Leukocyte Chemotactic Activity of Cyclophilin*

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During the purification of eosinophil chemotactic factors synthesized by the uterus in response to estrogen we isolated a protein having an N-terminal (15 amino acids) sequence identical to that of rat cyclophilin. Our data demonstrate that cyclophilin, a cytosolic protein isolated from bovine thymocytes, which specifically binds the immunosuppressive drug cyclosporin A, as well as recombinant human cyclophilin, displays eosinophil chemotactic activity. In addition to its chemotactic activity, cyclophilin stimulated the release of peroxidase activity from eosinophils. Maximal chemotactic activity of cyclophilin was achieved at a concentration of approximately 10 nM. At similar concentrations cyclophilin was also able to stimulate the migration of neutrophils. This chemotactic activity could be prevented by the addition of cyclosporin A, but not by a nonimmunosuppressive analog (1-furfuryl-cyclosporin A) at similar concentrations. This chemotactic activity may represent an additional mechanism by which immunosuppressive drugs function to prevent tissue rejection.

The cyclic variation in circulating concentrations of sex steroids results in the growth regulation of the uterus in preparation for possible pregnancy. Similarly, treatment of ovariec-tomized or immature rats with estradiol results in the stimulation of generalized uterine growth (1, 2). In many ways several aspects of the uterine response to estradiol resemble an inflammatory type of reaction, including the water inhibition and a cellular infiltration (3–5). This cellular infiltration has been well documented (4–6), and in the rat uterus is associated with sites of inflammation as well as with corneal and kidney graft rejection in humans (7–9). The molecular mechanism whereby estrogens stimulate the infiltration of eosinophils is not known. However, recent studies from our laboratory have described the steroid regulation of an eosinophil chemotactic factor found in the uterus (10, 11). The bulk of the uterine eosinophil chemotactic activity was associated with a protein having a molecular mass in the range of 20 kDa as determined by gel exclusion chromatography (10). During the isolation and characterization of uterine proteins displaying eosinophil chemotactic activity, an amino acid sequence analysis indicated that one of these proteins could be cyclophilin, a protein which specifically binds cyclosporin A (CsA). CsA has been shown to decrease eosinophil recruitment in rats stimulated by immune challenge (12). CsA has also been shown to inhibit the degradation of basophils mediated through an IgE receptor mechanism (13). The effects of CsA as well as other immunosuppressive drugs such as FK506 very likely result from the ability of these drugs to inhibit T cell activation (14) and modulate cytokine production (15). Good evidence exists to indicate that the function of these drugs is mediated through specific binding proteins such as cyclophilin or FKBP, the binding proteins for CsA and FK506, respectively (16, 17). Cyclophilin is a member of a family of abundant cytosolic binding proteins more generally termed immunophilins, which are widely distributed in tissues and found in most organisms (18).

In these studies we report that bovine as well as recombinant human cyclophilin demonstrates in vitro chemotactic activity for both eosinophils and neutrophils. Furthermore, this activity can be blocked by the immunosuppressive drug cyclosporin A. However, the role cyclophilin may play in the estradiol-stimulated infiltration of eosinophils into the uterus remains to be established.

MATERIALS AND METHODS

Chemicals—Bovine thymus cyclophilin (CyP) (16) was prepared as previously described. Recombinant cyclophilin (rCyP) was prepared from a vector kindly supplied by Dr. S. Schreiber (Harvard University) (19). 1-Furfuryl-cyclosporin A was a gift from Dr. P. Durette of Merck Sharp and Dohme Research Laboratories. CsA was a gift provided by Dr. C. Teuscher (Utah University). Aprotinin, leupeptin, phenylmethylsulfonyl fluoride, CAPS, butyric acid, dimethyl sulfoxide, and fMLP were purchased from Sigma.

