

Identification of Paramyosin as a Binding Protein for Calgranulin C in Experimental Helminthic Keratitis

Esen K. Akpek, Sammy H. Liu, Robert Thompson, and John D. Gottsch

PURPOSE. Calgranulin C (CaGC) is a protein released by activated neutrophils and involved in host defense against filarial infections. This study involved the identification of binding protein(s) of the helminth *Brugia malayi* to CaGC and the ability of binding complexes to induce keratitis.

METHODS. Parasitic extracts prepared from *B. malayi* microfilariae and adult worms were incubated with recombinant CaGC protein. Parasite binding protein-CaGC complex was isolated by affinity chromatography. A *B. malayi* microfilariae cDNA library was immunoscreened with antisera from rats immunized with the isolated parasitic CaGC-binding protein. All positive clones contained paramyosin sequences. Paramyosin was thus considered the major CaGC-binding protein in the parasite. To delineate the binding of CaGC to native and recombinant paramyosin, ^{125}I -CaGC was used as a binding tracer in SDS-PAGE analysis to identify a CaGC-binding complex. To determine whether the complex of CaGC and its binding protein could induce keratitis mimicking the onchocercal human corneal disease, BALB/c mice preimmunized with the binding complex were challenged with intracorneal binding complex or live *Brugia* microfilariae. In addition, splenocytes harvested from the same animals were assessed for their ability to elicit cellular immune responses to the binding complex by [^3H]thymidine assay.

RESULTS. In vitro binding of CaGC to paramyosin was confirmed using recombinant paramyosin and ^{125}I -CaGC. Test animals showed development of severe keratitis that mimicked, clinically and histopathologically, the human onchocercal corneal disease, demonstrating the antigenic specificity of the paramyosin-CaGC-binding complex.

CONCLUSIONS. Paramyosin is identified as a CaGC-binding protein in *B. malayi*. (*Invest Ophthalmol Vis Sci.* 2002;43:2677-2684)

More than 100 million people are infected with filarial nematodes, and hundreds of millions are at risk of infection worldwide.¹ How filarial parasites induce disease in their hosts is not well understood. Sclerosing keratitis, the blinding corneal complication of onchocerciasis, is thought to result from immunologically mediated reactions to intracorneal microfilariae.^{2,3} Using crude extracts of *Onchocerca volvulus* antigens, investigators have developed animal models of onchocercal keratitis that support this hypothesis.⁴⁻⁷

Mooren ulcer is a peripheral ulcerative keratitis of unknown cause. However, several studies from India and Nigeria have

reported an association with helminthic infections.⁸⁻¹⁰ Considerable evidence suggests that autoimmune responses to certain autoantigens may contribute to the pathogenesis of Mooren ulcer.¹¹⁻¹³ We have purified a corneal antigen (COAg) from stromal extracts and studied its role in the pathogenesis of Mooren ulcer and have demonstrated cellular and humoral immune responses to COAg in patients with Mooren ulcer.^{14,15} We have cloned both bovine and human COAg.^{16,17} The nucleotide sequences of the COAg cDNAs were found to be identical with those of bovine and human neutrophil calgranulin C (CaGC), a protein first identified on the surface of onchocercal worms in human subcutaneous nodules.¹⁸ Calgranulin C is believed to be involved in attacking and killing nematodes after its release by activated neutrophils.¹⁸ Using recombinant CaGC, we have shown a dose-dependent immobilization and killing of adult and microfilarial *Brugia malayi* in an in vitro culture system.¹⁹ Furthermore, immunohistochemical staining for CaGC in *B. malayi* demonstrated that the protein is localized near the surface in microfilariae and in the hypodermis-lateral chord in adult worms.¹⁹ We hypothesized that CaGC may exert its observed biological effects by binding to and subsequently regulating the activities of a parasite binding protein(s). In the present study we sought to identify CaGC-binding proteins (BP) and attempted to determine whether a complex of CaGC and its binding protein could induce keratitis in presensitized mice that mimics the onchocercal human corneal disease.

MATERIALS AND METHODS

Preparation of the Parasite Extracts

B. malayi microfilariae and adult worms were suspended in phosphate-buffered saline (PBS) and protease inhibitors (Sigma, St. Louis, MO) and homogenized on ice in a tissue grinder. The homogenate was centrifuged at 15,000 rpm for 60 minutes at 4°C. The supernatant was stored at -70°C. The tissue pellets were disrupted by sonication and then extracted twice for 12 hours at +4°C in PBS with 10 mM 3-([3-cholamidopropyl]dimethylammonio-2-hydroxy-1-propanesulfonate (CHAPS) detergent (Sigma, St. Louis, MO) and protease inhibitors. The extract was dialyzed against PBS, pooled with supernatant, and concentrated by ultrafiltration with a membrane (UM-3; Amicon, Beverly, MA). Protein concentration was determined with an extraction reagent (Bio-Rad Protein Assay; Bio-Rad, Richmond, CA).

Isolation of Parasite Binding Proteins by Affinity Chromatography

Recombinant CaGC protein was constructed and expressed to incorporate a hexohistidine sequence at the amino terminus of the fusion protein. This tag allows CaGC fusion protein to bind to nickel-nitrilotriacetic (Ni-NTA) magnetic agarose beads (Qiagen, Valencia, CA) in a uniform orientation, thus retaining CaGC's active conformation and giving optimal presentation to the parasite proteins. Ten milliliters hexohistidine-tagged CaGC (30 mg/mL) was added to 1 mL of 5% Ni-NTA beads and the suspension incubated in an end-over-end shaker for 1 hour at room temperature. The CaGC-Ni²⁺ beads settled at the bottom of the tube on a magnetic separator. The supernatant was removed and the beads washed once with the interaction buffer (50

From the Wilmer Eye Institute, Johns Hopkins School of Medicine, Baltimore, Maryland.

