Antimicrobial Proteins of Murine Macrophages

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Three murine microbicidal proteins (MUMPs) were purified from cells of the murine macrophage cell line RAW264.7 that had been activated by gamma interferon. Similar proteins were also present in nonactivated RAW264.7 cells, in cells of the murine macrophage cell line J774A.1, and in resident and activated murine peritoneal macrophages. MUMP-1, MUMP-2, and MUMP-3 killed *Salmonella typhimurium, Escherichia coli, Staphylococcus aureus, Listeria monocytogenes, Mycobacterium fortuitum,* and *Cryptococcus neoformans* in vitro. MUMP-1 resembled an H1 histone but was unusual because its N-terminal residue (serine) was not N acetylated. Although MUMP-2 was N terminally blocked, its high lysine/arginine ratio and its reactivity with an antibody to H1 histones suggested that it also belonged to the H1 histone family. MUMP-3 was identical to histone H2B in 30 of 30 amino-terminal residues. Although the antimicrobial properties of histones have been recognized for decades, this is the first evidence that such proteins may endow the lysosomal apparatus of macrophages with nonoxidative antimicrobial potential. Other MUMPs, including some with a more restricted antimicrobial spectrum and one that appeared to be induced in RAW264.7 cells after gamma interferon stimulation, were noted but remain to be characterized.

One of the main functions of macrophages in defense against infections is the ingestion and subsequent intracellular killing of microorganisms. Intracellular killing is mediated by oxidative and nonoxidative mechanisms. Macrophage oxidative antimicrobial mechanisms include the production of reactive oxygen and nitrogen intermediates (17, 33, 34). Reactive nitrogen intermediates have so far been shown to play a role in the antimicrobial activity of activated rodent macrophages only (34). The nonoxidative antimicrobial mechanisms of macrophages are less well defined. Presumably they are mediated by acidification of and nutrient deprivation in the phagolysosome and by antimicrobial molecules present in cytoplasmic granules. Such antimicrobial molecules have been extensively studied in granulocytes. Human neutrophils contain a large number of cytoplasmic granules in which several antimicrobial polypeptides have been identified, i.e., defensins, lysozyme, cathepsin G, elastase, proteinase 3, azurocidin, bactericidal/permeability-increasing protein, and lactoferrin (reviewed in references 9 and 23). It is known that after ingestion of microorganisms, these antimicrobial polypeptides are transferred from their storage sites in the granules to the phagolysosome, where they can kill ingested microorganisms. In addition to granule-associated antimicrobial polypeptides, neutrophils contain a cytosolic protein, calprotectin, with antimicrobial properties (47, 48).

In contrast to granulocytes, mature macrophages contain fewer cytoplasmic granules and retain an ability to synthesize granule proteins (13, 45). Little is known about the presence of antimicrobial polypeptides in these granules. Macrophages from humans and from most animal species have been shown to contain lysozyme, whereas rabbit alveolar macrophages also synthesize and contain defensins (9, 13). To study antimicrobial polypeptides in macrophages,

large numbers of cells are required. Since these cannot readily be obtained from mice, we selected the mouse macrophage cell line RAW264.7 as the main source for the study of such polypeptides. An advantage of the use of this cell line is that the antimicrobial activity of these cells can be enhanced by activation with gamma interferon (IFN-γ) (19). The present study was undertaken to determine whether antimicrobial polypeptides are present in these macrophages as well as in macrophages of the mouse cell line J774A.1 and mouse peritoneal macrophages.

