Schistosoma mansoni secretes a chemokine binding protein with antiinflammatory activity

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The coevolution of humans and infectious agents has exerted selective pressure on the immune system to control potentially lethal infections. Correspondingly, pathogens have evolved with various strategies to modulate and circumvent the host’s innate and adaptive immune response. Schistosoma species are helminth parasites with genes that have been selected to modulate the host to tolerate chronic worm infections, often for decades, without overt morbidity. The modulation of immunity by schistosomes has been shown to prevent a range of immune-mediated diseases, including allergies and autoimmunity. Individual immune–modulating schistosome molecules have, therefore, therapeutic potential as selective manipulators of the immune system to prevent unrelated diseases. Here we show that S. mansoni eggs secrete a protein into host tissues that binds certain chemokines and inhibits their interaction with host chemokine receptors and their biological activity. The purified recombinant S. mansoni chemokine binding protein (smCKBP) suppressed inflammation in several disease models. smCKBP is unrelated to host proteins and is the first described chemokine binding protein encoded by a pathogenic human parasite and may have potential as an antiinflammatory agent.

Schistosoma mansoni is a trematode parasite that infects humans. Infection of man involves initial skin penetration by cercariae that migrate, via the lungs, to develop into adult male and egg-laying female worms that may reside for up to 10 yr within the mesenteric vasculature. To achieve such chronic infections, schistosomes are particularly adept at manipulating the host’s immune system to the benefit of the parasite (1). This balanced host–parasite relationship ensures <10% of infected individuals develop severe disease. This close relationship between schistosomes and the host is illustrated by the granulomatous inflammation around parasitic eggs trapped in various organs, which, though a major cause of pathology, is evoked by the parasite to facilitate the expulsion of its eggs from the host. Thus, S. mansoni–infected mice with compromised immunity (e.g., CD4+ cell depleted) fail to excrete eggs in the feces, and S. mansoni–infected HIV+ patients with low CD4+ cell counts have impaired egg excretion (2, 3).

There is a spectrum of mechanisms whereby various pathogens can modulate the immune system. A currently unique aspect of viral immune evasion is modulation of cellular recruitment and activation via the virus-producing secreted chemokine binding proteins (CKBPs) that bind and neutralize chemokines (4–7). CKBPs are unrelated to host chemokine receptors and have not been identified to date in human hosts or other pathogens. In view of the dynamic selective modulation of local cell recruitment within the host by S. mansoni, we addressed whether this parasite also produces CKBPs.

RESULTS AND DISCUSSION

All the life cycle stages of S. mansoni that develop in man were tested for the presence of CKBP in a cross-linking assay. Using 125I-CXCL8 (IL-8) or 125I-CCL3 (MIP-1α) as target che-
mokines, we detected a single ∼44 kD complex in schistosome egg secretions (ES) but not in other life cycle stages (Fig. 1 A). The ∼44 kD complex from ES indicates that, after subtraction of the chemokine mass (∼8 kD), an ∼36 kD CKBP is secreted by schistosome eggs (S. mansoni CKBP [smCKBP]). ES and no other parasite stages inhibited the binding of 125I-CXCL8 or 125I-CCL3 to chemokine receptors expressed in U937 cells in a dose-dependent manner (Fig. 1 B). Cation exchange chromatography was used to purify a fraction containing an ∼36 kD doublet that specifically bound 125I-CXCL8 or 125I-CCL3 forming an ∼44 kD complex with ∼14 kD-chemokine after cross-linking (Fig. 1 C). The CKBP in S. mansoni eggs is produced by eggs from the two other major schistosome species that infect man, S. haematobium and S. japonicum (Fig. 1 D). Despite smCKBP being detectable in antigen extractions from eggs from the three schistosome species by Western blotting (Fig. 1 D), we could detect no chemokine binding activity using these whole egg antigen preparations (Fig. 1, A and B, and not depicted). smCKBP is present in egg homogenates at low concentrations; in 1 mg of soluble egg antigen protein there is ∼10 µg of smCKBP, whereas there is ∼150 µg of smCKBP in 1 mg of ES protein. However, when smCKBP was purified and concentrated from S. mansoni egg antigens, it bound chemokines (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20050955/DC1).

