INFECTION AND IMMUNITY, Nov. 1987, p. 2807–2815
0019-9567/87/112807-09$02.00/0

Vol. 55, No. 11

Proteinase Production by the Parasitic Cycle of the Pathogenic Fungus *Coccidioides immitis*

STEVEN RESNICK,[1] DEMOSTHENES PAPPAGIANIS,[2] AND JAMES H. McKERROW[3]*

Departments of Dermatology[1] and Pathology,[3] University of California, San Francisco, California 94143, and Department of Medical Microbiology, University of California, Davis, California 95616[2]

Received 30 April 1987/Accepted 3 August 1987

***Coccidioides immitis* is the causative agent of coccidioidomycosis (valley fever), a potentially disseminated fungal disease. We hypothesized that proteinases are expressed by the parasitic life cycle of *C. immitis* and that they might play an important role in the pathogenesis of coccidioidomycosis by facilitating spherule rupture, endospore dissemination, and tissue invasion and destruction. Filtrate from cultures of the parasitic life cycle of *C. immitis* was therefore assayed for proteolytic activity at neutral pH. The filtrate degraded 68% of a radiolabeled model of an elastin-rich extracellular matrix. The principal activity was against elastin and glycoprotein in the matrix. Degradation of purified elastin by filtrate was 222 μg/h per mg of filtrate protein at 37°C. Denatured type I collagen (Azocoll) degradation was 13.5 mg/h per mg of filtrate protein at 37°C. Proteinase activity peaked at 60 h of culture, correlating with release of endospores from mature spherules in the in vitro culture system. Elastase activity was attributed to a serine proteinase which exhibited an active-site preference for phenylalanine at the P1 site. The subunit molecular mass of the elastase determined by [^{3}H]diisopropylfluorophosphate labeling was approximately 25 kilodaltons. Inhibition of the azocollytic activity of crude filtrate by 2 mM 1,10-phenanthroline and 10 mM EDTA, and stimulation by 2 mM $CaCl_2$, suggested that a metalloproteinase was also present. Gelatin substrate gel electrophoresis with and without inhibitors confirmed that two proteinases were expressed, and they were separated by fast protein liquid chromatography.**

Coccidioides immitis is the etiologic agent of coccidioidomycosis, a fungal disease estimated to affect 100,000 new patients per year in the United States, where it causes 70 deaths annually (4). The infection is endemic in parts of the desert Southwest, California, and northern Mexico. Other endemic areas include parts of Guatemala, Venezuela, Paraguay, Colombia, and Argentina. The primary disease is usually a mild or moderately severe pulmonary infection that is self-limited and results in prolonged immunity. However, serious secondary forms of coccidioidomycosis develop in some patients (21). These forms include progressive, destructive pulmonary disease and dissemination to other organs.

C. immitis has two distinct life cycles. The first is a soil-living cycle that gives rise to arthroconidia. Human disease is initiated by inhalation of airborne arthroconidia. Once established in a human host, arthroconidia initiate a parasitic life cycle by enlarging into spherules which contain endospores. The mature spherules rupture and release numerous endospores that enlarge to form new spherules. Our studies have benefited from techniques for maintaining the endospore-spherule cycle in vitro (15).

Given the ability of endospores to disseminate from pulmonary alveolar spaces to the bloodstream and then to almost any organ, we hypothesized that proteinases were expressed in the parasitic life cycle of *C. immitis* and that they might play an important role in the pathogenesis of coccidioidomycosis. Such enzymes could facilitate spherule rupture, tissue invasion, and dissemination of endospores and could cause the initial tissue destruction which is later amplified by granulomatous inflammation. Using tissue-destructive pulmonary pathogens such as *Pseudomonas aeruginosa* (7) as clues, we further hypothesized that the proteolytic activity of *C. immitis*, like that of *P. aeruginosa*, might include a potent elastase, since elastin is a major structural component of lung interstitium.

To test these hypotheses, we first used an in vitro model of an elastin-rich extracellular matrix to assay filtrates from *C. immitis* endospore-spherule cultures for proteolytic activity capable of tissue damage. We identified, isolated, and characterized two major proteolytic activities: a potent serine elastase and a metalloproteinase. These two proteolytic activities, acting independently or in concert, may be in part responsible for the virulence of *C. immitis* infection.

(Portions of this work were presented in an abstract at the Western Section meeting of the Society for Investigative Dermatology, Carmel, Calif., February 1986 [S. Resnick, H. Levine, D. Pappagianis, and J. H. McKerrow, Clin. Res. **34**:163A, 1986].)

MATERIALS AND METHODS

Materials. Materials were obtained from the following sources: [^{3}H]acetic anhydride, [^{3}H]proline, [^{3}H]diisopropylfluorophosphate (DFP), and NaB^3H_4, Amersham Corp., Arlington Heights, Ill.; ox ligamentum nuchae elastin (E 60 mesh), Elastin Products Co., St. Louis, Mo.; type I collagen (Vitrogen), Collagen Corp., Palo Alto, Calif.; tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin, Worthington-Millipore Corp., Freehold, N.J.; electrophoresis molecular mass standards and sodium dodecyl sulfate (SDS), Bio-Rad Laboratories, Richmond, Calif.; succinyl–Ala–Ala–Pro–Phe–4-nitroanilide, Vega Biochemicals, Tucson, Ariz.; Triton X-100, α_1-proteinase inhibitor (α_1-PI), soybean trypsin inhibitor, leupeptin, EDTA, 1,10-phenanthroline (PT), *N*-ethylmaleimide, phenylmethylsulfonyl fluoride (PMSF), gelatin, Azocoll, clostridial collagenase, pancreatic elastase, and chymotrypsin, Sigma Chemical Co., St. Louis, Mo.; all chloromethylketone inhibitors and methylsuccinyl-

* Corresponding author.

Ala-Ala-Pro-Phe-thiobenzyl ester, Enzyme Systems Products, Livermore, Calif.

Culture of *C. immitis* and collection of endospore filtrate. Fungal cultures were maintained by the methods of Levine et al. (15). The culture medium was a simple synthetic medium with ammonium ion as the source of nitrogen and thus devoid of protein that might interfere with proteinase assays (15). Culture filtrate was collected at various intervals between 24 and 168 h in culture. The medium was filtered through Millipore filters (0.45 μm [pore size] once and 0.2 μm three times) to remove endospores and ensure its sterility before it was used in assays.

Ultimately, filtrate collected after 60 h in culture was found to have maximal proteolytic activity, and thus 60-h-old cultures were used for all subsequent experiments. Filtrate was stored at 4°C until use, and the proteolytic activity was unchanged after 2 weeks of storage. The endospore concentration of 60-h-old cultures was 10^8/ml.

