Characterization of Two Corneal Epithelium-Derived Antigens Associated with Vasculitis

Irena Reynolds, Sally L. John, Andrew B. Tullo, Shirley Ayad, Keith Morgan, Francis W. Ballardie, P. J. Lennox Holt, and M. Chantal Hillarby

PURPOSE. In a previous investigation into corneal autoimmunity, it was demonstrated that a putative autoantigen, a protein of 66 kDa, present in bovine corneal epithelium, binds circulating autoantibodies in approximately 60% of patients with Wegener’s granulomatosis (WG). The aim of the present study was to characterize and identify the 66-kDa protein.

METHODS. A purification protocol was established for the 66-kDa protein using standard chromatography techniques. During the purification procedure it became clear that the 66-kDa protein detected in patients’ sera was in fact two proteins, both running at 66 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, that eluted in different fractions on DE-52 chromatography columns. These two proteins have been labeled bovine corneal epithelial antigen-A and -B (BCEA-A and BCEA-B). Further investigations of antibody binding have demonstrated that patients’ sera bind to either one or the other of these proteins with no cross-reactivity between them. Separated BCEA-A and BCEA-B protein extracts were immunoblotted with 27 WG patients’ sera, 10 Churg-Strauss syndrome (CSS) patients’ sera, 31 rheumatoid arthritis (RA) patients’ sera, and 40 healthy control subjects’ sera from the blood bank.

RESULTS. Forty-six percent of WG patients’ sera had antibodies to one of the 66-kDa antigens, whereas none of the healthy control subjects’ sera had 66-kDa antibodies (P < 10^-5). In the WG group, 31% were positive to BCEA-A (versus controls, P = 0.0023), and 15% were positive to BCEA-B. WG patients with peripheral ulcerative keratitis (PUK) had a significant association with anti-BCEA-A antibodies when compared with healthy control subjects (50%, P < 10^-5). However, in the RA group with no eye disease there was an association with BCEA-A (25%, P = 0.011) but not in the RA group with PUK. The frequency of anti-BCEA-B antibodies was significantly increased in patients with CSS (60%, P < 10^-7).

CONCLUSIONS. In summary, it has been shown that vasculitis patients have antibodies to two 66-kDa corneal antigens and that autoantibodies to these antigens are mutually exclusive. It has also been shown that antibodies to BCEA-B are associated with CSS, whereas BCEA-A antibodies are associated with WG and RA.

From the 1Department of Rheumatology, the 2Arthritis and Rheumatism Council Epidemiology Research Unit, National Health Service, and 3The Wellcome Centre for Cell Matrix Research, University of Manchester, 4Manchester Royal Eye Hospital, and the 5Manchester Royal Infirmary, Central Manchester Healthcare NHS Trust, Manchester, UK.

Supported by a grant from the Arthritis and Rheumatism Council.

Submitted for publication March 18, 1998; revised July 1, 1998; accepted July 17, 1998.

Proprietary interest category: N.

Reprint requests: M. Chantal Hillarby, Department of Rheumatology, Stopford Building, University of Manchester, Oxford Road, Manchester, M13 9PT, UK.

Wegener’s granulomatosis (WG) is a rare disease of unknown etiology that is characterized by vasculitis of the upper and lower respiratory tracts, often in combination with glomerulonephritis. WG can also affect other organ systems, including the skin, eye, heart, nervous system, and gastrointestinal tract. Ophthalmic involvement may be present in as much as 58% of all WG cases. Sometimes it may constitute the major symptom, in other cases it can be the presenting clinical manifestation of the disease. Scleritis, proptosis, peripheral ulcerative keratitis (PUK), and conjunctivitis are among the most common ocular manifestations of WG.

Wegener’s granulomatosis has an autoimmune background, and a number of circulating autoantibodies have been identified in the sera of patients with the disease. The most widely studied among them are cytoplasmic anti-neutrophil antibodies (c-ANCA). A number of studies have shown that c-ANCA is a sensitive and specific marker for active WG (67%-95% depending on disease activity). It has also been suggested that the fluctuation in c-ANCA titer might correlate with disease activity.

Several non-neutrophil-related antibodies have also been found in patients with WG. Among others, antibodies against the basement membrane of the glomerulus, endothelial cells, smooth muscle, and cardiac muscle have been reported. With growing evidence that a number of autoantibodies may play an active part in the pathology of WG, the investigation into the role of putative autoantigens may help to elucidate the mechanisms underlying the disease process.

