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Identification of cyclophilin as a proinflammatory secretory product of lipopolysaccharide-activated macrophages

(cytokine/inflammation/chemotaxis/neutrophil/endotoxin)

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ABSTRACT **We have isolated an 18-kDa peptide (designated sp18, for 18-kDa secreted protein) from the conditioned medium of lipopolysaccharide-stimulated RAW 264.7 murine macrophages. Purified sp18 had *in vivo* inflammatory activity and *in vitro* chemotactic activity for human peripheral blood polymorphonuclear leukocytes and monocytes. Surprisingly, N-terminal sequencing and tryptic mapping studies revealed that sp18 and cyclophilin, an intracellular protein that binds the immunosuppressive drug cyclosporin A, are highly homologous. The *in vitro* chemotactic activity of sp18 on monocytes was blocked by cyclosporin A but not by cyclosporin H, a structural analog of cyclosporin A that does not bind cyclophilin. Like purified porcine cyclophilin, mouse sp18 exhibited peptidyl-prolyl cis–trans isomerase activity. Medium conditioned by lipopolysaccharide-stimulated resident peritoneal exudate macrophages isolated from C57BL/6 mice contained substantially higher levels of sp18/cyclophilin than medium conditioned by nonstimulated macrophages. The observation that sp18/cyclophilin exhibits proinflammatory activity and is secreted by macrophages in response to endotoxin suggests that this protein may function as a cytokine, and invites the hypothesis that the immunosuppressive action of cyclosporin A results in part from interaction with an extracellular form of cyclophilin released as a mediator of immune and inflammatory functions.**

In response to the stimuli of both infectious and neoplastic diseases, host macrophages synthesize and secrete a variety of proteins that signal other cells in the body to aid in combating the invasion. In addition, these proteins effect a series of critical physiological changes that, if normal regulatory mechanisms fail, can lead to the disruption of homeostatic processes both locally and systemically (1–3). Over the past decade, a number of these macrophage-derived cytokines (e.g., tumor necrosis factor, interleukins 1, 6, and 8) have been isolated and their biological activities assessed. In addition to signaling cells of the immune system, these mediator proteins have been found to evoke responses in many other cell types (e.g., endothelial cells, adipocytes, muscle cells).

We have identified an 18-kDa protein (designated sp18) specifically secreted by resident peritoneal exudate macrophages from C57BL/6 mice in response to bacterial endotoxin (LPS). Due to difficulty in obtaining large amounts from this source, we chose to isolate sp18 from medium conditioned by LPS-stimulated RAW 264.7 cells. We now have isolated this protein to homogeneity and found it to be proinflammatory in an *in vivo* footpad assay and chemotactic for both human peripheral blood polymorphonuclear leukocytes and monocytes *in vitro*. N-terminal sequencing and tryptic mapping showed the sequence of sp18 to correspond closely to the sequence predicted from a cDNA clone coding for mouse cyclophilin, a molecule known as a ubiquitous intracellular protein that binds the immunosuppressive agent cyclosporin A (CsA) and possesses peptidyl-prolyl cis–trans isomerase (PPIase) activity. The possibility that cyclophilin functions as a cytokine suggests an explanation for the selective action of CsA on the immune system.

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MATERIALS AND METHODS

Materials. LPS W, *Escherichia coli* 0127:B8, was obtained from Difco. CsA and cyclosporin H (CsH) were the gifts of Angela Piperno (Rockefeller University). Pig spleen minor cyclophilin and chicken anti-bovine cyclophilin were gifts of Robert Handschumacher (Yale University, New Haven, CT). Female C57BL/6 mice (20–25 g) were obtained from Charles River Breeding Laboratories.

Cell Culture and Generation of LPS-Stimulated Macrophage Conditioned Medium. Resident peritoneal macrophages were harvested from C57BL/6 mice by peritoneal lavage (4). Cells were plated in 6-well Linbro plates at 10^6 cells per ml. After a 2-hour attachment period at 37°C and 5% CO_2, monolayers were washed five times with serum-free RPMI 1640 culture medium (GIBCO/BRL), covered with serum-free RPMI 1640 with or without LPS (1 μg/ml), and incubated for 16 hr at 37°C and 5% CO_2 before the conditioned medium was harvested for analysis. The mouse macrophage-like cell line RAW 264.7 was obtained from the American Type Culture Collection. Conditioned medium from LPS-stimulated RAW 264.7 cells was generated in a similar manner (5, 6).