Preparation and radioactive labeling of extracellular matrix. An elastin-rich extracellular matrix, synthesized by rat vascular smooth muscle cells (R22), was prepared and radioactively labeled with ^{3}Hproline as described previously (12, 18, 24). Cells from stock cultures were seeded on 16-mm-diameter plastic tissue culture wells. [^{3}H]proline (1 μCi) and ascorbic acid (50 μg/ml) were added 5 days after seeding. The cells were cultured for another 7 days with two changes of medium containing fresh ascorbic acid and 1 μCi of [^{3}H]proline. Cells were then lysed by adding 0.5 ml of 0.25 M NH_4OH to the cultures and incubating them for 30 min at room temperature. The insoluble extracellular matrix which remained attached to the wells was washed with distilled water–70% ethanol and stored in 10% ethanol at 4°C. The entire procedure was performed under sterile conditions. Differential enzyme digestion, amino acid analysis, peptide analysis, radiolabeling, and morphologic studies have shown that a typical insoluble extracellular matrix contains 200,000 cpm of ^{3}H and is composed of 35% glycoprotein, 40% elastin, and 25% collagen (largely types I and III) (12, 24). Wells were washed with 1 ml of phosphate-buffered saline before assays were conducted.

Studies of extracellular matrix degradation. Sterile *C. immitis* filtrate (1 ml; 115 μg of protein per ml) was added to wells containing radiolabeled matrix, and the wells were incubated at 37°C. Control wells contained 1 ml of phosphate-buffered saline. The progress of degradation was monitored by putting 50-μl samples of reaction mixture at timed intervals into 7 ml of scintillation fluid and counting the radioactivity in a liquid scintillation spectrometer. After 48 h, the remaining reaction mixture was removed from the wells, and the composition of the residual matrix was determined as described previously (12, 18, 24). The total amount of radioactivity per well was determined by summing the radioactivity released by filtrate and the radioactivity released from the residual matrix by sequential enzyme digestion. Matrix degradation at timed intervals was expressed as a percentage of total radioactivity. Specific degradation of glycoprotein, elastin, and collagen in the matrix was determined as a percentage of the trypsin-sensitive, pancreatic elastase-sensitive, or clostridial collagenase-sensitive material solubilized by sequential enzyme digestion of controls. This matrix has been analyzed exhaustively both biochemically and ultrastructurally to confirm that, after enzyme digestion of residual matrices with TPCK-trypsin, pancreatic elastase, and clostridial collagenase, the material solubilized corresponds remarkably well with trypsin-labile glycoproteins (such as fibronectin and laminin), elastin, and interstitial collagen, respectively (12, 24). The structure of these matrix molecules is highly conserved among mammalian species, so this matrix from rat vascular smooth muscle cells has relevance for human matrix studies. Furthermore, when we studied the degradation of purified connective tissue macromolecules by schistosome larvae (13, 18), the specific macromolecules degraded correlated very well with what we had predicted from matrix degradation studies. Matrix degradation experiments were also performed with filtrate in the presence of either 5 mM PMSF or PT.

Elastase assay. Purified elastin was labeled with NaB^3H_4 and used to assay elastase activity by methods previously described (19). [^{3}H]elastin (200 μg) was added to Microfuge (Beckman Instruments, Inc., Fullerton, Calif.) tubes with 0.1 M Tris hydrochloride (pH 7) and 100 μl of *C. immitis* endospore filtrate (enzyme) in a total assay volume of 300 μl. Tubes were incubated at 37°C for 24 h and spun in a Beckman Microfuge for 5 min; 100-μl samples of supernatant were then added to vials containing 7 ml of liquid scintillation fluid. Radioactivity released, indicative of solubilized (i.e., degraded) elastin, was determined in a scintillation counter. Elastase activity was calculated relative to that of a control assay with an equal volume of buffer instead of enzyme. Total radioactivity available was determined by using 5 μl of pancreatic elastase in the same reaction volume. This completely degraded the elastin in the assay. Elastase activity was calculated by using counts per minute (cpm) as follows: [(filtrate cpm − background cpm)/(total cpm − background cpm)] × 200 μg. Activity could then be expressed as micrograms of elastin degraded per hour, per milligram of filtrate protein.

Azocoll assay. Azocoll is denatured collagen linked to an azo dye. Azocoll degradation was assayed as described previously (16). Azocoll (5 mg) was incubated with 25 to 100 μl of enzyme sample in 0.1 M Tris hydrochloride with 2 mM $CaCl_2$ at pH 8 and 37°C for 8 to 24 h. Samples were then centrifuged for 2 min in a Beckman Microfuge. Azocoll degradation was measured as the A_{540} of the supernatant. Clostridial collagenase (5 μl) was used to determine the total available substrate. Degradation of Azocoll was calculated as follows: filtrate A_{540}/total A_{540} × 5 mg. Activity could then be expressed as milligrams of Azocoll degraded per hour per milligram of filtrate protein.

Peptide substrate assays. Hydrolysis of the 4-nitroanilide peptide substrate succinyl–Ala–Ala–Pro–Phe–4-nitroanilide was determined by measurement at 410 nm on a Gilford spectrophotometer (model 262) (3). Reactions were initiated by adding 50 to 100 μl of enzyme sample to 0.5 ml of a solution of substrate in 0.1 M Tris hydrochloride (pH 7).

Hydrolysis of the thioester peptide substrate methoxysuccinyl-Ala-Ala-Pro-Phe-thiobenzyl ester was determined by measurement of the increasing A_{324} (3). Substrate in dimethyl sulfoxide and 4,4′-dithiopyridine in dimethyl sulfoxide were added to 1.8 ml of 0.1 M Tris hydrochloride (pH 7) to give a total volume of 2.0 ml. A 50-μl enzyme sample was added to start the reaction.

[^{3}H]collagen degradation studies. Type I collagen (Vitrogen) was labeled with [^{3}H]acetic anhydride by the methods of Gisslow and McBride (6). [^{3}H]collagen (10 μl; 16 μCi/mg; 2.5 mg/ml) was incubated for 12 h at 30°C with 10 or 20 μl of *C. immitis* endospore filtrate and 10 μl of buffer (0.1 M Tris hydrochloride, 2 mM $CaCl_2$ [pH 8]). Additional buffer (10 μl) or 100 μg of TPCK-trypsin per ml was substituted for filtrate as a control. Samples were added to sample buffer with 5% (vol/vol) 2-mercaptoethanol and boiled for 3 min. Samples

were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a 4% stacking gel and a 10% running gel (14). The gels were fixed, treated with En^{3}Hance (New England Nuclear Corp., Boston, Mass.), and dried. Gels were analyzed by autoradiography for 24 h at −70°C with Kodak XAR-5 X-ray film.

Enzyme inhibition studies. In assays with inhibitors, the inhibitor at an appropriate concentration was preincubated with the enzyme for 20 min at room temperature; substrate was then added, and proteolytic activity was assayed as described above. Chloromethylketone peptide inhibitors (20) were prepared as stock solutions in dimethyl sulfoxide. PMSF and PT were prepared as stock solutions in ethanol. An equal volume of dimethyl sulfoxide or ethanol alone was also tested with the enzyme sample, and inhibition was calculated relative to that of the appropriate solvent control. *N*-ethylmaleimide, α_1-PI, leupeptin, and EDTA were prepared as stock solutions in water.

The elastase and Azocoll assays for the various inhibitors, pH optimum, and calcium dependence were performed in quadruplicate. The data were taken as average values for four runs, and maximum variability was less than 4%.

