Autoimmunity to a Cornea-Associated Stromal Antigen in Patients With Mooren’s Ulcer


**Purpose.** To purify and characterize a cornea-associated antigen (CO-Ag) and to determine antibody levels to CO-Ag in patients with Mooren’s ulcer.

**Method.** Standard ion exchange and gel filtration chromatographies were used to isolate and purify CO-Ag from crude bovine stromal extracts. The serum of a patient with Mooren’s ulcer, containing a high level of antibodies directed against CO-Ag, was used to monitor isolation procedures. Using this newly purified CO-Ag, an enzyme-linked immunoabsorbent assay was used to detect the presence of antibodies to CO-Ag in the sera of other patients with Mooren’s ulcer.

**Results.** CO-Ag was purified to apparent homogeneity from bovine corneal stromal extracts by a series of ion exchange chromatographies and gel filtration. Polyacrylamide gel electrophoresis showed that CO-Ag was a tetramer with a molecular weight of 30,000 d that may dissociate under denaturing conditions into a monomer of 7000 d. Strong indirect immunofluorescent staining was demonstrated of the stroma by guinea pig anti-CO-Ag antibody. A statistically significant difference in the level of specific antibodies to CO-Ag between patients with Mooren’s ulcer and controls was found (P < 0.001). The antibody level was elevated in patients with Mooren’s ulcer (mean antibody level, 0.58 ± 0.13) compared with the controls (mean antibody level, 0.22 ± 0.04).

**Conclusion.** These results suggest that an autoantigen exists in the corneal stroma that reacts with serum antibodies from patients with Mooren’s ulcer. The availability of a purified corneal antigen could facilitate the diagnosis and define the pathogenetic mechanisms in Mooren’s ulcer.

Soluble corneal proteins have been the subject of much investigation. Immunochemical analysis has demonstrated that soluble extracts contain tissue-specific antigens as well as antigens originating in homologous serum. Immune recognition of these antigens has profound implications for the possible development of autoimmune corneal disease mediated by host response to these antigens. Many investigators have sought to identify such cornea-associated antigens and to demonstrate their involvement in certain diseases of the cornea. However, almost all these studies were performed with soluble antigens extracted from whole cornea.

Recently, we reported a case of Mooren’s ulcer after penetrating keratoplasty. Autoimmunity was suspected to be involved in the pathogenesis of this disease based on evidence of circulating antibodies to the corneal stroma and specific cell-mediated immune reactions toward a partially purified corneal antigen demonstrated by lymphocyte transformation assay. These studies led to the purification of a cornea-associated antigen with attention to the stromal protein.

Using the serum of the initial patient, the purification procedures were monitored and a cornea-associated antigen (CO-Ag) has been identified. The purification methods of CO-Ag are presented, and circulating antibody levels to CO-Ag in other patients with Mooren’s ulcer were tested.
MATERIALS AND METHODS

Identification of CO-Ag

The serum of a patient with Mooren’s ulcer after penetrating keratoplasty was used to identify CO-Ag by enzyme-linked immunosorbent assay (ELISA). This patient’s serum contained high levels of antibodies directed against corneal stroma.9 Among the sequential procedures to monitor the presence of CO-Ag in the different fractions and purification steps were: Fractions in every purification step were pooled according to 280-nm profile; pooled fractions were concentrated to 5 ml on an Amicon YM-10 membrane (Amicon, Danvers, MA); concentrated protein in every fraction was estimated by the method of Lowry et al10 using bovine serum albumin as the protein standard; and an aliquot of every protein fraction was diluted to 30 µg/ml with carbonate buffer. One hundred microliters of every diluted protein fraction was applied to the appropriate wells of Immulon-2 plates (Dynatech, Chantilly, VA) and was used as potential antigen coating. The plates were incubated overnight at 4°C. Unbound sites were blocked with 1% bovine serum albumin in 0.1 M, borate-buffered saline, pH 8.2 (BSA-BBS) for 2 hours at room temperature. The patient’s serum was diluted 1:200 with BSA—BBS. The patient’s serum (100 µl/well) was added to each well. The plates were incubated (2 hours, 37°C), and an alkaline phosphatase-conjugated goat anti-human immunoglobulin G (Sigma) was added (2 hours, 37°C) followed by the substrate p-nitrophenyl phosphate (Sigma). The plates were thoroughly washed with BBS containing 0.01% Tween 20 between the additions of reagents. The results were read with a Titertek Multiskan spectophotometer (Flow, Rockville, MD) at 405 nm.