MATERIALS AND METHODS

Cell culture and subcellular fractionation of macrophage cell lines. RAW264.7 and J774A.1 cells (both derived from BALB/c mice) were obtained from the American Type Culture Collection, Rockville, Md., and cultured in Dulbecco's minimal Eagle's medium (Flow Laboratories, Irvine, Scotland) supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), D-glucose (3.5 mg/ml) and 10% heat-inactivated fetal calf serum (Flow Laboratories) in 1-liter spinner bottles. When the cells reached a density of 1×10^6 to 2×10^6/ml, recombinant rat IFN-γ (49) (a generous gift from P. H. van der Meide, Institute of Applied Radiobiology and Immunology, TNO Rijswijk, The Netherlands) was added to a final concentration of 200 U/ml, and the cells were cultured for an additional 20 h. Next the cells were harvested, washed in phosphate-buffered saline (PBS) containing 0.5 U of heparin per ml, resuspended at a concentration of 10^8/ml in sucrose buffer [0.34 M sucrose, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 1 mM MgCl₂, 10 mM sodium phosphate buffer (NAPB) (pH 7.4)], and disrupted by nitrogen cavitation for 20 min at 750 lb/in² at 4°C by using a cell disruption bomb (Parr Instrument Co., Moline, Ill.). The cavitate was collected in a mixture of proteinase inhibitors: 2 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10

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μM pepstatin, 1 mM n-ethylmaleimide, and 2 mM EDTA (final concentrations are given; all proteinase inhibitors were obtained from Sigma Chemical Co., St. Louis, Mo.). The suspension was centrifuged for 10 min at 200 × g to remove intact cells, nuclei, and cellular debris, and the postnuclear supernatant was centrifuged for 20 min at 27,000 × g to obtain a granule-enriched sediment (granule fraction) and a cytosol-enriched supernatant (cytosol fraction). This cytosol fraction was extracted by dialysis against 5% (vol/vol) acetic acid in Spectrapor 3 tubing (Spectrum Medical Instruments Inc., Los Angeles, Calif.), and insoluble material was removed by low-speed centrifugation. The granule fraction was suspended in 50 mM NAPB (pH 6.5) containing the proteinase inhibitors, and the mixture was sonicated on ice twice for 15 s each time at maximum power (Bronwill Biosonik IV; VWR Scientific, Los Angeles, Calif.). The mixture was kept on ice for 30 min, and 30% acetic acid was added to a final concentration of 5% (vol/vol). Following sonication, the mixture was stirred for 18 h at 4°C to extract acid-soluble compounds. Next, this suspension was centrifuged for 20 min at 27,000 × g and the pellet was again extracted as described above. The cleared extracts were dialyzed in Spectrapor 3 tubing against 5% acetic acid and concentrated to 1.5 ml by vacuum centrifugation (Speed-Vac; Savant Instruments Inc., Hicksville, N.Y.).

Peritoneal macrophages and spleen cells. Specific-pathogen-free male BALB/c mice (Iffa-Credo, L'Arbresle, France) weighing 20 to 30 g were used. Peritoneal macrophages were collected by lavaging the peritoneal cavity with 2 ml of ice-cold PBS (pH 7.4) containing 50 U of heparin per ml (50). The composition of the cell suspension was evaluated by using Giemsa-stained cytospin preparations: 63% macrophages, 35% lymphocytes, and 2% granulocytes. Peritoneal macrophages were also obtained from specific-pathogen-free female C57BL/10J and CBA/J mice (Jackson Laboratories, Bar Harbor, Maine) and outbred female Swiss Webster mice (Harlan-Sprague Dawley, San Diego, Calif.). These cells were extracted by using the procedure outlined above for granules without previous subcellular fractionation. Spleen cells from BALB/c mice were enriched for T lymphocytes by depletion of B lymphocytes and macrophages, using adherence to nylon wool. The fraction of nonadherent cells consisted of 91% lymphocytes, 7% macrophages, and 2% granulocytes as determined in Giemsa-stained cytospin preparations. Extracts from the granule and cytosol fractions of these spleen cells were prepared after disruption of the cells by nitrogen cavitation as described above.