Supported by Research to Prevent Blindness.

Submitted for publication December 14, 2001; revised March 25, 2002; accepted April 9, 2002.

Commercial relationships policy: N.

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Corresponding author: John D. Gottsch, MD, Wilmer Eye Institute, Johns Hopkins Hospital, 600 N. Wolfe Street, Maumenee 321, Baltimore, MD 21287-9238; jgottsch@jhmi.edu.

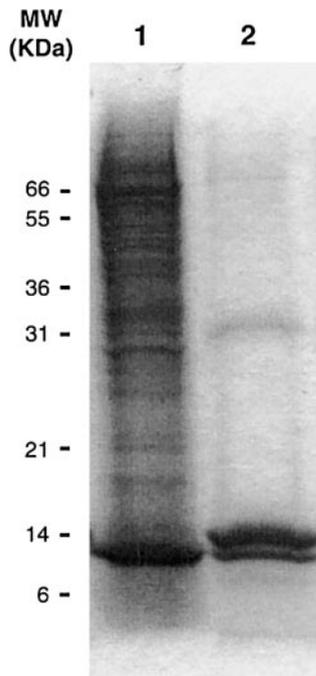


FIGURE 1. *B. malayi* crude extracts and purified CaGC-BPs analyzed by 4% to 15% SDS-PAGE followed by staining with Coomassie blue. *Lane 1:* crude extracts of *B. malayi* worms; *lane 2:* parasite-binding proteins eluted from a CaGC-affinity column. The presence of lower-molecular-weight bands than native paramyosin (97 kDa) was attributed to digestion occurring during the preparation of crude parasitic extracts.

mM phosphate buffer, 300 mM NaCl, 20 mM imidazole, [pH 8.0]). *B. malayi* extracts in interaction buffer were then added to the CaGC-Ni²⁺ beads, and incubated in an end-over-end shaker for 3 hours at room temperature. The beads were washed three times with the interaction buffer and eluted with the elution buffer (50 mM phosphate buffer, 300 mM NaCl, 250 mM imidazole [pH 8.0]).

Most of the parasite proteins did not bind to the CaGC-Ni²⁺ beads and were recovered in the supernatants. The proteins bound to CaGC-Ni²⁺ beads were then eluted as CaGC-BP complexes by 300 mM imidazole, which competes with the protein ligands. SDS-PAGE analysis of isolated CaGC-BP complex from *B. malayi* extracts showed four major protein bands with apparent molecular weights of 66, 31, 14, and 12 kDa (Fig. 1).

Preparation of Polyclonal Antibodies Directed against CaGC-BPs

Lewis rats were immunized with 20 mg CaGC-BP isolated from the *B. malayi* effluents as described, emulsified in complete Freund adjuvant (Difco, Detroit, MD). Three weeks later, the rats were challenged with 10 mg of the same solution. Sera were collected 2 weeks after the challenge and pooled. The sera were absorbed on five strips of 2 × 5 cm nitrocellulose membrane previously saturated in 20% *Escherichia coli* extracts. The absorbed sera were stored at -20°C until used for immunoscreening.

Immunoscreening of the cDNA Expression Library

The *B. malayi* microfilaria cDNA library was graciously provided by Lori Saunders and Steven A. Williams of Smith College (Northampton, MA). Portions of the cDNA library were plated at a density of 35,000 plaque-forming units per 135-mm Petri dish, and the plaques were induced to produce fusion protein, according to the protocol of Huynh et al.²⁰ After the plates had been incubated for 3 hours at 42°C, a

nitrocellulose filter (Schleicher & Schuell, Keene, NH) saturated with 10 mM isopropyl thiogalactose (Life Technologies, Gaithersburg, MD) was overlaid with the agar and left overnight at 37°C to induce the expression of β -galactosidase fusion proteins. The filters were then blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline plus 0.05% Tween 20 (TBS-Tween) for 5 hours at room temperature and incubated with 1:100 diluted anti-CaGC-BP antibody overnight at 4°C. After several washings with TBS-Tween, the bound antibodies were detached by incubation with anti-rat IgG conjugated with alkaline phosphatase (Sigma) for 2 hours at room temperature. The original positive plaque was preplated and rescreened sequentially until all progeny of plaques were recognized by the sera.

Two rounds of screening of 300,000 plaques identified 16 positive clones, which were sequenced on an automated DNA sequencer (PE-Applied Biosystems, Foster City, CA). Sequence analysis of the cDNA insert showed that all 16 recombinant phages contained paramyosin sequences of *B. malayi*.²¹ One clone contained the full-length cDNA of the paramyosin. The deduced mRNA sequence consists of 2640 nucleotides, and, from the deduced amino acid sequence, we predicted a paramyosin protein of 880 amino acids with a molecular weight of approximately 97 kDa. The other 15 phage clones contained partial-length paramyosin cDNAs ranging from 750 to 2210 bp. Paramyosin was thus considered the candidate for parasite CaGC-BP and was chosen for detailed characterization.