Purification of CO-Ag

Step 1: Preparation of Stromal Extracts. Bovine eyes were obtained from a local slaughterhouse and kept on ice until dissection. The epithelium and endothelium were denuded by gentle scraping of the cornea. Stromal tissue was stored at -20°C until used. The tissue was then cut into small pieces and suspended in 10% (wt/vol) in 0.01 M phosphate buffer, pH 8.0, containing 0.05 M ethylenediaminetetraacetic acid (EDTA). The suspension was stirred slowly for 18 hours at 4°C and centrifuged for 30 minutes at 30,000g. The supernatant was decanted and saved. The pellet was resuspended in 3 M KCl and 0.05 M EDTA in phosphate buffer, stirred for 18 hours, and centrifuged. The supernatant was dialyzed in the cold against several changes of phosphate buffer. The two supernatants were then pooled and concentrated to 50 mg protein/ml on an Amicon YM-10 membrane.

Step 2: Anion Exchange Chromatography. The crude extracts were dialyzed against several changes of 0.01 M phosphate buffer, pH 8.0. Fifty milliliters of the extracts containing 2.5 gm protein was applied to a 4 × 20 cm column of DE-53 (Whatman, Clifton, NJ) equilibrated with phosphate buffer. The column was eluted with a three-column volume of a stepwise gradient of 0.05, 0.1, 0.15, and 0.25 M NaCl in phosphate buffer. The column was run at room temperature at a flow rate of 150 ml/hour. An aliquot of each fraction was used to coat ELISA plates for the determination of its reactivity with the patient’s autoantibodies.

Step 3: Gel Filtration Chromatography. The active diethylaminoethyl (DEAE) cellulose fraction was concentrated and applied to a 2.5 × 120 cm Sephacryl S-300 column (Pharmacia, Piscataway, NJ). The column was calibrated with blue dextran (>500,000 d), bovine immunoglobulin G (150,000 d) and bovine serum albumin (68,000 d) as molecular weight (MWT) markers in phosphate-buffered saline (PBS) at a flow rate of 40 ml/hour at 4°C. The elute was monitored for absorbance at 280 nm, and fractions were assayed for reactivity with the patient’s autoantibodies by ELISA.

Step 4: Cation Exchange Chromatography. The active fractions from the Sephacryl S-300 column (Pharmacia) were pooled, concentrated, and dialyzed against several changes of 0.01 M, sodium acetate buffer, pH 5.0. The sample was applied to a 1.5 × 30 cm column of SE-52 (Whatman) equilibrated with acetate buffer. The column was eluted with a salt gradient of 0.05 to 0.8 M NaCl in acetate buffer. The eluted material from each gradient was titrated to pH 7.8 and assayed for reactivity with the patient’s autoantibodies.

Step 5: Rechromatography on Anion Exchange Column. The active SE-52 fraction was dialyzed against several changes of 0.01 M, sodium phosphate buffer, pH 8.0. The sample was applied to a 1.0 × 15 cm column of DE-53 (Whatman), equilibrated with phosphate buffer. The column was eluted with a salt gradient of 0 to 0.2 M NaCl in phosphate buffer. The elute was monitored for absorbance at 280 nm and assayed for reactivity with the patient’s autoantibodies by ELISA.

Polyacrylamide Gel Electrophoresis

Molecular size and homogeneity of purified CO-Ag was analyzed by polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli.11 PAGE was performed on precast 4% to 20% gradient gels that did not contain sodium dodecyl sulfate (SDS) and that had a 4% stacking gel (Novex, San Diego, CA). Co-Ag (5 µg) was prepared in Laemmli sample buffer with or without reducing agent (Novex). Proteins with known molecular weight served as internal markers. Electrophoresis was carried out under non-denaturing conditions (i.e., in the absence of 0.1% SDS) and denaturing conditions (i.e., in the presence
Antistromal Antibodies in Mooren's Ulcer

of SDS) at a constant 125 V at room temperature until the bromophenol blue marker reached the anodic end of the plate. The gels were stained with silver stain according to the manufacturer's instructions (Bio-Rad, Richmond, CA). The molecular weight of CO-Ag and its subunits were determined by comparison with known proteins.

