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Functional Chemotactic Factor CP-10 and MRP-14 Are Abundant in Murine Abscesses

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Murine abscesses induced by intraperitoneal injection of a mixture of *Escherichia coli*, *Bacteroides fragilis*, and bran are established models for the study of localized infectious and inflammatory lesions. Chemotactic factors are thought to mediate the directed migration of large numbers of leukocytes into the abscess. Microorganisms located within the encapsulated lesion are not readily eliminated by the leukocytes, but their numbers are controlled over many weeks. We report the presence of large amounts of two murine S100 proteins, CP-10 and migration inhibition factor-related protein 14 (MRP-14), in abscesses as demonstrated by immunohistochemistry and measured by enzyme-linked immunosorbent assay and Western blotting (immunoblotting). High levels of CP-10 (7.7 ± 1 mg/ml) and MRP-14 (5.5 ± 1 mg/ml) were found throughout the time course of abscess development from early acute-phase lesions, which are predominantly neutrophilic, to late chronic-phase lesions, which contained more mononuclear cells. Approximately one-third of these amounts occurred as monomers (2.0 mg/ml for MRP 14 and 2.2 mg/ml for CP-10). Abscess fluid was strongly chemotactic, and a portion of the activity was due to CP-10, indicating its important role in leukocyte recruitment. CP-10–MRP-14 complexes were present in abscess fluid, and the proteins were immunoabsorbed together. In analogy with the related human MRP-8–MRP-14 complex, these proteins could be involved in the inhibition of microbial growth. No growth inhibition occurred with 20 μg of CP-10 or MRP-14 per ml or with mixtures of both, but these concentrations may have been insufficient and were not representative of the high concentrations found within abscesses. CP-10 may contribute indirectly to the antimicrobial response in abscesses by virtue of its strong chemotactic properties and its capacity to modulate the activation state of recruited leukocytes.

Migration of cells from the blood to sites of infection in the extravascular tissue is crucial to abscess development. Gradients of chemotactic factors produced either by resident tissue cells or by invading microorganisms mediate the directed movement of leukocytes (22). The composition of the cellular infiltrate and the degree of activation are determined by the nature of the chemotactic factors within the lesion. In contrast to the classical chemoattractants LTB_4, fMLP, C5a (22), interleukin 8, and RANTES (33), picomolar levels of murine CP-10 recruit polymorphonuclear neutrophils (PMN) and monocytes-macrophages without concomitantly activating them (9). CP-10 belongs to the highly conserved S100 protein family of small calcium-binding proteins and is constitutively expressed in neutrophils (23). The function of most S100 proteins is unclear, but they have been associated with cell regulatory processes and differentiation (25). These proteins are characterized by two highly conserved calcium-binding EF-hand regions separated by a short hinge region which may confer functional specificity by interaction with effector proteins (15, 25). This region of CP-10 is chemotactic, supporting this hypothesis (27). CP-10 and CP-$10_{42–55}$ elicit a sustained inflammatory recruitment of neutrophils after 6 to 8 h (9) with monocytes, which express elevated levels of scavenger receptor and therefore may have more efficient antimicrobial properties, which are more evident over 24 h (27, 29). These observations confirm a fundamental extracellular function of CP-10 as a potent chemotactic agent.

CP-10 shares 59% amino acid identity with human migration inhibition factor-related protein 8 (MRP-8) (28), although the hinge region sequence of MRP-8 differs from that of CP-10 and the protein is not chemotactic. MRP-8 and a related S100 protein, MRP-14, occur as complexes (6, 12, 34, 45) and, like CP-10, are abundant cytoplasmic components of myeloid cells (19). Immunohistochemical studies indicate that macrophages from acutely inflamed human tissues express MRP-14 but not MRP-8, whereas macrophages in chronic lesions express both (13, 47). Relatively high concentrations of the MRP-8–MRP-14 complex have antimicrobial properties in vitro (31, 42).

Abscesses induced by intraperitoneal injection of a mixture of *Escherichia coli*, *Bacteroides fragilis*, and bran are good models of localized inflammatory lesions (32). Throughout their persistence (10 weeks or more), viable bacteria occur both extracellularly and inside cells within the abscess (32). Although the ability of PMN to participate in phagocytic bacterial killing is impaired (18), bacterial numbers within the abscess are relatively stable but mechanisms preventing uncontrolled proliferation are unclear. Abscess fluid from humans, mice (43), and rabbits (1a) inhibit microbial growth, and activity has been associated with the MRP-8–MRP-14 complex

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(43) and MRP-14 isolated from human neutrophils inhibits growth of *Candida albicans* in vitro (31). In this study, we found high levels of functionally active CP-10 and murine MRP-14 in abscesses and investigated their potential as antimicrobial agents.

MATERIALS AND METHODS

Mice. Male mice of the BALB/c strain, 4 to 8 weeks old, were obtained from the Animal Resource Center, Department of Agriculture, Adelaide, South Australia, Australia. The ethical guidelines prescribed jointly by the National Health and Medical Research Council, the Commonwealth Scientific and Industrial Research Organisation of Australia, and the Australian Agricultural Council were followed in all experiments.

Bacteria and fungi. Frozen stock suspensions of *E. coli*, *Proteus mirabilis*, and *B. fragilis* were obtained from murine gut flora isolates as previously described (18). A clinical isolate of *C. albicans* was cultured on Sabouraud agar plates, and cultures were resuspended in Sabouraud broth before storage in small aliquots at −70°C. For use in in vitro assays, small aliquots of thawed suspensions were inoculated into Sabouraud (*C. albicans*) or tryptic soy (*E. coli* and *P. mirabilis*) broth. Bacteria and fungi harvested during log phase growth were washed three times in saline and resuspended to 10^3 CFU/ml in RPMI 1640 containing 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.2) (RPMI-HEPES).

Inoculation of mice and harvesting of abscesses. Abscesses were induced by intraperitoneal injection of 50 μl of an abscess-inducing mixture of 5×10^6 CFU of *E. coli*, 5×10^8 CFU of *B. fragilis*, and 1 mg of bran in RPMI 1640 (24). Preparation of the abscess-inducing mixture was done as previously described (32). Briefly, bacteria were grown on agar plates, harvested, and stored at −70°C. They were thawed, combined directly with bran, and inoculated. Individual abscesses were excised, weighed, and snap-frozen in liquid nitrogen on various days after inoculation. Quantitative bacteriologic analysis of the abscess at various times after inoculation was typically done as described by Nulsen et al. (32).

