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Aspergillus fumigatus Complement Inhibitor: Production, Characterization, and Purification by Hydrophobic Interaction and Thin-Layer Chromatography

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***Aspergillus fumigatus* has previously been shown to produce a soluble extracellular inhibitor of the alternative complement pathway, called *Aspergillus* complement inhibitor, or CI. We now report an efficient method for production of CI which relies on the fact that poorly conidiating cultures yielded CI activity with approximately sevenfold-higher potency than CI produced by conidiating cultures. CI from poorly conidiating cultures provided 50% inhibition of alternative pathway-mediated binding of ^{125}I-labeled complement component C3 to cryptococcal blastoconidia at a mean concentration of 60 μg/ml. The ability of crude CI to inhibit the alternative complement pathway seemed to be independent of intact protein or polysaccharide structure, as evidenced by resistance of inhibitory activity to digestion by proteases, including subtilisin, α-chymotrypsin, papain, and pepsin as well as endoglycosidases F and H. Separation of the active inhibitory component of CI from contaminating materials contained in crude CI preparations was achieved by using Phenylsuperose hydrophobic interaction chromatography in a fast protein liquid chromatography system. The active material proved to be extremely hydrophobic, desorbing from the column only during elution with ethanol; it contained only 15% protein and 5% polysaccharide. Furthermore, results from preparative thin-layer chromatography indicated that lipids which comigrated with phosphatidylserine/phosphatidylinositol and phosphatidylethanolamine possessed significant complement-inhibitory activity. Taken together, these data suggested that phospholipids from *A. fumigatus* contributed to the functional activity of CI.**

Aspergillus fumigatus causes life-threatening invasive infections in immunocompromised hosts (6, 33). The fact that leukopenia and chronic granulomatous disease represent risks for these infections points to the importance of polymorphonuclear neutrophils and monocytes in host defense in vivo against invasive aspergillosis. There is substantial evidence that these phagocytes can kill or damage *Aspergillus* cells in vitro (7, 8, 23, 31, 32). Complement may play a significant role in host defense against this infection by generating C5a (29), the chemoattractant for peripheral blood phagocytes, and by opsonizing inhaled conidia for ingestion and killing by these leukocytes (23, 31, 32). We have been studying the ability of *A. fumigatus* culture supernatants to interfere with the complement cascade, thinking that anticomplementary activity could enhance virulence by interfering with chemotaxis and opsonophagocytosis. We called this inhibitory material *Aspergillus* complement inhibitor, or CI (32). At least two nonpathogenic fungi produce complement inhibitors (17, 18), and pathogenic species of *Candida* have previously been shown to possess surface receptors for complement component C3 fragments iC3b and C3d (3, 11, 12, 16). However, to our knowledge, CI was the first complement inhibitor described for a pathogenic fungus.

We have previously shown that the activity of CI was due to soluble nondialyzable extracellular material on fungal surfaces which inhibited activation of the alternative complement pathway (32). The major mechanism by which CI inhibited activation of complement was neither depletion of functional titers of complement component C3 nor chelation of calcium or magnesium, the divalent cations which are required for activation of the complement cascade. CI inhibited binding of C3 to blastoconidia of *Cryptococcus neoformans* but did not mask C3 binding sites on cryptococcal surfaces, suggesting that CI inhibited activation of complement. The inhibitor was approximately fivefold less active against the classical complement pathway in a hemolysin (CH50) system than against assembly of the alternative pathway C3 convertase on blastoconidia *C. neoformans*. Taken together, these data suggested that CI was probably a selective inhibitor of activation of the alternative complement pathway.

We now describe an efficient method for production of CI which takes advantage of the fact that poorly conidiating cultures of *A. fumigatus* yield inhibitor with higher potency than that produced by conidiating cultures. Biochemical characterization of CI and separation of the active component of CI from contaminating materials by Phenylsuperose hydrophobic interaction chromatography in a fast protein liquid chromatography (FPLC) system are described. Finally, we present direct evidence from a preparative thin-layer chromatography (TLC) system which demonstrates that *Aspergillus* phospholipids possess significant complement-inhibitory activity.

