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Increased Content of Microbicidal Cationic Peptides in Rabbit Alveolar Macrophages Elicited by Complete Freund Adjuvant†

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We measured the microbicidal peptides MCP-1 and MCP-2 in rabbit alveolar macrophages (AM), comparing rabbits pretreated with complete Freund adjuvant with untreated control animals. Levels of MCP-1 increased from 4.7 ± 0.6 $\mu g/10^7$ resident AM to 13.2 ± 0.1 $\mu g/10^7$ complete Freund adjuvant-elicited AM. MCP-2 levels rose from 1.8 ± 0.1 $\mu g/10^7$ resident AM to 7.3 ± 0.4 $\mu g/10^7$ complete Freund adjuvant-elicited AM. The activities of five lysosomal hydrolases (β-D-glucuronidase, β-D-galactosidase, acid phosphatase, *N*-acetyl-β-D-galactosaminidase, and *N*-acetyl-β-D-glucosaminidase) were 44 to 96% higher in complete Freund adjuvant-elicited AM, and lysozyme activity was three- to fourfold higher. As MCP-1 and MCP-2 are major constituents of rabbit AM and exhibit potent antibacterial and antifungal properties, they may contribute to the expression of microbicidal activity in both resident and activated states.

Alveolar macrophages (AM) from rabbits pretreated with complete Freund adjuvant (CFA) kill ingested *Candida albicans* more rapidly or effectively than do resident alveolar or peritoneal macrophages (13). The candidacidal mechanisms of rabbit AM differ from those described in other mammalian phagocytes by being independent of peroxidase-mediated metabolic processes (12, 13).

Recently, we described the presence of two microbicidal components, MCP-1 and MCP-2, in rabbit AM (16, 17). These highly cationic arginine and cysteine-rich peptides of low molecular weight were extremely active against *C. albicans*, but also killed other fungi and several species of gram-positive bacteria. Because macrophages from animals stimulated by mycobacterial antigens such as CFA often express nonspecifically enhanced microbicidal activity, we compared the levels of MCP-1 and MCP-2 in resident and CFA-elicited AM.

MATERIALS AND METHODS

Macrophages. AM were recovered from CFA-treated and untreated New Zealand White rabbits by previously described methods (16). Treated animals had received 1 ml of CFA (no. 0638-60; Difco Laboratories, Detroit, Mich.) intravenously 21 days earlier. CFA-elicited macrophages were purified to ≤1% granulocyte contamination by a Ficoll-Hypaque step gradient (15). Resident macrophage populations contained a mean of 1.25% granulocytes and were not further purified.

Cell fractionation. Purified macrophage populations, 1 × 10^8 cells/ml, were suspended in 0.34 M sucrose (pH 6.8) and homogenized as previously described (16). After sequential 400 × *g* and 27,000 × *g* centrifugations, the resulting pellets were extracted with approximately 5 ml of 0.1 M citric acid/10^8 macrophage-equivalents, and the supernatants were used for electrophoretic studies. In preliminary studies, we found that virtually all of the cellular content of MCP-1 and MCP-2 was present in the citric acid extract of the lysosome-rich, 27,000 × *g* sediment. Approximately 70% of the total cellular lysozyme in resident or CFA-elicited AM was recovered in the 27,000 × *g* pellet. Most of the remainder was present in the 27,000 × *g* sucrose supernatant.

Purified MCP-1 and MCP-2 were prepared from CFA-stimulated macrophages as previously described (17) and quantitated by the method of Lowry et al. (14), using hen egg white lysozyme standards. Standard densitometric curves were generated by electrophoresing known quantities of purified MCP-1, MCP-2, and lysozyme on native acid disc polyacrylamide gels(4 cm long, 5-mm internal diameter, 15% polyacrylamide) as previously described (17). After staining for 1 h in an aqueous solution containing 9% acetic acid, 45% methanol, and 0.2% amidoblack, the gels were destained by diffusion for 24 h against the same dye-free solvent. Gels were scanned at 580 nm in a Gilford model 2520 gel scanner, and peak areas were measured with a compensating polar planimeter (model 620005; Keuffel and Esser Morristown, N.J.). We quantitated the levels of MCP-1 and MCP-2 in AM densitometri-

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cally, using hen egg white lysozyme (Worthington Diagnostics, Freehold, N.J.) standards as previously described (17). Unknown samples were diluted appropriately to ensure that the components of interest were measured on the linear portions of their standard curves (e.g., 1 to 8 μg of lysozyme/gel). Reproducibility of the method within and between experiments was within 10%.