Autoantibodies have also been identified in inflammatory eye disease without systemic involvement. A recent report has identified an autoantigen specific for Mooren’s ulcer.

In a previous study, we investigated the presence of autoantibodies to the cornea in peripheral ulcerative keratitis (PUK) patients, both in isolation and in association with an
autoimmune disease such as WG or rheumatoid arthritis (RA). We demonstrated that a number of putative autoantigens present in the corneal epithelium are recognized by antibodies in the patients' sera. One of these antigens, identified as a 54-kDa aldehyde dehydrogenase, has been associated with PUK and eye injury.14,15 Another of these proteins, a 66-kDa antigen, is the subject of our present study. This antigen is clearly associated with WG irrespective of the presence or absence of eye disease, is rarely detected by sera from patients with RA, and is not detected at all by control blood bank sera.14 In this report, we further characterize this 66-kDa antigen and demonstrate the presence of two 66-kDa proteins. We investigated the association between antibodies against these proteins and different forms of systemic vasculitis, with and without ocular involvement.

**Materials and Methods**

Methods of securing human and animal tissues were in compliance with the National Institutes of Health Guidelines on the Care and Use of Animals in Research, the Declaration of Helsinki, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Patients**

Sera from patients with PUK and WG were compared with those from normal control subjects and several other control groups, including WG patients without PUK, to determine whether the autoantibodies were PUK or WG specific. Patients with Churg-Strauss syndrome (CSS) were used as a control group of patients with vasculitis similar to that of the test group but no eye complications. RA was used as a control group of patients for vasculitis and eye complications. Blood was taken from patients attending the Manchester Royal Eye Hospital or the Rheumatology Department of the Manchester Royal Infirmary. The diagnoses of WG, CSS, and RA were made according to the standard method. The demographic characteristics of patients are summarized in Table 1. Blood was collected, allowed to clot at room temperature, and then centrifuged at 1500g for 10 minutes. The serum was removed and stored at −20°C until required. Normal control sera were acquired from the blood bank.

**Detection of Anti-Neutrophil Cytoplasmic Antibodies**

The tests for c-ANCA and perinuclear ANCA were carried out in the Immunology Department of St. Mary's Hospital (Manchester, UK). Indirect immunofluorescence staining was performed using normal human neutrophils that were cytocentrifuged onto slides, fixed in ethanol, incubated with dilutions of patients' sera, and then stained with fluorescent secondary anti-immunoglobulin antibody.

**Preparation of Soluble Corneal Extract**

Corneal epithelium was scraped from the central region of bovine corneas. Three hundred micrometers of tissue collected from 20 eyes was homogenized in 5 mL 1 M NaCl 40 mM Tris/HCl, pH 7.8, containing 1 μL 10 mM phenylmethylsulfonyl fluoride (PMSF), using an Ultraturrax homogenizer. The homogenized extract was then centrifuged at 20,000g for 30 minutes. The supernatant was dialyzed against distilled water followed by 20 mM Tris/HCl, pH 8.0, for 48 hours. The concentration of the protein was estimated by spectrophotometer at a wavelength of 280 nm. The integrity of the corneal extract was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining to ensure that the proteins were not degraded. The resultant corneal extract was used as the substrate in the immunoblotting assay. With this same method, tissue extracts were prepared from bovine kidney, liver, esophageal tissue, the human skin epithelial cell line (NCTC-Flow Laboratories), and human neutrophils.

**Purification of BCEA**

The soluble corneal extract was dialyzed into 0.05 M NaCl 20 mM Tris/HCl, pH 7.8, and diluted to a protein concentration of 29 mg/mL in the same buffer. Five milliliters of the extract was loaded onto a DE-52 column. Unbound proteins were eluted from the column with 0.05 M NaCl 20 mM Tris/HCl, pH 7.8. Bound proteins were eluted with 0.5 M NaCl 20 mM Tris/HCl pH 7.8. The resultant fractions were dialyzed into 20 mM Tris/HCl, pH 7.8. The proteins in each fraction were analyzed by SDS-PAGE and immunoblotting with patients' sera containing antibodies to BCEA.