MATERIALS AND METHODS

Production of *Aspergillus* CI. A clinical isolate of *A. fumigatus* (National Institutes of Health strain B4132) was used to produce CI for these studies. The organism was grown on Czapek-Dox agar for 10 days at 30°C. This agar is a chemically defined medium which contains sodium nitrate

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as the only nitrogen source and glucose as the sole source of carbon. The synthesis of inducible proteases such as trypsin and chymotrypsin by *A. fumigatus* is represented under these growth conditions. Czapek-Dox agar possesses the additional advantage that it contains no high-molecular-weight constituents, so any contaminants from the medium are dialyzable. Following 10 days of growth, the confluent lawns of *A. fumigatus* were washed with sterile deionized water containing the protease inhibitor *p*-nitrophenyl-*p'*-guanidinobenzoate (NPGB; Sigma Chemical Co., St. Louis, Mo.) at 25 μM to inhibit possible trace contamination by protease. In preliminary experiments, NPGB was shown not to interfere with CI activity or with the complement binding assay (described below). The resulting crude supernatant was first filter-sterilized (0.45-μm-pore-size filters; Nalge Company, Rochester, N.Y.) and then dialyzed extensively against sterile distilled water at 4°C with dialysis tubing with a 3.5-kDa exclusion limit. Sodium azide (0.1%) was included in the first three of six changes of the dialysis bath in order to minimize the possibility that bacteria or their products might contaminate CI preparations. The dialyzed material was then lyophilized, and the resulting fluffy yellowish powder was stored at 4°C until it was used in experiments.

Radiolabeling of complement component C3. Complement component C3 was purified from human plasma by the method of Hammer et al. (14). Purified C3 was labeled with ^{125}I (Amersham, Arlington Heights, Ill.) to a specific activity of 200,000 cpm/μg of protein by using polystyrene beads coated with *N*-chloro-benzenesulfonamide (Iodobeads; Pierce Chemical Co., Rockford, Ill.). Radiolabeled C3 was separated from free ^{125}I with Sephadex G25 columns (PD10; Pharmacia, Piscataway, N.J.).

Complement component C3 binding assay. Binding of complement component C3 to the yeastlike fungus *C. neoformans* is known to be mediated principally by the alternative complement pathway (9, 19, 21). We have previously shown that CI inhibits alternative pathway-mediated binding of ^{125}I-labeled C3 to cryptococcal blastoconidia in a dose-dependent fashion (32). This system was therefore used in the current studies to monitor the complement-inhibitory activity of CI which had been either biochemically treated or chromatographically purified by the methods outlined below.

The C3 binding assay was performed as follows. A total of 10^8 blastoconidia of a small-capsule isolate of *C. neoformans*, serotype A, were mixed in 10% nonimmune human serum in Hanks balanced salt solution (HBSS) to which 200,000 cpm of ^{125}I-C3 had been added, with or without the simultaneous addition of inhibitor in a total volume of 2.5 ml. Duplicate tubes were assayed for each experimental condition outlined below. Incubation mixtures were rotated at 8 rpm and 37°C for 30 min, and cryptococci were washed three times with cold phosphate-buffered saline. After centrifugation at 1,000 × *g* and 4°C for 10 min, radioactivity in the final pellet was counted in a gamma counter (Packard Instruments Company, Inc., Downers Grove, Ill.). Following subtraction of background, 100% inhibition was calculated by subtracting the radioactivity which became nonspecifically bound in the presence of heat-inactivated serum (typically <400 cpm) from the amount bound when fresh serum was used (typically 6,000 to 12,000 cpm). The percent inhibition caused by CI preparations was determined by the formula $[(cpm_{FS} - cpm_{FS+CI})/(cpm_{FS} - cpm_{HIS})] \times 100$, where HIS is heat-inactivated serum and FS is fresh serum.

Heat treatment of CI. Susceptibility of the inhibitor to heat was assessed by heating CI which had been suspended in HBSS to 56 or 100°C for 30 min, followed by testing for activity in the cryptococcal ^{125}I-C3 binding inhibition assay.

Protein and polysaccharide assays of CI. The protein content of CI preparations was determined with the Pierce BCA protein assay with bovine serum albumin standards (Pierce Chemical Company) according to the manufacturer's instructions. Hexose concentrations were measured by the phenol-sulfuric acid method with glucose standards (10).

Preincubation of CI with solid-phase proteases. In order to assess the contribution of intact protein structure towards the functional activity of CI, the material was digested with several different proteases. In these experiments, CI (2.5 mg/ml) was preincubated for 60 min at 37°C with one of four different proteases conjugated to agarose beads (Sigma) in volumes of 1.2 ml. Control inhibitor was incubated with the same quantity of beads lacking proteases. Enzymes included subtilisin (0.2 U), α-chymotrypsin (5 U), papain (10 U), and pepsin (290 U). Following removal of solid-phase proteases by centrifugation at 1,000 × *g* and 4°C for 10 min, CI in the supernatants was diluted 10-fold and assayed for functional activity in the cryptococcal ^{125}I-C3 binding inhibition assay. Activity of the proteases was confirmed by digestion of bovine serum albumin under conditions identical to those outlined above; visualization of digestion fragments was achieved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining (data not shown).