Large-Scale Purification of sp18 from Medium Conditioned by LPS-stimulated RAW 264.7 Cells. Medium conditioned by LPS-stimulated RAW 264.7 cells was concentrated 20-fold (Amicon), dialyzed against excess 0.02 M Tris·HCl (pH 8.0), and fractionated by anion-exchange chromatography (Mono Q 10/10; Pharmacia) (5). The flow-through fraction from the Mono Q column was concentrated 10-fold, dialyzed against excess 0.05 M Mes (pH 6.7), and passed over a cation-exchange column (Mono S 10/10; Pharmacia) equilibrated with 0.05 M Mes (pH 6.7). Material that was eluted in the flow-through fraction was collected, dialyzed against excess 0.02 M Tris·HCl (pH 8.0), and fractionated on a heparin-Sepharose (Pharmacia) affinity column essentially as described (5). Material that was eluted at 0.3 M NaCl was collected, concentrated with a Centricon-3 microconcentrator (Amicon), and further fractionated on a size-exclusion column (Superose 12; Pharmacia) in 0.1 M ammonium acetate buffer (pH 7.4). Homogeneous preparations of the 18-kDa peptide obtained in this manner are referred to in the text

Abbreviations: LPS, lipopolysaccharide; PPIase, peptidyl-prolyl cis–trans isomerase; CsA and CsH, cyclosporins A and H.

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as "chromatographically pure" sp18. Samples were analyzed by SDS/PAGE (7). In several instances, Superose 12-purified sp18 was fractionated on a second gel filtration column in 0.1 M ammonium acetate (pH 7.4) (Superdex 75; Pharmacia). Protein concentrations were determined by Bradford assay (8).

***In Vivo* Inflammatory Activity.** Inflammatory cell infiltration was evaluated after footpad injections, according to Granstein *et al.* (9). In brief, female C3H/HeJ mice (6–12 weeks) were lightly anesthetized with Metofane (methoxyflurane; Pitman–Moore, Mundelein, IL) by inhalation. Each animal received in one hindlimb a subcutaneous footpad injection (50 μl) containing 2 μg of sp18 in RPMI 1640 supplemented with 0.1% fetal bovine serum. As a control, RPMI 1640 supplemented with 0.1% fetal bovine serum was injected in the contralateral hindlimb. Four hours after injection the mice were sacrificed and their hindlimbs were fixed in 10% formalin. Bones and cartilage were gently removed from each footpad with tweezers, and the remaining tissue was embedded in paraffin, sectioned, and then stained with hematoxylin and eosin. Slides were coded, and then scored by one of us (A.S., blinded as to treatment) for degree of inflammation. Each section was graded on a scale ranging from 0 to 3^+ for increasing inflammatory infiltration.

Amino Acid Sequence Analysis and Tryptic Mapping. Material for N-terminal sequencing was prepared according to Matsudeira (10). In brief, chromatographically pure sp18 was subjected to SDS/PAGE and transferred to a poly(vinylidene difluoride) (PVDF) membrane. N-terminal sequence was determined directly from sp18-containing PVDF membrane slice with an automated gas-phase sequenator (Applied Biosystems) at the Rockefeller University Protein Sequencing Facility.

Assay for PPIase Activity. PPIase activity was measured by modification of a published procedure (11). *N*-Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma) was used as substrate for the reaction, and assays were carried out at 18°C. First-order rate constants (k_{obs}) were derived from the first-order rate equation $A_{390} = A_\infty + A_0 e^{-kt}$, where k is the rate constant.

Western Blotting Analysis. Western blot (5) bands were visualized with the Protoblot System as recommended by the manufacturer (Promega).