Expression and Purification of Recombinant Δ Sal-Paramyosin

The Δ sal-paramyosin clone is a partial-length cDNA of *B. malayi* paramyosin that codes for the 5' sequence, nucleotides 1 to 650. The Δ sal-paramyosin cDNA insert was subcloned into the expression vector pMAL (New England BioLabs, Beverly, MA), which contains the maltose-binding protein (MBP) gene. The pMAL- Δ sal-paramyosin subclone was generously provided by Larry McReynolds of New England Biolabs. Recombinant Δ sal-paramyosin was expressed and purified according to the methods provided by the manufacturer (Protein Fusion and Purification Kit; New England Biolabs, Beverly, MA). Bacterial colonies with plasmid containing DNA inserts were grown to a density of 2×10^8 /mL. Isopropyl- β -D-thiogalactoside was added to a final concentration of 0.3 mM to induce production of fusion protein. After cultures were grown for an additional 2 hours at 37°C, the bacterial cultures were sonicated and centrifuged at 10,000g for 20 minutes. The crude extract was applied to a 2.5 × 10-cm column of amylose resin equilibrated with the column buffer (20 mM Tris buffer [pH 7.4], containing 200 mM NaCl and 1 mM EDTA). After extensive washings, the column was eluted with the column buffer plus 10 mM maltose. The eluate containing the fusion protein was dialyzed against PBS overnight and concentrated to 1 mg/mL by membrane ultrafiltration (Amicon).

Confirmation of Binding and Cross-linking of ¹²⁵I-CaGC to Native Paramyosin and Δ Sal-Paramyosin Fusion Protein

¹²⁵I-labeled CaGC (specific activity approximately 5 mCi/mg) was prepared using magnetic beads (Iodo-Beads; Pierce, Rockford, IL), according to the procedure recommended by the manufacturer. The ¹²⁵I-CaGC was kept at 4°C in PBS (pH 7.5) containing 1 mg/mL of BSA and 0.2% NaN₃. Nematode paramyosin was isolated from *B. malayi* microfilariae according to the method of Harris and Epstein.²² Native paramyosin or Δ sal-paramyosin (10 mg) was incubated at 4°C with 2.9 nM ¹²⁵I-CaGC, with or without a 100-fold excess of unlabeled CaGC for 2 hours. After incubation, a final concentration of disuccinimidyl suberate (DSS; Pierce) was added for 15 minutes at room temperature. Proteins were precipitated by 20% trichloroacetic acid. The pellet was washed twice with ice-cold acetone, air dried, solubilized in electrophoresis sample buffer, and analyzed by 7.5% SDS-PAGE. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250,

dried, and exposed to autoradiograph film (XAR; Eastman Kodak, Rochester, NY) at -80°C in the presence of the intensifying screen.

Isolation of the Binding Complex of ΔSal -Paramyosin and CaGC by Gel Filtration

The interaction of ΔSal -paramyosin and CaGC was cross-linked with DSS as described. The mixture was applied to a gel-filtration chromatography column (1.0×200 cm; Sephacryl S-200; Sigma) equilibrated in 0.1 M formic acid. The absorbance of column effluents was monitored at 280 nm and counted aliquots in a gamma counter. The binding complex fractions were pooled and the formic acid removed by freeze drying. The binding complex was dissolved in Tris-buffered saline (pH 8.0) at 50 mg/mL.

Induction of Keratitis by the Binding Complex of ΔSal -Paramyosin and CaGC

Fifty female BALB/c mice weighing 18 to 20 g (Jackson Laboratory, Bar Harbor, ME) were housed under standard conditions and maintained on laboratory chow and water ad libitum. An emulsion was prepared by mixing 2.0 mL of Tris-buffered saline containing 0.1 mg of the binding complex, isolated by performing gel filtration, with monophosphoryl lipid A and synthetic trehalose dicorynomycolate emulsion (MPL+TDM, R-700; Corixa, Hamilton, MT). The formic acid was removed by freeze drying. Twenty mice were immunized with three biweekly subcutaneous injections of 0.2 mL of this emulsion and comprised the test group. One week after the final immunization, 10 of the mice were injected intracorneally with the binding complex (10 mg in 10 mL saline) and 10 mice with live *B. malayi* microfilariae (20–30 per 10 mL) in their right eyes. Thirty control mice were immunized with ovalbumin (OA) in adjuvant. Ten of the control mice were challenged with intracorneal OA, 10 control mice with binding complex, and 10 with live *B. malayi* microfilariae in their right eyes in the same way as the test group. Animals underwent weekly slit-lamp biomicroscopic examinations and intensity of opacification (defined as degree of corneal clouding based on the visibility of iris details) and extent of neovascularization (defined as extension of vessels across the limbus toward pupillary area) were scored as follows: -, absent; \pm , no corneal opacification/neovascularization at the limbus; +, corneal opacification clinically detectable but not obscuring the iris visualization/corneal neovascularization crossing the limbus and extending onto the clear cornea; ++, corneal opacification decreasing the iris details, but pupil visible/corneal neovascularization not extending onto the pupil; +++, corneal opacification obscuring the details of iris/corneal neovascularization crossing the papillary border. Animals were killed at various times after the antigen challenge, and eyes were removed for histopathologic and immunohistochemical examination. Treatment of animals adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Histologic Assessment of Stromal Keratitis

Eucleated globes of the test animals were either fixed in 10% neutral buffered formalin and embedded in paraffin for routine light microscopic examinations or immersed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) and 5% sucrose for immunohistologic staining. Paraformaldehyde-fixed specimens were rinsed twice in cool (4°C) 0.1 M PB with 20% sucrose and incubated overnight in the same solution. The specimens were placed in plastic biopsy molds containing a 2:1 mixture of 0.1 M PB with 20% sucrose and optimal cutting temperature (OCT) compound (Miles Laboratories, Elkhart, IN). The plastic molds were frozen with a mixture of dry ice and methylbutane, and the blocks were stored at -70°C overnight. Serial frozen sections (8 μm), cut across the center of the cornea along the optic axis, were placed on gelatinized slides, and the slides were kept at -70°C until staining. Slides were allowed to warm up at room temperature for 1 hour before staining.