For fluid, abscesses (18 pooled, collected after 7 days, approximately 500 mg/ml in RPMI 1640 [Gibco, Grand Island, N.Y.]) were forced through a coarse wire mesh. Debris was pelleted by centrifugation at 13,000 × *g* for 15 min, and the fluid was filtered through a 0.22-μm-pore-size filter and stored at −80°C. Alternatively, a second pool of 11 abscesses (3 from day 4 and 8 from day 6) were snap-frozen immediately after harvest, stored at −80°C, and then homogenized (100 mg/ml in Hanks balanced salt solution (Cytosystems, Castle Hill, New South Wales, Australia) with the protease inhibitors used as described below for Western blotting (immunoblotting). Triton X-100 (Calbiochem, San Diego, Calif.) was added to a final concentration of 0.2%, disruption was completed with three freeze-thaw cycles, and the lysate was cleared by centrifugation for 15 min at 16,000 × *g*. Protein concentrations were determined with bicinchoninic acid and copper(III) sulfate (both from Sigma, St. Louis, Mo.) in 96-well microtiter plates with bovine serum albumin (BSA; Pierce, Rockford, Ill.) as the standard.

Production of polyclonal antibodies. New Zealand White rabbits were immunized by multiple intradermic dorsal injections with a total of 50 μg of purified murine native MRP-14 (35a) bound to nitrocellulose particles (20) in 500 μl of 0.9% saline mixed with an equal volume of Freund's complete adjuvant and boosted with the same amount 2 and 6 weeks after the initial injection by using Freund's incomplete adjuvant. Antisera were collected at 2-week intervals thereafter and tested for reactivity to purified MRP-14 and CP-10 on Western blots with a Multiscreen apparatus (Immunetics, Cambridge, Mass.). Immunoglobulin G (IgG) from normal rabbit serum and from anti-MRP-14 and anti-CP-10 sera (23) was purified by protein A affinity chromatography (Pharmacia, Uppsala, Sweden) in accordance with the manufacturer's instructions, and aliquots were biotinylated (3).

Immunohistochemistry. Abscesses from mice injected with an abscess-inducing mixture 7 days earlier were harvested and fixed in 10% neutral buffered formalin for 48 h at room temperature and subsequently embedded in paraffin. Six-micrometer sections were dewaxed with xylene (5 min) and then 100% (twice for 2 min each time), 90%, 80%, and 70% (for 2 min each time) ethanol. Hydrated sections were then treated with mouse osmolarity phosphate-buffered saline (PBS) containing 10% normal goat serum and 10% fetal calf serum for 10 min at room temperature (RT) before addition of the primary antibody. Anti-CP-10 IgG, anti-MRP-14 IgG, or normal rabbit IgG was diluted in diluent (PBS containing 1% normal goat serum, 1% fetal calf serum, and 1% normal mouse serum) and added to sections for overnight incubation at RT. Slides were washed three times in washing buffer (PBS containing 2% [wt/vol] gelatin), and biotinylated sheep anti-rabbit IgG (Silenius, Hawthorn, Victoria, Australia) was added. After 1 h at RT, slides were washed three times and treated for 15 min with methanol containing 0.09% (wt/vol) H_2O_2. Slides were washed, an avidin-horseradish peroxidase (HRP) conjugate (Sigma) was added, and the mixture was incubated for 1 h at RT. 3,3′-Diaminobenzidine was used as the peroxidase substrate, and sections were counterstained with hematoxylin.

Enzyme-linked immunosorbent assay (ELISA). A double-sandwich method was used for specific detection of CP-10. Flat-bottom 96-well microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated at 50 μl per well with anti-CP-10 IgG at 5 μg/ml (23) in 0.05 M sodium carbonate buffer, pH 9.6; incubated for 2 h at 37°C; washed four times with 10 mM Tris-HCl–150 mM NaCl–0.1% Tween 20, pH 7.4 (wash buffer), with a microplate washer (Titertec S8/12; Flow, Irvine, Scotland); blocked with 0.1% ovalbumin (Sigma) in wash buffer for 1 h at 37°C; washed four times, and stored for up to 2 months at −20°C. Samples diluted in RPMI 1640 containing 0.1% BSA (Sigma), 100 μl per well, were incubated overnight at 4°C. Purified CP-10 (final concentration, between 1 and 50 ng/ml) diluted in RPMI–0.1% BSA was included as the standard. After four washes, 100 μl of biotinylated anti-CP-10 IgG (4 μg/ml) per well was added and plates were incubated for 1 h at 37°C, washed four times, and incubated with a streptavidin-HRP conjugate (1:1,000 dilution; Amersham, Buckinghamshire, England) for 1 h at 37°C. After washing, plates were incubated with ABTS [2,2′-azinobis(3-ethylbenzthiazoline sulfonic acid)] and H_2O_2 (Kirkegaard & Perry, Gaithersburg, Md.) for 10 min at RT, the color reaction was stopped with 1% sodium dodecyl sulfate (SDS; Kirkegaard & Perry), and A_{405} was measured (Titertec Multiscan MCC/340).

Western blotting. Single-cell suspensions of spleen cells, bone marrow cells, and thioglycolate-elicited (TG) peritoneal exudate cells collected as previously described (39) were lysed in a final concentration of 0.2% Triton X-100–5 μg of leupeptin (Sigma) per ml–10 mM *N*-ethylmaleimide (Sigma)–5 U of nuclease (Benzonase; Merck, Darmstadt, Germany) per ml–10 mM phenylmethylsulfonyl fluoride (Sigma) in Hanks balanced salt solution. Samples were prepared to final concentrations of 4% SDS (Bio-Rad, Hercules, Calif.) and 10% glycerol (Merck). Reduced samples containing 100 mM dithiothreitol (Bio-Rad) were heated to 96°C for 2 min, and free sulfhydryl groups were alkylated with 0.15% iodoacetamide (Sigma) for 1 h in the dark at RT. *N*-Ethylmaleimide (10 mM) was maintained for nonreduced samples, and 0.001% pyronin Y–0.002% bromophenol blue (both from Bio-Rad) was used as a tracking dye. Samples were separated in SDS- and Tricine-containing 10% polyacrylamide gels (40) and transferred to polyvinylidene difluoride membranes (Immobilon, Bedford, Mass.) by electrophoresis (46). Blots were reacted with antiserum or purified IgG followed by a goat anti-rabbit IgG-HRP conjugate (Bio-Rad) or with biotinylated IgG followed by a streptavidin-HRP conjugate (Amersham), and bands were visualized by chemiluminescence in accordance with the manufacturer's instructions (Amersham ECL system with Amersham Hyperfilm). For quantitative analysis, the film was scanned (XRS 6cx; Omnimedia, Torrance, Calif.) and integrated densities of bands were quantitated (NIH Image 1.4, Macintosh version). For figures, scanned blots were arranged by computer (Canvas 3.5 for Macintosh; Deneba, Miami, Fla.) and printed. Stock solutions of high-performance liquid chromatography (HPLC)-purified CP-10 (26) and native murine MRP-14 (35a) were diluted, and samples of 20 to 100 ng of CP-10 and 10 to 40 ng of MRP-14 for standard curves were separated under reducing conditions.

