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Microbicidal Cationic Proteins of Rabbit Alveolar Macrophages: Amino Acid Composition and Functional Attributes†

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We purified two microbicidal cationic proteins, MCP-1 and MCP-2, from rabbit alveolar macrophages. MCP-1 was remarkably rich in arginine (25.5 mol%) and half cystine (18.7 mol%) residues and constituted approximately 1.5% of the total protein content of Freund adjuvant-elicited alveolar macrophages. MCP-2 was approximately half as abundant as MCP-1 and contained relatively less arginine (14.9 mol%) and half cystine (9.8 mol%). The amino acid compositions of MCP-1 and MCP-2 resembled those reported for the lysosomal cationic proteins of rabbit granulocytes, but were distinct from those of any known histone. MCP-1 (1 μg/ml) killed 99.6% of *Candida albicans* in 20 min, whereas MCP-2 killed approximately 80% under similar conditions. Both proteins rapidly suppressed O_2 consumption by *C. albicans* and induced a rapid loss of intracellular $^{86}Rb^+$. Although more information is needed about the biological origin, distribution, and roles of macrophage microbicidal proteins, it seems likely that MCP-1 and MCP-2 contribute to the microbicidal efficacy of rabbit alveolar macrophages.

Phagocytic cells have evolved two general molecular strategies that enable them to kill ingested microorganisms. One general mechanism uses the oxidative metabolism of the phagocyte to produce oxygen metabolites, including H_2O_2, O_2^-, and OH·, that are directly or indirectly microbicidal (1). The other general mechanism is oxygen independent and reflects the existence of microbicidal lysosomal components that are translocated to phagosomes containing ingested organisms. Some microbicidal components, such as lysozyme (2, 7) or permeability factor (23), have a relatively narrow microbicidal spectrum, whereas others, such as the lysosomal cationic proteins of rabbit or guinea pig granulocytes (24, 25) or the chymotrypsin-like cationic proteins of human neutrophils (6, 18), have a broad spectrum of efficacy.

Well-aerated, uninfected lungs provide an oxygen-rich environment for alveolar macrophages. However, these cells may also encounter microorganisms in alveoli that are poorly ventilated, hypoperfused, or filled with transuded or exuded fluid. Although one might expect alveolar macrophages to possess microbicidal mechanisms that function effectively under the wide range of oxygen tensions likely to occur in health or disease, relatively little is known of their operation.

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We recently reported that rabbit alveolar macrophages contain two microbicidal proteins that are highly active against *Candida albicans*, *C. parapsilosis*, and several species of gram-positive bacteria (20). We have successfully purified these proteins and now report on their molecular characteristics, amino acid compositions, and additional aspects of their effects on *Candida* spp.

MATERIALS AND METHODS

Extraction of lysosome-rich fraction. Elicited rabbit alveolar macrophages were purified (≤1% granulocytes), and subcellular fractions were prepared as previously described (20). A 27,000 × *g* fraction, derived from approximately 10^9 purified macrophages, was extracted with 10 ml of 0.1 M citric acid by stirring the suspended organelles for 2 h at 4°C. The preparation was centrifuged at 27,000 × *g* for 20 min at 4°C, and the sediment was extracted as before. The combined supernatants contained approximately 2 mg of protein per 10^8 macrophages. The citric acid extracts were concentrated to approximately 1 ml by using an Amicon ultrafiltration unit with a UM 2 Diaflo membrane (Amicon Corp., Lexington, Mass.).

Precipitation of cationic proteins. The concentrates were dialyzed against two changes, 1 liter each, of 0.05 M HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Sigma Chemical Co., St. Louis, Mo.), pH 7.5, for 24 h at 4°C. This yielded a white, flocculent, biologically active precipitate, the "HEPES precipitate," which we recovered by centrifugation at 27,000 × *g* for 20 min at 4°C. Neither

increasing the concentration of HEPES buffer (0.01 to 0.1 M) nor increasing the dialysis time (up to 72 h) increased the quantity of the precipitate. We dissolved the HEPES precipitate in 0.1 M HCl and dialyzed it against two changes, 1 liter each, of 0.01 M HCl for 24 h at room temperature. Dialyzed HEPES precipitate withstood storage at −20°C for at least 2 months without losing microbicidal activity. Occasionally, a translucent precipitate, insoluble in 0.1 M HCl, appeared after freezing and thawing. This was removed by centrifugation without concomitant loss of microbicidal activity.