Substrate gel electrophoresis. Gelatin copolymerized with SDS-polyacrylamide was used for simultaneous detection of proteinases and determination of molecular mass as described previously (8, 19). Polyacrylamide (10%) gels were polymerized in the presence of 0.1% swine skin type I gelatin. Boiling and reducing agents were not used, but otherwise the procedure was that of Laemmli (14), with a 4% nongelatin stacking gel. Gels were prepared on a Hoefer small-slab gel apparatus (0.75-mm thickness). A Tris-glycine running buffer (pH 8.3) was used. Enzyme samples (approximately 5 μg of protein in 2.5 μl water) were diluted 1:1 in sample buffer (5% SDS, 2% sucrose, 0.0005% bromphenol blue in the stacking gel buffer 0.5 M Tris hydrochloride [pH 6.8]–0.4% SDS). Electrophoresis was run at 15 mA per gel. After electrophoresis, the gel was washed once in 100 ml of 2.5% Triton X-100 for 2 h to remove excess SDS. The gel was then incubated in 0.1 M Tris hydrochloride (pH 7) for 24 h at 37°C. The reaction was stopped by washing the gel for 1 h in 50% trichloroacetic acid at room temperature. The gel was stained with Coomassie brilliant blue R250 for 1 h and then destained in 45% methanol–10% acetic acid until proteolytic bands that clear the dye from the gel were optimal for photography (about 30 min).

Inhibitors were also used in this procedure. Enzyme samples were preincubated with either 5 mM PMSF or 5 mM PT before electrophoresis. The inhibitor was also included at the same concentration in the postelectrophoretic incubation. Equal volumes of ethanol were used as controls.

Column chromatography. To separate the two major proteinase activities and determine their approximate isoelectric points (pIs), we used a Mono P HR 5/20 chromatofocusing column (Pharmacia, Uppsala, Sweden) with a fast protein liquid chromatography (FPLC) system. Filtrate (1,000 ml) was concentrated by precipitation with 70% ammonium sulfate. Precipitated protein was collected by centrifugation at 19,000 × *g*, suspended in 3 to 4 ml of distilled H_2O, and dialyzed overnight against 4 liters of 20 mM Tris-acetate (pH 8.3) at 4°C. Concentrated samples were then applied to the column in 20 mM Tris-acetate (pH 8.3) at a flow rate of 0.25 ml/min and eluted by pH gradient with 70% Polybuffer 74-acetate (Pharmacia)–30% Polybuffer 96-acetate (Pharmacia) (pH 5.0) at a flow rate of 0.5 ml/min. The elution pH was measured continuously by a Pharmacia flowthrough pH monitor.

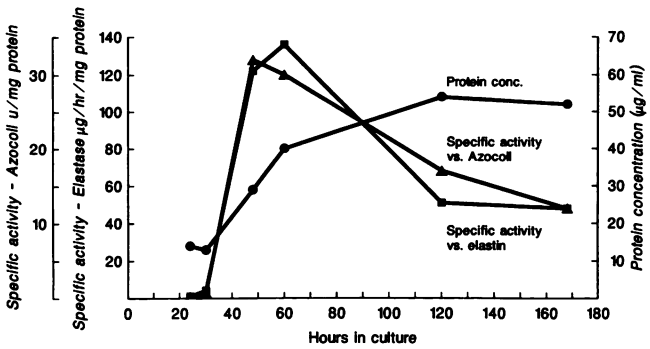

FIG. 1. Time dependence of secretion of elastinolytic and azocollytic activities and total protein in endospore filtrate at intervals following initiation of in vitro endospore-spherule culture.

Ion-exchange chromatography of selected active fractions from chromatofocusing was carried out on a Mono Q HR 5/5 column (Pharmacia) in 20 mM Tris hydrochloride (pH 8.5). Samples were applied at 0.25 ml/min and eluted with a linear gradient (0 to 500 mM NaCl; total volume, 30 ml) at 0.5 ml/min with an FPLC gradient programmer.

Reversed-phase chromatography of selected active fractions from chromatofocusing was carried out on an Altex C3 column (Beckman) in 0.1% trifluoroacetic acid. A linear gradient of acetonitrile in 0.1% trifluoroacetic acid was used to elute fractions (0 to 50% acetonitrile; 1% per min) at 0.5 ml/min.

For all chromatographic runs, the A_{280} was monitored with a Pharmacia UV-1 monitor. The elastase and azocollytic activities of fractions were measured as described above. In addition, samples of crude culture filtrate were labeled with [^{3}H]DFP to mark the serine proteinase, and tritium counts were monitored in chromatography fractions.

Labeling of serine proteinase with [^{3}H]DFP. DFP binds covalently to the active site of serine proteinases. [^{3}H]DFP (5 μl; 5 mCi/ml; 4 Ci/mmol) was added to 1 ml of crude concentrated filtrate (pH 7.8). After incubation at 22°C for 3 h, the sample was exhaustively dialyzed to remove unreacted [^{3}H]DFP. The labeled filtrate was then used to detect the serine proteinase in SDS-PAGE followed by autoradiography and in column chromatography fractions.

RESULTS

Degradation of R22 extracellular matrix. Initial experiments were performed to determine whether filtrate from *C. immitis* endospore cultures contained proteolytic activity capable of degrading a model of the elastin-rich extracellular matrix of lung interstitium. One milliliter of 168-h endospore filtrate degraded 68% of the matrix after 48 h of incubation at 37°C. Analysis of specific matrix component degradation revealed that 98% of the elastin, 92% of the glycoprotein, and 30% of the collagen had been degraded.

The assays were repeated with filtrate plus specific inhibitors of metalloproteinases (PT) or serine proteinases (PMSF). PMSF inhibited 47% and PT inhibited 30% of the extracellular matrix degradation by filtrate.

Time course of proteinase expression in *C. immitis* endospore-spherule cultures. Filtrate was collected from endospore-spherule cultures at selected intervals of 24 to 168 h. The specific activities of elastin and Azocoll degradation measured at each time interval are shown in Fig. 1. Both activities peaked at 48 to 60 h and then declined over

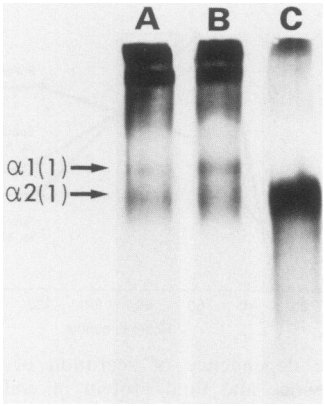

FIG. 2. Type I collagen degradation by *C. immitis* endospore filtrate. [^{3}H]collagen was incubated for 12 h at 30°C with 10 μl (lane C) of filtrate plus buffer. Samples were electrophoresed, and autoradiography was performed. Lanes A and B represent control incubations with 10 μl of buffer and 100 μg of TPCK-trypsin per ml, respectively. The $\alpha_1(1)$ and $\alpha_2(1)$ subunit chains seen in intact type I collagen are indicated in the control lanes.

subsequent days in culture. Total secreted protein, in contrast, continued to increase until 120 h and then plateaued. Interestingly, the peak of proteinase activity corresponded to the maturation and rupture of spherules and release of endospores in this in vitro system. Maturation is a roughly synchronous event that occurs only once under these culture conditions. The results indicated the optimal time (60 h) for subsequent collections of material for studying the proteinases.