Immunoabsorption. Anti-CP-10 and anti-MRP-14 IgGs were bound to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) in accordance with the manufacturer's instructions. The Sepharose beads contained 2.15 mg of normal rabbit serum-IgG per ml, 2.12 mg of anti-CP-10 IgG per ml, or 5.53 mg of anti-MRP-14 IgG per ml. Abscess fluid (100 μl) diluted in Hanks balanced salt solution containing 500 mM NaCl was incubated with 100 μl of gel overnight with slow rotation and washed, and bound material was eluted with 500 μl of 200 mM glycine, pH 2. After adjustment of the pH to 7.0 with 1 M Tris, samples were diluted 10-fold in RPMI 1640–0.1% BSA and tested in the chemotaxis assay. The total protein contents of samples were measured as described above. Proteins in the eluate were precipitated with 5% trichloroacetic acid, washed with ice-cold acetone, and analyzed by Western blotting.

Chemotaxis assays. Abscess fluid serially diluted in RPMI 1640–0.1% BSA was tested for chemotactic activity as described by Ryan and Geczy (39), with TG peritoneal exudate cells which were harvested after 16 h and comprised 30 to 40% PMN and approximately 50% monocytes, as indicated by differential staining. Chemotactic activity was quantitated microscopically by counting cells which had migrated through the pores of the membrane (PC 5 μm; Costar, Cambridge, Mass.) in four grid fields each of triplicate wells at a magnification of ×100. Endotoxin-activated mouse serum (5%), a source of C5a, was used as a positive control. Results were expressed as the mean number of cells per field.

Growth inhibition assays. CP-10, MRP-14, or abscess fluid was added to 20 CFU of *C. albicans*, *E. coli*, or *P. mirabilis* in a final volume of 200 μl of RPMI-HEPES in 96-well flat-bottom tissue culture plates (Falcon; Becton Dickinson, Lincoln Park, N.J.) and incubated for 18 h at 37°C in a humidified atmosphere containing 5% CO_2. Samples were serially diluted in saline and plated overnight onto Sabouraud or MacConkey agar plates, and colonies were counted for determination of CFU per milliliter. Results were expressed as Δlog CFU per milliliter, defined as the difference between the log CFU per milliliter at 18 h and the log CFU per milliliter at the beginning of the incubation.

Statistical analysis. Student's *t* test was used to analyze the data for statistical significance.

RESULTS

CP-10 and MRP-14 in abscesses. The antibody to CP-10 (23) reacted strongly with the 10-kDa band of purified CP-10 and cross reacted with MRP-14 in a weight-to-weight ratio of approximately 2:1 (CP-10–MRP-14) on Western blots (Fig. 1)

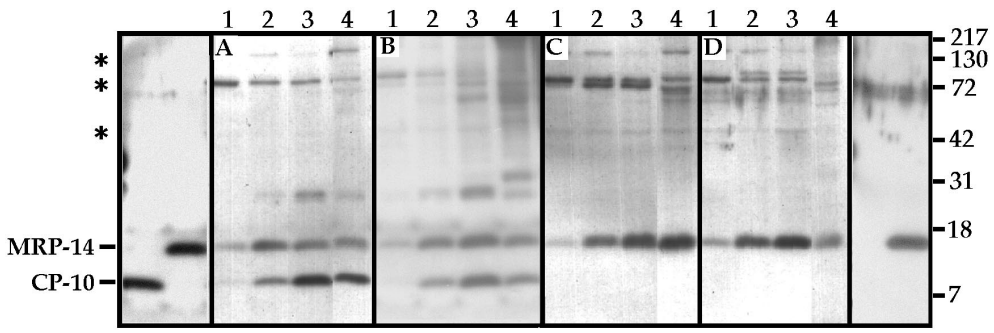

FIG. 1. Western blot of abscess fluid and different cell types. Lysates of spleen cells (lane 1), bone marrow cells (lane 2), TG PMN (lane 3), and abscess fluid (lane 4) were separated under reducing (A and C) and nonreducing (B and D) conditions as described in Materials and Methods. Proteins (1 μg per lane) were visualized with biotinylated anti-CP-10 (A and B) or anti-MRP-14 (C and D) antibody. Pure CP-10 and MRP-14 are indicated on the left, and the reactivity of each protein is given in the far right (anti CP-10) and far left (anti-MRP14) panels. Asterisks denote nonspecific bands reacting with the streptavidin-HRP conjugate alone. Molecular masses are indicated in kilodaltons on the right.

so that twice as much MRP-14 was required to produce a band with an intensity equivalent to that of CP-10. In contrast, cross reactivity was about 3,000:1 in ELISAs (data not shown). Anti-CP-10 recognized CP-10 homo- and heterodimers (23), whereas the antibody to MRP 14 was less reactive with dimeric complexes. The polyclonal antibody raised against pure MRP-14 did not cross react with CP-10 (Fig. 1) and failed to bind MRP-14 in ELISAs.

In lysates of bone marrow cells or TG PMN and in abscess fluid, the major band detected by anti-CP-10 antibody was CP-10 at 10 kDa (Fig. 1A and B). MRP-14 (at 14 kDa) was also evident, as well as a less intense band, possibly the heterodimer, at 24 kDa. TG PMN and abscess fluid samples contained higher-molecular-weight complexes (Fig. 1B) that were not due to nonspecific reactivity with the second antibody. One major band at 14 kDa was evident in abscess fluid and cell samples incubated with anti-MRP-14 (Fig. 1C and D). Spleen cells contained only minor amounts of both proteins. Streptavidin-HRP alone reacted nonspecifically with some high-molecular-weight bands (marked by the asterisks in Fig. 1). In all of the samples tested, the CP-10 band increased following reduction (Fig. 1A), indicating the possibility of disulfide-dependent complex formation. Reduction of abscess samples (lane 4) also increased the band intensity of MRP-14, but this was not apparent with spleen cells, bone marrow cells, or TG PMN (Fig. 1C and D, lanes 1, 2, and 3).

CP-10 was detected throughout abscesses collected after 7 days, both in the neutrophil-rich core region and in the surrounding outer region, whereas neither anti-MRP-14 nor rabbit IgG stained significantly (Fig. 2A to C). At higher magnification, staining was quite diffuse and not distinctively cell associated in the central core (data not shown). In the surrounding tissue of the capsule, which contained mononuclear cells, fibroblasts, and many neutrophils, staining was more intensely associated with specific cells and appeared perinuclear. On the basis of morphology, neutrophils appeared to be the most predominant of the stained cells.

To determine whether the relative amounts of the proteins changed during abscess development, band intensities from samples of individual abscesses over the time course (six abscesses each from days 4, 6, and 30 and five from day 55) were measured. Total protein concentrations of these samples were between 0.4 and 3.4 mg/ml, but no significant changes in the levels of total CP-10 and MRP-14 (per microgram of total protein separated under reducing conditions) were detected in acute-phase (4 to 6 days) or chronic-phase (30 to 55 days) abscesses. Typical samples from each time point are shown in Fig. 3. A 24-kDa band of varying intensity detected with anti-CP-10 was partially reduced to components with masses typical of the monomeric forms following reduction (data not shown).