**Preparation of Extracts from Human Neutrophils**

Heparinized whole human blood mixed 1:1 with Hanks' balanced salt solution was layered onto a Histopaque density gradient, specific gravity 1.083. Cells were centrifuged, collected from the interface, and washed three times in Hanks' balanced salt solution, according to the standard method. In total, 10^6 cells were resuspended in 0.1 M NaCl 20 mM Tris/HCl, pH 7.8, containing 1 μL 10 mM PMSF and 0.1% Nonidet P40. The proteins were extracted from the neutrophils as described above.

**SDS-PAGE and Immunoblotting**

Protein from tissue extracts or antigen-enriched protein preparations were separated by SDS-PAGE on 10% acrylamide slab gels with 3.5% stacking gels. The proteins were visualized by staining the gels in 0.075% Coomassie blue, 45% methanol, and 10% glacial acetic acid and then destained with 10% glacial acetic acid. Alternatively, the proteins were transferred to a nitrocellulose membrane by electroblotting. The membrane

<table>
<thead>
<tr>
<th>Demographic Characteristics of Patients and Healthy Control Group</th>
<th>No. of Patients</th>
<th>M-F Ratio</th>
<th>Mean Age</th>
<th>Age Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>WG without eye disease</td>
<td>13</td>
<td>5:8</td>
<td>46</td>
<td>21-81</td>
</tr>
<tr>
<td>WG + PUK</td>
<td>8</td>
<td>8:0</td>
<td>56</td>
<td>24-83</td>
</tr>
<tr>
<td>WG + non-PUK eye disease</td>
<td>6</td>
<td>4:2</td>
<td>53</td>
<td>41-61</td>
</tr>
<tr>
<td>CSS</td>
<td>10</td>
<td>5:5</td>
<td>56.5</td>
<td>45-70</td>
</tr>
<tr>
<td>RA without eye disease</td>
<td>12</td>
<td>3:9</td>
<td>56.5</td>
<td>49-73</td>
</tr>
<tr>
<td>RA + PUK</td>
<td>17</td>
<td>6:11</td>
<td>64.5</td>
<td>50-90</td>
</tr>
<tr>
<td>RA + non-PUK eye disease</td>
<td>7</td>
<td>2:5</td>
<td>63.8</td>
<td>55-72</td>
</tr>
<tr>
<td>RA + vasculitis</td>
<td>27</td>
<td>11:6</td>
<td>61.5</td>
<td>35-85</td>
</tr>
<tr>
<td>Healthy control group</td>
<td>40</td>
<td>23:17</td>
<td>41</td>
<td>24-65</td>
</tr>
</tbody>
</table>

WG, Wegener's granulomatosis; PUK, peripheral ulcerative keratitis; CSS, Churg-Strauss syndrome; RA, rheumatoid arthritis.
A BCEA-A-enriched extract was separated by SDS-PAGE and Two-Dimensional Gel Electrophoresis to identify the relevant band. The horizontal strip of nitrocellulose was washed in PBS/Tween and then washed in veronal acetate buffer (0.15 M sodium acetate, 0.15 M sodium barbitone, pH 9.6). The final enzymatic reaction occurred when the strips were incubated in 10 ml substrate (0.122 mM nitroblue tetrazolium, 0.115 mM 5-bromo-4-chloroindol phosphate, 4 mM MgCl2 in veronal acetate buffer, pH 9.6). The strips were allowed to develop until a brown precipitate indicated where antibody had bound to the protein. The reaction was stopped by washing the nitrocellulose in distilled water to remove the excess substrate. One patient’s serum was included as a standard in each assay to maintain consistency between assays. For the cross-reactivity experiment, the antibodies bound to the relevant band were eluted with 0.2 M glycine, pH 2.2 (before the enzymatic reaction), and immediately neutralized with 1 M Tris/HCl, pH 7.5.

Two-Dimensional Gel Electrophoresis

Two-dimensional (2D) gel electrophoresis was performed on epithelial cells and antigen-enriched extracts. The ampholine gradient for the isoelectrofocusing of the proteins for the first dimension was as follows: pH 3.5 to 9.5 broad range and pH 6.0 to 8.0 narrow range. The 2D separation was performed after the SDS-PAGE procedure described above. After the 2D separation, proteins were visualized by silver staining. Alternatively, the 2D gel was electroblotted onto nitrocellulose, and the transferred proteins probed with the patients’ sera (1/100) after the procedure described above.