Degradation of purified elastin and Azocoll. Elastin degradation was 222 μg/h per mg of protein in 60-h endospore filtrate in 24-h assays at 37°C. This amount corresponded to nearly half of the available elastin in the assay as determined by digestion with pancreatic elastase. Azocoll degradation by filtrate was 13.5 mg/h per mg of protein in endospore filtrate in 24-h assays at 37°C. For comparison, the elastase activity of filtrate from 10^9 endospores was equal to the elastase activity of secretions of 5×10^{10} murine macrophages or secretions of 2,000 schistosome cercariae in the same assay (2, 19).

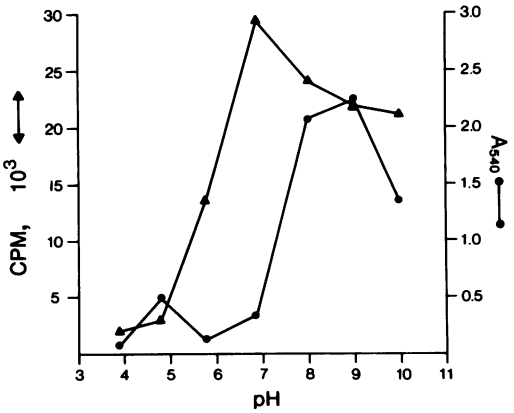

FIG. 3. pH optimum of proteinases in *C. immitis* filtrate. Activity was measured as counts per minute in the elastase assay (▲) and A_{540} in the Azocoll assay (●), as described in Materials and Methods.

Degradation of type I collagen. Crude endospore filtrate produced detectable type I collagen degradation under conditions in which the native triple-helical structure was intact (Fig. 2), but the degradation products were 50,000 or greater molecular weight. The 3/4-1/4 cleavage pattern seen with a vertebrate metallocollagenase was not observed.

Characterization of proteolytic activities. Since elastases characteristically degrade a number of other substrates besides elastin, our next studies were aimed at determining whether the azocollytic and elastase activities of crude *C. immitis* filtrate were due to the same or different enzymes. The pH optimum and calcium dependence of each of the two activities was studied. Calcium ions had strikingly different effects on the two activities. The elastase activity was inhibited by calcium, whereas the azocollytic activity was stimulated at 2 mM $CaCl_2$. The pH optima were 7 for the elastase activity and 9 for the azocollytic activity (Fig. 3).

In addition, each activity was assayed in the presence of a battery of proteinase inhibitors (Fig. 4 and 5). The elastase activity in crude filtrate was inhibited by 10 mM PMSF (81% inhibition) and 100 μg of α_1-PI per ml (51% inhibition), suggesting that elastase activity could be attributed to a serine proteinase. Thiol inhibitors (*N*-ethylmaleimide and leupeptin) had no effect on elastase activity. EDTA (10 mM) had an inhibitory effect (44% inhibition), but the specific metalloproteinase inhibitor PT had no significant effect on elastase activity. The azocollytic activity, on the other hand, was inhibited 65% by 2 mM PT and 93% by 1 mM EDTA, suggesting that at least some of the activity against this nonspecific proteinase substrate was due to a metalloproteinase.

Peptide inhibitor studies (Fig. 4) showed that the *C. immitis* elastase activity was inhibited more by the tetrapeptide chloromethylketone derivative succinyl-Ala-Ala-Pro-Phe-chloromethylketone than by acetyl-Ala-Ala-Pro-Ala-chloromethylketone. These findings led us to test the filtrate against the tetrapeptide substrates succinyl–Ala–Ala–Pro–Phe–4-nitroanilide and methoxysuccinyl-sucrose-Ala-Ala-Pro-Phe-thiobenzyl ester. Both were cleaved, thus

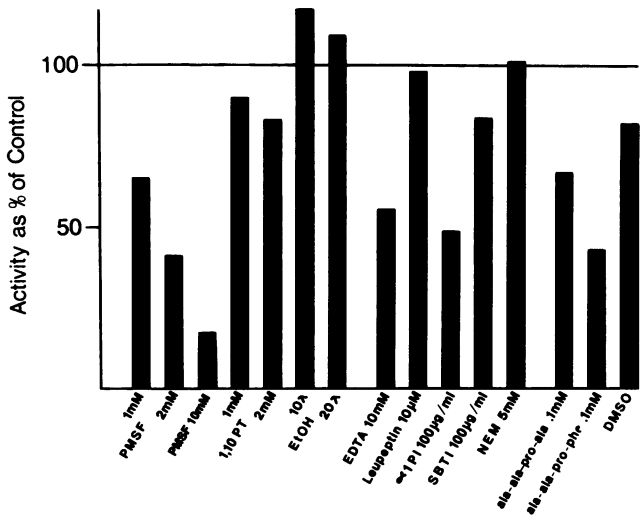

FIG. 4. Inhibition of elastase activity in *C. immitis* filtrate by various proteinase inhibitors. The greatest inhibition was by PMSF. Abbreviations: EtOH, ethanol; SBTI, soybean trypsin inhibitor; NEM, *N*-ethylmaleimide; DMSO, dimethyl sulfoxide.

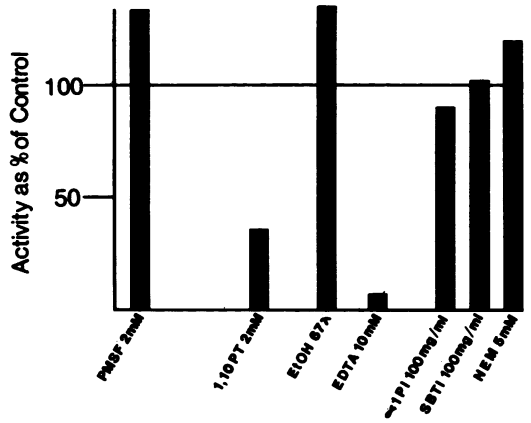

FIG. 5. Inhibition of azocollytic activity in *C. immitis* filtrate by various proteinase inhibitors. The greatest levels of inhibition were produced by EDTA and PT. For definitions of abbreviations, see the legend to Fig. 4.

providing preliminary information about the active-site specificity of the serine elastase activity.

Substrate gel electrophoresis. To further substantiate the presence of two proteinases, *C. immitis* endospore filtrate was electrophoresed in the presence of SDS on polyacrylamide gels that had been copolymerized with gelatin. Bands indicative of proteolytic activity were evident at approximately 200 and 85 kilodaltons (kDa) (Fig. 6). These molecular masses represent native protein complexes, since boiling and reducing agents were not used before electrophoresis. Substrate gel electrophoresis was also performed in the presence of PMSF or PT. PMSF partially inhibited the proteolytic band at 200 kDa, whereas PT inhibited the proteolytic band at 85 kDa (Fig. 6).

[^{3}H]DFP labeling of endospore filtrate. To confirm that the elastase was a serine proteinase, crude endospore filtrate was concentrated 60-fold by ammonium sulfate precipitation and labeled with [^{3}H]DFP. SDS-PAGE in the presence of reducing agents and boiling showed a tight doublet, following autoradiography, at a molecular mass of approximately 24 to 26 kDa (Fig. 7).