CP-10–MRP-14 complexes in abscesses. Immunoabsorption with immobilized antibodies against CP-10 and MRP-14 was used to investigate the presence of complexes. Western blotting of bound material indicated that both CP-10 and MRP-14 were eluted from each sample of specific-antibody-coated beads (Fig. 4). Biotinylated anti-CP-10 (Fig. 4A) and anti-MRP-14 (Fig. 4B) antibodies were used for detection. Immobilized normal rabbit IgG and nonconjugated Sepharose beads did not bind detectable amounts of either protein. The integrated densities of the bands were used to measure the amounts of each protein eluted. For each milligram (0.1 μmol) of CP-10 bound to the anti-CP-10 Sepharose beads, 1.7 mg (0.12 μmol) of MRP-14 was eluted. When bound to the equivalent amount of anti-MRP-14 antibodies, 0.3 mg of CP-10 and 0.7 mg of MRP-14 were eluted. Weak reactivity of anti-CP-10 with a protein at 24 kDa was sometimes evident under reducing conditions, suggesting that noncovalent interactions also contribute to dimerization.

CP-10 in abscess fluid is chemotactic. The two curves in Fig. 5 indicate that abscess fluid contains substances affecting directed neutrophil migration (chemotaxis) and motility (chemokinesis) and that the activity was distributed over a wide range of concentrations. Optimal activity was observed with a 1:10 dilution (226 ± 116 cells per field compared with 71 ± 34 cells per field for control samples; $P = 0.008$, $n = 6$) and a weaker, but statistically significant, activity (106 ± 49 cells per field compared with 71 ± 34 cells per field for control samples; $P = 0.003$, $n = 6$) was evident at a dilution of $1{:}10^6$. About half of the activity at the 1:10 dilution, and all of the activity at the $1{:}10^6$ dilution, was blocked by addition of the same concentration of abscess fluid to the cells in the upper chamber (Fig. 5), indicating true chemotaxis. Inhibition was not observed at the 1:100 dilution, and cell migration increased in some experiments (data not shown), indicating that chemokinesis predominated at this concentration.

Incubation of fluid with immobilized anti-CP-10 antibodies reduced, but did not totally deplete, chemotactic activity compared with that in samples incubated with immobilized normal rabbit IgG (Table 1). The eluate from the anti-CP-10 antibody beads was strongly chemotactic, with a typical bell-shaped dose-response curve and maximal activity at approximately 1 ng of CP-10 monomer per ml (10 ng of total protein per ml; see below), whereas the control eluate had minimal activity. Both neutrophils and macrophages present in the mixed TG infil-

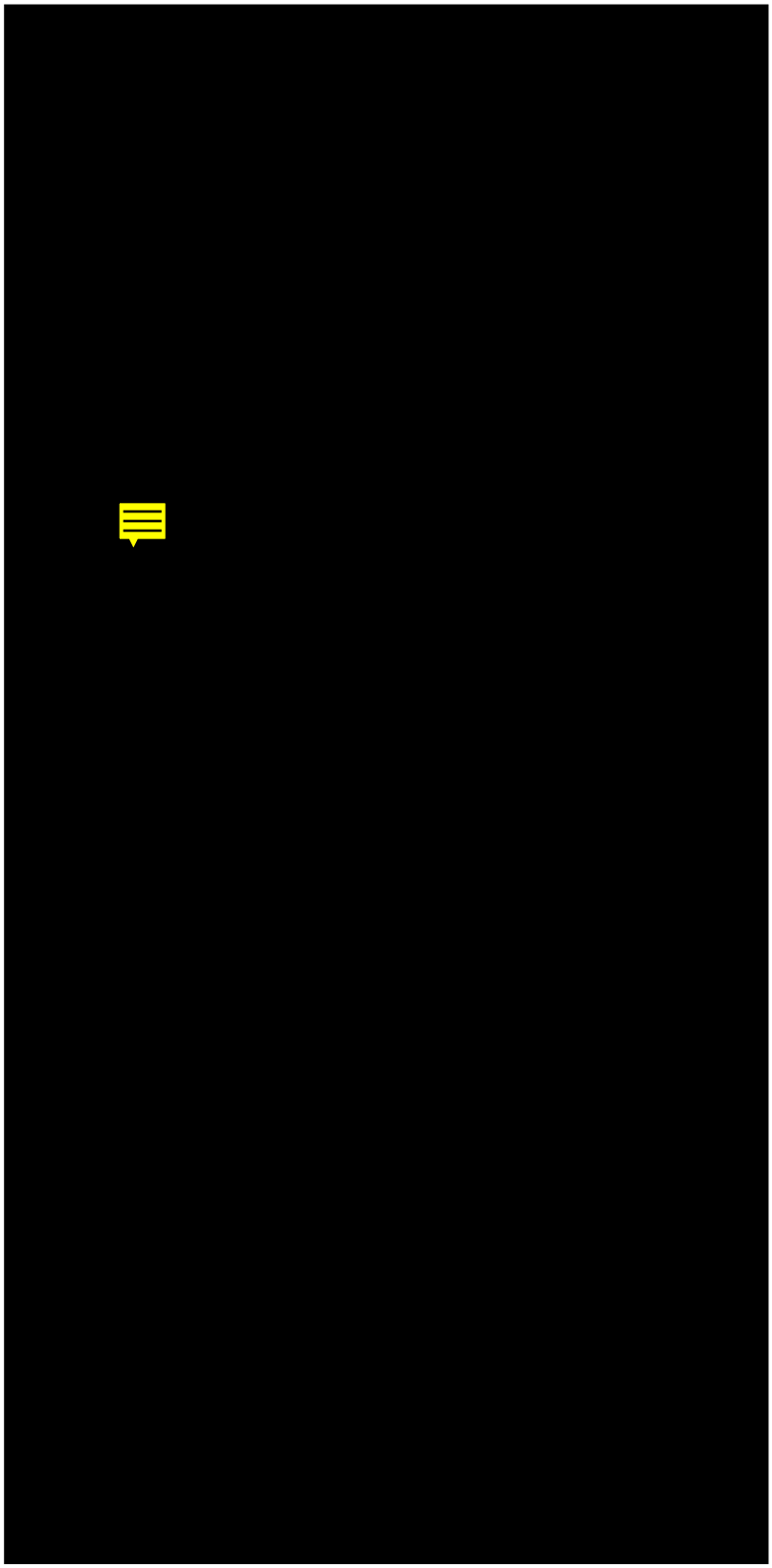

FIG. 2. Immunohistochemistry. Formalin-fixed sections were paraffin embedded, and 6-μm sections were examined immunohistochemically for the binding of rabbit anti-CP-10 (A) or anti-MRP-14 (B) or nonspecific rabbit IgG (C). The peroxidase-diaminobenzidine detection system produces a brown reaction product, and sections were counterstained with hematoxilin. The large arrow in an area with diffuse extracellular staining and the small arrow in an area with cell-associated staining (A) point in the direction of the necrotic core.