Protein Purification—Uterine extracts were obtained from mature rats which had received estradiol implants for 1 week. Tissues were homogenized (50 mg/ml) at 4°C in 10 mM Tris·HCl buffer, pH 7.4 (Tris·HCl), containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μM phenylmethylsulfonyl fluoride using a Kinematica Polytron homogenizer (Brinkman Instruments, Westbury, NY) set at 7. The homogenate was centrifuged at 30,000 × g for 30 min, and the supernatant passed through a Blue Sepharose/G-25 Sephadex column in 10 mM Tris·HCl buffer, pH 7.4. After washing with 5 bed volumes of buffer, proteins bound to the Blue Sepharose were eluted with 2 M NaCl. Fractions demonstrating chemotactic activity were pooled and applied to a DEAE-ion-exchange column, and the column was developed using a NaCl gradient from 0 to 300 mM. Chemotactic activity was associated with a protein having a molecular mass in the range of 20 kDa as determined by gel exclusion chromatography (10). During the isolation and characterization of uterine proteins displaying eosinophil chemotactic activity, an amino acid sequence analysis indicated that one of these proteins could be cyclophilin, a protein which specifically binds cyclosporin A (CsA). CsA has been shown to decrease eosinophil recruitment in rats stimulated by immune challenge (12). CsA has also been shown to inhibit the degradation of basophils mediated through an IgE receptor mechanism (13). The effects of CsA as well as other immunosuppressive drugs such as FK506 very likely result from the ability of these drugs to inhibit T cell activation (14) and modulate cytokine production (15). Good evidence exists to indicate that the function of these drugs is mediated through specific binding proteins such as cyclophilin or FKBP, the binding proteins for CsA and FK506, respectively (16, 17). Cyclophilin is a member of a family of abundant cytosolic binding proteins more generally termed immunophilins, which are widely distributed in tissues and found in most organisms (18).

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1 The abbreviations used are: CsA, cyclosporin A; CAPS, 3-(cyclohexylamino)propanesulfonic acid; CaP, cyclophilin; rCyP, recombinant cyclophilin; Eos, eosinophils; Neu, neutrophils; fMLP, fMet-Leu-Phe; SDS, sodium dodecyl sulfate.

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observed in the effluent fractions and in the 0–30 mM NaCl eluate fractions. Fractions containing chemotactic activity were analyzed by SDS-polyacrylamide gel electrophoresis.

Electrotransfer—SDS-polyacrylamide gel electrophoresis (12%) gels were prerun at 200 V for 30 min, 0.82% thioglycolic acid was added to the upper running buffer, and then the partially purified protein sample in reducing buffer was applied to the gel. Gels were run for 18 h at 12-mA constant current and then removed and soaked in 10 mM CAPS buffer for 5 min. Proteins were transferred to ProBlott membranes (Applied Biosystems) at 400 mA using a semidyry electrophoretic buffer with 10 mM CAPS buffer, pH 11.8, containing 10% methanol. Following 2-h transfer, the membrane was removed, rinsed with water, stained in 0.2% Ponceau S/1% acetic acid for 1 min, and destained in water until the protein bands were clear. The 18-kDa protein band was subjected to N-terminal sequence analysis.

Chemotaxis Assays—Differentiated eosinophils (Eos) and neutrophils (Neu) were induced in vitro from human leukemia cell line HL-60/C15 as previously described (28). Briefly, HL-60/Eos were induced using 0.3 mM butyric acid, and HL-60/Neu were induced with 1.25% dimethyl sulfoxide in RPMI-1640 media containing 10% fetal calf serum for 7 days. These protocols resulted in over 80% eosinophil or neutrophil differentiation and a viability of over 90% in all studies. The differentiated leukemia cells responded to fMLP and other stimulators in the same manner as normal cell types in the chemotaxis assays as has been previously shown (20).

Chemotaxis assays were performed as previously described (10, 11). Briefly, each sample, run in triplicate, was added to the lower chamber of a 48-well modified Boyden chamber (Neuro Probe, Cabin John, MD) overlaid with a 5-μm polycarbonate membrane (Nuclepore, Pleasanton CA), and preincubated for 20 min at 37 °C. Fifty μl of a HL-60/Eos or HL-60/Neu cell suspension at concentration of 1–2 × 10⁶ cells/ml were added to the upper wells. The chamber was then incubated for 1 h at 37 °C in 5% CO₂ after which the membrane was washed in phosphate-buffered saline, fixed in methanol, and stained with Wright Giemsa (Hemacolor, EMI Diagnostics, Gibbstown, NJ). Chemotaxis was assessed by counting the cells which had migrated through the membrane and were fixed to the surface adjacent to the lower wells. The variability between assays i.e., the total number of cells which migrate in response to chemotactic substances may be due to differences in the extent of cell differentiation for each specific cell preparation. All experiments were repeated at least three times.

Cyclophilin was tested at serial dilutions in 10 mM Tris·HCl/100 mM sodium chloride/5 mM β-mercaptoethanol/0.01% sodium azide (TSB buffer). CyP was inactivated by heating at 95 °C for 15 min. CsA was dissolved in 100% ethanol to give a final concentration of <1%. fMLP, at a maximally active concentration of 10⁻⁸ M in TSB, was used as a positive control, and TSB buffer alone was used as a negative control. The addition of sodium azide at the maximal concentration encountered (0.01%) did not alter the chemotactic activity of either the positive or negative controls.