After brief fixation in acetone and quenching of endogenous peroxidase with 0.03% H_2O_2 , the sections were hydrated in PBS and

stained by an enhanced four-step avidin-biotin-peroxidase complex (ABC) method (Vectastain Elite ABC kit; Vector Laboratories). A 15-minute incubation with blocking agent (Vector) was followed by application of primary monoclonal antibodies for T lymphocytes, T helper cells, T suppressor cells, IL-4 (PharMingen, San Diego, CA), and IFN- γ (Biosource International, Camarillo, CA). After a 1-hour incubation in a moist chamber at room temperature, the sections were washed with PBS. The secondary, biotin-labeled antibody was applied for 30 minutes. The sections were washed and ABC reagent 1:100 was added for 30 minutes. The slides were washed again and developed in 3-amino-9-ethyl-carbazole (AEC) solution. Mayer hematoxylin 0.1% (Sigma) was used for counterstaining. For each staining run and each antibody, positive (frozen sections of mouse spleen) and negative (normal mouse serum substituted for primary antibody) control experiments were performed to ensure quality control.

Serial sections were reviewed under a standard binocular light microscope (Carl Zeiss, Oberkochen, Germany). Positive-stained cells, identified by dense reddish brown color were scored as follows: -, no cells; \pm , 1 to 3 cells; +, 4 to 7 cells; ++, 8 to 12 cells; +++, more than 12 cells.

Lymphoproliferation

Spleens from immunized animals were removed 14 days after immunization and mechanically dispersed to yield a single-cell suspension. Dead cells and erythrocytes were removed by centrifugation over a gradient (Lympholyte; Accurate, Westbury, NY). The isolated spleen cells were washed in complete medium (RPMI 1640 supplemented with 10% fetal calf serum [FCS], 15 mM HEPES buffer, 2 mM glutamine, 0.1 mM nonessential amino acids, 50 U/mL penicillin, 50 U/mL streptomycin, 50 μM 2-mercaptoethanol, and 1 mM sodium pyruvate) and dispersed into 96-well flat-bottomed microtiter plates at a concentration of 2×10^5 /well. The optimal concentration of antigen used to stimulate splenocytes was predetermined for the binding complex (50 $\mu\text{g}/\text{mL}$), ΔSal -paramyosin (25 $\mu\text{g}/\text{mL}$), CaGC (30 $\mu\text{g}/\text{mL}$), and OA (50 $\mu\text{g}/\text{mL}$). Control wells contained only splenocytes without antigen. All cultures were in a final volume of 0.2 mL and were incubated at 37°C in 5% CO_2 for 3 days. Cultures were pulsed with 1 $\mu\text{Ci}/\text{well}$ [^3H]thymidine (DuPont-NEN, Boston, MA) for the final 16 hours, harvested the cultures, and determined incorporated radioactivity by liquid scintillation counter. The results of triplicate wells were averaged and reported as the stimulation index (SI) \pm SD: SI = (counts per minute in stimulated wells)/(counts per minute in control wells).

RESULTS

Binding and Cross-linking of ^{125}I -CaGC to Native and Recombinant Paramyosin

SDS-PAGE analysis of the binding complex showed one major band with an apparent molecular weight of 125 kDa and several minor bands (Fig. 2A, lane 1). The major and minor bands were not seen when a 100-fold excess of unlabeled CaGC was included in the binding mixture (Fig. 2A, lane 2), confirming the binding of ^{125}I -CaGC to paramyosin. If we assume that the binding complex is a one-to-one association of the monomeric CaGC (23 kDa) with the native paramyosin (97 kDa), the resultant molecular weight of 120 kDa would correspond to the major binding band in the autoradiograph. The lower-molecular-weight bands probably represent CaGC-bound paramyosin fragments resulting from enzymatic digestion of the molecule during the isolation procedure.

A similar result was obtained from the binding of ^{125}I -CaGC to a recombinant protein expressed from a fusion plasmid containing an inserted paramyosin restriction fragment. The fragment was liberated from paramyosin cDNA by digestion with *SalI* and subcloned into the expression vector pMAL-c2X, containing the MBP gene. The MBP- ΔSal -paramyosin fusion protein (molecular weight 70 kDa) was purified on an amylose