A proteomic approach was used to identify the gene encoding smCKBP. Each of the two bands of ∼36 kD (Fig. 2 A) were excised from an SDS-PAGE gel for mass spectrometry and peptide sequence analysis. The two bands were confirmed to be the same protein with both bands having the same peptide mass and fragmentation patterns, indicating that the difference in size is caused by posttranslational modification of the protein. One of the smCKBP peptides obtained (ITGLGHGTCIDDFDTK) was found to match an expressed sequence tag (EST; available from GenBank/EMBL/DDBJ under accession no. AY820476) clone from an S. mansoni egg cDNA library, and its entire nucleotide sequence was determined. Despite having chemokine-binding activity, smCKBP shares no amino acid sequence similarity to known viral CKBPs or mammalian proteins. As smCKBP was initially identified in eggs isolated from mouse liver, we have confirmed that smCKBP is an S. mansoni–specific gene, as it is amplified by PCR from genomic DNA prepared from schistosome cercariae from snails but not from mouse DNA (unpublished data). Recently, the same S. mansoni gene (available from GenBank/EMBL/DDBJ under accession no. AY028436) was expressed in Escherichia coli and the recombinant protein was shown to induce human basophil degranulation (8); however, we have not seen this activity in insect cell–expressed smCKBP (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20050955/DC1). The open reading frame present in the cDNA was expressed with a COOH-terminal 6xHis tag using the baculovirus and vaccinia virus expression systems. In both systems, the recombinant protein (r-smCKBP) was secreted as a doublet of ∼32 kD in size, smaller than the ∼36 kD natural molecule, with anti-smCKBP rabbit sera recognizing the natural and recombinant protein and both bound chemokines (Fig. 2 A and not depicted). Purified r-smCKBP expressed in the baculovirus system was used in further experiments. Binding assays confirmed that r-smCKBP specifically bound 125I-CXCL8, 125I-CCL3, and 125I-CX3CL1 (fractalkine; Fig. 2 B). More extensive competition assays with 10 cold competitor chemokines showed that r-smCKBP also binds CCL2 (MCP-1) and CCL5 (regulated on activation, normal T cell expressed and secreted; unpublished data). smCKBP is glycosylated, with the difference in size between native (∼36 kD) and r-smCKBP (∼32 kD) a reflection of differences in glycosylation after insect cell expression. Despite the differences in

**Figure 1.** Presence of a CKBP in S. mansoni ES. (A) Cercarial homogenates (1) or homogenates (2, 4, and 6) or secretions (3, 5, and 7) from schistosomula (2 and 3), worms (4 and 5), or eggs (6 and 7) or binding media (8) were cross-linked to 125I-CXCL8 or 125I-CCL3. All schistosome antigen preparations had nonspecific binding (*) to iodinated chemokines (55–60 kD) that was also detectable in the binding media. (B) CXCL8 and CCL3 binding to U937 cells in the presence of a range of protein concentrations of homogenates (open circles and dotted line) or secretions (closed circles and continuous line) from different life cycle stages of S. mansoni. (C) Cross-linking of 125I-CXCL8 or 125I-CCL3 to binding media (1 and 4) or smCKBP in the absence (2 and 5) or presence (3 and 6) of excess unlabeled chemokine. (D) Anti-smCKBP rabbit sera probe of Western blot of homogenates of S. mansoni cercariae (1), schistosomula (2), worms (3), and eggs (4), S. japonicum eggs (5), S. haematobium eggs (6), or secretions from S. mansoni schistosomula (7), worms (8), or eggs (9).
size, the ability of smCKBP to bind chemokines was glycan independent, with deglycosylated natural smCKBP or baculovirus-expressed r-smCKBP remaining completely bioactive in binding CXCL8 and blocking its activity (unpublished data). r-smCKBP also inhibited the specific binding of 125I-CXCL8 or 125I-CCL3 to U937 cells (Fig. 2 C). The biological function of r-smCKBP was also determined by its ability to block the CXCL8-elicited migration of human neutrophils in a chemotaxis assay (Fig. 2 D). In contrast, a control baculovirus-expressed and 6xHis-tagged protein, a truncated variant of the ectromelia virus–encoded tumor necrosis factor receptor CrmD (CRD), containing only two cysteine rich domains (9) and purified according to the same protocol as for r-smCKBP did not alter chemokine binding or biological activity (Fig. 2, C and D). r-smCKBP activity was chemokine specific, as it did not block migration induced by the bacterial chemotactic peptide N-formylmethionyl-leucyl-phenylalanine (n-FMLP; Fig. 2 D).