Gel filtration. Dialyzed HEPES precipitate (1 ml, 2 to 3 mg of protein) was applied to a plastic column (1.2 by 28 cm) containing Bio-Gel P-10, 50–100 mesh (Bio-Rad Laboratories, Richmond, Calif.) that had been equilibrated at room temperature in 0.01 M HCl. Loading and elution were accomplished at a constant linear flow rate, 6 cm/h, of 0.01 M HCl, and 1-ml fractions were collected. The effluent was monitored by measuring its optical density at 215 nm (OD_{215}) and OD_{225} in a Gilford Model 222A spectrophotometer and then calculating the difference ($OD_{215} - OD_{225}$). Column fractions were also examined for fungicidal activity (see below). The single candidacidally active peak (peak 3) was pooled, lyophilized (this removed the HCl), suspended in 1 ml of water, and stored at −20°C. Preparations destined for subsequent microbicidal testing were stabilized by adding 0.1 mg of crystalline bovine serum albumin per ml before freezing. Such stabilized preparations retained full microbicidal activity for several months despite repeated freeze-thawing. Fractions to be further purified were stored without bovine serum albumin.

Fungicidal activity of column eluates was monitored by minor modifications of a dye exclusion assay (11). Briefly, *C. parapsilosis* was grown for 48 h in tryptose phosphate broth (Difco Laboratories, Detroit, Mich.) at 32°C, harvested by centrifugation, and washed three times with sterile distilled water. The fungi were adjusted to 2.5×10^7 cells per ml in 0.1 M sodium citrate buffer, pH 6.3. A 50-μl sample of *C. parapsilosis* suspension was added to 100 μl of each column eluate fraction in a microtest plate (no. 3040; Falcon Plastics, Oxnard, Calif.) and incubated overnight at room temperature. Then 30 μl of an aqueous solution of trypan blue (0.2%) and eosin (0.1%), pH 6.4, was added to each well. After 1 h, the percentage of stained yeast cells was determined by examining wet mounts microscopically.

Polyacrylamide gel electrophoresis. Analytical-disc native tube gels were cast with 15% acrylamide and electrophoresed at pH 4.3, 2 mA/gel, by using previously described modifications (11) of the procedure of Reisfeld et al. (21). Long urea gels, as described by Panyim and Chalkley (19), were used for the final purification step. Briefly, 15% polyacrylamide gels (0.5 by 25 cm) containing 2.5 M urea were polymerized with ammonium persulfate and pre-electrophoresed at 2 mA/gel for 18 h with 2.5 M urea in 0.9 M acetic acid. These retained their resolving power for as long as 1 month when stored, with running buffer overlays, at room temperature. After pre-electrophoresis, a stacking gel (0.4 ml) containing 2.5 M urea was photopolymerized with riboflavin as described by Reisfeld et al. (21). Samples applied to the urea gels contained 100 μg (Lowry protein) of the fungicidal proteins eluted from the Bio-Gel P-10 column, 10% (vol/vol), glycerol, 2.5 M urea, and 0.001% methyl green as tracking dye. Electrophoresis, 2 mA/gel, was performed until the dye front was 1 cm from the bottom of the gel. Analytical gels were stained for 1 h in 0.2% amido black dissolved in water containing 45% methanol and 9% acetic acid, and destained, by diffusion, in the same solvent. Alternatively, they were stained by the rapid Coomassie blue G-250 method of Reisner et al. (22).

Localization of protein bands in preparative urea gels. After electrophoresis, the gels (usually four in number) were removed from their tubes, and one was quartered longitudinally. This indicator strip was stained with Coomassie blue G-250 until the protein bands were visible (a few minutes). Approximate R_f's for microbicidal cationic proteins 1 and 2 (MCP-1 and MCP-2) were 0.82 and 0.78, respectively.

Using the stained gel slice as a guidestrip, a 2-cm section from the unstained gels containing the two protein bands was removed and sliced transversely into 2-mm sections that were individually immersed in 250 μl of water. After 12 h, 50 μl of the eluted proteins plus 10 μl of 0.1 M $NaHCO_3$ was re-electrophoresed on small analytical gels and stained to localize the bands. Gel eluates containing a single protein species were appropriately pooled and frozen. Eluates containing mixtures of the two proteins were re-electrophoresed on long urea gels and reprocessed as above. The pooled MCP-1 and MCP-2 acrylamide segments were separately pulverized and thoroughly eluted by twice agitating the suspension at room temperature for 12 h with 5 ml of water. The gel pieces were removed by centrifugation, washed twice with water, and filtered through an Amicon XM-100 membrane. The filtrates were combined and dialyzed against two changes, 1 liter each, of 1 mM HCl for 24 h at room temperature. The dialyzed proteins were concentrated to approximately 1 ml in a Speed Vac concentrator-100 (Savant Instruments, Inc., Hicksville, N.Y.) and stored at −20°C.

Densitometry. MCP-1 and MCP-2 (eluted from 2.5 M urea gels) and hen egg white lysozyme were quantitated by gel densitometry. The protein concentration of the individual cationic protein samples was measured by the method of Lowry et al. (12), with egg white lysozyme, ($E^{1\%}_{281.5} = 26.4$) as a standard (3). The proteins were applied to analytical 15% acrylamide gels (pH 4.3), electrophoresed, stained, and destained as described for amido black. The gels were scanned in a Gilford gel scanner, model 2520, at 580 nm at a scan rate of 0.5 cm/h and a chart speed of 150 cm/h. Densitometric traces were measured with a Keuffel & Esser model 62 0000 compensating polar planimeter (Keuffel & Esser, Morristown, N.J.). Estimates of concentration were done on gels whose samples were in the linear portion of the densitometric standard curve, as described below.