Enzyme assays. Intact macrophages were suspended in 0.34 M sucrose at 1×10^7 cells/ml and kept frozen at −20°C until needed. For enzyme assays, the macrophages were thawed and subjected to six cycles of freezing and thawing, and samples were diluted 100-fold in 0.34 M sucrose that contained 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.).

Lysozyme (EC 3.2.1.17) was measured by radial diffusion in agar impregnated with *Micrococcus leisodeikticus* (19). The following enzymes were measured fluorometrically, employing appropriate 4-methylumbelliferyl (4-Mu) substrates (Research Products International, Elk Grove Village, Ill.): β-D-glucuronidase (EC 3.2.1.31), β-D-galactosidase (EC 3.2.1.23), acid phosphatase (EC 3.1.3.2), *N*-acetyl-β-D-galactosaminidase (EC 3.2.1.49), and *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30).

Stock solutions of 4-Mu substrates were prepared in 0.1 M sodium acetate or sodium citrate buffer, as follows: pH 4.5 acetate, 4-Mu-β-D-glucuronide and 4-Mu-β-D-galactopyranoside; pH 5.0 acetate, 4-Mu-phosphate; pH 5.0 citrate, 4-Mu-2-acetamido-2-deoxy-β-D-galactopyranoside and 4-Mu-2-acetamido-2-deoxy-β-D-glucopyranoside.

Assay mixtures, 0.2 ml, contained 2.5×10^{-4} M substrate, 50 mM acetate or citrate buffer (pH 4.5 or 5.0), and 10 μl of "enzyme" (10^3 macrophage-equivalents). Incubations were conducted for 15 and 30 min at 37°C and stopped by the addition of 1.8 ml of cold 0.1 M sodium carbonate-bicarbonate buffer, pH 10.7. The fluorescent product, 4-methylumbelliferone, was quantitated with an Aminco fluorocolorimeter (American Instruments Co., Silver Spring, Md.). Standard curves for 4-methylumbelliferone were linear between 0 to 300 pmol of product. Each enzyme was measured under conditions experimentally shown to be linear with time and enzyme concentration and which resembled conventional conditions employed by others who have used these substrates (3, 10, 18). Appropriate controls (enzyme free, substrate free, and zero time) were included with each assay.

In preliminary experiments we found the acid phosphatase activity of both resident and CFA-stimulated macrophages to be completely inhibited by 2 mM NaF (2), confirming the specificity of 4-Mu-phosphate for the lysosomal acid phosphatase (18).

RESULTS

Distribution of protein. Resident and CFA-elicited AM were homogenized in sucrose and fractionated by the procedures summarized in Table 1. Although both types contained virtually identical amounts of protein per cell, the lysosome-rich 27,000 × *g* sediment was approximately twice as large in the CFA-elicited cells. Whether derived from resident or CFA-elicited macrophages, approximately two-thirds of the protein content of that sediment was soluble in 0.1 M citric acid.

TABLE 1. *Protein content of resident and elicited AM fractions*

Supernatant	Protein content[a]	
	Resident	Elicited
Whole homogenate	1,248 ± 148 (3)	1,232 ± 280 (3)
400 × *g* Supernatant		
27,000 × *g* Supernatant	667 ± 33 (2)	685 ± 17 (3)
27,000 × *g* Sediment		
Citric acid extract	126 ± 21 (4)	228 ± 26 (3)
Citric acid residue	64 ± 19 (2)	123 ± 4 (3)
400 × *g* Sediment		
Citric acid extract	84 ± 8 (4)	84 ± 4 (2)
Citric acid residue	366 ± 21 (2)	135 ± 62 (2)
Total protein recovered	1,301	1,255
% Recovery	104.2	101.9

[a] Protein content is expressed as micrograms of protein per 10^7 macrophages. Values shown represent mean ± standard deviation (*n*).

Quantitation of constituents. Because MCP-1 and MCP-2 lack known enzymatic activity, we quantitated them by gel densitometry (Table 2). Macrophage lysozyme was measured densitometrically and by radial enzyme diffusion (Table 2). The results indicate that both MCP-1 and MCP-2 are major constituents of rabbit AM and that their combined amount closely approximates that of lysozyme in both resident and CFA-elicited cells. Note that CFA-stimulated cells contained three to four times the levels of these proteins present in resident cells.