Production of Rabbit Polyclonal Antibodies to BCEA-A

A BCEA-A-enriched extract was separated by SDS-PAGE and electroblotted onto a pure nitrocellulose membrane. The membrane was stained with Ponceau S to identify the relevant band. The identified protein band was excised from the membrane with a sterile scalpel blade. The horizontal strip of nitrocellulose containing the BCEA-A was washed in sterile PBS/Tween to remove the Ponceau S. Then the dried strip of nitrocellulose was crushed with a mortar and pestle in a small amount of liquid nitrogen. The fine powder obtained was suspended in 100 µl sterile PBS and stored at -80°C. Four nitrocellulose strips (prepared as described above) were used for one immunization.

A female New Zealand white rabbit was bled before immunization, and 100 µl serum was diluted 1:10 with 1% Marvel/phosphate-buffered saline (PBS)/Tw cen (pH 7.5) for 1 hour at room temperature and then cut into strips. The strips were then fed to patients’ sera or control sera diluted 1:1000 in Marvel/PBS/Tween (pH 7.5) for 1 hour at room temperature. After washing in PBS/Tween, the strips were incubated with alkaline phosphatase-conjugated rabbit anti-human IgG (DAKO Ltd.), diluted 1:2000 in Marvel/PBS/Tween for 1 hour at room temperature. The nitrocellulose was washed in PBS/Tween and then washed in veronal acetate buffer (0.15 M sodium acetate, 0.15 M sodium barbitone, pH 9.6). The final enzymatic reaction occurred when the strips were incubated in 10 ml substrate (0.122 mM nitroblue tetrazolium, 0.115 mM 5-bromo-4-chloroindol phosphate, 4 mM MgCl2 in veronal acetate buffer, pH 9.6). The strips were allowed to develop until a brown precipitate indicated where antibody had bound to the protein. The reaction was stopped by washing the nitrocellulose in distilled water to remove the excess substrate. One patient’s serum was included as a standard in each assay to maintain consistency between assays. For the cross-reactivity experiment, the antibodies bound to the relevant band were eluted with 0.2 M glycine, pH 2.2 (before the enzymatic reaction), and immediately neutralized with 1 M Tris/HCl, pH 7.5.

Tissue Localization of BCEA-A by Immunofluorescence Staining

Fresh bovine eyes were obtained from an abattoir and brought back to the laboratory on ice. The corneal disc, including 2 mm of the surrounding sclera, was removed using a trephine and scissors. The disc was then cut into blocks of approximately 2 x 10 mm, ensuring that the limbus was included. The tissue was then snap-frozen in liquid nitrogen-cooled isopentane, mounted in ornithine carbamoyltransferrase compound, and cut perpendicular to the corneal surface into 5-µm sections using a cryostat. The sections were mounted onto 3-aminopropyltriethoxysilane-coated microscope slides, air-dried, and stored at -20°C until required. The same procedure was also used to prepare human corneal sections. The eyes were obtained from the eye bank. Eyes unsuitable for corneal transplant were stored under sterile physiological conditions until the corneal disc was removed and the sample processed. Morphology of the sections was visualized by hematoxylin and eosin staining. Patient serum positive for anti-BCEA-A antibodies or the rabbit antiserum was used to detect the presence of BCEA-A in human or bovine tissue sections, respectively. The patient serum chosen for this study did not detect any antigen other than BCEA-A in the bovine corneal epithelium protein extract. Frozen sections were fixed in acetone for 5 minutes. After washing for 5 minutes in PBS, pH 7.5, the sections were incubated with rabbit, patient, or control sera (diluted in PBS) for 30 minutes at room temperature. A serum dilution of 1/20 was used routinely to screen sera, and some positive sera were titrated out in doubling dilutions to determine the end point.

![FIGURE 1. Soluble corneal epithelial protein extract was loaded onto a DE52 column, and proteins were eluted with 5 M NaCl. Unbound and bound proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (lanes a and c, respectively). Lanes b and c show immunoblotting with sera from two patients with Wegener's granulomatosis revealed a 66-kDa antigen in both preparations, which were recognized by different patient sera. Bovine corneal epithelial antigen-A (BCEA-A) in the unbound fraction (lane b). BCEA-B in the bound fraction (lane d).](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/732454/)
Statistical Methods

Chi-square analysis (with Yates' correction) was used to determine the significance of a frequency difference between the test groups and the normal control group (significance level 5%).