Amino acid analysis. Amino acid analyses were performed on a Durrum D-500 automatic amino acid analyzer by the method of Moore and Stein (14) employing single-column methodology. Triplicate samples were hydrolyzed in 1.0 ml of 6 N HCl containing 20 μl of 5% (wt/vol) phenol for 24 h at 110 ± 2°C

in vacuo. Half-cystine content was determined as cysteic acid on separate duplicate samples after performic acid oxidation (13). Threonine and serine contents were corrected upward 5 and 10%, respectively, for destruction during acid hydrolysis. Tryptophan content was not determined.

Microorganisms and microbicidal assays. *Salmonella typhimurium* SL 4045 and *Listeria monocytogenes* were grown as previously described (20). *C. albicans* 820 was cultivated in Sabouraud dextrose broth (BBL Microbiology Systems, Cockeysville, Md.). Overnight cultures (5 ml) were grown in liquid medium with agitation at 37°C. A 1:50 dilution was made into 50 ml of prewarmed medium contained in a 125-ml Ehrlenmeyer flask, and this subculture was shaken at 37°C until the midphase of exponential growth was attained. The mid-log-phase organisms were recovered by centrifugation, washed three times in assay buffer (see below), and suspended to a final cell density of 5×10^6/ml in that buffer. The assay buffer (ionic strength, 0.02) contained 0.01 M sodium citrate, 5×10^{-4} M $MgSO_4$, 1.5×10^{-4} M $CaCl_2$, and 0.056 M glucose (pH 6.3). Reaction mixtures (0.5 ml) contained 5×10^5 colony-forming units in buffer at 37°C. Microbicidal proteins were added last. Timed samples were removed and serially diluted in 0.05 M phosphate buffer (pH 7.4) containing the aforementioned additives. The dilution buffer was found experimentally to block the lethal expression of unadsorbed macrophage cationic proteins, allowing precise timing of the kinetics of microbicidal activity. The ionic strength of all buffers was measured with a Yellow Springs Instruments model 31 conductivity bridge, using NaCl solutions as standards.

^{86}Rubidium release. *C. albicans*, prepared as described above, was labeled with ^{86}Rb by modifications of the method of Drazin and Lehrer (5). ^{86}Rb (2×10^6 cpm/ml, 8.5 mCi/mg; New England Nuclear Corp., Boston, Mass.) was added to 2×10^7 cells per ml in assay buffer and incubated with shaking for 1 h at 37°C. The labeled cells were centrifuged, washed three times, and suspended in assay buffer. Reaction mixtures contained 2.3×10^7 labeled yeast cells in a total volume of 1.3 ml, and 10 μg of microbicidal protein was recovered from the P-10 column. At intervals after addition of the P-10 proteins, samples were removed, and the yeast cells were centrifuged at 8,000 × *g* for 1 min in an Eppendorf model 3200 microfuge. Radioactivity of the supernatant fluid was analyzed in a Beckman gamma counter (Beckman Instruments, Inc., Fullerton, Calif.).

Oxygen consumption. Oxygen uptake of *C. albicans* (5×10^6 cells per ml) in assay buffer was measured at 37°C in a Gilson Oxygraph with a Clark-YSI electrode (GME, Middletown, Wis.). After an initial rate of oxygen consumption was recorded, selected concentrations of microbicidal cationic proteins were added, and monitoring was continued for 10 min.

RESULTS

Purification of MCP. Our starting material, a 27,000 × *g* fraction obtained from highly purified alveolar macrophages, was subjected to a four-stage purification procedure consisting of: citric acid extraction, precipitation by HEPES, column chromatography on Bio-Gel P-10, and preparative electrophoresis on long urea gels.

Initial citric acid extracts contained approximately 20 mg of total protein and 1.6 mg of MCP per 10^9 macrophages. The MCP content was measured by gel densitometry as described below. After concentration and dialysis, the resultant HEPES precipitate was substantially enriched in its content of MCP, although MCP recovery from the citric acid extract was incomplete (Fig. 1). Other buffers, including 0.05 M potassium phosphate (pH 7.4) and 0.2 M sodium borate (pH 9.0), also precipitated MCP, but were not more efficient than HEPES.