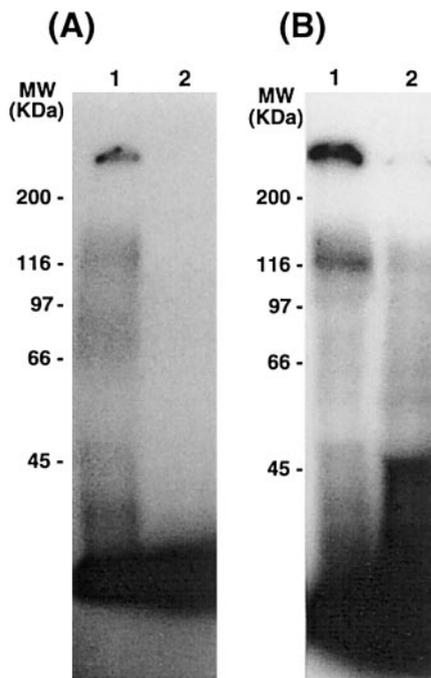


FIGURE 2. Binding and cross-linking of ^{125}I -CaGC to native and recombinant paramyosin. Native or recombinant paramyosin was incubated with ^{125}I -CaGC for 2 hours at 4°C in the absence or presence of a 100-fold excess of unlabeled CaGC. Bound ^{125}I -CaGC was cross-linked, using DSS. The reaction mixture was analyzed by 7.5% SDS-PAGE and autoradiography. (A) Native paramyosin incubated with ^{125}I -CaGC alone (lane 1) and in the presence of a 100-fold excess of unlabeled CaGC (lane 2). (B) Recombinant Δsal -paramyosin fusion protein incubated with ^{125}I -CaGC alone (lane 1) and in the presence of a 100-fold excess of unlabeled CaGC (lane 2). The positions of the marker proteins that were run in parallel are indicated.

affinity resin and used in the binding assays. Cross-linking studies on this recombinant Δsal -paramyosin protein using ^{125}I -CaGC and DSS, as described earlier, demonstrated a single band with a molecular weight of 95 kDa (Fig. 2B, lane 1), the appearance of which was completely blocked by a 100-fold excess of unlabeled CaGC (Fig. 2B, lane 2).

Gel-filtration chromatography (Sephacryl S-200 column; Sigma) was used to isolate the binding complex from the

reaction mixture. The binding complex was eluted in the void volume, well resolved from the unbound proteins (Fig. 3).

Experimental Keratitis

The results of slit lamp examination of the mice eyes are summarized in Table 1. Living, immotile microfilariae were visible (by slit lamp) in the right corneas of all test animals (immunized with binding complex) 2 days after the intracorneal injection. However, nonmotile, straightened, and opaque microfilariae were seen beginning 4 days after injection. Corneal opacity progressed gradually in this group, reaching a maximum on days 7 to 10 and decreasing by day 14. Neovascularization peaked at day 14 and persisted through days 18 to 21. Histologic changes correlated well with the clinical appearance of corneal disease. A mass of inflammatory cells consisting of mononuclear and polymorphonuclear cells with eosinophils was observed at the site of injection. In contrast, control mice (immunized with OA) showed development of mild opacity that persisted through day 21. Neovascularization was not observed in these eyes. Histologic examination on day 21 showed mild corneal edema and rare inflammatory cell infiltrates around dead microfilariae.

The test animals challenged with intracorneal binding complex showed stromal edema 1 day after the injection. Corneal opacity progressively increased in intensity, reaching a maximum on days 7 to 10 (Fig. 4A). These eyes also showed a progressive increase in neovascularization from days 12 to 14 (Fig. 4B). Histologic examination of the eyes on day 14 showed an inflammatory cell infiltrate composed of mononuclear and polymorphonuclear cells, along with eosinophils and stromal neovascularization. In contrast, the right eyes of mice in the control group (immunized with OA) showed minimal stromal edema after the intracorneal binding complex challenge that gradually resolved by days 5 to 7. By day 21, corneas were transparent. Neovascularization did not develop in any of these eyes. Histologic examination of corneas from this group on day 14 showed no inflammatory cell infiltrate. The control animals challenged with intracorneal OA showed mild keratitis that manifested by day 7 and completely subsided by day 14. Histopathologic examination of the corneas performed at day 8 demonstrated only rare mononuclear cell infiltration at the injection site. These data demonstrate the antigenic specificity of the binding complex to elicit keratitis.

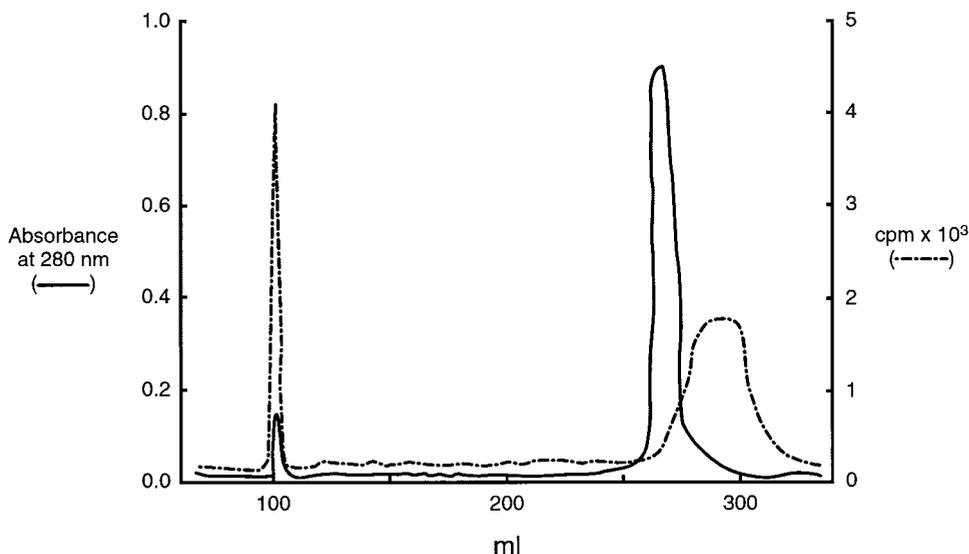


FIGURE 3. Gel filtration of the reaction mixture of ^{125}I -CaGC and recombinant Δsal -paramyosin on a gel-filtration chromatography column. Column effluents were monitored for absorbance at 280 nm (solid line), and aliquots were counted. The binding complex of ^{125}I -CaGC and Δsal -paramyosin (broken line) eluted in the void volume.