Resolution of proteolytic activity by chromatofocusing. To separate the two proteolytic activities, we applied concentrated filtrate to an FPLC chromatofocusing column. Elastase activity eluted at pIs 6.5 to 7.0 and 5.5 to 6.0 (Fig. 8A). Azocollytic activity eluted as a peak without significant elastase activity at an approximate pI of 7.5 and overlapped with elastase activity at pIs 6.5 to 7.0 and 5.5 to 6.0 (Fig. 8B). The elastase activity peak eluting at a pI of 5.5 to 6.0 was inhibited 100% by 10 mM PMSF. By contrast, the first azocollytic peak (pI 7.5) was inhibited 100% by 10 mM PT. [^{3}H]DFP-labeled filtrate was also separated by chromatofocusing (Fig. 9) under conditions identical to those used for unlabeled filtrate (Fig. 8). Tritium was detected in the same peaks at pIs 6.5 to 7.0 and 5.5 to 6.0 that had elastase activity but not in the Azocoll peak at pI 7.5.

Further purification of the elastase. The foregoing results showed that crude filtrate could be separated by chromatofocusing into three major peaks of proteinase activity: an azocollytic peak at pI 7.5, completely inhibited by PT and associated with a minor protein shoulder (Fig. 8 and 9, peak I); a peak at pI 6.5 to 7.0 with both azocollytic and elastinolytic activities, associated with a major protein peak that was labeled with [^{3}H]DFP (Fig. 8 and 9, peak II); and a peak at pI 5.5 to 6.0 with elastase activity, completely inhibited by PMSF, and associated with a smaller protein peak that also incorporated [^{3}H]DFP (Fig. 8 and 9, peak III).

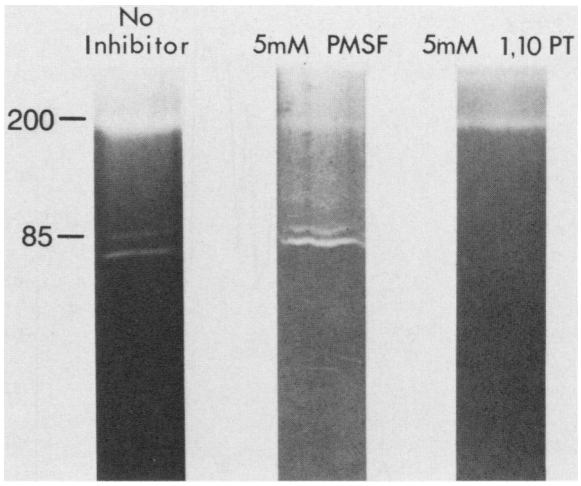

FIG. 6. Substrate gel electrophoresis of crude *C. immitis* endospore filtrate with and without proteinase inhibitors. Proteinases degrading gelatin in the SDS-polyacrylamide gel are visualized as clear bands in the dark background. The numbers on the left indicate molecular size in kilodaltons.

Given that the autoradiograph of [^{3}H]DFP-labeled filtrate (Fig. 7) showed only one tight doublet band, we hypothesized that filtrate contained a single serine proteinase. But elastase activity and [^{3}H]DFP eluted in two chromatofocusing peaks (II and III). This could have been due to differences in charge or association with other proteins. Peak III had the highest elastase specific activity and was labeled by [^{3}H]DFP, and the elastase activity was entirely inhibited by 10 mM PMSF. Peak II, in contrast, although containing most of the [^{3}H]DFP-labeled protein, was contaminated by metalloproteinase activity, as suggested by the high Azocoll activity (Fig. 8B) which was inhibited by PT. Complex of the elastase with another protein(s) would explain why the small DFP molecule could still react with the active site even though the enzyme in peak II had lower specific activity toward the large, insoluble elastin substrate. Subsequent

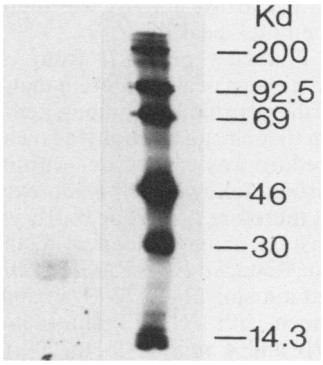

FIG. 7. Autoradiography of [^{3}H]DFP-labeled filtrate that was electrophoresed through a 10% SDS-polyacrylamide gel. A doublet is evident at 24 to 26 kDa. ^{14}C-labeled methylated protein standards are shown in the accompanying lane. Kd, Kilodaltons.

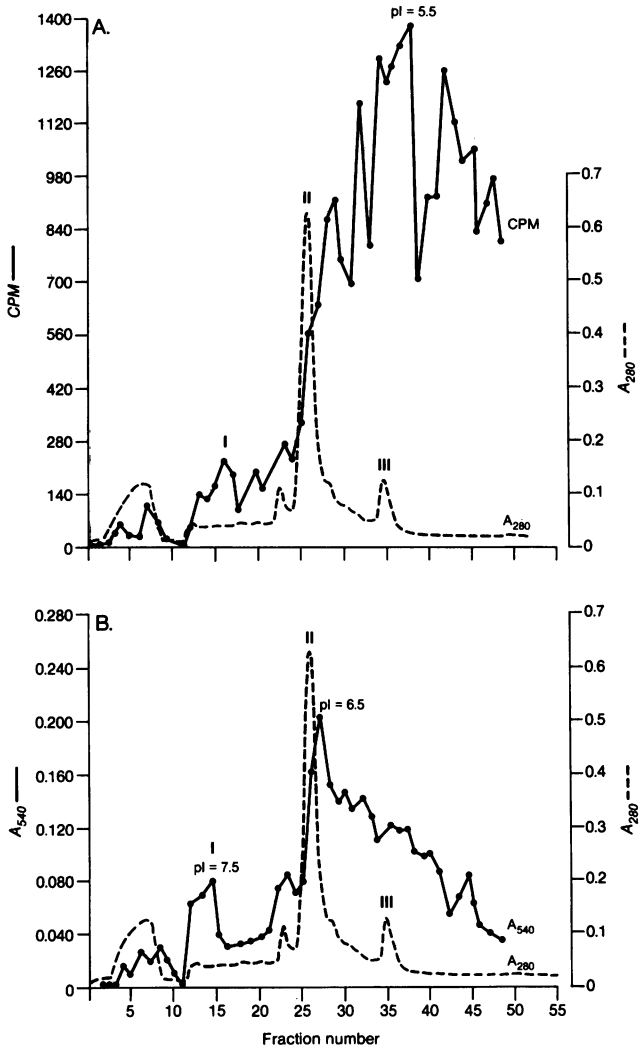

FIG. 8. FPLC chromatofocusing of *C. immitis* filtrate. Filtrate was applied to a 0.5-by-20-cm Mono P column in 20 mM Tris-acetate buffer (pH 8.3) and eluted with a pH 8.3 to 5 gradient by using 70% Polybuffer 74 and 30% Polybuffer 96. One-milliliter fractions were collected. The elastase (A) and azocollytic (B) activities of fractions are indicated. The isoelectric points of major activity peaks are shown.

purification of the elastase activity was therefore undertaken with the less complex peak III.