Neutrophil Chemotaxis. Human polymorphonuclear leukocytes were obtained from heparinized venous blood (12). Neutrophil chemotaxis was assessed in modified Boyden chambers (12). The two compartments of the Boyden chamber were separated by a polyvinylpyrrolidone (PVP)-free polycarbonate filter with a 3-μm pore size (no. 155844; Nuclepore). Neutrophils [2.5×10^6 cells per ml in Gey's balanced salts solution supplemented with 2% bovine serum albumin and 20 mM Hepes (chemotaxis medium)] were added to the compartment above the filter, and test samples of possible chemoattractants diluted in chemotaxis medium were present below (for chemotaxis assays) or above and below (for chemokinesis assays) the filters. Chambers were incubated at 37°C and 5% CO_2 for 45 min; then the filters were recovered, fixed, and stained with Giemsa reagent. The number of cells appearing on the lower face of the filter was recorded in four high-power fields for each well, and each experimental condition was assayed in triplicate wells.

Monocyte Chemotaxis. Human peripheral blood monocytes were obtained as described (13, 14). Monocyte chemotaxis was assessed in a manner identical to that described for neutrophil chemotaxis except that the two wells were separated by a filter with a 5-μm pore size (no. 155845; Nuclepore), and the chambers were incubated at 37°C and 5% CO_2 for 90 min. CsA and CsH blocking experiments were conducted similarly, with the exception that all experimental samples were tested in both the presence and the absence of either 1 μM CsA or 1 μM CsH.

RESULTS

Purification of 18-kDa Inflammatory Peptide. Comparison of the SDS/PAGE profiles of medium conditioned *in vitro* by nonstimulated and LPS-stimulated resident peritoneal macrophages isolated from C57BL/6 mice led to the identification of an LPS-inducible macrophage secretory peptide of apparent molecular mass 18 kDa (designated sp18, for 18-kDa secreted protein). We used a more convenient source, conditioned medium from LPS-stimulated RAW 264.7 cells (a macrophage-like cell line), as starting material for the large-scale purification of sp18. To purify this component, conditioned medium was fractionated by sequential anion-exchange, cation-exchange, heparin affinity, and gel filtration chromatography. Conditions were chosen such that sp18 failed to bind either anion- or cation-exchange resins and was recovered in the flow-through fraction in each case. Murine sp18 exhibited weak affinity for heparin-Sepharose and was eluted at 0.3 M NaCl. Heparin-affinity-purified fractions containing sp18 were concentrated and then applied to a gel filtration (Superose 12) column. Samples from each Superose 12 fraction were electrophoresed in SDS/polyacrylamide gels, and a single fraction at about 2.2 void volumes contained a single protein band at 18 kDa (Fig. 1, lane 2). Comparison with the migration pattern of gel filtration standards indicated a relative molecular mass of 17,000, suggesting that purified sp18 occurs as a monomer.

***In Vivo* Proinflammatory Activity of sp18.** The LPS-induced secretion of sp18 by primary murine macrophages suggested that it might participate in the host inflammatory response. To test for proinflammatory activity, chromatographically purified sp18 was injected intradermally into the footpads of C3H/HeJ mice (known to be resistant to LPS, a possible contaminant of the peptide preparations). A markedly more intense inflammatory response was observed surrounding the site of sp18 injection compared with footpads receiving vehicle alone. In a representative experiment, footpads injected with 2 μg of sp18 were graded 1.75^+ for inflammation (on a scale of 0 to 3^+), while contralateral control footpads were graded 0.35^+.

N-Terminal Sequencing and Tryptic Mapping of sp18. Electrophoretically pure sp18 gave a single N-terminal sequence, which could be read out to 19 amino acids (Fig. 2*A*). Comparison of that sequence with those in the translated GenBank database (accession no. X52803) revealed that it was nearly identical to the amino acid sequence deduced from a mouse cyclophilin cDNA clone (15). Preparations of the macrophage-derived peptide were then digested with trypsin, the cleavage products were separated by reverse-

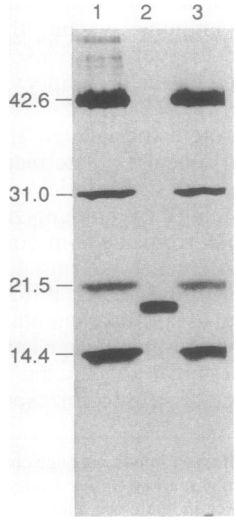

FIG. 1. SDS/PAGE analysis of chromatographically purified sp18. Lanes 1 and 3, molecular weight standards with sizes (kDa) indicated at left; lane 2, chromatographically pure sp18. Excision of such bands provided electrophoretically pure sp18.