Peroxidase Release—Peroxidase release from eosinophils was determined using guaiacol and H₂O₂ as substrates as previously described (21). In this assay 100 μl, containing 1 × 10⁶ HL-60/Eos, was incubated overnight with 10⁻⁶ M CyP in the presence or absence of 10⁻⁸ M CsA. Following the incubation cells were removed by centrifugation, and the supernatant was assayed for peroxidase activity. Cyclophilin itself demonstrated no peroxidase activity. In these assays CyP was diluted in Tris-buffered saline without sodium azide. Controls included cells incubated with buffer alone.

RESULTS

In the course of purification of the uterine eosinophil chemotactic factor from rat uteri we employed chromatography on Blue-Sepharose and DEAE ion-exchange resin. These steps resulted in an approximately 80–109-fold purification of the uterine chemotactic activity. Analysis of the DEAE fraction displaying chemotactic activity on SDS-polyacrylamide gel electrophoresis revealed four protein bands one of which had a molecular mass of 18 kDa (Data not shown). While other of the four proteins possess chemotactic activity, the 18-kDa protein was further characterized since it had a molecular mass similar to that previously estimated for the uterine eosinophil chemotactic factor (10). The N-terminal sequence of this protein was determined after electrotransfer to a polyvinylidene fluoride membrane. The resulting 15-

![FIG. 1. Chemotactic activity of bovine cyclophilin. Results are expressed as the percentage of chemotaxis evoked by fMLP (10⁻⁸ M) as described under "Materials and Methods." Data in this and other figures represent the means ± S.D. of at least three different experiments with triplicate wells for each determination.](image1)

![FIG. 2. Inhibition of eosinophil chemotaxis by cyclosporin derivatives. CsA or 1-furfuryl CsA were added to a solution of 10⁻⁸ M CyP and chemotactic activity toward HL60/Eos determined as described under "Materials and Methods." Negative controls were TSB buffer. Results are expressed as the percentage of the maximal chemotactic activity of CyP.](image2)

![FIG. 3. Chemotactic activity of recombinant cyclophilin. The eosinophil chemotactic activity of recombinant cyclophilin (rCyP) was examined. CsA (10⁻⁸ M) was added to 10⁻⁸ M CyP. fMLP (10⁻⁸ M) and buffer are presented. Results are expressed as the number of eosinophils per well.](image3)

amino acid sequence, VNPTVEFDIADGEPL, was 100% identical to the corresponding N terminus of rat cyclophilin. Three other proteins identified as having 86% or greater sequence identity were bovine cyclophilin, human cyclophilin,
and pig peptidylprolyl isomerase (EC 5.2.1.8). The latter enzyme was previously shown to be identical to cyclophilin (22).

To determine if cyclophilin possessed eosinophil chemotactic activity we tested highly purified bovine thymus cyclophilin in the chemotactic assay. The major isoform of bovine cyclophilin demonstrated a dose-dependent eosinophil chemotactic activity having a maximal activity at a concentration of between $10^{-7}$ and $10^{-6}$ M (Fig. 1). The shape of the dose-response curve for chemotactic activity was the typical bell-shaped curve seen with a variety of chemotactic factors (22–24). Heating the protein to 95°C for 15 min reduced the chemotactic activity by 84 ± 3% in parallel with the decrease in CsA binding and isomerase activities (16). Additionally, when the concentration gradient across the membrane of the chemotactic chamber was destroyed by the addition of $10^{-6}$ M cyclophilin to both the upper and lower chambers, chemotactic activity was not seen (data not shown). This result suggests that the effect is due to chemotaxis and not just to an increase in chemokinesis.

The addition of CsA to $10^{-8}$ M cyclophilin reduced the chemotactic activity of bovine cyclophilin (Fig. 2). Concentrations of either $10^{-9}$ or $10^{-10}$ M did not significantly modulate the chemotactic activity demonstrated by cyclophilin, whereas concentrations between $10^{-8}$ and $10^{-6}$ M significantly reduced chemotactic activity. The 1-furfuryl-cyclosporin A derivative, an analog of CsA which has approximately 1% of the binding activity of CsA, (25) was correspondingly less effective as an inhibitor of chemotactic activity.

To demonstrate that cyclophilin itself was responsible for the eosinophil chemotactic activity concentrations of $10^{-6}$ to $10^{-12}$ M recombinant cyclophilin were tested. Chemotactic activity was essentially the same as that from isolated bovine thymus and could be inhibited by the addition of CsA (Fig. 3).