The [^{3}H]DFP-labeled peak III from chromatofocusing (Fig. 9) yielded a sharp peak of protein that coeluted with ^{3}H label when further purified by anion-exchange FPLC (Fig. 10). To confirm that elastase activity also eluted in this peak, the same procedure was carried out with unlabeled filtrate. Chromatofocusing, followed by anion-exchange FPLC of unlabeled (and therefore active) peak III, yielded a symmetric peak of elastase activity identical to the [^{3}H]DFP peak seen in Fig. 10. Reduced SDS-PAGE of this peak (Fig. 11, lane C) showed a major 21- to 22-kDa band (resembling the tight doublet seen with [^{3}H]DFP autoradiography of crude filtrate [Fig. 7]) and a minor 48- to 50-kDa band. Amino terminus sequencing was performed on this sample (10) and yielded a single strong signal for an amino terminus. To further confirm our assumptions about the number of proteinase species, another purification scheme was carried out by first applying concentrated filtrate to anion-exchange FPLC, followed by chromatofocusing of a pool of all elastinolytic fractions. The protein and activity profile of this chromatofocusing run was virtually identical to that of chromatofocusing of crude filtrate, yielding the major peak of the elastase activity associated with protein eluting at pI 5.5 to 6.0 (similar to peak III described in the initial scheme). This peak was 90% inhibited by 2 mM PMSF but unaffected by 2 mM PT. Nonreduced substrate gel electrophoresis showed a single proteolytic species with a native molecular mass of 200 kDa, just like the serine elastase activity of crude filtrate (Fig. 11, lane D). Reduced SDS-PAGE of this material again revealed a prominent band (tight doublet) at 21 to 22 kDa and a minor band at 48 to 50 kDa (identical to Fig. 11, lane C). Reversed-phase chromatography was used to separate the ampholyte-containing buffer from the protein fraction. Amino terminus sequencing of the protein fraction (10) yielded a single strong signal for the same sequence obtained from the first purification scheme (manuscript in preparation).

Further studies on the other proteinase species in endospore filtrate. The azocollytic metalloproteinase was not present in sufficient quantity to be further purified after one column step, but a 33-kDa band was relatively concentrated in SDS-PAGE (Fig. 11, lane A) from the pI 7.5 peak (peak I), and there was none of the 21- to 22-kDa species seen in elastase peak fractions. As noted above, the inhibition profile of this azocollytic peak suggested that its proteolytic activity was of the metalloproteinase class.

The pI 6.5 to 7.0 peak (peak II) contained a mixture of the metalloproteinase and serine elastase activities. Anion-exchange FPLC of this heterogeneous peak from chromatofocusing yielded a single major symmetric protein peak (data not shown). SDS-PAGE of this fraction showed minor bands at 21 to 22 and 33 kDa, and a major band at 48 to 50 kDa (Fig. 11, lane B). In summary, the results of two purification schemes indicated that peaks I, II, and III from

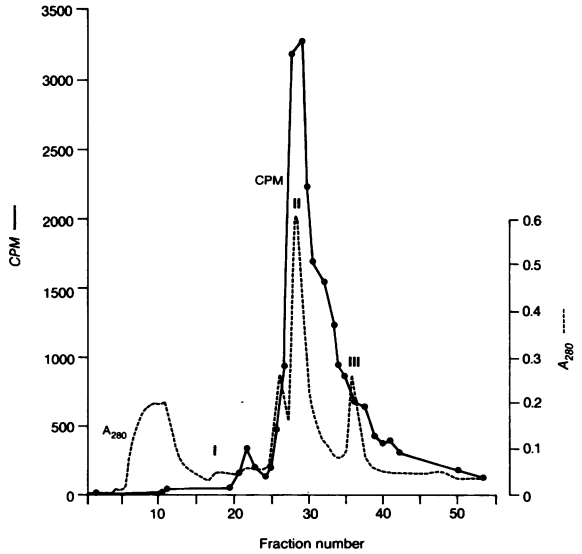

FIG. 9. FPLC chromatofocusing of [^{3}H]DFP-labeled *C. immitis* filtrate. Filtrate was applied to a 0.5-by-20-cm Mono P column in 20 mM Tris-acetate buffer (pH 8.3) and eluted with a pH 8.3 to 5 gradient by using 70% Polybuffer 74 and 30% Polybuffer 96. One-milliliter fractions were collected. Tritium counts of fractions are indicated.

chromatofocusing were relatively enriched for the 33-, 48- to 50-, and 21- to 22-kDa species, respectively (Fig. 11, lanes A, B, and C).

DISCUSSION

We have demonstrated degradation of connective tissue macromolecules in an in vitro model of extracellular matrix by filtrate from *C. immitis* endospore cultures. This finding supports our hypothesis that *C. immitis* produces proteinases that could lead to tissue destruction and dissemination of infection. The advantage of the in vitro matrix model is that the various connective tissue macromolecules are present in an interactive framework like that found in vivo. It is important to show that organisms can degrade this type of substrate, since a macromolecule that proteolytic enzymes can degrade when it has been purified and isolated may be less accessible to enzymatic degradation when it is present in a matrix in vivo. The principal activity of the filtrate was against elastin and glycoprotein in the matrix. The glycoprotein composition is complex, but a major component is fibronectin.

The elastinolytic activity in *C. immitis* endospore filtrate had an inhibition profile of a serine proteinase. The inhibition observed with EDTA is consistent with the known dependence of most serine proteinases on at least some divalent cation in the reaction buffer for maximal activity. The lack of inhibition by the specific metalloproteinase inhibitor PT and inhibition by calcium concentrations of 1 mM or more support the assumption that the elastase activity in crude filtrate is limited to proteolytic activity of the serine class. This was further substantiated by showing that [^{3}H]DFP-labeled protein and elastase activity copurified following chromatofocusing and ion-exchange chromatography.

Further analysis of the elastase with tetrapeptide chloromethylketone inhibitors indicated an active-site preference for phenylalanine at the P1 site (the amino acid on the N-terminal side of cleavage). This active-site specificity was also observed in the ability of endospore culture filtrate to cleave 4-nitroanilide and thioester tetrapeptide substrates with phenylalanine at P1. This pattern of P1 site specificity

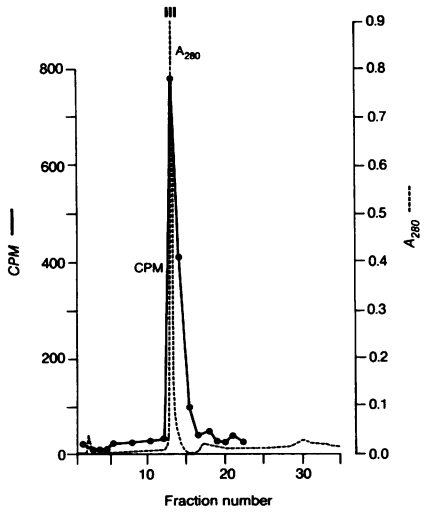

FIG. 10. Anion-exchange FPLC of peak III from chromatofocusing of [^{3}H]DFP-labeled *C. immitis* filtrate. Filtrate was applied to a 0.5-by-5-cm Mono Q column in 20 mM Tris hydrochloride (pH 8.5) and eluted with a 0 to 500 mM salt gradient. One-milliliter fractions were collected.