TABLE 1. Slit Lamp-Examination of Keratitis and Neovascularization

	Day 3 O/N	Day 7 O/N	Day 14 O/N	Day 21 O/N
BC-P	+++	+++	+++	+++
OA-P	±/-	±±/-	+/-	±/-
BC-BC	+++	+++	+++	+++
OA-BC	+/-	±±	-/-	-/-
OA-OA	±/-	±±/-	-/-	-/-

$n = 10$ in each group. O, corneal opacity; N, corneal neovascularization; BC, paramyosin-CaGC-binding complex; P, *B. malayi* microfilaria; OA, ovalbumin; BC-BC, test animals immunized with binding complex and challenged with intracorneal binding complex; BC-P, test animals immunized with binding complex and challenged with intracorneal *B. malayi* microfilaria; OA-BC, control animals immunized with ovalbumin and challenged with intracorneal binding complex; OA-P, control animals immunized with ovalbumin and challenged with intracorneal *B. malayi* microfilariae; OA-OA, control animals immunized with ovalbumin and challenged with intracorneal ovalbumin.

Immunohistochemical Assessment of Stromal Keratitis

Results of the immunohistochemical studies performed on the test animals challenged with intracorneal paramyosin-CaGC complex or parasite (outlined in Table 2), showed an intense infiltration of mononuclear cells positively stained with CD3 at the site of injection (Fig. 5). Most of these cells displayed CD4 antigens in the cell surface, with only a few expressing CD8 antigen. There was also a marked expression of IL-4 at the injection sites. IFN- γ -positive cells were very few.

Lymphoproliferative Analysis

The ability to elicit cellular immune responses was compared in binding complex-immunized and OA-immunized BALB/c mice. Splenocytes from mice immunized with the binding complex in adjuvant gave a strong response to the binding complex or Δ sal-paramyosin, a weaker response to CaGC, and no response to OA (Fig. 6A). In contrast, splenocytes from OA-immunized mice did not show significant proliferative responses to the binding complex, Δ sal-paramyosin, or CaGC, but they responded well to OA (Fig. 6B). The data demonstrate the ability of these cells to respond to specific antigenic stimuli.

DISCUSSION

In this study we identified paramyosin, a component of the thick filaments of parasite muscle, as a CaGC-BP. Paramyosin is involved in the "catch mechanism" of muscle contraction, which allows prolonged and stronger contractions with reduced energy consumption.²³ In earlier work we showed that a low concentration of CaGC can immobilize *Brugia* micro-

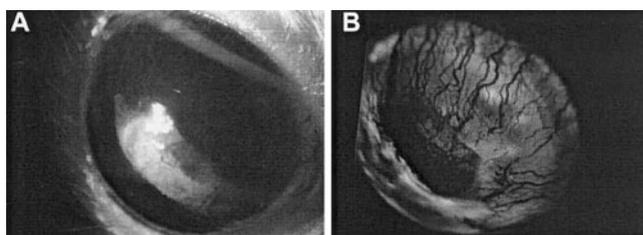


FIGURE 4. Paramyosin-CaGC complex-preimmunized mouse cornea 14 days after challenge with intrastromal injection of binding complex, showing severe corneal stromal infiltration (A) and neovascularization (B).

TABLE 2. Immunohistologic Analysis of Corneal Inflammatory Cells at Day 14 in the Two Test Groups

	Pan T	T Helper	T Suppressor/Cytotoxic	IL-4	IFN- γ
BC-BC	+++	++	+	++	-
BC-P	++	++	±	++	±

$n = 10$ in each group. BC, paramyosin-CaGC-binding complex; P, *B. malayi* microfilaria; OA, ovalbumin; BC-BC, test animals immunized with binding complex and challenged with intracorneal binding complex; BC-P, test animals immunized with binding complex and challenged with intracorneal *B. malayi* microfilaria.

lariae and that a high concentration can kill them.¹⁹ Immunohistologic analysis has localized paramyosin of larval and adult *B. malayi* to somatic muscle within the body wall and just below the cuticle.²⁴ These findings are consistent with our immunolocalization of CaGC binding in adult *Brugia*.¹⁹ After exposure to recombinant CaGC, immunostaining was most intense in the hypodermis-lateral chord and below the cuticle. In addition, we found that microfilariae exposed to CaGC were positively stained near the surface. The combined results of the present study and the immunolocalization of CaGC suggest that the filaricidal and filaristatic effects of CaGC could be due to the interruption of helminthic contractile elements.

