
<td>Total No.</td>
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<tr>
<td>Specificity (%)</td>
<td>72</td>
<td>96.5</td>
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*Calculated as ratio of positive readings to the sum of positives and false negatives, multiplied by 100.
†Calculated as ratio of true negatives to sum of true negatives and false positives, multiplied by 100. Specificity of stratified squamous epithelium of tongue and esophagus are not calculated because control sera consisted in part of patients with pemphigus vulgaris and pemphigus foliaceus, both of which cannot be reliably discriminated from PNP with these substrates. Bladder epithelium gave best balance between sensitivity and specificity, but a substantial number of false negative readings occurred.

Initially this technique appeared to be highly reliable, but these reports involved only a limited number of cases. For example, Liu et al. estimated the specificity of urinary bladder for detection of these autoantibodies at 98.9%. Their conclusions were based on the study of just four patients with confirmed PNP, and they stated that "many patients with paraneoplastic pemphigus need to be identified and studied before the exact sensitivity of IIF can be determined." Our own initial report included only five patients with the disease. Now that additional cases have been identified and referred to our laboratory for diagnosis, we have been able to evaluate more thoroughly the usefulness and limitations of IIF screening of these patients' autoantibodies. Additionally, we chose to examine the usefulness of IF with the use of two nonepithelial tissues that contain desmoplakins in morphologically identifiable structures: murine myocardium and murine liver.

In our study of 28 patients, IIF with urinary bladder epithelium is a reasonably specific test but is not very sensitive. Fully one fourth of PNP sera had reactivity that was not greater than that of some control sera and were not positive in blinded readings. Its sensitivity can be improved from 75% to 90% with the C3 amplification method. This method is a three-step procedure that amplifies the detection of complement binding antibodies that are present in low titers in the patient's serum, and it is routinely used for detection of antibodies against the BP 180 antigen in herpes gestationis (the HG factor). Even with the use of complement amplification, 10% of the patients with PNP had a false-negative IIF result with murine urinary bladder. In view of these results, we advise cautious interpretation of negative IIF results with urinary bladder. Some investigators have questioned whether the use of bladder epithelium from certain species may be more sensitive in detecting these antibodies, but there is no indication that urinary bladder tissue from any species increases the test's usefulness. Desmoplakins are highly conserved proteins, and monoclonal and polyclonal antibodies to desmoplakins have been shown to react equally well in all vertebrate species.

We have examined patients' autoantibody reactivity with urinary bladder from guinea pig, mouse, rat, and human bladder carcinoma cell lines. The sera with high levels of autoantibodies against desmoplakins reacted well with all tissues: no tissue was superior in detecting those sera with lower levels of antidesmoplakin autoantibodies (data not shown).

The usefulness of IIF screening on other tissues that are known to contain desmoplakins also had significant limitations. Reactivity with desmoplakins in the intercalated disks of myocardium was found in far too many control sera, so this tissue was sensitive but not specific. The opposite was true when autoantibody binding to desmosomes in liver was
examined: this was observed almost exclusively with PNP sera, but more than half the cases were not detected with this tissue.

In the initial report that described this disease, numerous sera from other diseases were screened for the presence of high titers of antidesmoplakin antibodies, including 60 sera from patients with erythema multiforme, and none was found. However, Fodinger et al.\textsuperscript{18} recently identified six patients with erythema multiforme major, not associated with any neoplasm, who had antidesmoplakin antibodies. These sera stained urinary bladder, myocardium, and liver in a pattern indistinguishable from that of patients with PNP, and recognized desmoplakins I and II by immunoblotting. These antibodies were detectable only during periods of disease activity and were not present during convalescence. Although no similar sera were identified in the control sera used in this study, false-positive IIF may additionally be seen in some cases of erythema multiforme major, a disease that may clinically be mimicked by some cases of PNP.

Finally, in all cases considered in this study, in cases of PNP in which the characteristic autoantibodies were not detectable by IIF, they were demonstrated by immunoprecipitation. Recently we evaluated two cases of suspected PNP associated with a lymphoid malignancy in which serum autoantibodies could not be detected by any technique. Despite this, the patients fulfilled all other clinical, histologic, and direct immunofluorescence criteria for the syndrome, including relentless progression and death. It is possible that more such cases will be identified, and at present these are considered to be suspected but not proved cases of antibody-negative PNP.

The results of this study reinforce the principle that no single laboratory test should be relied on to establish a diagnosis; a complete clinical pathologic correlation must be considered. This is true for PNP. Although screening serum by IIF on substrates such as urinary bladder epithelium is useful in a majority of cases, both false-negative and false-positive results are observed in a frequency greater than that at which we had originally estimated. In suspected cases in which preliminary IIF screening fails to detect autoantibodies reactive with stratified squamous epithelium and/or bladder epithelium, further immunochromatographic studies should be pursued if the clinical presentation warrants it. In our opinion immunoprecipitation with metabolically labeled keratinocytes remains the standard for laboratory diagnosis, but even this technique cannot be assumed to identify all cases of PNP.

REFERENCES