Estimates of cellular lysozyme activity, as measured by the radial diffusion enzyme assay, were somewhat higher than those obtained by gel densitometry. However, similar differences were detected between resident and CFA-elicited AM. As the densitometric method detects protein content and the radial diffusion assay measures enzyme activity, their mildly discrepant results most likely reflect the previously described different specific activities of rabbit and hen egg white enzymes (4).

By fluorometric analysis, the levels of five lysosomal enzymes were 44 to 95% higher in CFA-elicited cells than in the control resident AM (Table 2).

DISCUSSION

We measured the amounts of MCP-1 and MCP-2 in rabbit AM so that we could determine how the in vivo administration of a mycobacterial preparation (CFA) affected the cellular contents of these microbicidal peptides.

AM from unstimulated rabbits contain substantially more lysozyme and lysosomal hydro-

TABLE 2. *Selected components of resident and CFA-elicited rabbit AM*

Component	Method[a]	Sp act[b]/10^7 macrophages		Ratio of elicited/ resident
		Resident	Elicited	
MCP-1	A	4.7 ± 0.6 (3)	13.2 ± 0.8 (3)	2.81
MCP-2	A	1.8 ± 0.1 (3)	7.3 ± 0.4 (3)	4.05
Lysozyme	A	4.7 ± 0.8 (3)	19.5 ± 0.6 (3)	4.15
Lysozyme	B	7.6 ± 0.2 (4)	25.7 ± 1.2 (3)	3.38
β-D-Glucuronidase	C	5.7 ± 1.2 (4)	11.2 ± 0.9 (4)	1.96
Acid phosphatase	C	37.9 ± 9.8 (4)	59.7 ± 8.5 (4)	1.58
β-D-Galactosidase	C	52.5 ± 10.7 (4)	82.9 ± 8.0 (4)	1.58
N-Acetyl-β-D-galactosaminidase	C	85.0 ± 17.4 (4)	129.7 ± 12.3 (4)	1.53
N-Acetyl-β-D-glucosaminidase	C	309.3 ± 85.6 (4)	445.3 ± 55.8 (4)	1.44

[a] The following methods were employed: A, gel densitometry; B, radial enzyme diffusion (lysoplates); C, fluorometric enzyme analysis.

[b] The units employed in these methods were as follows: (A) micrograms of protein (14), (B) micrograms of hen egg white lysozyme equivalent, and (C) nanomoles of 4-methylumbelliferone released per minute. Results are expressed as mean ± standard error of the mean (*n*).

lase activity than do comparable peritoneal macrophages (6). Intravenous or intratracheal instillation of mycobacteria is followed by further increases in the AM content of these components (4, 7). Thus, our finding a two- to threefold increment in the cellular content of lysozyme and less marked increases in a family of lysosomal hydrolases is entirely consistent with previous reports by other investigators (4, 7, 11).

The increased protein content of 27,000 × *g* sediments in CFA-elicited AM (Table 1) no doubt reflects their increased content of lysosomes and other electron-dense cytoplasmic organelles, also noted by previous investigators (5, 7, 11).

The present experiments confirmed our earlier finding that MCP-1 and MCP-2 are substantial constituents of rabbit AM and revealed that the cellular content of these peptides rose substantially in AM obtained after intravenous administration of CFA. CFA-elicited AM contained 2.8 times as much MCP-1 and 4.1 times as much MCP-2 as did their unelicited counterparts. Together, MCP-1 and MCP-2 constituted approximately 1.7% of the total protein content of CFA-elicited AM. In both resident and CFA-elicited cells, the joint content of these microbicidal peptides closely approximated that of lysozyme, long recognized to be a major component of the AM.

AM, by virtue of their ability to ingest and kill bacteria and fungi, are important defenders of the lung's sterility (8, 9). In part, their microbicidal effectiveness may derive from metabolic processes that generate reactive oxygen species (see reference 1 for review). On the other hand, microbicidal components such as the recently described peptides MCP-1 and MCP-2 may equip them with microbicidal mechanisms that operate independently of the aforementioned oxidative processes. Further investigation is required to assess the functional contribution of microbicidal peptides such as MCP-1 and MCP-2 to the effector mechanisms of microbicidally competent macrophages or to the enhanced antimicrobial effectiveness of "activated" or immune macrophages.

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