Although the initial citric acid extracts were not candidacidal, 4 to 8 μg of HEPES precipitate per ml reduced the number of *C. albicans* colony-forming units by approximately 90% in 10 min. When HEPES precipitate was subjected to chromatography on Bio-Gel P-10 columns, three principal peaks were recovered in the effluent (Fig. 2). The last to emerge, peak 3, had a very low OD_{280}/OD_{225} ratio, suggesting a paucity of aromatic amino acids. Peak 3 contained both MCP components and, alone among the recovered fractions, killed *C. albicans* when tested by the screening dye exclusion assay. Candidacidal activity was confirmed by colony counting. As shown in Fig. 3, 1 μg of three different peak

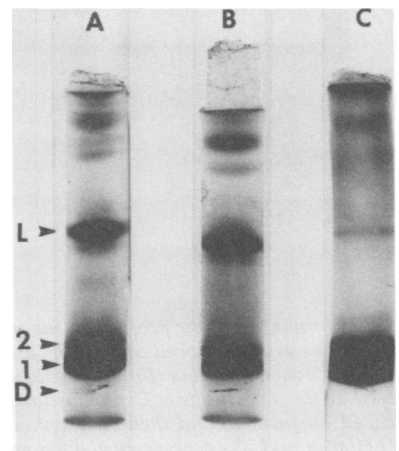

FIG. 1. *Polyacrylamide gels of the citric acid extracts and HEPES precipitate. A, Citric acid extract of the 27,000 × g fraction of alveolar macrophages; B, supernatant fluid after removal of HEPES precipitate; and C, the resuspended HEPES precipitate. Approximately 200 μg of protein was applied to each gel. L, Lysozyme; 1, MCP-1; 2, MCP-2; D, cathodal dye front marked with india ink.*