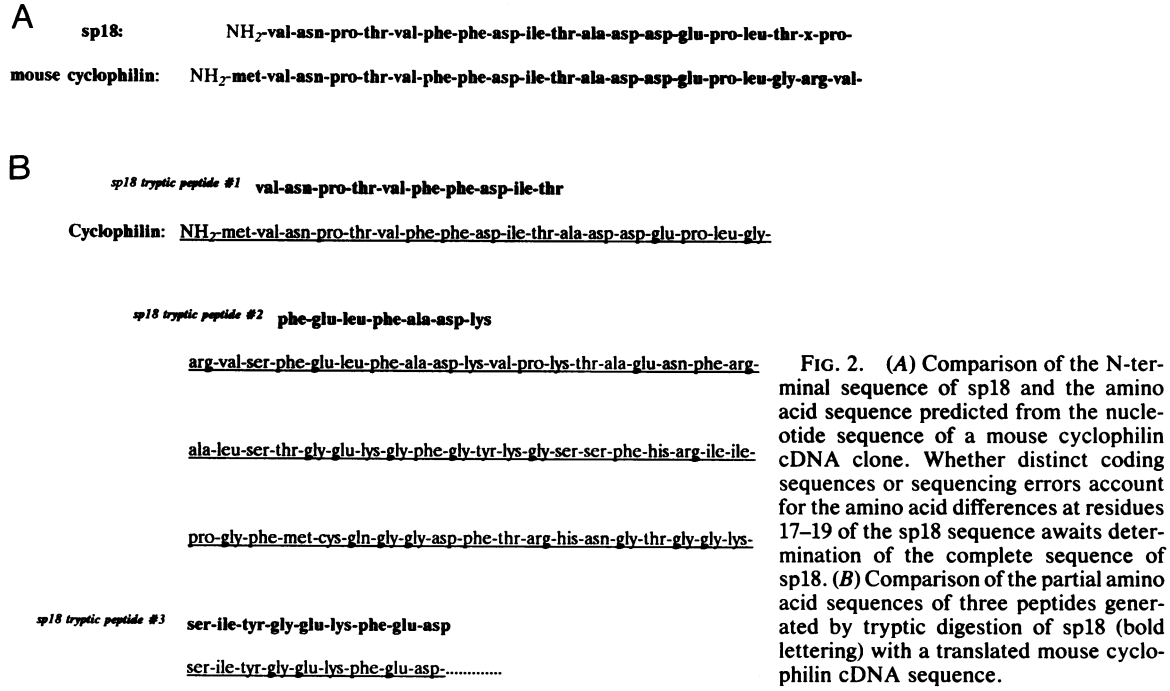

FIG. 2. (*A*) Comparison of the N-terminal sequence of sp18 and the amino acid sequence predicted from the nucleotide sequence of a mouse cyclophilin cDNA clone. Whether distinct coding sequences or sequencing errors account for the amino acid differences at residues 17–19 of the sp18 sequence awaits determination of the complete sequence of sp18. (*B*) Comparison of the partial amino acid sequences of three peptides generated by tryptic digestion of sp18 (bold lettering) with a translated mouse cyclophilin cDNA sequence.

phase HPLC, and three of the larger peptides were sequenced. The sequences of all three cleavage products (Fig. 2*B*) correspond exactly to the amino acid sequence inferred from the cloned mouse cyclophilin cDNA, strongly suggesting that the inflammatory peptide that we had isolated from the conditioned medium of LPS-stimulated murine macrophages was a mouse form of cyclophilin.

PPIase Activity. A chymotrypsin-coupled assay with the tetrapeptide substrate *N*-succinyl-Ala-Asp-Pro-Phe-*p*-nitroanilide confirmed that sp18 possessed PPIase activity (Table 1). The observed catalytic efficiency of sp18 was an order of magnitude lower than that observed with the positive control, pig spleen minor cyclophilin. Whether this was due to tissue or species differences, or to partial loss of activity during purification, is not known. In conjunction with CsA binding, the PPIase activity of cyclophilin is inhibited. Similarly, PPIase activity of sp18 was reduced by 60% in the presence of 1 μM CsA, providing further evidence that sp18 may be a mouse form of cyclophilin (data not shown).