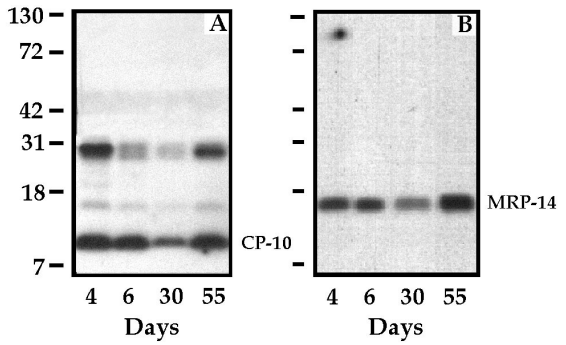

FIG. 3. Western blot of individual abscess lysate samples. Lysates (1 μg of protein per lane) from abscesses obtained on the days indicated were separated and transferred to polyvinylidene difluoride as described in Materials and Methods. Reactivity with biotinylated anti-CP-10 (A) and anti-MRP-14 (B) was visualized with a streptavidin-HRP conjugate. Bands quantitated by densitometry are indicated. Molecular masses are indicated in kilodaltons on the left.

trate migrated in response to the CP-10-adsorbed material, whereas the unfractionated fluid was apparently more chemotactic to leukocytes with morphological characteristics of neutrophils. The eluates tested were equivalent to those analyzed by Western blotting and shown in lane 3 of Fig. 4, which contained CP-10 and MRP-14, and lane 1, which contained minor protein bands detected by silver staining (data not shown) but no CP-10 or MRP-14. The potencies of abscess fluid (~8 μg of total protein per ml) and the anti-CP-10 eluate at the optimal activity were similar to that of 5% endotoxin-activated mouse serum.

Quantitative determination of CP-10 and MRP-14. HPLC-purified recombinant (23) and native (26) CP-10 samples were used as standards in the ELISA. The lower limit of CP-10 detection was approximately 1 ng/ml, and the linear range extended to 50 ng/ml. Serial dilutions (1:500 to 1:100,000) of abscess fluid prepared from pooled frozen abscesses were assayed six times, and fresh abscess fluid was measured once. The values obtained indicated that the level of CP-10 in abscesses was 90 ± 23 μM or 902 ± 227 μg/ml (mean ± standard deviation [SD] of seven measurements). Virtually all of the reactivity was attributed to CP-10, because cross reactivity with pure MRP-14 in this ELISA was 3,000:1 (CP-10–MRP-14, wt/wt). Three- to fourfold more CP-10 homodimer than monomer was required to give equivalent absorbance readings by ELISA (data not shown), suggesting that complexed CP-10 is not efficiently quantitated by this assay. Furthermore, previous estimates based on PMN (23) and bone marrow cells and abscess samples analyzed by Western blotting indicated higher levels of CP-10 than those detected by ELISA, and because MRP-14 could not be quantitated by this technique, the levels of CP-10 and MRP-14 were also measured by Western blotting. The chemiluminescence reaction of bands separated with 1 or 5 μg of total protein (Fig. 1 and 3) was so strong that these levels were unsuitable for accurate quantitative determination. Antibody reactivity measured by using 0.2 μg of total reduced protein (Fig. 6) or 0.4 μg of native proteins (data not shown) generated band intensities within the linear range of the standards (20 to 100 ng of CP-10 and 10 to 40 ng of MRP-14 per lane). The results (mean ± SD of seven determinations of abscess fluid pools and a sample of an individual abscess) indicated that CP-10 monomer levels were 2.2 ± 0.6 mg/ml (220 ± 60 μM), and these increased approximately threefold to 7.7 ± 1 mg/ml (770 ± 100 μM) following reduction of samples. MRP-14 monomer levels were 2.0 ± 1.26 mg/ml (140 ± 90 μM), which increased to 5.5 ± 0.98 mg/ml (390 ± 70 μM) after reduction. Thus, the molar ratio of CP-10 to MRP-14 was approximately 2:1. In terms of total protein, abscesses contained approximately 22% (6% monomer) CP-10 and 16% (5% monomer) MRP-14 (wt/wt). Taken together, quantitative assays indicated that a typical abscess of 50 mg would contain 1.75 mg of total protein, which comprised 385 μg of CP-10 and 275 μg of MRP-14.

Effects of CP-10, MRP-14, and abscess fluid on microbial growth in vitro. The effects of CP-10 and MRP-14 on microbial growth were initially tested with *C. albicans*, which was previously used to show the growth-inhibitory activity of the human MRP-8–MRP-14 complex (31, 42). In a series of three experiments with 20 μg of CP-10 or MRP-14 per ml or the pooled proteins (each at 10 μg/ml), we were unable to show a significant change in the growth of *C. albicans* (Table 2). Similar experiments with *E. coli*, one of the abscess-inducing bacteria used in our model, in which 40 μg of CP-10 per ml and 20 μg of MRP-14 per ml were used, indicated no significant inhibition with either protein, alone or in combination (Table 2). Abscess fluids identical to those used for quantitation of CP-10 and MRP-14 were titrated in microbial inhibition assays using *C. albicans* and *P. mirabilis*. Inhibition of growth was observed with dilutions of the two organisms of up to 1:64 and 1:80, respectively (Table 3). In a single experiment, rabbit anti-CP-10 IgG (30 μg/ml) did not significantly reduce the growth inhibition observed with *C. albicans* (data not shown).

DISCUSSION

Abscess development is characterized by a predominantly PMN infiltration which walls off a focus of infection. By 6 days, the abscess is established and comprises an outer capsule of vascularized connective tissue, an inner layer of mononuclear cells, and the PMN-rich necrotic core (Fig. 2). Our earlier reports showed that CP-10 is optimally chemotactic at 10^{-12} M for PMN and monocytes in vitro, making it one of the most potent chemoattractants reported. Furthermore, CP-10 elicits an inflammatory response in vivo (27) and the infiltrate after 6 to 8 h is composed predominantly of CD11b-positive neutro-

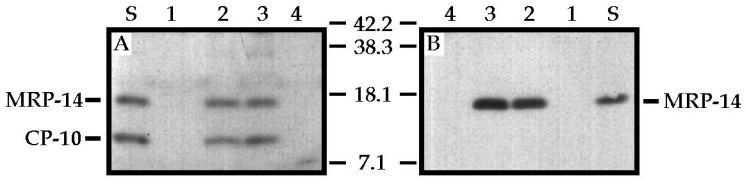

FIG. 4. Immunoabsorption of CP-10–MRP-14 complex. Proteins from abscess lysate (1 μg of total protein, lane S) absorbed by immobilized nonspecific rabbit IgG (lane 1), anti-MRP-14 IgG (lane 2), anti-CP-10 IgG (lane 3), or Sepharose 2B (lane 4). Eluted proteins were separated by SDS-Tricine-polyacrylamide gel electrophoresis under reducing conditions and Western blots stained with biotinylated anti-CP-10 (A) or biotinylated anti-MRP-14 (B) antibodies and streptavidin-HRP and visualized by chemiluminescence. CP-10 and MRP-14 are indicated by arrows, and molecular masses are indicated (in kilodaltons) between the panels.