Studies performed with *B. malayi*,²⁵ *Dirofilaria immitis*,²⁶ and *O. volvulus*²⁷ suggest that paramyosin is an immunodominant antigen, and it has been proposed as a candidate for a vaccine against various helminths.²⁸⁻³⁰ Indeed, mice vaccinated with extracts of *Schistosoma mansoni* produced antibodies, predominantly against paramyosin.³¹ It has long been known that schistosomes adsorb the host Fc portion of immunoglobulins onto the tegument, but the molecular mechanisms by which they achieve this process has not been elucidated. Recently, it has been shown that paramyosin, a vaccine candidate antigen that elicits protection against schistosomiasis in laboratory and domestic animals, is an Fc-binding protein.³² Because paramyosin is a muscle protein found only in invertebrates, the protein and its fragments are likely to become highly immunogenic in vertebrate animals used to generate antibodies to the binding protein. The reason that paramyosin was the main clone detected from the cDNA library could be that there was an abundance of mRNA rather than any connection with CaGC. The other possibility is that, because paramyosin is an Fc binder, it may have interfered with the adsorption of antibodies directed against the parasitic CaGC-BP. Evidence that paramyosin is indeed a binding protein of CaGC includes the binding and cross-linking of ¹²⁵I-CaGC to native and recombinant paramyosin, the immunolocalization of CaGC binding to muscle,¹⁹ and the finding that all clones identified by antibody screening (16/16) were paramyosin. This study also confirmed the immunogenic potential of paramyosin in lymphoproliferative assays, but the paramyosin-CaGC complex was found to be most potent in stimulating the lymphocytes in this assay.

It is known that invasion of the cornea with intact *O. volvulus* microfilariae produces very little acute inflammation in the eye.^{33,34} Perhaps soluble antigens, including paramyosin-CaGC complex, are released from decaying worms, initiating the pathologic reactions observed in ocular onchocerciasis. Onchocercal keratitis is clinically characterized by inflammation and vascularization of the cornea progressing to complete opacification, a condition referred to as sclerosing keratitis. In our experiments, the binding complex of paramyosin-CaGC produced an intense inflammation with marked corneal infiltration and neovascularization, similar to the human sclerosing keratitis. Although paramyosin is a prime candidate antigen for a vaccine against several helminths, the fact that the CaGC-paramyosin complex induces keratitis in presensitized animals

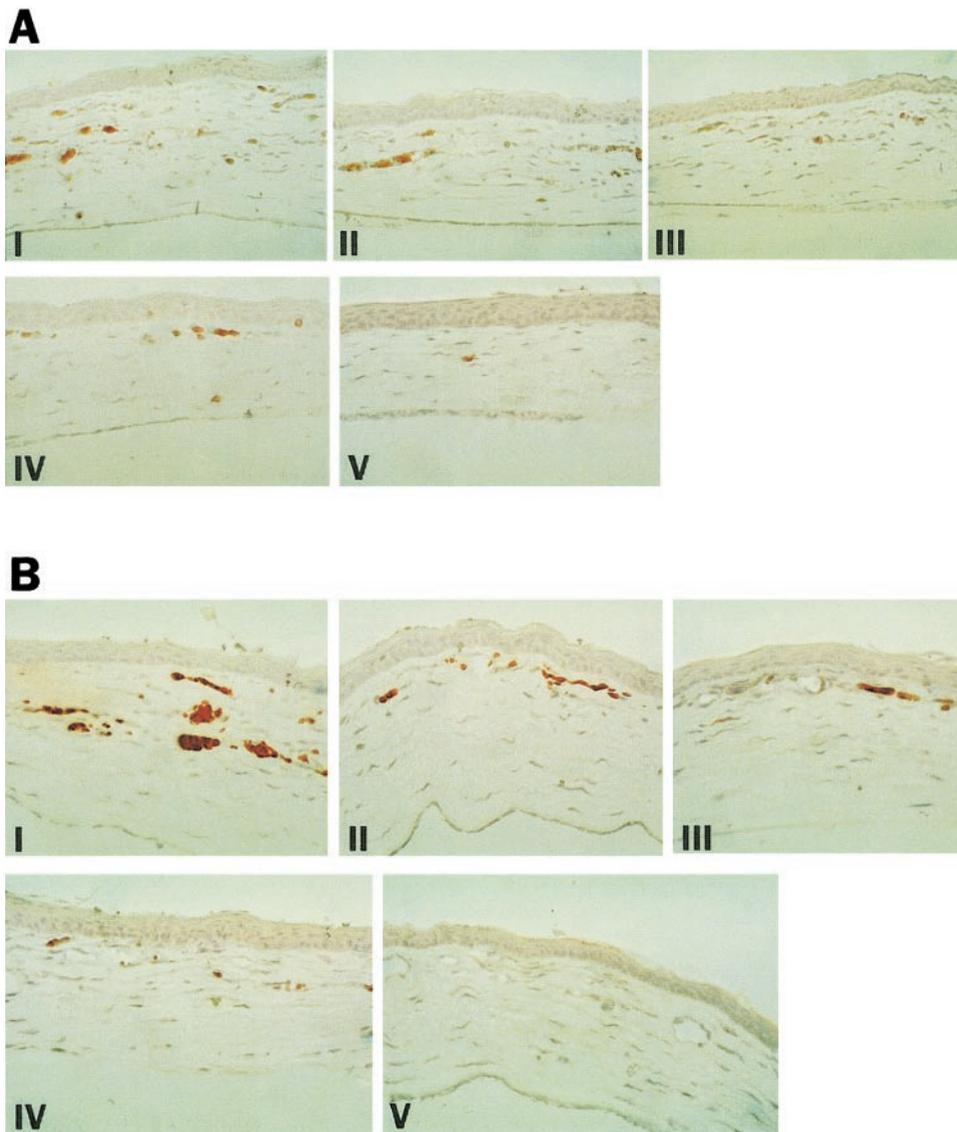


FIGURE 5. Immunohistochemistry of corneal sections from (A) binding-complex-preimmunized mouse challenged with intracorneal injection of binding complex and (B) binding-complex-preimmunized mouse challenged with intracorneal live *Brugia* injection. The positive cells stained reddish brown with AEC. I, anti-CD3 antibody (pan T cells); II, anti-CD4 antibody (T helper cells); III, anti-CD8 antibody (T-suppressor/cytotoxic cells); IV, anti-IL-4 antibody; and V, anti-IFN- γ antibody. Original magnification, $\times 64$.

raises concerns about immunizing with paramyosin. However, it is also possible that molecules other than proteins (e.g., carbohydrates that were present in the parasitic extracts) might also have directly contributed to or skewed the immune responses in our experimental model.