Pretreatment of CI with endoglycosidases. CI (5 mg in 1 ml of PBS) was treated with endoglycosidase F (Endo F, 2.8 U) or H (1.5 μg) (endo-β-*N*-acetylglucosaminidase H [28]) for 60 min at 37°C (New England Nuclear, Boston, Mass.). As a control, a third tube of CI was incubated in parallel without endoglycosidase. After the incubation was complete, the mixtures were diluted 10-fold and assayed for CI functional activity. Tenfold dilutions of endoglycosidases without CI were shown in preliminary experiments not to significantly affect binding of ^{125}I-C3 to cryptococci (data not shown). The enzymes were functionally active, as judged by digestion of the glycoprotein ovalbumin, followed by SDS-PAGE.

Autoradiographic assay for proteolysis of ^{125}I-C3 by CI. We have previously shown that CI possesses only minimal proteolytic activity against casein and that it depletes hemolytic titers of C3 in human serum by no more than 15% (32). In the current study, we wished to exclude more rigorously the possibility that the major effect of CI could be due to proteolysis. We therefore examined directly for digestion of C3 by CI. In this experiment, ^{125}I-C3 (18,000 cpm) was incubated at 37°C in HBSS containing 10% fresh normal human serum with or without CI (100 μg/ml), a concentration which produced 50% inhibition in the cryptococcal C3 binding assay. SDS–8% PAGE under dissociating conditions was then performed on these samples to examine for digestion products of the 126-kDa α-chain and 75-kDa β-chain of C3 (15) by standard autoradiographic techniques.

Preincubation of CI in protease inhibitors. We reasoned that if CI's major mechanism of action is proteolysis, then the activity of CI in the cryptococcal ^{125}I-C3 binding system should be susceptible to protease inhibitors. In order to test this hypothesis, we incubated samples of CI (1 mg/ml) for 30 min at 37°C either alone or with one of nine different protease inhibitors (Sigma). These preparations were then diluted 10-fold and assessed for their ability to inhibit binding of ^{125}I-C3 to cryptococcal surfaces. The protease inhibitors included 10 mM EDTA (metalloprotease inhibitor; chelates calcium and magnesium), 0.1 mM *o*-phenanthroline (metalloprotease inhibitor; chelates zinc and cobalt), 1 μM leupep-

tin (broad-spectrum protease inhibitor), 1 mM phenylmethylsulfonyl fluoride (serine esterase inhibitor), 25 μM NPGB (serine esterase inhibitor), 1 μM pepstatin (acid protease inhibitor), aprotinin (10 μg/ml; trypsin inhibitor), 0.01 mM diazoacetyl norleucine methyl ester (inhibitor of proteases employing a carbonyl residue for catalytic action), and 20 mM iodoacetamide (inhibitor of proteases relying on free sulfhydryl groups for proteolysis). In preliminary experiments, protease inhibitors at 10% of these concentrations were found not to interfere with binding of ^{125}I-C3 to cryptococci in our assay system.

Hydrophobic interaction chromatography. Others have demonstrated that hydrophobic interaction chromatography is useful for purification of *A. fumigatus* polysaccharides and proteins (26). We therefore used this method to separate the active component of CI from extraneous materials in our crude CI preparations. During hydrophobic interaction chromatography, hydrophilic materials desorb from the column matrix during elution with high-salt buffer, whereas relatively hydrophobic molecules desorb during elution with low-salt buffer. Extremely hydrophobic compounds, including many lipids, remain adherent to the column even during elution with low-salt buffers but can subsequently be eluted by organic solvents such as methanol or ethanol. In a preliminary study done with conventional Phenylsepharose CL-4B hydrophobic interaction chromatography (Pharmacia), the majority of CI functional activity was found to be extremely hydrophobic. CI was therefore separated from contaminating materials in our crude lyophilized preparations with a Phenylsuperose column (HR 10/10) in an FPLC system. The column was first equilibrated with the starting buffer, 4 M sodium chloride buffered to pH 7.6 with 0.05 M Trizma base (Sigma). Lyophilized CI (60 to 80 mg) which had been suspended in 2 ml of the starting buffer was loaded onto the column at a flow rate of 0.8 ml/min while 1.6-ml fractions were collected. Optical density was monitored at 280 nm (OD_{280}). The column was subsequently eluted with a discontinuous salt gradient, pH 7.6 (4 column volumes for each salt concentration), beginning with buffered 4 M NaCl, followed by 2, 1, 0.5, and 0.15 M NaCl, and finally with deionized water. Following elution with these aqueous salt solutions, a band of dark yellow material remained adherent to the top of the hydrophobic column matrix. The column was therefore next washed with 25% ethanol followed by 50% ethanol, resulting in elution of the yellow hydrophobic material. Finally, the column was regenerated according to the manufacturer's directions by washing sequentially with 5 column volumes each of 40% acetic acid, 100% acetonitrile with 0.1% trifluoroacetic acid, and 0.1 M sodium hydroxide, with intervening rinses of deionized water. The column was stored in 25% ethanol between experiments to prevent bacterial growth.