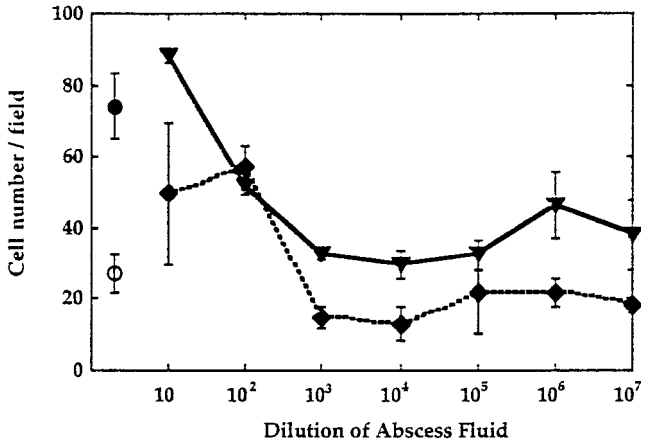

FIG. 5. Chemotaxis of TG neutrophils by abscess fluid. Tenfold serial dilutions of abscess fluid were tested for chemotactic activity towards TG neutrophils as described in Materials and Methods. Symbols: ▼, abscess fluid in the lower chamber only; ◆, the same concentration of abscess fluid in the lower and the upper chambers; ○, migration of unstimulated cells in controls; ●, cells attracted by 5% endotoxin-activated mouse serum.

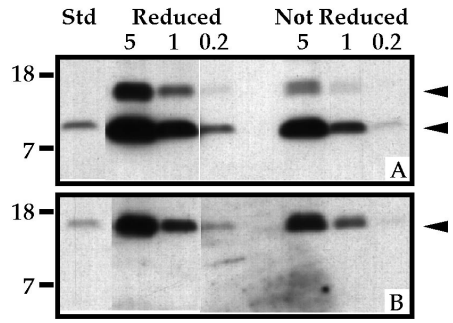

FIG. 6. Levels of CP-10 and MRP-14 in abscess fluid. Lysates from a freshly prepared abscess fluid pool were separated and transferred to polyvinylidene difluoride, and proteins were detected as described in Materials and Methods, with anti-CP-10 (A) or anti-MRP-14 (B) serum. The total amounts of abscess protein indicated in micrograms above the lanes were separated under reducing or nonreducing conditions. Purified CP-10 and MRP-14 (both 40 ng) were used as standards (Std), and their positions are indicated by arrowheads. Positions of molecular masses are indicated in kilodaltons on the left.

phils (9), followed by mononuclear cells after 24 h (27, 29). Our studies confirm the chemotactic nature of murine abscess fluid (Fig. 5) and strongly suggest a role for CP-10 in leukocyte recruitment.

Murine bone marrow, blood, and TG neutrophils contain high levels of CP-10 (23) and MRP-14 (Fig. 1), and both are products of activated spleen cells (27, 35a). Although we have scant evidence for expression of CP-10 in normal murine monocytes, Lagasse and Weissman have reported reactivity in blood and newly recruited monocytes, but not in TG or tissue macrophages, when an antibody to human MRP-8 was used (28). Although CP-10 and MRP-8 share 59% amino acid identity, MRP-8 has no chemotactic activity for PMN (14a, 27) and the functional relationship of the proteins is still unclear. Homologs of the highly conserved S100 proteins usually share more than 90% amino acid identity between species (25, 27). The MRP-8–MRP-14 complex is found in fluid of human abscesses (43), and MRP-14 is the antimicrobial component in vitro (31). The antibodies to CP-10 and MRP-14 used in the studies reported here failed to cross react with native or recombinant human MRP-8 or native human MRP-14 by Western blotting or ELISA (data not shown), confirming specificity for the murine proteins.

Levels of CP-10 and MRP-14 comparable to those found in TG PMN and bone marrow cells, but higher than those detected in spleen cells, were evident on Western blots of murine abscesses induced in the peritoneum by injection of an abscess-inducing mixture (Fig. 1), and these levels, relative to the total protein concentration, were maintained during the time course of abscess development (Fig. 3). Immunohistochemical staining showed CP-10 throughout the abscess after 7 days (Fig. 2), located extracellularly and within a large proportion of cells in the outer region (Fig. 2), which is a defined zone of mononuclear cells distinguishable from the surrounding granular tissue (32). Because the anti-MRP-14 antibody failed to detect MRP-14 by immunohistochemical staining, we were unable to demonstrate colocalisation of MRP-14 with CP-10 (Fig. 2A and B). CP-10 may diffuse from decaying cells in the core, in which the predominant CP-10-positive cells appeared to be neutrophils (Fig. 2), or could be actively released from neutrophils infiltrating the abscess in the early stages. The half-life of CP-10 in this situation is unknown, and it could be maintained throughout the chronic phase. Alternatively, it may be

TABLE 1. CP-10-specific chemotactic activity of abscess fluid

Dilution[a] (10-fold)	Mean no. of cells/field[b] ± SD				
	Untreated fluid	Anti-CP-10 IgG[c]		Control IgG[c]	
		Depleted	Bound	Depleted	Bound
1	409 ± 105	284 ± 57	67 ± 21	437 ± 86	112 ± 24
2	180 ± 6	177 ± 29	131 ± 27	207 ± 13	126 ± 13
3	166 ± 22	112 ± 10	429 ± 96	170 ± 45	142 ± 8
4	133 ± 36	168 ± 17	168 ± 67	340 ± 82	102 ± 10
6	195 ± 15	128 ± 16	186 ± 17	129 ± 15	90 ± 16

[a] Sample dilutions were to the power of 10 as shown.

[b] Results are means of triplicate wells of duplicate chambers of two experiments adjusted to a control value of 134 cells per field. Chemotactic activity was determined as described in Materials and Methods. Untreated fluid and normal rabbit serum-treated fluid contained approximately 39 μg of total protein. Total protein eluted from the anti-CP-10 adsorbent was 5 μg, and that eluted from the normal rabbit serum was <0.1 μg in 500 μl. Control medium, 134 ± 24 cells per field; 5% endotoxin-activated mouse serum-treated fluid, 346 ± 61 cells per field.

[c] Abscess fluid was absorbed with Sepharose-bound rabbit anti-CP-10 IgG or normal rabbit IgG as described in Materials and Methods.

TABLE 2. CP-10 and MRP-14 do not affect growth of *C. albicans* and *E. coli* in vitro

Reagent(s)[a]	Mean $\Delta\log_{10}$ CFU/ml ± SD at 18 h relative to control value[b]	
	C. albicans	*E. coli*
CP-10	0.20 ± 0.63	0.09 ± 0.09
MRP-14	−0.04 ± 0.84	0.03 ± 0.12
CP-10 + MRP-14	−0.27 ± 0.78	0.00 ± 0.08

[a] For triplicate assays against *C. albicans*, CP-10 and MRP-14 were tested at 20 μg/ml and together at 10 μg of each protein per ml. For *E. coli*, the concentrations were 40 and 20 μg/ml, respectively.