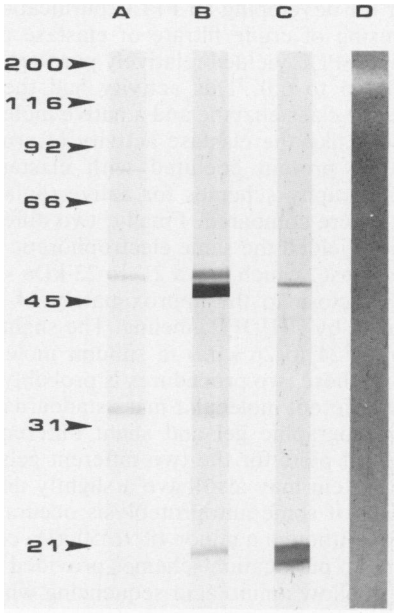

FIG. 11. Lane A, 10% SDS-PAGE of the pI 7.5 peak (I) from chromatofocusing of *C. immitis* filtrate. The 33-kDa band may correspond to the azocollytic metalloproteinase detected in crude filtrate. Lane B, 10% SDS-PAGE of the pI 6.5 to 7.0 peak (II) from chromatofocusing of *C. immitis* filtrate, further purified by anion-exchange FPLC, showing bands at 48 to 50, 33, and 21 to 22 kDa. This fraction contained both metallo- and serine proteinase activities. Lane C, 10% SDS-PAGE of the pI 5.5 to 6.0 peak (III) from chromatofocusing of *C. immitis* filtrate, further purified by anion-exchange FPLC. This purified fraction had serine elastase activity, and the predominant 21-22-kDa band corresponds to the band seen on autoradiography of [^{3}H]DFP-labeled filtrate. Lane D, Substrate gel electrophoresis of the pI 5.5 to 6.0 peak (III) from chromatofocusing of *C. immitis* filtrate. Proteinase activity is visualized as a clear band at 200 kDa in the dark background.

for phenylalanine has been noted in a subclass of serine elastases, including human and rat pancreatic elastase II and *Schistosoma mansoni* cercarial elastase (M. J. Banda, Z. Werb, and J. H. McKerrow, Methods Enzymol., in press).

Elastases are known to have broad substrate specificity (2, 19). The elastase activity in *C. immitis* filtrate, like other elastases, also had some activity against Azocoll. This explains the azocollytic activity in peak III, as well as the azocollytic activity in peak II, that was not inhibited by PT (Fig. 8). FPLC peaks with 1 μg of elastase activity per h could degrade 18 μg of Azocoll per h. Consequently, both serine elastase activity and the azocollytic metalloproteinase should have been detectable in a gelatin substrate gel (gelatin and Azocoll are both denatured type I collagen). Crude filtrate produced bands of clearing at approximately 200 and 85 kDa on a gelatin substrate gel. PMSF partially inhibited the 200-kDa species, identifying the serine elastase, while PT inhibited the 85-kDa species, identifying the metalloproteinase.

The serine elastase activity in *C. immitis* filtrate was further studied by labeling the crude starting material with [^{3}H]DFP, providing a specific radioactive probe irreversibly bound to the active site of the serine proteinase in the filtrate. SDS-PAGE under reducing conditions, followed by autoradiography, revealed a single species of approximately 24 to 26 kDa, confirming the presence of a single serine proteinase in the filtrate. The labeled filtrate also provided a

stable marker for developing an FPLC purification scheme. Chromatofocusing of crude filtrate or elastase peaks from anion-exchange FPLC yielded relatively pure serine elastase activity at pI 5.5 to 6.0. This activity had the inhibition profile of a serine class enzyme and a native molecular mass of 200 kDa, just like the elastase activity of crude filtrate. [^{3}H]DFP-labeled protein coeluted with elastase activity when chromatography schemes for active (unlabeled) and labeled filtrate were compared. Finally, two different purification schemes yielded the same electrophoretic pattern for the purified elastase, which had a 21- to 22-kDa subunit that was reasonably close to the approximately 24- to 26-kDa protein identified by [^{3}H]DFP labeling. The slight difference (21 to 22 versus 24 to 26 kDa) in subunit molecular mass calculated from these two procedures is probably not significant because different molecular mass standards were used for the autoradiographic gel and slight differences in the slope of standard plots for the two different gels occurred. DFP-labeled protein may also have a slightly different mobility, especially if some autoproteolysis occurs with unlabeled samples. Although a minor 48- to 50-kDa contaminant was present, both purification schemes provided sufficiently pure protein to allow amino acid sequencing which yielded the same amino terminus sequence (manuscript in preparation).

The data, therefore, indicate that the *C. immitis* serine elastase has an approximately 23-kDa subunit that complexes to a 200-kDa multimer under nonreducing or native conditions.

The metalloproteinase had a native molecular weight of 85 kDa. It was resolved from serine elastase activity as the pI 7.5 peak on chromatofocusing. Reduced SDS-PAGE of this peak showed a relatively abundant 33-kDa species that may represent the metalloproteinase subunit.

Since endospore filtrate degraded 30% of the collagen in the R22 matrix, we tested whether the metalloproteinase might be a metallocollagenase. While we did observe some degradation of type I collagen by endospore filtrate on SDS-PAGE, the pattern of degradation was not the specific 3/4-1/4 cleavage produced by true vertebrate collagenases nor the total degradation to small peptides seen with clostridial collagenase.

A 48- to 50-kDa protein was the major species present in the pI 6.5 to 7.0 chromatofocusing eluent of filtrate. This peak also contained both metalloproteinase and serine elastase activities. Reduced SDS-PAGE of this material, in fact, showed both the 33- and 21- to 22-kDa species discussed above, as well as the predominant 48- to 50-kDa band. The 48- to 50-kDa protein may represent a carrier molecule for the fungal proteinases, given its tendency to copurify with them.

We have, therefore, identified in *C. immitis* endospore filtrate two types of proteinase that are active at neutral pH. Inhibitors of these proteinases can inhibit 77% of in vitro extracellular matrix degradation by endospore filtrate. The lack of 100% inhibition by PMSF and PT in these matrix assays may be explained by hydrolysis of the PMSF during the assay or nonspecific binding of PMSF to other proteins, reducing the effective PMSF concentration. This latter explanation is supported by the observation that the purified (i.e., free of other proteins) elastase was completely inhibited by PMSF. Dissemination of endospores in vivo requires invasion of elastin-rich tissue of a lung and blood vessels. The potent tissue-destroying capacity of *C. immitis* has long been recognized (5), and endospores released from rupturing spherules may produce localized tissue destruction even before a significant inflammatory response is present. These observations can be explained by the action of the proteinases we have identified in vitro. In vivo, as with other proteinase-mediated diseases like emphysema and *Pseudomonas* bronchopneumonia, an interplay between *C. immitis* proteinases and host proteinase inhibitors like α_1-PI might take place. Whereas the serine elastase could be inhibited by α_1-PI, metalloproteinases are capable of inactivating α_1-PI (1). Thus, the two fungal enzymes may act synergistically in a lung in a way similar to what has been proposed for the pathogenesis of emphysema (1). Whereas our observations of proteolytic activity by *C. immitis* support, but do not prove, the hypothesis of proteinase-mediated dissemination, preliminary characterization of the active-site specificity of the elastase opens the way to future studies of the effects of specifically designed proteinase inhibitors on experimental *C. immitis* infections in laboratory animals. Furthermore, the proteolytic activity of other strains of *C. immitis*, in addition to the Silveira strain used in these studies, could be analyzed. Variable virulence among strains is documented (21), and a correlation of proteolytic activity with virulence would also support our hypothesis.