Column effluents which had desorbed from the column during elution with each of the different solvents were pooled by OD_{280}. Materials which desorbed from the column during elution with decreasing concentrations of sodium chloride were subsequently concentrated by using collodion membranes with a 10-kDa exclusion limit (Schleicher & Schuell, Dossel, Federal Republic of Germany), and finally desalted with Sephadex G25 columns (Pharmacia). In contrast, the yellow materials which desorbed from the column during elution with ethanol were evaporated to dryness in conical polypropylene centrifuge tubes (Sarstedt, Nümbrecht, Federal Republic of Germany) under a vacuum and suspended in 1 ml of phosphate-buffered saline. Each of the pools was then assayed in duplicate for complement-inhibitory activity (0.25 ml per assay tube) as well as protein and polysaccharide content. As a control, reagent ethanol was treated in parallel in an identical fashion; it possessed no activity in our complement inhibition assay.

SDS-PAGE. SDS-PAGE was performed on CI starting material and on pooled column fractions with 10% polyacrylamide gels and reducing conditions by the method of Laemmli (22). Bands were visualized by silver staining.

TLC. Lipids were extracted from CI (10 to 20 mg of starting material for analytical TLC; 40 mg for preparative TLC) by a modification of the method of Bligh and Dyer (1). The lipid-containing chloroform phase was dried under a stream of nitrogen (N_2), suspended in a small volume of chloroform-methanol (2:1, vol/vol), and spotted onto Silica Gel-60 plates (E. Merck, Darmstadt, Federal Republic of Germany). Individual CI lipids were separated side by side with lipid standards (Serdary Research Laboratories, London, Ontario, Canada) in a solvent system of chloroform-methanol-glacial acetic acid-water (75:48:12.5:3.5, by volume). Lipid standards included ergosterol, phosphatidic acid, phosphatidylcholine, lysophosphatidylcholine, phosphatidylethanolamine, lysophosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and sphingomyelin. CI lipid fractions from 10 to 20 mg of starting material were visualized by Coomassie brilliant blue R-250 staining (Bio-Rad Laboratories, Richmond, Calif.) and identified by comparison with the migration of known lipid standards. The resulting R_f values were used to determine the positions of CI lipids on the unstained preparative side of the silica plate. CI lipid-containing regions were scraped and eluted from the preparative side of the plate with chloroform-methanol-water (10:25:5, by volume), evaporated under N_2, and suspended in HBSS (0.7 ml) for measurement of complement-inhibitory activity.

RESULTS

Efficiency of CI production by *A. fumigatus*: effect of poor conidiation. Through serial passage in vitro, the *Aspergillus* isolate began to conidiate poorly and concomitantly to produce CI more efficiently than the conidiating parent strain from which it was derived. The poorly conidiating isolate, which produced 500-fold fewer conidia per 32-oz (ca. 930 ml) agar culture flask than the original isolate, synthesized CI of approximately sevenfold higher potency in the cryptococcal ^{125}I-C3 binding inhibition assay (n = 10 lots of CI). Fifty percent inhibition could be achieved at a mean concentration of 60 μg/ml with CI derived from poorly conidiating cultures versus 410 μg/ml with CI from conidiating cultures (Fig. 1). We therefore performed the remainder of our studies with CI prepared from poorly conidiating cultures.

Heat treatment of CI. After being heated to 56°C for 30 min, the inhibitor retained 92% of its original activity. In contrast, CI proved to be partially susceptible to heating to 100°C, losing 72% of its ability to inhibit binding of ^{125}I-C3 to cryptococci. This result was compatible with the possibility that intact protein, polysaccharide, or lipid structures could be required for optimal functional activity of the inhibitor.

Protein and polysaccharide measurements. Following suspension in phosphate-buffered saline, lyophilized CI contained 33 to 43% protein and 36 to 46% polysaccharide (n = 5 different lots of crude CI). We consistently found that approximately 20% of the dry weight of the material could not be accounted for by the protein and polysaccharide assays, raising the possibility that additional classes of compounds such as lipids could be present within crude preparations of CI.

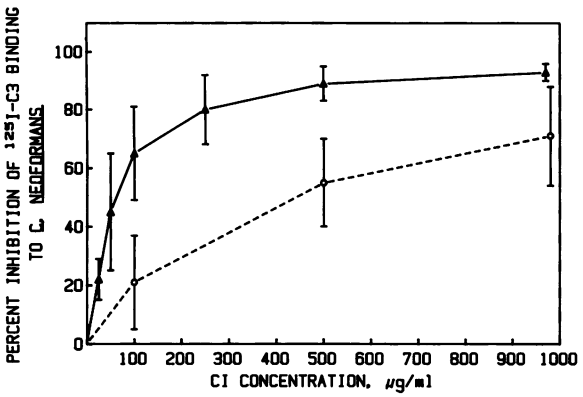

FIG. 1. Comparison of dose-response curves for *Aspergillus* (CI) which was obtained either from poorly conidiating fungal cultures (▲, n = 10) or conidiating cultures (○, n = 10). Assay was performed as described in Materials and Methods. Data represent mean ± SD.