RESULTS

Purification of the 66-kDa BCEA

After purification of BCEA on a DE-52 cellulose column and examination of the resultant fractions by SDS-PAGE (Fig. 1, lanes a and c) and immunoblotting (Fig. 1, lanes b and d), it became clear that two 66-kDa proteins were present in the extract. One did not bind to the column (BCEA-A), and the other was bound and could be eluted with an increased salt concentration (BCEA-B). BCEA-A and BCEA-B both were detected by antibodies present in patients' sera. To confirm that the two antigens were distinct, both fractions were tested for cross-reacting antibodies. The extract was also examined by 2D electrophoresis.

Lack of Cross-reactivity of Antibodies to BCEA-A and BCEA-B

An antibody cross-reactivity test was performed on three sera positive for anti-BCEA-A antibodies and three sera positive for anti-BCEA-B antibodies. Antibodies bound to BCEA-A in the immunoblotting assay were eluted from the nitrocellulose strip and used for immunoblotting BCEA-B. Antibodies eluted from BCEA-B were also used in immunoblotting assays of BCEA-A. There was no cross-reactivity between antibodies binding to the two 66-kDa proteins. This suggested that BCEA-A and BCEA-B are two different proteins.

2D Electrophoresis and Immunoblotting of BCEA-A and BCEA-B

To confirm that BCEA-A and BCEA-B are two different proteins, the corneal epithelial cell extract was separated by 2D electrophoresis. The 2D gel was stained with silver nitrate to examine

Table 2. Detection of Antibodies to the BCEA-A and BCEA-B Proteins in Patient Sera

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>BCEA-A</th>
<th>BCEA-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>WG without eye disease</td>
<td>4 (31)*</td>
<td>2 (15)</td>
</tr>
<tr>
<td>WG + PUK (n = 8)</td>
<td>4 (50)</td>
<td>0</td>
</tr>
<tr>
<td>WG + other eye disease</td>
<td>2 (33)</td>
<td>2 (33)</td>
</tr>
<tr>
<td>CSS (10)</td>
<td>0 (0)</td>
<td>6 (60)</td>
</tr>
<tr>
<td>RA without eye disease</td>
<td>3 (25)</td>
<td>0</td>
</tr>
<tr>
<td>RA + PUK (17)</td>
<td>2 (12)</td>
<td>4 (23.5)</td>
</tr>
<tr>
<td>RA + other eye diseases</td>
<td>2 (28.5)</td>
<td>0</td>
</tr>
<tr>
<td>RA + vasculitis (27)</td>
<td>0</td>
<td>3 (11)</td>
</tr>
<tr>
<td>Controls (40)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

BCEA-A (and -B), bovine corneal epithelial antigen-A (and -B); WG, Wegener's granulomatosis; PUK, peripheral ulcerative keratitis; CSS, Churg-Strauss syndrome; RA, rheumatoid arthritis.

Values are number of antibodies, with percentages in parentheses. Statistical comparisons were made to normal control frequencies: *P = 0.0023; † = 0.000007; ‡ = 0.0078; § = 0.000001; || = 0.011; ′ = 0.0089.
FIGURE 3. Detection of bovine corneal epithelium antigen-A (BCEA-A) in human and bovine corneal epithelia. Human (A, B) and bovine (D, E) corneal sections were screened with either rabbit anti-BCEA-A sera (human sections) or human anti-BCEA-A sera (bovine sections). The sections were then incubated with fluorescein isothiocyanate-conjugated secondary antibody. Results are shown as phase contrast (A, E) and under fluorescence (B, D). BCEA-A was detected in a cellular pattern in the epithelium (E) in both the human (B) and bovine (E) sections. No BCEA-A was detected in the stroma (S) in either human or bovine cornea. No positive staining was detected when the secondary antibodies were used alone on human (C) and bovine (F) sections.