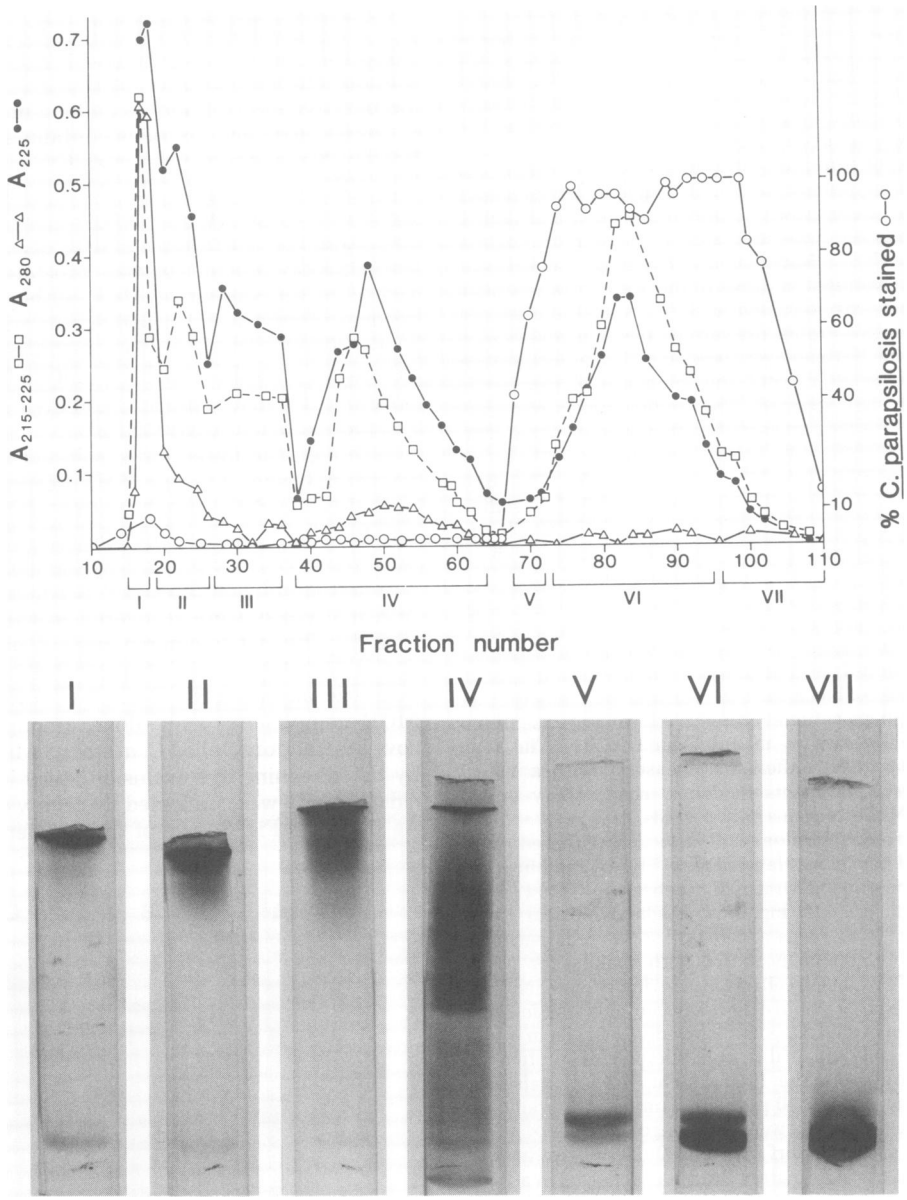

FIG. 2. *Chromatographic profile of 3 mg of resuspended HEPES precipitate on Bio-Gel P-10. The OD (-A) of the fractions was monitored at 280, 225, and 215 nm. The algebraic difference, $OD_{215} - OD_{225}$, estimates the peptide bond concentrations (15). Candidacidal activity was measured by dye exclusion, and the results are expressed as the percentage of C. parapsilosis stained. Fractions (roman numerals) were pooled as indicated, lyophilized, suspended, and then electrophoresed on analytical polyacrylamide gels. Except for fractions IV and VI (160 μg and 22 μg, respectively) the entire fractions were applied to these gels.*

3 preparations per ml reduced the number of *C. albicans* colony-forming units by approximately 70% in 10 min.

Although minor resolution of MCP-1 and MCP-2 was apparent when appropriate subfractions of peak 3 were pooled (see Fig. 2) the proteins were not well separated by gel filtration. Their resolution was not improved by alterations in column dimension, flow rate, or load, by rechromatographing the fractions on Bio-Gel P-10, or by using phosphocellulose, Biorex-70, or CM-Sephadex columns.

We succeeded in separating MCP-1 and MCP-2 by electrophoresing the peak 3 proteins on

long polyacrylamide-urea gels. The excellent resolving power of these gels relative to standard (4-cm) analytical gels is shown in Fig. 4. Recovery of proteins from the long gels was also excellent. By applying 100 μg of peak 3 protein to the gel, we could recover 51 μg of MCP-1 and 25 μg of MCP-2. These data were obtained by the Lowry protein method.

We also used gel densitometry to quantitate these proteins in partially purified extracts. When stained with amido black, their absorption proved to be a linear function of MCP-1 and MCP-2 concentrations, up to 5 μg of protein (Lowry) per gel. Egg white lysozyme, quantitated by its spectrophotometric extinction coefficient, was run as a known standard on companion gels. Its staining intensity was also linear with protein concentration. The two MCPs exhibited five to seven times as much affinity per microgram of Lowry protein for the amido black stain as did lysozyme. Consequently, their staining intensity on gels was exaggerated relative to their actual concentration incrude extracts.

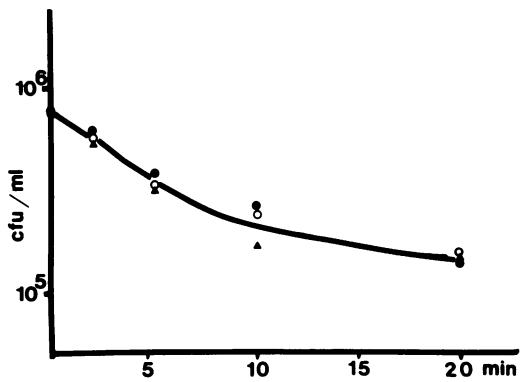

FIG. 3. *Candidacidal activity of peak 3. C. albicans cells (10^6) were incubated for 20 min with 1 μg of peak 3 protein per ml. At intervals, samples were assayed for viable yeasts by colony counting. Each symbol represents a different peak 3 preparation. Control yeast, incubated in buffer, remained fully viable.*

Characterization of cationic proteins. The amino acid composition of MCP-1 and MCP-2, purified by electrophoresis on long urea gels, is presented in Table 1. Both proteins contained a high content of arginine and half cystine and had unusually high arginine/lysine ratios. They were relatively deficient in tyrosine, consistent with their low absorbance at 280 nm relative to that at 225 nm (Fig. 2). Tryptophan content was not measured. MCP-1 and MCP-2 also differed substantially from each other (Table 1). In the MCP-1 hydrolysates, we noted a small but distinct amino acid peak eluting approximately 3 min before the lysine peak

TABLE 1. *Amino acid composition of MPCs*

Amino acid	mol% of:	
	MCP-1	MCP-2
Asp	1.26	3.44
Thr[a]	0.66	2.01
Ser[a]	3.69	11.20
Glu	5.33	9.34
Pro	6.05	4.72
Gly	9.30	12.30
Ala	9.31	9.74
Cys (half)[b]	18.70	9.82
Val	2.39	3.02
Met	0.05	0.95
Ile	4.02	3.17
Leu	8.01	8.02
Tyr	0.20	0.75
Phe	2.63	2.48
His	2.57	2.93
Lys	0.31	1.16
Arg	25.50	14.90
Trp	ND[c]	ND

[a] Threonine and serine contents were corrected 5 and 10%, respectively, for destruction during acid hydrolysis.

[b] Determined as cysteic acid after performic acid oxidation.

[c] ND, Not done.