Treatment of CI with proteases. The functional activity of CI proved to be resistant to protease digestion. At a concentration of 250 μg/ml, CI which had been incubated with agarose control beads for 1 h at 37°C yielded 50% inhibition in the ^{125}I-C3 binding assay. Treatment with subtilisin-, α-chymotrypsin-, papain-, or pepsin-conjugated agarose beads left this inhibitory activity intact; complement inhibition by protease-treated CI ranged between 54.4 and 60.4%.

Treatment of CI with endoglycosidases. CI activity was not susceptible to digestion by endoglycosidases. In this experiment, untreated CI yielded 67.4% inhibition at a concentration of 500 μg/ml, and CI pretreated with Endo F or Endo H gave 68.5 and 73.7% inhibition, respectively. These negative results, combined with those from protease digestions of CI outlined above, suggested that intact polysaccharide or protein structure was not likely to be required in order for CI to exert its effect.

Lack of ^{125}I-C3 digestion by CI. The ^{125}I-C3 autoradiogram, performed as described in Materials and Methods, demonstrated neither any change in the electrophoretic mobility of the C3 α- or β-chain following incubation with CI nor any lower-molecular-weight digestion fragments (data not shown).

Lack of inhibition of CI's effect by incubation in protease inhibitors. Although CI did not digest complement component C3, the possibility remained that the inhibitor might digest other proteins required for alternative pathway activation, such as factor B, factor D, or properdin. To assess this possibility, CI was tested for activity following treatment with a spectrum of different protease inhibitors; none of the nine protease inhibitors listed in Materials and Methods significantly altered CI's subsequent function in the ^{125}I-C3 binding inhibition assay. The inhibitory activity of protease inhibitor-treated CI varied by no more than 22% from that of control CI. Taken together, the negative data from this experiment and from the autoradiographic examination for digestion of ^{125}I-C3 rendered highly unlikely the possibility that CI's major effect could be due to proteolysis of alternative pathway components.

Hydrophobic interaction chromatography. Results from a representative Phenylsuperose column run are shown in Fig. 2. The chromatogram (Fig. 2A) shows that the majority of material which absorbed UV light at a 280-nm wavelength desorbed from the column during elution with either 4 or 2 M sodium chloride; thus, the bulk of crude lyophilized CI consisted of hydrophilic material. Peaks of hydrophobic material were subsequently obtained during elution of the column with ethanol. Similar chromatograms were obtained from a total of seven different lots of crude lyophilized CI. The majority of complement-inhibitory activity proved to be hydrophobic, desorbing from the column during elution with ethanol at concentrations of either 25 or 50%. Recovery of CI activity from the column was consistently >80%.

The major peak of complement-inhibitory activity, pool 10, was obtained during elution with 50% ethanol and yielded 96% inhibition of ^{125}I-C3 binding in our cryptococcal alternative complement pathway system (Fig. 2B). Pool 10 was extremely potent, providing 76% inhibition and 33% inhibition at dilutions of 1:5 and 1:25, respectively. The dry weight of pool 10 was 1 mg, but it contained only 150 μg of protein and 54 μg of hexose (Fig. 2C and D). Thus, on a weight basis, the majority of the active hydrophobic material apparently fell into yet another category of compound. This notion was supported by the fact that SDS-PAGE of pool 10 revealed only very faint bands after staining with silver stain (see below).

Schønheyder and Andersen demonstrated that catalase activity from *A. fumigatus* is hydrophobic (26), so we examined for an effect of that enzyme in our complement system. Reagent catalase (6,000 U; Sigma) possessed no activity (data not shown); the enzyme therefore seemed unlikely to account for the functional activity of CI.

SDS-PAGE. Silver staining revealed that CI starting material contained numerous bands having molecular weights which ranged between 15,000 and 75,000 (Fig. 3). The majority of these polysaccharides and glycoproteins proved to be hydrophilic, desorbing from the column during elution with either 4 or 2 M sodium chloride. This result was consistent with the observation by Schønheyder and Andersen that the majority of *A. fumigatus* somatic antigens are hydrophilic (26). In contrast, the most hydrophobic materials in CI, which were contained in pools 8 through 11, possessed very little stainable material. Pool 9 had a definite band with a molecular weight of approximately 15,000, but the relevance of the band to inhibitory activity was doubtful since pools 8, 10, and 11, which were highly active in the complement inhibition assay, lacked the band.