Detection of Antibodies to BCEA-A and BCEA-B

The BCEA-A- and BCEA-B-enriched extracts were separated by SDS-PAGE and immunoblotted with patients' sera. Several defined groups of patients were used to study the association between disease group and the presence of anti-BCEA-A or anti-BCEA-B antibodies. The results are summarized in Table 2.
In brief, BCEA-A antibodies were detected in significantly more WG and RA patients than in the normal control group. The group of WG patients with PUK showed the most significant association with anti-BCEA-A antibodies. However, no CSS patient had detectable anti-BCEA-A antibodies. Antibodies to BCEA-B were not significantly increased in WG, RA, or WG with PUK; however, the presence of these antibodies was significantly increased in the RA patients with PUK and the WG patients with non-PUK eye complications. There was a significant ($P < 10^{-6}$) increase of anti-BCEA-B antibodies in CSS patients compared with the normal control group.

Sera that previously contained antibodies to the unpurified BCEA detected either BCEA-A or BCEA-B protein, but no sera detected both BCEA-A and BCEA-B. The sera previously shown to contain no antibodies to the unpurified BCEA did not bind to either of the two proteins.

**Localization of BCEA-A in Human and Bovine Corneas**

Immunofluorescence staining of human corneal sections with rabbit anti-BCEA-A polyclonal antibodies showed BCEA-A present in the corneal epithelium and intracellular localization (Fig. 3a). No staining was present in either the stroma or the endothelium. Similar results were shown with bovine corneal sections and patients’ sera positive for anti-BCEA-A antibodies (Fig. 3b).

**Localization of BCEA-A and BCEA-B in Other Non-Corneal Tissues by Immunoblotting**

Serum, which detected the BCEA-A protein in the corneal extract, was tested against other tissue extracts. No protein at 66 kDa was detected by sera from patients with WG in bovine liver, kidney, and esophageal lining. However, there was binding to a 66-kDa band on a blot containing proteins from human skin epithelial cells (Fig. 4a).

The procedure described above was also applied to sera recognizing BCEA-B protein. This serum recognized a similar band in an esophageal epithelium extract and kidney extract (Fig. 4b).

**BCEA Proteins in Association with ANCA**

To investigate the possibility that c-ANCA-positive sera contained cross-reacting antibodies, detecting components in neutrophils and corneal epithelium, an immunoblotting assay was performed using BCEA-A- and BCEA-B–positive patients’ sera on neutrophil extract. No protein of 66 kDa could be detected in the extract, nor could any protein of a similar molecular weight to any of the known ANCA-reactive proteins be detected.

All the WG and CSS patients positive to either of the two 66-kDa proteins were examined for the presence of ANCA. Table 3 shows the summary of the results. This study indicates that there is no association between anti-BCEA-A and anti-BCEA-B antibodies and ANCA for any of the patient groups analyzed in this study.

**DISCUSSION**

In this study we demonstrated that sera from patients with systemic vasculitis and eye disease detect two bovine corneal epithelial proteins of 66 kDa. Two-dimensional gel electrophoresis has confirmed that BCEA-A and BCEA-B are distinct proteins, each with a molecular weight of 66 kDa but different isoelectric points. Immunofluorescence staining with rabbit anti-BCEA-A antibodies has demonstrated that BCEA-A is localized to the human cornea, specifically to the epithelium.

Circulating antibodies against the two 66-kDa proteins were detected in a significant number of patients with WG, RA, and CSS but not in the normal control group. The strongest association with anti-BCEA-A antibodies was in a group of patients with WG and PUK, whereas anti-BCEA-B antibodies were not found in this group of patients. Anti-BCEA antibodies were detected at a similar frequency in WG (31%) and RA (25%) without eye complications. In the WG group with PUK, the frequency was increased (to 50%), but in RA with PUK there was a decrease (to 12%). However, because of the low number of patients with PUK there was no significant difference between WG with PUK and WG without PUK or between the WG with PUK group and the RA with PUK group. Antibodies to BCEA-B were strongly associated with CSS, suggesting that antibodies to the two antigens are associated with different aspects of vasculitic disease. It is unclear whether BCEA is associated with the vasculitic disease process, because in RA this antigen is associated with nonvasculitic disease. Anti-BCEA-A antibodies may be associated with PUK in vasculitic disease but not RA–associated PUK, whereas anti-BCEA-B antibodies are associated with vasculitis. It therefore appears that BCEA-A and BCEA-B are unrelated proteins that share the same molecular weight, both are corneal derived, and both are detected by antibodies in the sera of patients with different forms of rheumatoid and vasculitic diseases.