Western Blot Analysis of Macrophage-Derived sp18 and Cyclophilin. To further test the correspondence between sp18 and cyclophilin, Western blot analysis with anti-cyclophilin antiserum was performed. When aliquots of concentrated, but otherwise untreated, conditioned media from nonstimulated and LPS-stimulated resident peritoneal macrophages were electrophoresed in SDS/polyacrylamide gels, transferred to nitrocellulose, and probed with cyclophilin-specific antiserum, an LPS-inducible band was observed at 18 kDa (Fig. 3*A*, compare lanes 2 and 3). Likewise, an 18-kDa immunoreactive band was observed when conditioned medium from LPS-stimulated RAW 264.7 cells was identically analyzed by Western blot techniques (Fig. 3*A*, lane 5). Although little or no sp18 was detectable without LPS induction of primary mouse macrophages, medium conditioned by noninduced RAW 264.7 cells sometimes contained as much immunoreactive sp18 as parallel cultures induced with LPS. This difference is not understood, but the transformed phenotype of RAW 264.7 cells may contribute. Western blot analysis of the final fractionation step in sp18 purification, gel filtration chromatography, is shown in Fig. 3*B*. Two distinct immunoreactive bands are observed in the partially purified preparation (product of anion-exchange, cation-exchange, and heparin affinity fractionation steps) of sp18 loaded on the gel filtration column (Fig. 3*B*, lane 3). The lower band (≈18 kDa) corresponds to sp18—that is, the immunoreactive band observed with chromatographically purified preparation of sp18 (Fig. 3*B*, lane 4). The upper band (Fig. 3*B*, lane 5) represents a second, higher molecular mass immunoreactive species, whose identity is not known. Pig spleen minor cyclophilin (Fig. 3*A*, lane 4; Fig. 3*B*, lane 6), included for comparison, migrates slightly faster than murine sp18.

Table 1. PPIase activity

Test sample	Rate of cis–trans isomerization
Buffer alone	1.9 ± 0.2
sp18 (20 nM)	2.0 ± 0.2
sp18 (100 nM)	3.6 ± 0.3
Pig spleen cyclophilin (5 nM)	5.9 ± 0.1

sp18/Cyclophilin Is Chemotactic for Human Peripheral Blood Neutrophils and Monocytes. *In vivo* inflammation assays indicated that the predominant cell type mobilized to the footpad following injection of sp18/cyclophilin was the neutrophil. As an indication of whether sp18/cyclophilin acted directly or indirectly in this regard, the purified peptide was tested as a chemoattractant *in vitro* and was found sufficient to attract neutrophils in the absence of other cell types (Fig. 4*A*). Chemotaxis was dose-dependent, peaking at a concentration of 0.5 μg/ml in the lower chamber. Chemokinesis (defined as migration in the absence of an sp18 gradient) was not increased relative to controls (data not shown). Macrophage-derived sp18 was also chemotactic for monocytes. The sp18-mediated monocyte chemotaxis was dose-dependent, peaking at 0.5 μg/ml. The enhanced movement of monocytes in response to sp18, like that of neutrophils, was attributed to the stimulation of directed (as opposed to random) migration in a gradient of sp18 (data not shown).

A purified preparation of pig spleen minor cyclophilin was assayed *in vitro* for its ability to stimulate the directed migration of human peripheral blood neutrophils and monocytes, and this porcine cyclophilin was also chemotactic for both types of inflammatory cells (data not shown). Porcine cyclophilin attracted neutrophils and monocytes at a slightly lower optimal concentration (0.125 μg/ml) than murine sp18 (0.5 μg/ml).