TLC. The above results seemed compatible with the possibility that *Aspergillus* lipids could contribute to the activity of CI. We chose to examine that possibility by using a modification of the lipid extraction procedure of Bligh and Dyer (1). In preliminary experiments, significant complement-inhibitory activity was observed to partition into the lipid-containing chloroform phase during the extraction procedure (results not shown). We therefore turned our attention toward identification of individual CI lipids by TLC. TLC and Coomassie brilliant blue staining revealed the presence of several individual CI lipids (Fig. 4A). The following lipid-containing regions were identified: CI material which remained at the origin of the TLC plate was designated region 1; region 2 contained CI lipids which comigrated with phosphatidylcholine, sphingomyelin, lysophosphatidylethanolamine, and lysophosphatidylcholine; one well-resolved CI lipid in region 3 comigrated with phosphatidylethanolamine and another with phosphatidylserine/phosphatidylinositol; and finally, CI region 4, the area nearest the solvent front, contained lipids having R_f values corresponding to those of ergosterol and phosphatidic acid.

The R_f values of individual lipids from the stained sides of TLC plates were used to guide subsequent scraping and

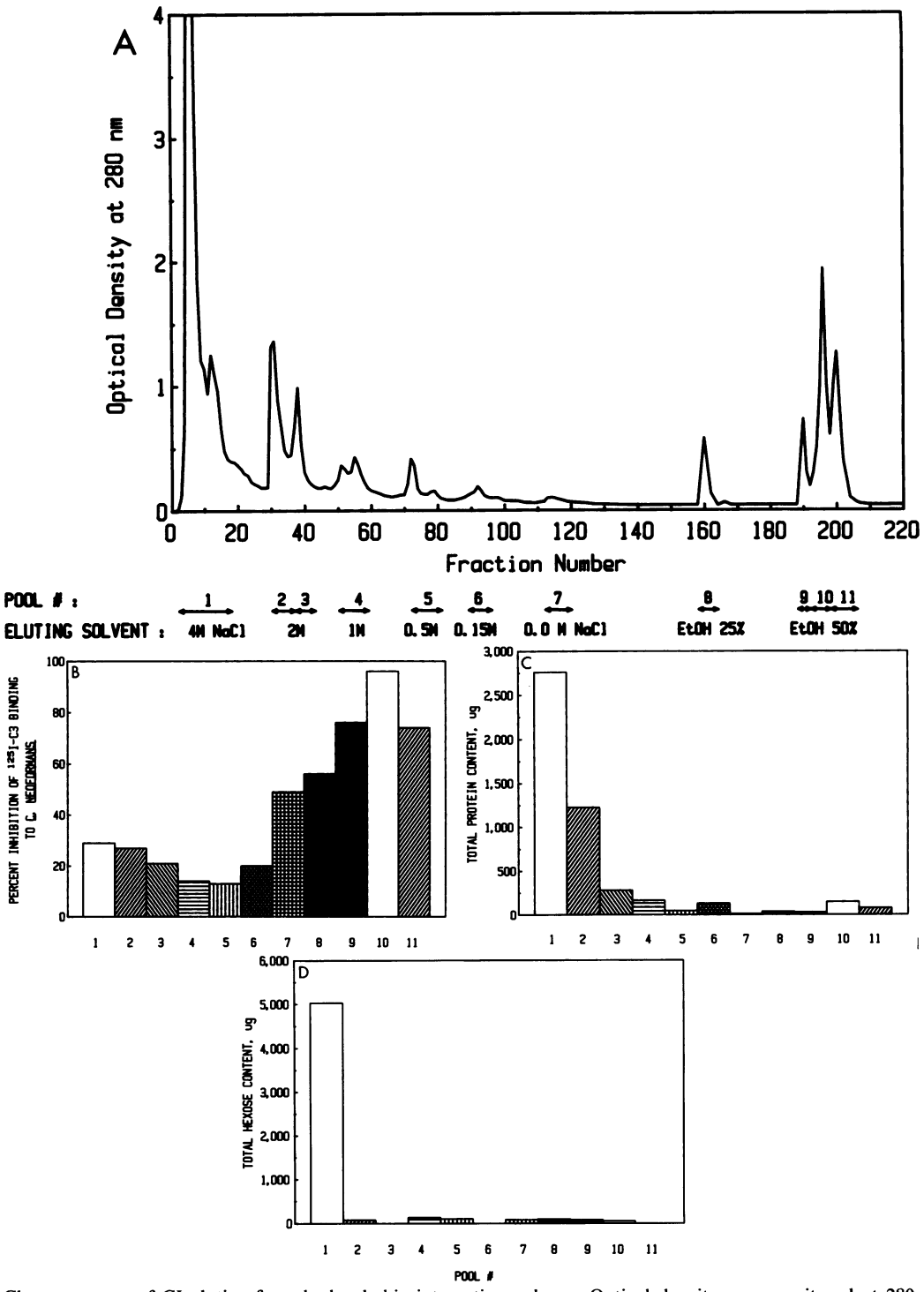

FIG. 2. (A) Chromatogram of CI eluting from hydrophobic interaction column. Optical density was monitored at 280 nm. The most hydrophilic material desorbed from the column during elution with 4 M NaCl buffered to pH 7.6 with 0.05 M Trizma base. Hydrophobic material was later eluted by washing the column with ethanol (EtOH). (B) Complement-inhibitory activity of pooled column effluents. Data represent duplicate assays of 0.25-ml samples of the indicated pools in final incubation volumes of 2.5 ml. Pools 8 through 11 contained hydrophobic materials which desorbed from the column during elution with ethanol. (C and D) Total protein (C) and polysaccharide (D) contents of pools 1 through 11 measured by the Pierce BCA protein assay (Pierce Chemical Company) with bovine serum albumin standards and by the phenol-sulfuric acid assay with glucose standards, respectively.

eluting of lipid-containing regions 1 through 4 for measurement of complement-inhibitory activity ($n = 4$ experiments). Results are shown in Fig. 4B. Region 3 (phosphatidylserine/phosphatidylinositol and phosphatidylethanolamine) possessed the greatest amount of activity, yielding 64% ± 9% (mean ± standard error of the mean) inhibition in our C3 binding assay. Region 2 made a relatively minor and more variable contribution, giving 33% ± 17% inhibition. Regions