Indirect Immunofluorescence

A Hartley guinea pig was intradermally injected at multiple sites of the back with 2.0 mg of purified CO-Ag in complete Freund's adjuvant. Four weeks after sensitization, the guinea pig was challenged with 2.0 mg of CO-Ag in incomplete adjuvant. Antisera were collected 2 weeks after challenge. These investigations conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Indirect immunofluorescent staining was used to locate CO-Ag in sections of fresh bovine corneas. The cornea was sectioned at 5 μm in a cryostat. The corneal sections were fixed in acetone for 5 minutes and incubated for 1 hour at room temperature with normal rabbit serum (1/10 dilution in PBS) to block nonspecific absorption of the corneal tissue. The slides were subsequently incubated with antiserum against CO-Ag and then with fluorescein-labeled rabbit anti-guinea pig immunoglobulin G (Sigma) after the standard washing procedures with PBS at room temperature. In the control incubations, the staining specificity of CO-Ag was tested by replacing the primary anti-CO-Ag antibody with normal guinea pig serum at the same dilution.

Immunodiffusion Test

Immunodiffusion analysis was used to determine the tissue specificity of CO-Ag. The diffusion plates were prepared in 1% agarose in 0.05 M BBS, pH 8.2, using 25-μl wells spaced 10 mm apart. Bovine retina, liver, lacrimal gland, iris and ciliary body were extracted with PBS and 3 M KCl and then underwent chromatography on a DE-53 column as described. The 0.1-M salt fraction was concentrated (10 mg/ml) for use as control antigens. Precipitation lines, developed after 48 hours of incubation in a moist chamber at room temperature, were stained with amide black and photographed.

Mooren's Ulcer and Normal Control Patients

Fifteen patients were diagnosed with Mooren's ulcer; progressive centripetal corneal ulceration occurred with no evidence of collagen vascular disease or immune hypersensitivity. Fourteen controls were identified from a population of patients undergoing cataract surgery who had no active corneal disease or collagen vascular disorder. Control patients reviewed and signed an institutional review board-approved informed consent form for a venipuncture to obtain approximately 5 ml of blood in a nonheparinized tube. In this manner, the tenets of the Declaration of Helsinki were followed.

RESULTS

Purification of CO-Ag

The serum of a patient with Mooren's ulcer after penetrating keratoplasty, containing a high titer of autoantibodies directed against stromal antigen(s), was used to monitor the isolation of CO-Ag. The crude extracts were first subjected to ion exchange chromatography on a DE-53 column. The column was eluted with stepwise increasing concentrations of NaCl from 0.05 M to 0.25 M. Each fraction was used as antigen for coating enzyme-linked immunosorbent assay plates. A positive antibody level was found in the reference serum to 0.1 M salt fraction.

FIGURE 1. Anion exchange chromatography of bovine corneal stromal extracts. The column was eluted with stepwise increasing concentrations of NaCl from 0.05 M to 0.25 M. Each fraction was used as antigen for coating enzyme-linked immunosorbent assay plates. A positive antibody level was found in the reference serum to 0.1 M salt fraction.
FIGURE 2. Gel filtration of the active diethylaminoethyl cellulose fraction II on a Sephacryl S-300 column. The column was eluted with phosphate-buffered saline. Each fraction was used as antigen for coating enzyme-linked immunosorbent assay plates to determine reactivity with the reference serum antibody.

5% to 7% of the protein applied to the Sephacryl column was recovered in the CO-Ag fraction.

The active fraction from the gel filtration step was further purified by cation ion exchange chromatography. The column was eluted with a linear gradient of 0.05 M to 0.8 M NaCl in acetate buffer. The active material reactive with the patient's autoantibodies was eluted by a high salt gradient (Fig. 3). Approximately 20% to 25% of the protein applied to the SE-52 column was recovered in the active fraction.