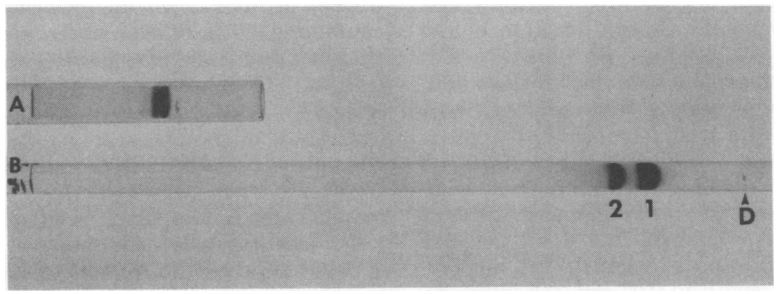

FIG. 4. *Polyacrylamide gels of peak 3 protein electrophoresed on (A) analytical gels (pH 4.3) and on (B) 25-cm-long urea gels. Gel A received 12 μg of protein, gel B received 45 μg; 1 and 2 indicate macrophage cationic proteins; D, dye front marked with india ink.*

emerged. This peak, not monomethyl lysine, urea, or a known amino sugar, was not further identified.

Our attempts to estimate the molecular weights of the peak 3 cationic proteins by electrophoresis or chromatography were initially unsuccessful. The proteins, precipitated by sodium dodecyl sulfate, failed to enter sodium dodecyl sulfate-acrylamide gels. They displayed anomalous chromatographic behavior on Sephadex G-50 and Bio-Gel P-10, eluting after the very-low-molecular-weight markers, bacitracin and ^{125}I.

We subjected the peak 3 fraction to graded ultrafiltration and dialysis. Over 93% of the proteins were retained after filtration on PM-10 Diaflo membranes (molecular weight cutoff, 10,000), whereas almost 94% was lost after 22 h of dialysis within a Spectrapor 2 membrane (molecular weight cutoff, 12,000 to 14,000). More accurate determinations of molecular weight, now in progress, will be communicated at a later date.

Microbicidal properties of macrophage cationic proteins. We have reported that partially purified macrophage cationic proteins eluted from polyacrylamide gels are microbicidal for several gram-positive and gram-negative bacteria as well as for two species of yeast (20). This activity was retained by the more purified peak 3 preparation, predominantly composed of MCP-1 and MCP-2. Once again, *C. albicans* was most sensitive to the lethal effect of peak 3 proteins. Although *L. monocytogenes* was also susceptible, it required approximately 40-fold more protein to achieve equivalent killing (Fig. 5). *S. typhimurium* was substantially more resistant.

We tested the effect of pH on the candidacidal activity of peak 3. So long as ionic strength was kept equal (0.024), we saw equivalent effectiveness over a broad range of pH, from 3.0 to 6.4. Higher pH levels were not tested, due to the tendency for a precipitate to form at or above pH 7 in a variety of buffers. In all of the above experiments (data not shown), control yeasts remained fully viable for the duration of the experiments.

Ionic strength profoundly affected the candidacidal activity of peak 3. We conducted most of our experiments at an ionic strength approximating 0.02. In a representative experiment, 3 μg of peak 3 per ml reduced the number of viable *C. albicans* by over 99% in 5 min under standard conditions. This was unaffected if we included 0.1 M mannitol in the assay buffer. Yet, increasing the ionic strength to 0.05 reduced killing to approximately 60%, and increasing it to 0.08 completely prevented candidacidal activity. Precisely the same results were obtained whether we used KCl, NaCl, or KBr to alter the ionic strength of the assay buffer. The viability of our control yeasts was unaffected by these various manipulations. Together with the mannitol data, these findings indicate that killing was modulated by ionic strength rather than by osmolality or specific effects of anions or cations.

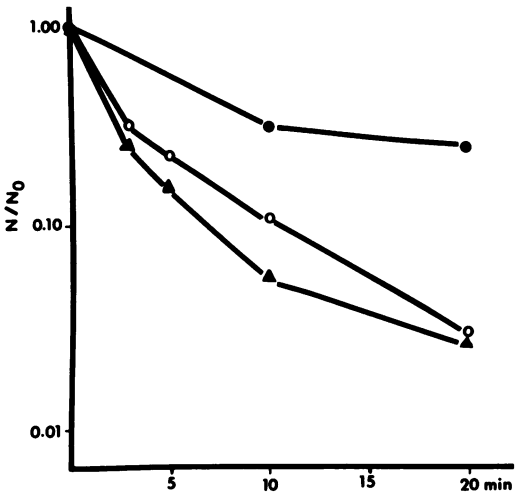

FIG. 5. *Killing activity of Bio-Gel P-10 peak 3 proteins. Approximately 10^6 colony-forming units of S. typhimurium (●, 20 μg/ml) and L. monocytogenes (○, 20 μg/ml) were incubated with 20 μg of peak 3 proteins per ml for 20 min. C. albicans (▲, approximately 10^6 cells per ml) was incubated with 0.5 μg of this preparation per ml. N/N_0 indicates the number of viable organisms at each sampling time divided by the starting cell density.*

The candidacidal activity of peak 3 proteins was not altered when the proteins were tested after they had been heated at 100°C for 10 min in a concentration of 10 μg/ml in assay buffer.