Our present studies have focused on local host tissue destruction by *C. immitis* endospore proteinases. Proteinases may have other roles in the host-parasite relationship in coccidioidomycosis. Spherule rupture and subsequent endospore release may be proteinase mediated, and in fact we have shown that proteinase activity peaks at 48 to 60 h, corresponding to the time of spherule rupture in the roughly synchronous in vitro endospore-spherule cultures. There is also evidence for proteinase production in the mycelial phase of *C. immitis* (17, 22), which gives rise to arthroconidia. A 36-kDa serine proteinase has been isolated from conidial walls (L. Yuan and G. T. Cole, Abstr. Ann. Meet. Am. Soc. Microbiol. 1987, F-58, p. 398). The relationship, if any, of the proteinase to those we identified in the parasitic life cycle is uncertain.

The products of elastin degradation have been shown to be chemotactic for inflammatory cells (11), and elastin-derived peptides are particularly chemotatic for mononuclear cells (23). Thus, fungal elastase activity could also initiate the destructive inflammatory response seen in this mycosis. The *C. immitis* endospore proteinases, like similar enzymes in other microbial pathogens (7, 9, 19, 25), may therefore be virulence factors in the pathogenesis of coccidioidomycosis by several mechanisms.

ACKNOWLEDGMENTS

This study was supported by National Science Foundation grant PCM 8304160 and a Dermatology Foundation fellowship award to S.R. sponsored by Herbert Laboratories.

LITERATURE CITED

1. **Banda, M., E. Clark, and Z. Werb.** 1980. Limited proteolysis by macrophage elastase inactivates human α_1-proteinase inhibitor. J. Exp. Med. **152**:1562–1570.
2. **Banda, M., and Z. Werb.** 1981. Mouse macrophage elastase. Biochem. J. **193**:589–605.
3. **Castillo, M. J., K. Nukajima, M. Zimmerman, and J. C. Powers.** 1979. Sensitive substrates for human leukocyte and porcine pancreatic elastases: a study of the merits of various chromophoric and fluorogenic leaving groups of assay for serine proteases. Anal. Biochem. **99**:53–64.
4. **Drutz, D. J., and A. Catanzaro.** 1978. Coccidioidomycosis: state of the art. Am. Rev. Respir. Dis. **117**:559–585.
5. **Forbus, W. D., and A. M. Besterbreurtje.** 1946. Coccidioidomycosis: a study of 95 cases of the disseminated type with special reference to the pathogenesis of the disease. Mil. Surg. **99**:

653–719.
6. **Gisslow, M. T., and B. C. McBride.** 1975. A rapid sensitive collagenase assay. Anal. Biochem. **68:**70–78.
7. **Heck, L. W., K. Morihara, W. B. McCrae, and E. J. Miller.** 1986. Specific cleavage of human type III and IV collagens by *Pseudomonas aeruginosa* elastase. Infect. Immun. **51:**115–118.
8. **Heussen, C., and E. B. Dowdle.** 1980. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Anal. Biochem. **102:**196–202.
9. **Hotez, P., N. Trang, J. H. McKerrow, and A. Cerami.** 1985. Isolation and characterization of a proteolytic enzyme from the adult hookworm *Ancylostoma caninum*. J. Biol. Chem. **260:** 7343–7348.
10. **Hunkapiller, M. W., R. M. Hewick, W. J. Dreyer, and L. Hood.** 1983. High-sensitivity sequencing with a gas-phase sequenator. Methods Enzymol. **91:**399–412.
11. **Hunninghake, G. W., J. M. Davidson, S. Rennard, S. Szapiel, J. E. Gadek, and R. G. Crystal.** 1981. Elastin fragments attract macrophage precursors to diseased sites in pulmonary emphysema. Science **212:**925–927.
12. **Jones, P. A., T. Scott-Burden, and W. Gevers.** 1979. Glycoprotein, elastin, and collagen secretion by rat smooth muscle cells. Proc. Natl. Acad. Sci. USA **76:**353–357.
13. **Keene, W. E., K. Jeong, J. H. McKerrow, and Z. Werb.** 1983. Degradation of connective tissue matrix by larvae of *Schistosoma mansoni*. II. Degradation by newly transformed and developing schistosomula *in vitro*. Lab. Invest. **49:**201–207.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
15. **Levine, H. B., J. M. Cobb, and C. E. Smith.** 1960. Immunity to coccidioidomycosis induced in mice by purified spherule, arthrospore, and mycelial vaccines. Trans. N.Y. Acad. Sci. **20:**436–499.
16. **Lindquist, R. L., A. W. Senft, M. Petitt, and J. H. McKerrow.** 1986. *Schistosoma mansoni*: purification and characterization of the major acidic proteinase of adult worms. Exp. Parasitol. **61:**398–404.
17. **Lupan, D. M., and P. Nziramasanga.** 1985. Collagenolytic activity of *Coccidioides immitis*. Infect. Immun. **51:**360–361.
18. **McKerrow, J. H., W. E. Keene, K. Jeong, and Z. Werb.** 1983. Degradation of extracellular matrix by larvae of *Schistosoma mansoni*. I. Degradation by cercariae as a model for initial parasite invasion of host. Lab. Invest. **49:**195–200.
19. **McKerrow, J. H., S. Pino-Heiss, R. L. Lindquist, and Z. Werb.** 1985. Purification and characterization of an elastinolytic proteinase secreted by cercariae of *Schistosoma mansoni*. J. Biol. Chem. **260:**3703–3707.
20. **Powers, J. C., and P. M. Tuhy.** 1973. Active-site specific inhibitors of elastase. Biochemistry **12:**4767–4772.
21. **Rippon, J. W.** 1982. Medical mycology, p. 154–248. The W.B. Saunders Co., Philadelphia.
22. **Rippon, J. W., and D. P. Varadi.** 1968. The elastases of pathogenic fungi and actinomycetes. J. Invest. Dermatol. **50:** 54–58.
23. **Senior, R. M., G. L. Griffin, and R. P. Mecham.** 1980. Chemotactic activity of elastin-derived peptides. J. Clin. Invest. **66:** 859–862.
24. **Werb, Z., M. J. Banda, and P. A. Jones.** 1980. Degradation of connective tissue matrices by macrophages. I. Proteolysis of elastin, glycoproteins, and collagen by proteinases isolated from macrophages. J. Exp. Med. **152:**1340–1357.
25. **Werb, Z. M., J. Banda, J. H. McKerrow, and R. Sandhaus.** 1982. Elastases and elastin degradation. J. Invest. Dermatol. **79:**154–159.