The purification of CO-Ag was finally accomplished by rechromatography on a DE-53 column. CO-Ag was eluted in the breakthrough peak of the column (data not shown). The protein content of the crude stromal extracts was approximately 2.5 gm. The CO-Ag obtained after the four-step chromatography yielded approximately 1.5 mg.

Polyacrylamide Gel Electrophoresis
Polyacrylamide gel electrophoresis was used to determine the molecular weight of CO-Ag and to investigate the subunit structure of CO-Ag. No bands were stained by Coomassie blue when the gel was run with or without SDS. A sensitive silver staining procedure was used because it is capable of detecting less than 1.0 ng of protein per mm² of gel. CO-Ag was considered to be a homogeneous protein that migrated as a single major band corresponding to a molecular weight of 30,000 d under nondenaturing conditions. However, when CO-Ag was treated with 0.5% SDS and then electrophoresed on 4% to 20% gradient gels in the presence of 0.1% SDS, it migrated with a single, much lower molecular weight of 7000 d (Fig. 4). These data suggest the presence of a dissociated subunit structure for CO-Ag.

Immunofluorescent Localization of CO-Ag
Indirect immunofluorescent staining was used to determine the cellular localization of CO-Ag within the cornea. Stromal lamellae clearly are visible (Fig. 5), and they demonstrate a fluorescent laminar pattern.
FIGURE 5. Immunofluorescent localization of cornea-associated antigen (CO-Ag) in the bovine cornea. CO-Ag immunofluorescence is detected throughout the stroma (ST), with staining in a laminar pattern. Epithelium (EP) and endothelium (ED) are negative.

The staining was maximal beneath Bowman's membrane. However, this membrane itself showed no staining. Immunofluorescence was not detectable in the epithelium, Descemet's membrane, or endothelium with anti-CO-Ag antibodies. The control sections exposed to normal guinea pig serum did not produce staining of any corneal layer.

**Immunodiffusion Test**

Antiserum directed against purified CO-Ag was used in immunodiffusion analysis of tissue specificity of CO-Ag. Antiserum to CO-Ag showed only a single immunoprecipitant band when reacted with CO-Ag and partially purified CO-Ag. There was no positive reaction with the similar preparation obtained from other ocular and nonocular tissues, such as retina, iris and ciliary body, liver, or lacrimal glands (Fig. 6). The data support the cornea-associated nature of CO-Ag.

**Circulating Antibodies to Cornea-Associated Antigen in Patients With Mooren’s Ulcer**

Cornea-associated antigen was purified from the crude stromal extracts according to its reactivity with only one patient’s serum antibodies. This finding led us to search, in other patients with Mooren’s ulcer, for the prevalence of circulating antibodies directed against purified CO-Ag. Sera from 15 patients with Mooren’s ulcer and 14 controls were assayed by ELISA for their relative antibody levels to purified CO-Ag (Fig. 7). Antibody levels were expressed as the absorbance values at 1:200 dilution of each test serum. Sample sera from patients with Mooren’s ulcer demonstrated higher antibody levels than those from the controls. The mean antibody level of serum samples from 15 patients with Mooren’s ulcer was 0.58 ± 0.13, with the controls demonstrating lower antibody levels.

FIGURE 6. Immunodiffusion analysis demonstrates the tissue-specificity of cornea-associated antigen (CO-Ag). Central well contains antiserum against CO-Ag. Peripheral wells (1) contain purified CO-Ag; 0.1 M salt fraction of diethylaminoethyl cellulose chromatography of (2) retina; (3) iris and ciliary body; (4) corneal stroma; (5) lacrimal gland; and (6) liver.

FIGURE 7. Serum samples from 15 patients with Mooren’s ulcer and 14 controls were tested by enzyme-linked immunosorbent assay to determine their relative antibody levels to cornea-associated antigen (CO-Ag). The bar indicates the mean ± 95% confidence intervals. Sample sera from patients with Mooren’s ulcer had significantly higher ($P < 0.001$) antibody levels to CO-Ag than sera from the controls.
These results suggest that humoral and cellular immune responses to CO-Ag may play a role in initiating and perpetuating Mooren's ulcer.