The keratitis elicited by the paramyosin-CaGC complex was histopathologically similar to inflammation elicited by *B. malayi* itself, characterized by a mononuclear and polymorphonuclear cell infiltrate with eosinophils. Immunohistochemical evaluations of corneal sections from both binding complex-injected and live parasite-injected test animals were comparable and demonstrated an intense CD3⁺ T lymphocytic infiltration. Most of these cells expressed CD4 in the cell surface, with lesser numbers of CD8⁺ cells. Expression of IL-4 was more pronounced than that of IFN- γ . These results are in accordance with the recent animal models of onchocerciasis.^{6,35}

In human populations exposed to infection with *O. volvulus*, a small minority of individuals seems to be able to control the infection despite ongoing exposure and transmission. In contrast to individuals with detectable active infection, these putative immune individuals are characterized by proliferative T helper 1-like cell responses to parasite antigen.³⁶ It is known that certain antigens are likely to induce a certain subset of T cells.³⁷ Studies on the differences in parasite-specific T cell

subpopulations in *O. volvulus*-infected and putatively immune subjects³⁸ showed hyporesponsiveness in the actively infected individuals, with lower levels of IL-2 production. Furthermore, treatment seems to have a modulating effect on cell-mediated responses. Therapy with microfilaricidal diethylcarbamazine results in nondetectable infection after several years, associated with high blastogenic responses to parasite-related antigens.³⁹ This indicates that elimination of microfilariae, the predominant form of the parasite in the body, may lead to activation of the cell-mediated immune response.

In earlier work examining the fine specificity of the human immune response to filarial paramyosin, epitope mapping demonstrated preferential recognition of the amino-terminal end of the molecule coded by nucleotides 1 to 360, both in immune individuals and in patients with lower microfilarial levels.⁴⁰ The Δ sal-paramyosin clone codes for the 5' mRNA nucleotide sequence, nucleotides 1 to 650 and is the immunodominant epitope recognized by serum antibodies of patients infected with *O. volvulus*. This particular fragment from *S. mansoni* paramyosin is well known to induce T cells of immunized mice to secrete IFN- γ that activates macrophages to kill schistosomes.²⁹

Other helminthic proteins or binding complexes may also be involved in the immunopathogenesis of onchocercal kera-

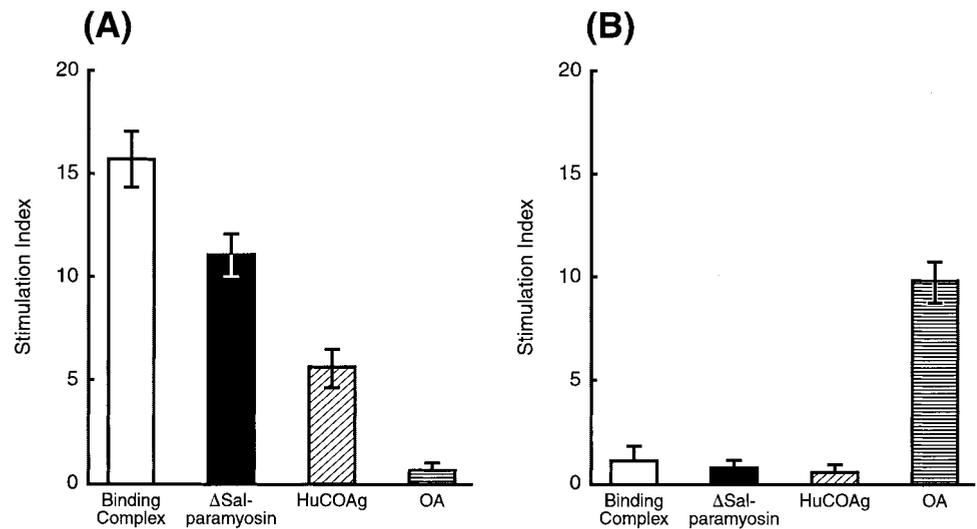


FIGURE 6. Antigen specificity of lymphoproliferation. Proliferative responses of splenocytes obtained from BALB/c mice immunized with the binding complex (A) and with OA (B) were measured by [³H]thymidine incorporation. SIs of the spleen cells were obtained after stimulation with binding complex, Δsal-paramyosin, CaGC, or OA. The data presented are the mean ± SD of three separate experiments.

titis. One group of candidate proteins is the defensins, which also have been identified on the surface of onchocercal worms,⁴¹ and anti-defensin antibodies have been detected in the sera of patients with hyperreactive onchocerciasis (Sowda disease). Defensins bound to the worm surface may also elicit an intense immune reaction in the cornea, and keratocytes may be cytokine-induced to express defensins *in vitro*.⁴²

In conclusion, by localizing and defining, at the structural level, the specific epitopes that induce particular response, it should be possible to generalize about the mechanisms that have evolved in parasites to evade or induce particular host immune responses. By extending these studies further at the T cell level, we could directly address the role of paramyosin in inducing lymphokines or T-cell subsets that may mediate immunity.

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