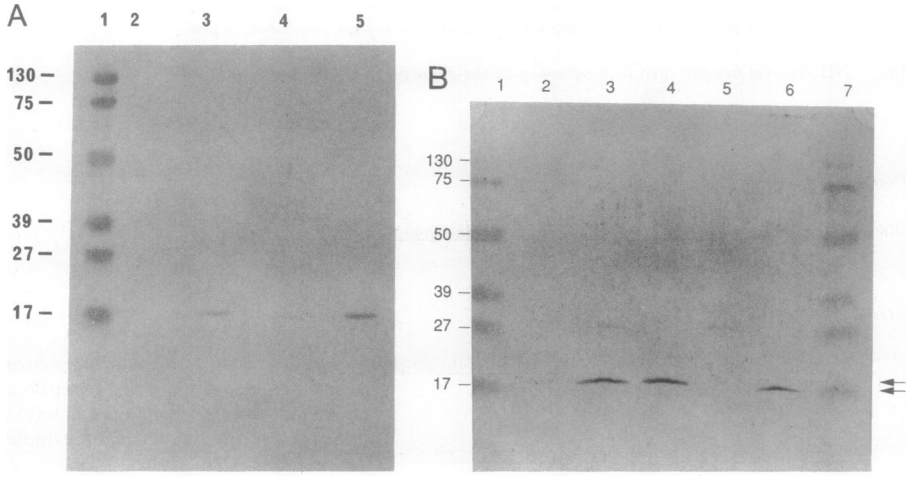

FIG. 3. (*A*) Anti-bovine cyclophilin antiserum recognizes an 18-kDa, LPS-inducible protein in the conditioned medium of C57BL/6 resident peritoneal macrophages. Lane 1, prestained molecular weight standards with sizes (kDa) indicated at left; lane 2, conditioned medium from non-stimulated C57BL/6 macrophages; lane 3, conditioned medium from LPS-stimulated C57BL/6 macrophages; lane 4, pig spleen cyclophilin; lane 5, conditioned medium from LPS-stimulated RAW 264.7 cells. (*B*) Anti-cyclophilin antiserum recognizes sp18 isolated from the conditioned medium of RAW 264.7 cells. Lanes 1 and 7, prestained molecular weight standards; lane 2, conditioned medium from LPS-stimulated RAW 264.7 cells; lane 3, sample applied to gel filtration column; lane 4, Superdex 75 fraction containing homogeneous sp18; lane 5, a second Superose 12 fraction containing a higher molecular weight immunoreactive species, whose identity is not known; lane 6, pig spleen cyclophilin. Arrows at right point to slight differences in the electrophoretic mobility of porcine and murine forms of cyclophilin.

CsA Inhibition of Chemotactic Activity. To further examine the relationship between sp18 and cyclophilin, we tested whether coincubation of sp18 with CsA would block its chemotactic properties. *In vitro* chemotactic activity of macrophage-derived sp18 for monocytes was completely inhibited in the presence of 1 μM CsA in the assay mixture (Fig. 5). CsH, a non-binding structural homolog of CsA, was not effective in this regard. Neither CsA nor CsH was chemotactic for monocytes (data not shown).

DISCUSSION

We report the isolation of an LPS-inducible 18-kDa macrophage secretory protein (sp18) that apparently is a mouse form of the ubiquitous intracellular protein known as cyclophilin. Macrophage-derived sp18/cyclophilin is inflammatory when injected *in vivo* in mouse footpads and is chemotactic for human peripheral blood neutrophils and monocytes *in vitro*. Identification of sp18 as a form of cyclophilin was confirmed by N-terminal sequence analysis, tryptic mapping studies, and the demonstration that sp18 has PPIase activity. Porcine spleen cyclophilin exhibited a similar ability to attract neutrophils and monocytes *in vitro* as did the 18-kDa mouse peptide.

Cyclophilin is a mammalian protein originally identified by its ability to tightly bind CsA (16, 17). The high affinity of CsA for cyclophilin suggests that it is the primary *in vivo* target, and therapeutic site of action, of CsA (18–21). Cyclophilin is identical to PPIase, which catalyzes the slow cis–trans isomerization of proline peptide bonds (11, 22). Numerous studies suggest that cyclophilin plays a critical intracellular role in regulating T-lymphocyte activation and proliferation (23–28).