[b] For *C. albicans*, the data from three separate experiments are given, and for *E. coli*, data from a single experiment are presented. Both *C. albicans* and *E. coli* were added at a final concentration of 20 CFU per well, and the assay was carried out at 37°C for 18 h. The difference between the increases in $\log_{10}$ CFU per milliliter over 18 h for each test group and the appropriate control group is shown. The increases in $\log_{10}$ CFU per milliliter for the two control groups were as follows: *C. albicans*, 3.31 ± 0.40; *E. coli*, 6.31 ± 0.06. No significant differences between control and test sample values were found.

TABLE 3. Effect of abscess fluid on growth of *P. mirabilis* and *C. albicans* in vitro

Concn of abscess fluid[a]	Mean $\Delta\log_{10}$ CFU/ml ± SD (18 h)[b]	
	C. albicans	*P. mirabilis*
1:32	0.05 ± 0.02[c]	ND[d]
1:40	ND	0.46 ± 0.05[c]
1:64	3.19 ± 1.05[c]	ND
1:80	ND	0.51 ± 0.11[c]
1:160	ND	0.60 ± 0.12
Control[e]	5.05 ± 0.29	0.84 ± 0.12

[a] Abscesses (0.5 g/ml) were added to RPMI 1640, and the material was forced through a coarse wire mesh. Supernatant was clarified by centrifugation at 13,000 × *g* for 15 min, filtered through a 0.22-μm-pore size filter, and stored at −70°C. Assays were performed in triplicate in a total volume of 205 μl containing 2.5% fetal calf serum.

[b] *P. mirabilis* and *C. albicans* were at 20 CFU per well at the start of the experiment.

[c] Statistically significantly different from control group at $P = 0.05$.

[d] ND, not done.

[e] The control consisted of RPMI 1640.

released by activated macrophages within the abscess. Our recent studies show that lipopolysaccharide-primed monocytoid cell lines stimulated by lipopolysaccharide challenge express and release CP-10 monomer, whereas only minor amounts are evident within the activated cells (21a). This may be different from complexed MRP-8–MRP-14, which translocates to plasma membranes in a calcium-dependent manner (30, 38), is expressed on the surface (5), and may be released from myelomonocytic cells (21). Many mononuclear cells in the outer region of the abscess were negative for CP-10, and although perinuclear staining was evident in specific cells, there was no clear distinction between monocytes and PMN about to transmigrate across this zone. It is possible that a subset of macrophages, activated by toxins from the bacteria in the abscess, may maintain high levels of CP-10 expression in abscesses older than 1 week to yield the relatively constant amounts detected over several weeks (Fig. 3).

Monomers of both CP-10 and MRP-14, detected in the absence of metal ion chelators, were separated by SDS-polyacrylamide gel electrophoresis under nonreducing conditions which leave disulfide bonds intact (Fig. 1 and 6). Although reactivity of anti-CP-10 with the heterodimer was variable (Fig. 1 and 3), there was little evidence of homodimers. Together, the proteins constituted approximately 38% of the total protein in the abscess, and about one-third of each was monomeric (Fig. 6). This is unusual for S100 proteins in which complex formation may be essential for function (25). For example, the extracellular neurite extension factor activity of S100β is dependent on dimerization via disulfide bonds (2). Human MRP-8–MRP-14 normally occurs as a noncovalently associated Ca^{2+}-dependent complex (12, 37) variously described as the L1 complex (8), cystic fibrosis antigen (10), calgranulin (7), or calprotectin (44). Molar ratios of the MRP-8 and MRP-14 subunits of 2:1 (45), 1:1 (12), 1:2 (4), and higher multimeric forms (12, 37) have been found. Monomers of MRP-8 and MRP-14 have been detected in subsets of macrophages involved in chronic renal allograft rejection (16). Our results indicate that approximately 71% of CP-10 and 64% of MRP-14 formed disulfide-dependent complexes, although Ca^{2+}-dependent complex formation may also occur, as reduction did not totally disrupt the complexes (Fig. 1). Total concentrations, in samples from abscesses separated under reducing conditions were 770 μM for CP-10 and 390 μM for MRP-14, of which 550 μM CP-10 and 250 μM MRP-14 were detected following reduction (Fig. 6). Proteins were absorbed by affinity to both anti-CP-10 and anti-MRP-14 antibodies, and the lack of cross reactivity of CP-10 with anti-MRP-14 (Fig. 1 and 4) suggests that CP-10 eluted from immobilized anti-MRP-14 was initially bound as a complex to MRP-14. The variable levels of complexes (Fig. 1 and 3) detected in the absence of cross linking were possibly due to the weaker reactivity of antibodies with the complexed forms of these proteins on Western blots.

CP-10 may contribute to the recruitment of PMN and monocytes into inflammatory lesions, such as abscesses, but does not directly activate these cells (9). In contrast, classical chemoattractants like C5a, interleukin 8, and MCP-1 recruit and activate leukocytes by generating an oxidative burst and releasing granular enzymes (33), thereby triggering their microbicidal abilities. Human neutrophil granules contain cationic proteins with both chemotactic (e.g., CAP37; 35) and antimicrobial (e.g., defensins; 14) properties and intracellular human MRP-8 and MRP-14 may be involved in the calcium-dependent intracellular events leading to cytoskeletal reorganization, migration, granule release, and oxidative burst (7, 11, 30). Nevertheless, neutrophil microbicidal function is poor in chronic abscesses (18), and abscess fluid inhibits neutrophil microbicidal activity (1a). Furthermore, abscess-derived neutrophils have impaired ability to kill abscess-derived organisms, particularly gram-negative bacteria in vitro, although these are phagocytosed in vivo (18, 24). However, the failure of the infecting microorganisms to multiply within the abscess environment indicates mechanisms which limit microbial growth, and abscess fluid itself is microbistatic (43). The human MRP-8–MRP-14 complex may contribute to host defense by controlling the growth of pathogens that escape killing (41, 43).