Cornea-associated antigen has been sequentially purified from crude stromal extracts by anion exchange chromatography, gel-filtration on Sephacryl S-300, cation, and anion exchange chromatography. The first step includes ion exchange chromatography on DEAE-cellulose. The CO-Ag was retained by a high binding strength of DEAE-cellulose (DE-53) column. This fractionation was able to eliminate the majority of serum proteins and permitted concentration of CO-Ag. The remaining, larger molecular weight contaminants were removed by gel filtration. The purification of CO-Ag was finally accomplished by cation ion exchange chromatography. Considerable losses occurred during each ion exchange chromatography, which was thought to be caused by strong binding of CO-Ag to anion and cation exchange resins. In the current study, it was not possible to determine the degree of purification of CO-Ag on the basis of activity per weight of protein because the starting crude stromal extracts did not produce significant activity over the controls. The CO-Ag activity could not be demonstrated until the crude extracts were fractionated by column chromatography. This lack of activity may result either from the presence of nonspecific binding proteins in the crude extracts or a low concentration of CO-Ag in the whole stromal preparation.

One of the striking features of CO-Ag is its electrophoretic behavior in native and SDS polyacrylamide gels. On native gels, CO-Ag showed a single band with a molecular weight of 30,000 d. On SDS gels, CO-Ag showed a much smaller size, with a molecular weight of 7000 d. The molecular weight differences suggest that the native CO-Ag protein is composed of four subunit polypeptides of 7000 d noncovalently associated, which can be disrupted to yield the smaller monomer protein. Because SDS not only destroys the tertiary structure of proteins and disaggregates protein–protein complexes, this agent was useful to probe the subunit structure of CO-Ag.

A protein antigen capable of reacting with serum antibodies obtained from patients suffering from Mooren's ulcer has been purified from bovine corneal epithelium. Although the molecular weight of CO-Ag is similar to the one isolated from the corneal epithelium (designated as BCP 11/24), our CO-Ag appears to differ from BCP 11/24 in terms of subunit structure, cellular localization, net charge, and immunologic properties. In contrast to CO-Ag, which is a tetramer of 7000 d each, BCP 11/24 is a dimer of 11,000 d. CO-Ag was localized in the stroma based on immunofluorescent staining, whereas BCP 11/24 was confined to the corneal epithelial layer. The net charge of the protein between CO-Ag and BCP 11/24 was different. CO-Ag was tightly bound to the DEAE column, whereas BCP 11/24 was loosely bound. BCP 11/24 exists as an abundant protein in the corneal epithelium, whereas CO-Ag is present in only minute amounts in the stroma even though the stroma represents the bulk of the cornea.

The cause of Mooren's ulcer remains uncertain, although defects in the immune system, such as altered ratios of helper and suppressor T cells and helminthic or viral infection, have been suggested. Recently, Wilson and coworkers reported an association of Mooren's ulcer and hepatitis C infection. In their article, the authors suggested the hepatitis C may stimulate an autoimmune response to corneal antigens by sharing cross-reacting epitopes. Our data may spur further studies of infectious agents that mimic CO-Ag sequences and initiate immune events.

Mooren's ulcer is diagnosed by excluding other causes of peripheral melting disorders, such as rheumatoid peripheral ulcerative keratitis and staphylococcal hypersensitivity disease. Studies are ongoing to determine the specificity of the CO-Ag antibody assay to exclude other peripheral ulcerative diseases.

The biologic function of CO-Ag in the cornea remains unknown. That cellular and humoral immune responses to CO-Ag can occur in patients with Mooren's ulcer strongly suggests that this antigen may serve as an immunogenic stimulus to initiate and perpetuate this debilitating, destructive corneal disease. Understanding the nature of the antigenic stimulus will aid in early detection of this destructive process and could lead to the development of more targeted treatment regimens.

**Key Words**

autoantibodies, cornea-associated antigen, Mooren's ulcer, stromal antigen
References