Most cyclophilins that have been characterized to date have been localized to the cytosol. Exceptions include the

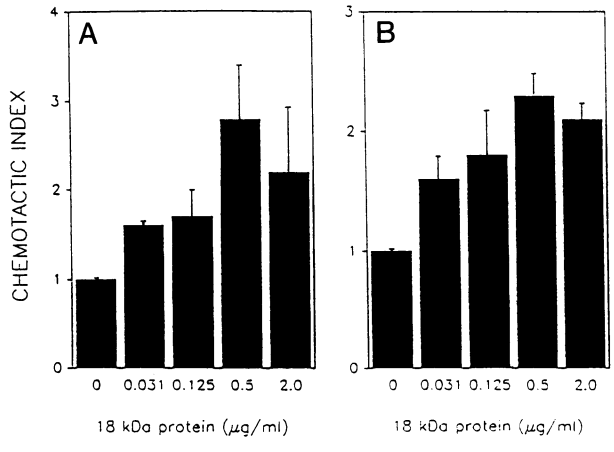

FIG. 4. (*A*) Murine sp18 is chemotactic for human peripheral blood polymorphonuclear leukocytes. Chemotaxis was measured in response to concentrations of sp18 ranging from 0.031 μg/ml to 2.0 μg/ml. Chemotactic index was derived by dividing the mean number of neutrophils migrating across the filter in response to the experimental sample by the mean number of neutrophils migrating in response to chemotaxis buffer alone. Bars represent the mean ± SEM of five independent experiments. (*B*) Murine sp18 is chemotactic for human peripheral blood monocytes. Concentrations of sp18 ranged from 0.031 μg/ml to 2.0 μg/ml. Bars represent the mean ± SEM of five independent experiments.

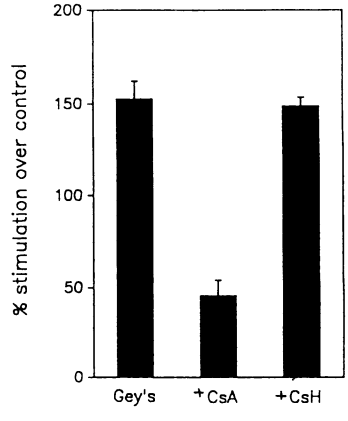

FIG. 5. Monocyte chemotaxis to sp18 *in vitro* is blocked by the presence of CsA. Data are presented as percent stimulation of human peripheral blood monocyte migration over control. For these experiments, the lower compartment of the Boyden chamber contained Gey's balanced salts solution with sp18 (0.5 μg/ml) alone or plus either 1 μM CsA or 1 μM CsH.

CsA-binding protein of *Neurospora crassa*, which is targeted to the mitochondria by its signal sequence, and the *ninaA*-encoded protein of *Drosophila*, which has a putative signal sequence and may be present as a membrane protein (29, 30). Recently, an endoplasmic reticulum-specific form of cyclophilin, as well as a secreted form of cyclophilin present in human milk, have been identified and characterized (31–33). The present report indicates that macrophages can be induced to secrete cyclophilin in response to an inflammatory stimulus—in this instance, LPS. The stimulated release of cyclophilin by macrophages appears to be specific and not the result of general cell lysis, in that the activity of several cytoplasmic "housekeeping" enzymes was not elevated in the supernatant fluids of LPS-stimulated cells (data not shown). The mechanism of cyclophilin release is unknown but is reminiscent of the cytokine interleukin 1, which, though lacking a classical signal sequence, is actively secreted (2). Preliminary evidence suggests that, in contrast to other cytokines, the level of cyclophilin mRNA in the macrophage is not affected by LPS stimulation. That released cyclophilin might function as a cytokine, carrying information between immune cells and other cells in the body, is an intriguing possibility that requires further investigation and implies the existence of a cyclophilin receptor.

It has been postulated that the immunosuppressive action of CsA is related directly to its binding of intracellular cyclophilin. CsA, a cyclic undecapeptide, is thought to gain entry into cells by partitioning through the phospholipid bilayer and subsequently forming a complex with cyclophilin in the cytosol. The results reported here, however, suggest alternative explanations. The loss of chemotactic activity by sp18/cyclophilin following incubation with CsA, but not with a nonbinding homolog, CsH, suggests that CsA might act by inhibiting the cytokine-like transcellular action of cyclophilin. This possibility offers an explanation for the selective nature of CsA treatment. Although cyclophilin is present in large amounts in many cell types, CsA might exert its unique immunosuppressive effects by interfering not only with intracellular cyclophilin, but also with the signaling properties of extracellular cyclophilin. Such a mechanism offers avenues for the design of immunosuppressive agents and strategies.

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