Eosinophils often degranulate after tissue infiltration and release specific proteins. Several factors that induce the migration or chemotaxis can also stimulate this degranulation process. To examine this possibility eosinophils were incubated with $10^{-8}$ M cyclophilin, and peroxidase activity released from the eosinophil was determined. The release of eosinophil peroxidase activity stimulated by the addition of CyP was blocked by the addition of CsA (Fig. 4).

Many factors such as fMLP, which are chemotactic for eosinophils are also chemotactic for neutrophils; therefore, we examined the chemotactic activity of cyclophilin using HL-60/C-15 cells which had been differentiated to neutrophils by dimethyl sulfoxide (Fig. 5). Cyclophilin at concentrations as low as $10^{-12}$ M was as active as $10^{-8}$ M fMLP. Similar results were obtained using neutrophils isolated from human blood by means of a Percoll gradient (data not shown).

These responses are specific to the differentiated state of the target cell since cyclophilin ($10^{-8}$ M) did not cause the chemotaxis of HL-60/C-15 cells which had not been differentiated to either the eosinophil or neutrophil phenotype.

**DISCUSSION**

The chemotactic activity of cyclophilin whether isolated from the bovine thymus or produced through recombinant methods (16, 19) toward eosinophil or neutrophil phenotypes of HL-60 cells provides yet another means by which tissue infiltration by these cells can be achieved.

The cyclophilin-stimulated peroxidase release from eosinophils, and its sensitivity to CsA complement the observation that CsA can prevent the IgE receptor-mediated degranulation of human basophils and rat basophilic leukemia cells (13). The molecular mechanisms underlying the inhibition of T cell activation have not been fully established; however, many reports indicate that this process involves the inhibition of cytokine expression at the level of the nuclear transcription factors, particularly the nuclear factor of activated T cells (14, 15, 26). Other studies had shown that the hyperesoinophilia and the peritoneal infiltration of eosinophils associated with an immune challenge could be prevented by treatment with CsA (12). This activity of CsA was attributed to the inhibitory effect of CsA on interleukin-5 production by T cells (12). A recent report indicates that CsA may act as an inhibitor of signal transduction in immune reactions by complexing with cyclophilin and subsequently inhibiting the protein phosphatase, calcineurin, in a CsA-dependent reaction (27). The ability of CsA to modulate chemotaxis of phagocytic cells such as pulmonary alveolar macrophages or polymorphonuclear leukocytes has also been reported by Darby and Kahan in 1983 (28) who suggested that CsA may have a general effect on the cell membrane. The current results suggest that both processes, degranulation and chemotactic activity, may be directly mediated by the CsA binding protein, cyclophilin.

The cytosolic localization of the predominant form of cy-
cyclophilin (18 kDa) makes it difficult to envisage how this protein could easily function as a chemoattractant under physiological conditions. However, additional forms of cyclophilin have been characterized which are either associated with membrane fractions (29) or contain an amino acid sequence similar to a signal peptide (30). Other data indicate that released forms of cyclophilin exist (31, 32). The existence of such forms of cyclophilin would be consistent with our findings of a possible role for one or more members of this family of proteins in leukocyte chemotaxis.

Although our initial studies were designed to examine the regulation of eosinophil infiltration into the rat uterus following stimulation by estradiol it remains to be established whether uterine cyclophilin is the chemotactic factor responsible for the estradiol-stimulated influx of eosinophils into the uterus of immature rats (10, 11), and whether it is regulated in this activity by steroid hormones. Previous studies regarding the abundant, stable, and ubiquitous nature of cyclophilin mRNA (33) would suggest that hormonal regulation most likely would control the release or secretion of cyclophilin not its synthesis. A sex-associated and sex steroid-modulated survival time of skin grafts has been reported in rats (34). These authors also suggest that estradiol abrogated the immunosuppressive activity of CsA and accelerated graft rejection. Whether sex steroids can modulate the expression or release of specific members of the immunophilin families or their natural ligands remains an open question.

In summary we have demonstrated that the immunosuppressive binding protein cyclophilin possesses eosinophil and neutrophil chemotactic activity which is inhibited by CsA. The ubiquitous tissue distribution of cyclophilin may reflect the ability of various tissues to respond to injury or to stimuli of tissue remodeling processes through the release of cyclophilin resulting in leukocyte infiltration. Furthermore, CsA, which has been shown to decrease eosinophil infiltration at sites of graft rejection may modulate cellular chemotaxis, directly by interaction with cyclophilin. This mechanism may be complementary to the several other mechanisms mediating the effects of CsA on T cells.

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