As several important human diseases are associated with inappropriate activation of chemokines (10), any molecule that can specifically block chemokine activity has potential use as a therapeutic (11). Because initial functional data on smCKBP chemokine-binding activity involved in vitro studies (Figs. 1 and 2), we have evaluated its role in vivo. In a murine air pouch model, mice that had been given an intravenous injection of r-smCKBP had significantly reduced CXCL8-induced neutrophil infiltration (P < 0.001; Fig. 3 A). Interestingly, r-smCKBP did not block eosinophil infiltration of the air pouch induced by CCL11 (eotaxin), which was consistent with smCKBP being unable to bind CCL11 in cross-linking studies (unpublished data). In a contact hypersensitivity model, hapten-sensitized mice treated with r-smCKBP had significantly reduced ear swelling 24 h after challenge (P < 0.001; Fig. 3 A). Isolation of the inflammatory infiltrate from the ears of r-smCKBP–treated mice showed that r-smCKBP–treated mice were devoid of the marked neutrophilia observed in cells isolated from the ears of the control mice (Fig. 3 C). We have also tested the effect of treatment of mice with r-smCKBP while undergoing experimental autoimmune encephalomyelitis or arthritis. In both chronic inflammatory models, r-smCKBP treatment had no effect on modulating disease (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20050955/DC1). Therefore, smCKBP has specific in vivo activity in suppression of immediate or local inflammation, which is consistent with its bioactivity within the egg granuloma, as described later in this section.

Data on administering r-smCKBP in vivo indicated a preferential effect on CXCL8 and neutrophils (Fig. 3, A and B). As antagonism of CXCL8 and neutrophilia is a potential therapeutic strategy for pulmonary diseases (10, 11), we evaluated whether r-smCKBP could suppress CXCL8-induced pulmonary inflammation and resulting compromised
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Lung function. Mice treated intranasally with CXCL8 develop acute pulmonary inflammation with significant cell infiltration in the lung and difficulty in breathing, as shown by the significant elevation in baseline Penh values (P < 0.005; Fig. 3 D). Flow cytometry on bronchoalveolar cells showed that the major lung infiltrating cell in CXCL8-treated mice were neutrophils (Fig. 3 D). Intravenous injection of r-smCKBP completely ablated all the CXCL8-induced pulmonary inflammation and changes in lung function (Fig. 3 D). When administered systemically, r-smCKBP is therefore a highly efficacious inhibitor of chemokine-induced pulmonary inflammation.