Studies with purified proteins. We performed several experiments with electrophoretically purified MCP-1 and MCP-2. Figure 6 demonstrates the candidacidal activity of 1 μg of MCP-1 or MCP-2 per ml, as well as a mixture containing 0.5 μg of each of these per ml. Note that MCP-1 was considerably more active, whereas the mixture was intermediate in its effect. To more precisely study the relative effectiveness of the two proteins against *C. albicans*, we did the dose-response study shown in Fig. 7. The data suggest that MCP-1 is approximately three to four times as effective as MCP-2 against this yeast.

Respiration by *C. albicans* was adversely affected when 5×10^6 yeast cells were incubated with 5 μg of each of the macrophage proteins per ml (Fig. 8). Note that substantial inhibition of

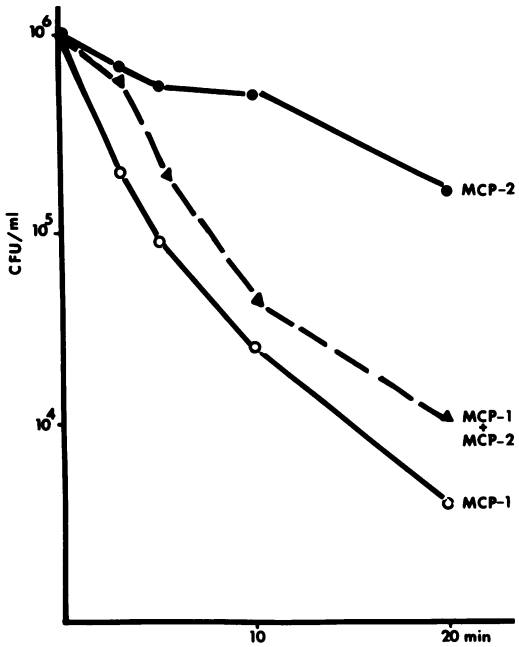

FIG. 6. *Candidacidal activity of MCP-1 and MCP-2; 1 μg of MCP-1 or MCP-2 or 0.5 μg of MCP-1 plus 0.5 μg of MCP-2 was incubated with 10^6 C. albicans cells per ml.*

O_2 consumption occurred within 2 min after the addition of peak 3, and that complete inhibition was established by 10 min. MCP-1 acted more promptly and completely than did an equivalent concentration of MCP-2, consonant with their relative candidacidal potencies as demonstrated by Fig. 6 and 7.

$^{86}Rb^+$ release. We have previously reported that release of $^{86}Rb^+$ from prelabeled *Candida* sp. cells is a sensitive indicator of membrane damage induced by amphotericin B (5) or the chymotrypsin-like cationic (microbicidal) protein of human neutrophils (6). In the present experiments, control prelabeled *C. albicans* cells released only 4% of intracellular ^{86}Rb in the 20-min incubation (baseline release). In contrast, cells exposed to peak 3 lost approximately 85% of their ^{86}Rb content in this time interval.

DISCUSSION

In this report, we describe properties of two microbicidal proteins purified from Freund adjuvant-elicited rabbit alveolar macrophages. We used gel densitometry, appropriately standardized for relative staining affinity, to estimate the amount of these proteins in such macrophages. We found that 10^8 macrophages (approximately 12 mg of total cellular protein) contained 180 μg of MCP-1 and MCP-2, with the former species being twice as abundant as the latter. Thus, the microbicidal proteins constituted approximately 1.5% of total macrophage proteins. MCP-1 and MCP-2 stained five to seven times as intensely as lysozyme with amido black, resulting in exaggerated prominence of these proteins in gels of our initial, unpurified extracts from macrophages (Fig. 1).

Our laborious but effective purification scheme owed its success in separating MCP-1 from MCP-2 to the outstanding resolving power of long polyacrylamide gels containing 2.5 M urea. Panyim and Chalkley used such long urea gels to resolve two species of calf thymus histone IV, which differed only by acetylation of a single lysine residue (19).