The anti-BCEA antibodies were not restricted to the patients with eye involvement. The presence of circulating anti-

**Figure 4.** Detection of bovine corneal epithelium antigen-A and -B (BCEA-A and BCEA-B) in noncorneal tissue extracts by immunoblotting. Protein extracts from human epidermis (lane 1), liver (lane 2), kidney (lane 3), esophagus (lane 4), and corneal epithelia (lane 5) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver stained (C). Gels were blotted and probed with anti-BCEA-A antibodies (A) and anti-BCEA-B antibodies (B). Immunoblotting indicates that BCEA-A is expressed in corneal epithelium and skin, whereas BCEA-B is expressed in kidney, esophagus, and corneal epithelium.
bodies against corneal proteins in WG patients without ocular involvement raises the question of how the sequenced antigens can be presented to the immune system to trigger antibody production. Patients with systemic disease may have antibodies that recognize tissue components in other parts of the body and can cross-react with antigens in the cornea. In this study, we demonstrated cross-reactivity of anti-BCEA-A antibodies with a component of skin epidermis, and anti-BCEA-B antibodies with proteins present in the esophagus and kidney. This cross-reactivity may have different consequences in the pathology of systemic disease. The antibodies against BCEA-A are present in 50% of patients with WG and PUK. Because PUK is often a presenting problem in WG, the circulating cross-reacting antibodies may contribute to, if not initiate, the autoimmune response in the vulnerable eye. On the other hand, patients with RA develop PUK much later, 10 to 15 years into the ongoing systemic disease. At such a late stage, the anti-BCEA-A antibodies are among a wide spectrum of circulating autoantibodies. They may contribute to the autoimmune reaction in the eye, but more likely are an epiphenomenon of advanced systemic disease. The different characteristics of PUK in RA and WG may account for the difference in the frequency of anti-BCEA-A antibodies in these two groups of patients.

Cross-reactivity with ANCA was addressed because of the large number of proteins identified to which ANCA will bind. Immunoblotting assays using BCEA-A- and BCEA-B-positive patients’ sera did not detect a 66-kDa protein in neutrophil extract. Several proteins are known to be ANCA-reactive, including proteinase-3 (27 kDa) and myeloperoxidase (130 kDa); however, no other band corresponding to the known weight of any ANCA-reactive proteins was detected, confirming that BCEA-A and BCEA-B are not members of the group of proteins recognized by ANCA.

It is of note that no patient had antibodies to both proteins. This mutual exclusivity may be explained by a complementary relationship between the two groups of antibodies, as has been demonstrated with the ANCA group of antibodies. In systemic vasculitis, c-ANCA does not occur with p-ANCA. A second antisera reported to have a complementary relationship with ANCA in systemic vasculitis is AECA, with AECA being present more often in ANCA-negative patients.18 This suggests that any one of these antibodies could trigger vasculitis.

The present study showed a high level of association between anti-BCEA-A antibodies and PUK complications in WG patients. If this association is confirmed in a larger group of patients, the presence of anti-BCEA-A antibodies may be a useful diagnostic marker for PUK in WG. At present the distinction between PUK, as the presenting symptom of WG as opposed to Mooren’s ulcer in which no systemic disease is present, is difficult. In the early stages of WG, c-ANCA antibodies may not be present, and the vasculitic process may not be advanced enough to be detected by biopsy. To have anti-BCEA-A antibodies as an additional diagnostic criteria may be valuable in such circumstances.

Anti-BCEA-B antibodies have a 60% association with CSS, but are present in only 13% of patients with WG. This is the first time that a significant association between an antigen and CSS has been shown. Therefore, the presence of anti-BCEA-B antibodies may be suitable as an additional criterion in the diagnosis of CSS and may help to distinguish CSS from WG in the early stages of the disease process.

To fully understand the relevance of these two new antigens, both in the disease process and as diagnostic tools, further characterization is under way. The identification of the two antigens will help to elucidate their role in disease pathology. Longitudinal studies are also needed to understand the role of antibodies to these antigens in disease activity and outcome. Depending on the results of such studies, anti-BCEA-A and anti-BCEA-B antibodies may be useful diagnostic tools in the management of vasculitis.

References