Schistosome eggs produce a CKBP for a biological purpose. The schistosome egg is a viable organism and the egg secretes antigens as it matures within the mammalian host and stimulates the formation of a granuloma around the egg. That smCKBP is specifically secreted from live eggs was shown by the formation of a precipitate around eggs cultured with anti-smCKBP sera in vitro (Fig. 4 A). Furthermore, smCKBP was detected within the egg itself and within the granuloma surrounding intact eggs in the liver or intestine of mice with S. mansoni infections (Fig. 4 B), with no sm-CKBP detected in older granulomas or within dead eggs (not depicted). The secretion of a CKBP by live schistosome eggs within the granuloma would suggest that the smCKBP may block certain chemokines to facilitate granuloma formation and preferentially alter the cellularity of the granuloma. To address this we tested the in vivo role of smCKBP in the setting of the genesis of the egg granuloma. Mice were injected intravenously with live schistosome eggs and were treated with anti-smCKBP rabbit sera. This serum specifically recognized smCKBP and no other schistosome antigens (Fig. 1 D) and in vitro reduced sm-CKBP ability to block CXCL8 activity (Fig. 5 A). In mice injected with normal rabbit sera (NRS), there was the characteristic granuloma surrounding eggs in the lungs (Fig. 5 B). In contrast, mice treated with blocking smCKBP sera had a significant (P < 0.001), approximately twofold increase in granuloma size relative to the granulomas in animals treated with NRS (Fig. 5, B and C). The increase in granuloma size in anti-smCKBP–treated mice was associated with profound changes in the cell content of the granuloma. Compared with granulomas in NRS–treated mice, animals with smCKBP blocked had a significant increase in the proportion of neutrophils and macrophages (P < 0.01 and P < 0.005, respectively) within the granuloma (Fig. 5, D and E). Despite the marked eosinophil infiltration into granulomas of anti-smCKBP–treated mice, the numbers of eosinophils present were significantly reduced (P < 0.001; Fig. 5, D and E). Although the formation of the pulmonary egg granuloma involves the Th2 cytokines IL-4 and IL-13 (12), the modulation of egg granuloma size by anti-smCKBP treatment was independent of these cytokines, with an increased size granuloma persisting even when smCKBP was blocked in double IL-4 and IL-13 gene–deficient mice (unpublished data). When we injected dead schistosome...
eggs or studied secondary granulomas in presensitized mice, blocking smCKBP had no effect on granuloma formation or cellularity (unpublished data). Therefore, in an experimental granulomatous inflammation model, secretion of smCKBP by live eggs profoundly modulates the differential recruitment of cells and the size of the egg granuloma. A fundamental question that remains to be addressed is what role does smCKBP have in schistosome infection. To address this definitively, the ideal strategy will be to infect mice with an S. mansoni mutant with the smCKBP gene deleted, as undertaken previously with certain viral CKBPs (6); however, for technical reasons this is not currently achievable in schistosomes.

Chemokine inhibitors have been described in viruses (4–6), whereas the parasite Toxoplasma gondii produces a chemokine mimic (13), and recently a Staphylococcus aureus protein has been shown to inhibit other leukocyte chemoattractants (14). We now show that schistosome eggs secrete a molecule that blocks activity of certain chemokines both in vitro and in vivo. smCKBP is the first CKBP identified in a human pathogen and is the only one identified in a parasite to date. smCKBP is a potent suppressor of inflammatory responses in acute murine inflammation models. This study highlights the potential for using pathogen-derived immune modulating molecules as novel therapeutics for inflammatory diseases.

MATERIALS AND METHODS
Parasite antigen preparations
S. mansoni eggs were obtained as described previously (15). For in vitro cultures of live eggs and preparation of ES, all reagents were sterile and endotoxin free, and procedures were performed under aseptic conditions. Cercariae homogenates and secretions or homogenates of schistosome worms and schistosomula were prepared as described previously (16). S. haematobium egg antigens were obtained from the Schistosome Biological Supply Center, and S. japonicum eggs were provided by M. Johansen (Danish Bilharziasis Laboratory, Charlotte-
DNA was isolated (20). DNA was isolated from mouse tail snips using the Wiz-form method in compliance with Irish Department of Health and Children regulations. Recombinant chemokines were purchased from PeproTech. Chemokine cross-linking and cell binding assays were performed as described previously (17). For cell migration assays, 100–1,000 ng/ml CXCL8 and smCKBP were mixed together for 15 min at 37°C before addition to transwell migration chambers (ChemoTX), and neutrophils were purified from human PBMCs were added. Triplicate wells were used per dilution and negative (no CXCL8, blank) and positive (CXCL8, alone) controls were included. 100 nM of the chemotactic peptide n-FMLP was also used as a nonchemokine inducer of neutrophil migration. Data are presented as mean percent inhibition of total CCL5-elicited Ca\(^{2+}\) flux and Cell Quest software (Becton Dickinson). Data are presented as the mean percent inhibition of total CCL5-elicited Ca\(^{2+}\) flux.