In a previous report, we presented indirect evidence that led us to conclude that macrophage microbicidal proteins were distinct from histones (20). Histones are also highly cationic proteins that can exert microbicidal activity in vitro (8). Occurring as several distinct molecular

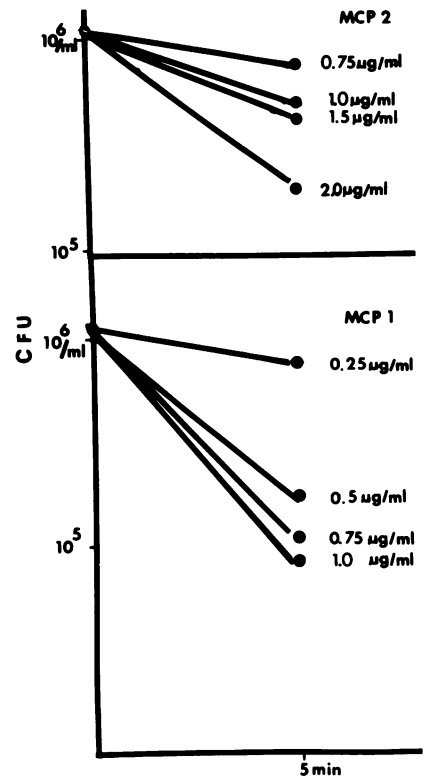

FIG. 7. *Candidacidal activity of various concentrations of MCP-1 and MCP-2, with C. albicans as test organism. Protein concentrations were estimated by the method of Lowry et al. with hen egg white lysozyme standards.*

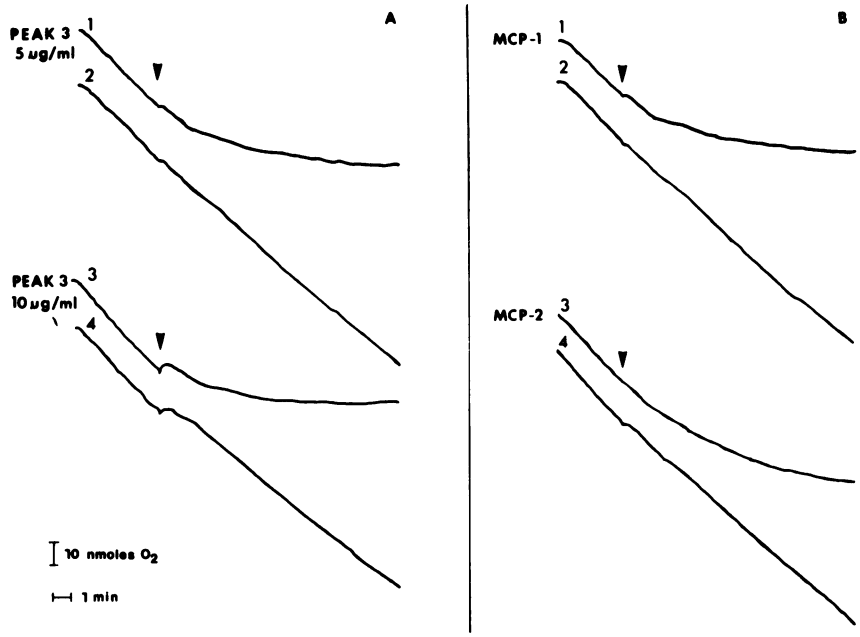

FIG. 8. *Oxygen electrode tracings illustrating the effect of peak 3, MCP-1, and MCP-2 on the respiration rate of C. albicans (5 × 10^6 cells of yeast per ml of assay buffer). At the arrows, the indicated amount of peak 3, MCP-1 (5 µg/ml) or MCP-2 (5 µg/ml) was added. Controls (even numbers) received an equivalent addition of buffer alone.*

species, histones show an extraordinary degree of conservation of amino acid composition and typically contain approximately 25 mol% of arginine plus lysine (4). However, even the so-called arginine-rich histones have a lysine/arginine ratio of >0.7, in contrast to the ratios of 0.01 (MCP-1) and 0.078 (MCP-2) of the rabbit macrophage proteins. Moreover, with the exception of mammalian histone IV (which contains two cysteinyl residues), histones characteristically lack cysteine (4). In contrast, both macrophage proteins were cysteine rich, containing 18.9 and 9.8 mol%, respectively.

Zeya and Spitznagel discovered the existence of a family of at least five lysosomal cationic proteins in rabbit granulocytes (24, 25). By subjecting these proteins to electrophoresis on sucrose density gradients and pooling the fractions closest to the cathodal front, they achieved a partial purification. The granulocyte proteins displayed high arginine (31 mol%) and cystine (14 mol%) contents and contained low levels of aromatic amino acids. This composition, clearly similar to that of MCP-1 and MCP-2, suggests that the granulocyte and macrophage proteins are related in structure and function. Further studies of both sets of proteins will be required to delineate this relationship more precisely.

Although the past decade has seen a great increase in our understanding of the microbicidal mechanisms of granulocytes (10), the comparable processes in macrophages are relatively poorly understood. Recent studies have demonstrated the presence of oxygen-dependent microbicidal processes in murine peritoneal macrophages (9, 16, 17). The present study clearly demonstrates that rabbit alveolar macrophages possess broadly effective microbicidal proteins of a type heretofore presumed to exist only in granulocytes. Much additional research will be needed to chart the distribution, regulation, interaction, and significance of such potentially complementary mechanisms in the host defense function of mononuclear phagocytes.

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