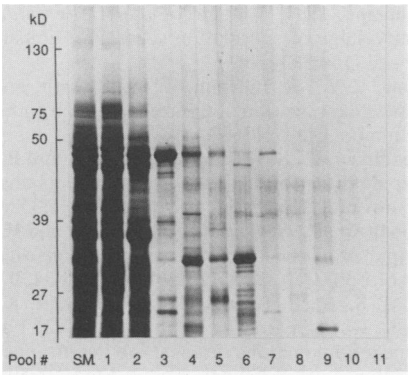

FIG. 3. SDS-PAGE (10% polyacrylamide) profiles of CI starting material (S.M., 80 μg of protein) and Phenylsuperose pools 1 through 11, visualized with silver staining. kD, Kilodalton.

1 and 4 were virtually devoid of activity. None of the regions contained detectable protein, and hexose concentrations were consistently <60 μg/ml, as judged by the phenol-sulfuric acid method. Taken together, these data suggested that phospholipids contributed to the inhibitory activity of CI.

DISCUSSION

The observation of a complement inhibitor produced by a fungus which causes human disease is novel. However, others have shown that a saprophytic soil fungus, *Stachybotrys complementi* sp. nov. K-76, produces a complement inhibitor called K-76 (17, 24). This inhibitor appeared in broth culture supernatants in a time-dependent fashion and, like CI, was hydrophobic. The mechanism of action of K-76 appeared to differ from CI's in that K-76 showed quantitatively greater inhibitory activity against the classical pathway than against the alternative pathway. Thus, CI and K-76 do not seem to be identical.

There are several potential mechanisms by which complement might help to protect the human host from invasive aspergillosis. The most likely of these would be to provide a chemotactic stimulus for neutrophils and monocytes, the effector cells which, by electron microscopic criteria, damage *Aspergillus* hyphae (7, 8). In addition, complement might play another important role in host defense against this fungus by opsonizing inhaled conidia for phagocytosis and killing by these leukocytes (23, 31, 32). It is not known whether complement helps to protect the host against aspergillosis, but this probably depends at least in part on the ability of *Aspergillus* cells to activate the complement cascade. Thus, an extracellular complement inhibitor produced by hyphae could potentially function to the advantage of the invading fungus by interfering both with production of the chemotaxin C5a and with opsonophagocytosis. It is known that resting conidia and hyphae of *A. fumigatus*, in contrast to swollen conidia, are inefficient activators of complement (29). In addition, Kozel and colleagues have recently shown that hyphae are inefficiently opsonized via the alternative complement pathway (20). Potentially, *Aspergillus* CI, a selective inhibitor of the alternative complement pathway which elutes readily from hyphal surfaces, could contribute to these inefficient interactions between *Aspergillus* hyphae and the alternative complement pathway.