Isolation, cloning, and expression of smCKBP

ES were fractionated by cation exchange chromatography and eluted using an NaCl gradient. Purified smCKBP was resolved by SDS-PAGE, and both bands were excised for mass spectrometry analysis using an LCQ Classic (ThermoFinnigan) with a nanospray interface (Protana). The sequences obtained were used in conventional database searches for sequence homology. An EST (available from GenBank/EMBL/DDJB under accession no. A820476) cloned from an S. mansoni egg cDNA library, predicted to encode an open reading frame with sequence similarity to an smCKBP peptide, was provided by G. Oliveira, Centro de Pesquisas Rene Rachou-Fiocruz, Belo Horizonte, Brazil. The smCKBP gene was PCR amplified, cloned into a baculovirus expression vector (pBAC-1) under the control of the strong polyhedrin promoter, cloned into a vaccinia virus expression vector (pMf601) under the control of a synthetic late promoter, and fused to a COOH-terminal 6xHis tag, with or without CD33 signal peptide. Recombinant viruses were constructed according to standard procedures.

The r-smCKBP protein was secreted from insect cells and purified from baculovirus-infected cells was purified using affinity chromatography in nickel chelate columns according to standard procedures. A construct comprising the two NH\(_2\)-terminal cysteine-rich domains of the tumor necrosis factor receptor CrmD from ectromelia virus (CRD) (9) was produced and purified in the same manner as r-smCKBP and used as a control baculovirus-expressed and 6xHis-tagged purified protein for functional studies. All batches of purified r-smCKBP and CRD were tested for endotoxin contamination and confirmed to have <1 EU/mg of protein (BioWhittaker).

smCKBP antisera

Rabbit sera against r-smCKBP was prepared by Harlan Sera Labs. To test the ability of the anti-smCKBP to block the chemokine binding activity of smCKBPs, U937 cells were used in 12/3I-chemokine cell binding assays. A range of dilutions of the anti-smCKBP sera or NRS were preincubated with r-smCKBP. These sera + smCKBP solutions were added to 12/3I-CXCL8 for 1 h before addition to U937 cells. Bound 12/3I-chemokine was determined by pthalate oil centrifugation (19).

Detection of smCKBP gene in schistosome cercarial DNA

A Puerto Rican strain of S. mansoni was maintained by passage in CD1 strain mice and albino Biomphalaria glabrata snails. All animal experiments were performed in compliance with Irish Department of Health and Children regulations. Cercariae were shed from individual patent infected snails, and genomic DNA was isolated (20). DNA was isolated from mouse tail snips using the Wizard SV Genomic DNA Purification System (Promega). Based on the smCKBP sequence, specific primers were designed and used in PCR. The band detected in cercarial DNA was excised from gel, purified with a gel extraction kit (QiAquick; QIAGEN), and sequenced to confirm it was smCKBP.