Despite the lack of activity of the pure proteins, growth of *C. albicans* and *P. mirabilis* was inhibited by abscess fluid (Table 3). The MICs would have been approximately 60 μg of CP-10 per ml and 48 μg of MRP-14 per ml. Preliminary studies on the effect of zinc ions, which inhibit the growth-inhibitory activity of human MRP-8–MRP-14 (43), on the antimicrobial activity of abscess fluid showed that addition of 20 μM $ZnCl_2$ had little effect on the growth of *C. albicans*, and further studies on the effects of zinc on fluids immunodepleted of CP-10 and MRP-14 are warranted. Nevertheless, we were unable to obtain reproducible growth inhibition of either *C. albicans* or *E. coli* by using up to 40 μg of CP-10 or MRP-14 per ml, either alone or in combination (Table 2). Pure preparations of native CP-10 and MRP-14 were of limited availability, and the levels used may have been below those required for activity. Similarly, 30 μg of rabbit anti-CP-10 IgG per ml failed to specifically reduce the growth inhibition of abscess fluid and stimulated the growth of *C. albicans* in a nonspecific manner. Purification could have caused some denaturation, resulting in loss of antimicrobial activity without affecting the chemotactic activity. Furthermore, and as described for MRP-8–MRP-14 (43), saturation of the proteins with zinc could have inhibited their activity, and mass spectral studies of pure native CP-10 and MRP-14 have provided evidence of zinc chelation (1). It is possible that synergistic reactivity with other factors in the complex abscess milieu (36) contributes to growth inhibition. Like CP-10, the MRP-8-rich fraction (150 μg/ml) of human neutrophil lysates does not affect the growth of *C. albicans*, whereas complexed MRP-8–MRP-14 is inhibitory, and in contrast to murine MRP-14, human MRP-14 (at 20 μg/ml and higher concentrations) is the key component mediating growth inhibition (31). The potential function of MRP-8, which, unlike CP-10, is not chemotactic (27), in abscess fluid is unknown.

CP-10 recruits macrophages with upregulated levels of scavenger receptor (29), which has an important role in bacterial

clearance via its ability to bind endotoxin (17). Our studies show that abscess fluid, which contains both monomeric CP-10 and MRP-14, was strongly chemotactic for PMN and macrophages (Fig. 5), as are fractions at various stages of the purification protocol of CP-10 (26) which also contain MRP-14 (35b). Depletion of fluid with immobilized anti-CP-10 antibodies markedly decreased its chemotactic activity (Table 1), and the contribution of other chemoattractants, including lipid mediators, complement fragments, and chemokines, is worthy of investigation in this context. The highly chemotactic eluate contained both CP-10 and MRP-14 (equivalent to the sample analyzed by Western blotting after immunoabsorption [Fig. 4, lane 3]), some of which was complexed. The C-terminal region of human MRP-14 has amino acid homology with neutrophil-immobilizing factor, and thus could potentially counteract chemotactic migration (11). The amino acid identity between murine MRP-14 and human MRP-14 in this region is only 48% (28), and our preliminary observations indicate that MRP-14 has no effect on migration when added directly to chambers containing chemotaxins, as described for neutrophil-immobilizing factor, whereas disulfide-linked heterodimers of CP-10 and MRP-14 have reduced activity. Nevertheless, if CP-10–MRP-14 complex formation modulated chemotactic activity, sufficient monomeric CP-10 was present in abscesses to maintain the low concentrations (10^{-11} to 10^{-13} M) required for leukocyte recruitment.

We suggest that CP-10 may contribute to the antimicrobial response in abscesses by virtue of its strong chemoattractant properties for macrophages and neutrophils and its capacity to modulate the activation state of recruited cells, thereby indirectly mediating growth inhibition and final resolution. Future work using specific neutralizing antibodies and gene targeting will provide further insight into the role of CP-10 and MRP-14 in the pathogenesis of infectious and inflammatory lesions.

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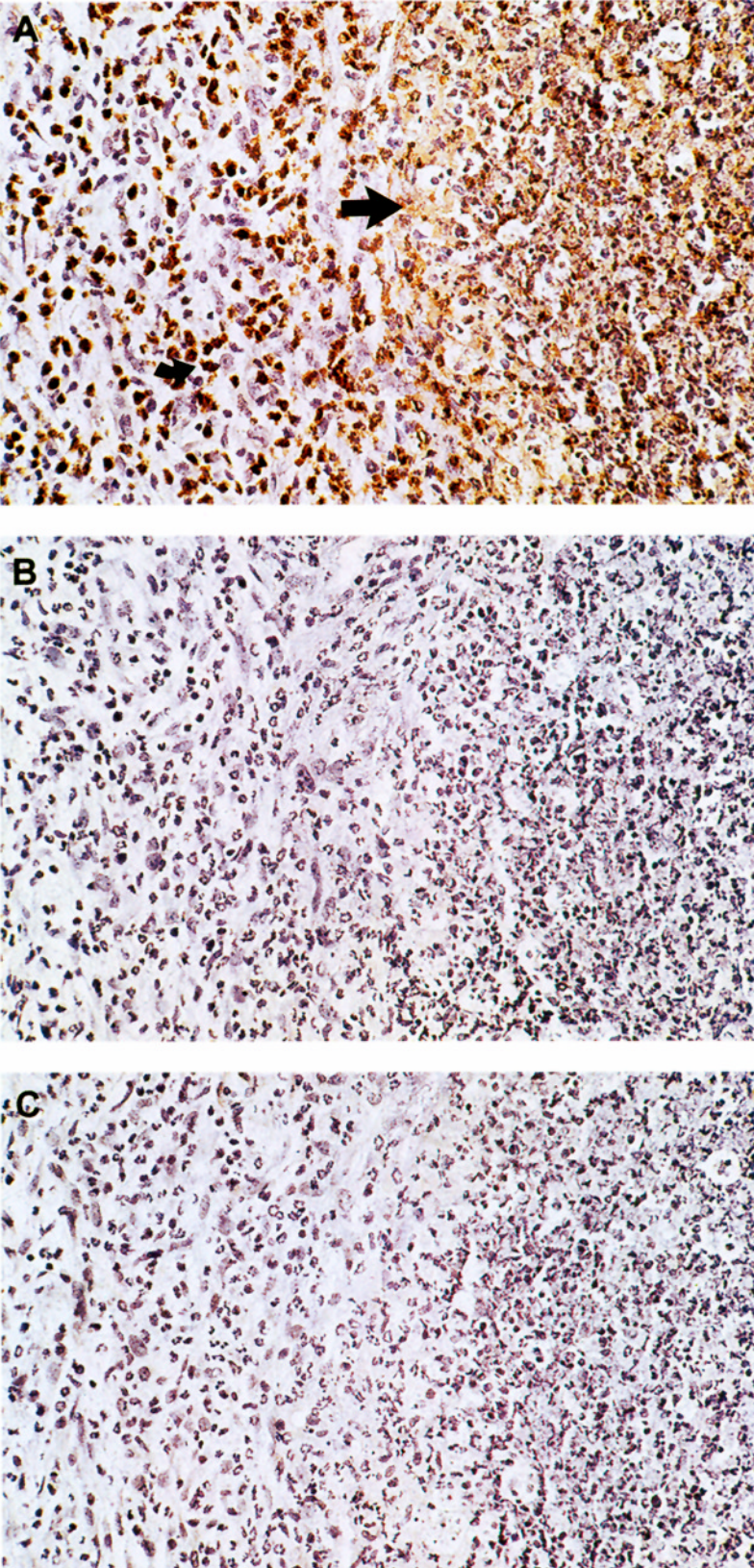
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