For the current studies, *Aspergillus* culture conditions were specifically chosen in order to minimize the possibility

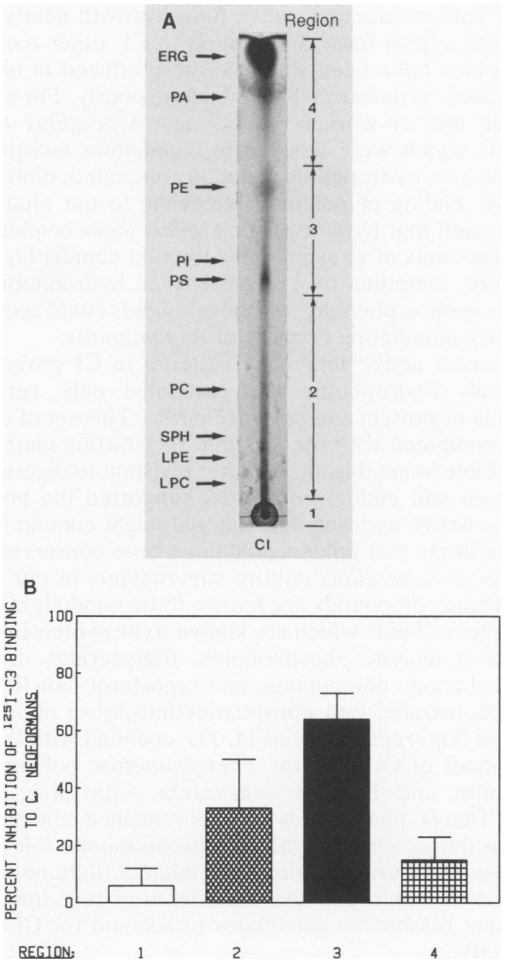

FIG. 4. (A) Thin-layer chromatogram, prepared as described in Materials and Methods. Abbreviations for lipid standards (positions shown on left): ERG, ergosterol; PA, phosphatidic acid; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; SPH, sphingomyelin. (B) Complement-inhibitory activity of individual CI lipid-containing regions from preparative TLC. Regions were eluted from the silica gel, evaporated under N_2, and suspended in HBSS (0.7 ml) for the complement inhibition assay. Error bars show standard errors of the means.

that proteases could contribute to the inhibitory activity of CI. Negative results from the autoradiographic assay for proteolysis of ^{125}I-C3, combined with the inability of nine different protease inhibitors to block CI's interference with ^{125}I-C3 binding to cryptococci, provided strong evidence against the possibility that proteolysis could represent the major mechanism of action of CI.

Early attempts to chromatographically purify the active component of the inhibitor were limited by the low inhibitory potency of CI which was prepared from conidiating cultures of *A. fumigatus*. This problem has now been circumvented by exploiting the observation that CI from poorly conidiating cultures reliably provides more potent inhibition than that obtained from conidiating cultures; 50% inhibition in the cryptococcal ^{125}I-C3 binding assay can be routinely achieved at CI concentrations of 50 to 100 μg/ml. Efficient production of CI by poorly conidiating cultures of *A. fumigatus* enhances the likelihood that CI could be produced in vivo

during human infection, when fungal growth nearly always takes the hyphal form. In addition to CI, other *Aspergillus* metabolites have been shown to be produced in high concentrations by isolates which conidiate poorly. For example, Butnick and co-workers found that *Aspergillus nidulans* mutants which were blocked in conidiation secreted large amounts of a hydrophobic phenolic compound, diorcinal (2). Another finding of potential relevance to our study is the observation that hyphae of *Aspergillus niger* contain higher concentrations of phospholipids than do conidia (4, 5). It is therefore tempting to speculate that hydrophobic compounds such as phenolics or phospholipids could accumulate in poorly conidiating cultures of *A. fumigatus*.

The most active inhibitory material in CI proved to be extremely hydrophobic and contained only very small amounts of protein and polysaccharide. This set of observations, combined with the fact that the starting material was susceptible to heating at 100°C but resistant to digestion with proteases and endoglycosidases, supported the possibility that the active hydrophobic material might contain lipids. It seemed likely that lipids would have been conserved during dialysis of *Aspergillus* culture supernatants in our studies, since these compounds are known to be nondialyzable (27). Examples of lipids which are known to be synthesized by *A. fumigatus* include phospholipids, triglycerides, free fatty acids, phenolic compounds, and ergosterol (30). Recently, we demonstrated that phosphatidylinositol, a phospholipid found in *Aspergillus* species (4, 13), coeluted with the active component of CI from the Phenylsuperose column (R. G. Washburn and B. J. Bryant-Varela, Clin. Res. **38**:15A, 1990). That is, phosphatidylinositol remained adherent to the column during elution with the discontinuous salt gradient but desorbed during elution with ethanol (data not shown). This result reinforced the consideration that lipids could represent reasonable candidates to account for CI's inhibitory activity.

Results from preparative TLC indicated that *Aspergillus* lipids which possessed significant inhibitory activity in our alternative complement pathway system comigrated with phosphatidylserine/phosphatidylinositol and phosphatidylethanolamine. This observation is relevant because individual phospholipids are known to be capable of either inhibiting or enhancing activation of the alternative complement pathway (25). Further studies will be required to determine the relative contributions made by the various *Aspergillus* lipids to the ability of CI to inhibit the alternative complement pathway.

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