Egg pulmonary granuloma model

Live eggs were isolated from the livers of infected mice as described previously (15). BALB/c strain mice, or double IL-4 + IL-13–deficient mice (12), were injected intravenously with 5,000 live eggs. On day 0 and every 3 d after, mice were injected intraperitoneally with NRS or anti-smCKBP sera. Mice were killed on day 15 and lungs were removed. Lungs were fixed in formaldehyde saline and processed for histology. The size (volume assuming a sphere) and percentage of eosinophils, neutrophils, and macrophages within the granuloma was evaluated in >30 individual egg granulomas per mouse, as described previously (21). Dead eggs were prepared by storing live eggs frozen and were used in the above protocol. Secondary granulomas were elicited as described previously, with a modification that live eggs were used in the secondary challenge (21).

Air pouch model

Dorsal air pouches were induced in mice using previously described methods (22). In brief, 4 ml of sterile-filtered air was injected subcutaneously into the back of female BALB/c mice, and the pouch was reinflated with 3 ml of sterile air 3 d later. The dorsal air pouches of groups of 5–6 mice were either injected with 1 ml PBS or 1 ml PBS with 1 μg CXCL8 3 d later. At the same time, mice were injected intravenously with 200 μl PBS or 200 μl PBS containing 20 μg of r-smCKBP or OVA. Mice were killed and air pouches were lavaged with PBS 3 h later. The aspirate was centrifuged and cells were counted. Differential counts were performed on stained cytopsins.

Contact hypersensitivity

Female BALB/c mice (6–8 wk old) were sensitized by topical application of 0.5% 2,4-dinitrofluorobenzene (DNFB; Sigma-Aldrich) to the shaved abdomens. After 5 d, 0.02% DNFB was added to the right ear and the vehicle (acetone/olive oil = 4:1) was applied to the left ear. r-smCKBP or OVA (10 μg in 100 μl) were injected intravenously into mice before the application of DNFB to the ears. Ear swelling was measured with a dial thickness gauge (Mitutoyo) 24 h after application of DNFB. Ears were removed from mice, fixed, and cross sections of ears were hematoxylin and eosin (H&E)–stained. The cellular infiltrate of the ear pinnas were isolated and counted, and differential cell counts were performed on DiffQuik-stained cytopsins.

CXCL8–induced pulmonary neutrophilia model

25 μg CXCL8 was administered intranasally by droplet to BALB/c mice (23). Mice were then injected intravenously with 200 μl of 20 μg r-smCKBP or CRD. 18 h later, lung function was analyzed as described in the next section.

Lung function studies

Unrestrained conscious animals were subjected to barometric whole-body plethysmography to determine lung function (Emka). Mice were acclimated to the chamber, and breathing was recorded over 6 min. Baseline enhanced pause (Penh) was determined as a measurement of lung function (24). Data are means of 4–7 mice per group, and all experiments were repeated at least twice. Bronchoalveolar lavage, cytopsins, flow cytometry for neutrophils (non–B cells, non–T cells, Gr-1+ cells, and Mac-1+ cells), and quantification of myeloperoxidase were by standard methods.

Detection of smCKBP secretion from eggs

Circumoval precipitin test. S. mansoni eggs were isolated from the livers of infected mice as previously described (15). Live eggs were cultured at 37°C with NRS or anti-smCKBP sera. Eggs were examined for the presence of precipitates around eggs 24–48 h later.

Immunocytochemistry. Liver and intestines were isolated from infected mice and frozen in OCT compound. Tissue was cryosectioned. Slides were
probed with NRS or anti-smCKBP sera. Detection was with horseradish peroxidase-conjugated anti-rabbit-IgG (Sigma-Aldrich). Diaminobenzidine was used as substrate, and slides were counterstained with H&E.

**Statistical analysis**

Significant differences between groups were analyzed by Student’s t test. P < 0.05 was considered significant.

**Online supplemental material**

Fig. S1 shows that smCKBPs isolated from *S. mansoni* ES and soluble egg antigens can bind CXCL8. In Fig. S2, r-smCKBP-2 does not induce basophil degranulation. In Fig. S3, r-smCKBP does not alter disease in experimental autoimmune encephalomyelitis or in collagen-induced arthritis. Supplemental Materials and methods are also included. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050958/DC1.

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