Purification of antimicrobial polypeptides. Concentrated granule extract from 2 × 10⁹ cells (approximately 7 mg of protein) was applied to a column of Bio-Gel P-60 (1.5 by 64 cm; Bio-Rad Laboratories, Richmond, Calif.) that was equilibrated in 5% acetic acid. The material was fractionated at a flow rate of 4.5 ml/h, and 2.25-ml fractions were collected. Selected fractions containing antimicrobial activity were lyophilized by vacuum centrifugation and further purified by reverse-phase high-performance liquid chromatography (RP-HPLC) on a C18 column (4.6 by 250 mm; Vydac, The Separations Group, Hesperia, Calif.) with a linear water-acetonitrile gradient that contained 0.1% trifluoroacetic acid as the ion-pairing agent. Final purification was achieved by repurification of the active fractions on the same column with a more shallow water-acetonitrile gradient in the region where the proteins of interest eluted; in these purification steps 0.13% heptafluorobutyric acid instead of trifluoroacetic acid was used as the ion-pairing agent.

The purity of the antimicrobial polypeptides was assessed by Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (41). The reactivity of these polypeptides with monoclonal antibodies directed against mouse histones (51) was analyzed by immunoblotting. Briefly, following separation of approximately 1 μg of the purified polypeptides by SDS-PAGE, these polypeptides were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (30) (Immobilon-P; Millipore Corp., Bedford, Mass.). Next, each polyvinylidene difluoride sheet was incubated with the anti-histone monoclonal antibodies (CLB-ANA 105, anti-histone H10; CLB-ANA 108, anti-histone H1A and/or H1C; CLB-ANA 102, anti-histone H2B) and bound antibodies were detected with an alkaline phosphatase-conjugated second antibody directed against mouse immunoglobulins. The anti-histone antibodies were a generous gift from R. J. T. Smeenk, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands. As a positive control for these immunoblot experiments, nuclear extract from IFN-γ-activated RAW264.7 cells was used. For this purpose we used the pellet containing intact cells, nuclei, and cellular debris that was obtained as described above after disruption of the cells by nitrogen cavitation. This pellet was boiled in SDS-PAGE sample buffer and was designated the nuclear extract.

Antimicrobial assays. The antibacterial activity of the extracts and fractions was investigated by using *Escherichia coli* ML-35p (22), mouse-virulent *Salmonella typhimurium* 14028S (*phoP⁺*) and its avirulent mutant 7953S (*phoP*) (10, 11) (a generous gift from E. A. Groismann and F. Heffron, Scripps Clinic Research Institute, La Jolla, Calif.), *Staphylococcus aureus* 42D and 502A, and *Listeria monocytogenes* EGD. To obtain bacteria in the mid-logarithmic phase, 50 to 200 μl of an overnight culture made in Trypticase soy broth (TSB; Becton-Dickinson, Cockeysville, Md.) was added to 50 ml of TSB and incubated for 2.5 h at 37°C with shaking. Next, the bacteria were washed in 10 mM NAPB (pH 7.4), and their concentration was estimated by measuring their A_{620} and using the relationship A_{620} 0.2 = 5 × 10⁷/ml. To study the antibacterial activity against *Mycobacterium fortuitum*, we cultured *M. fortuitum* for 3 days at 37°C in Bacto-Middlebrook 7H9 broth enriched with Bacto-Middlebrook ADC (Difco Laboratories, Detroit, Mich.) and 0.05% (vol/vol) Tween 80. This culture was diluted 10-fold in the same medium and cultured overnight at 37°C. Next, the bacteria were washed in 10 mM NAPB (pH 7.4) containing 0.01% Tween 80, clumps of mycobacteria were removed by filtration through sterile cotton wool, and their concentration was estimated by spectrophotometry as described above. The antifungal activity of the extracts and fractions was investigated by using *Cryptococcus neoformans* A383 (kindly provided by D. H. Howard, University of California, Los Angeles). Cryptococci were cultured for 2 days at 37°C in Sabouraud 2% dextrose broth (Difco) and washed twice in 10 mM NAPB (pH 7.4), and their concentration was estimated by measuring their A_{450} and using the relationship A_{450} 0.2 = 5 × 10⁷/ml. The antimicrobial activity of samples against these microorganisms were investigated by using two recently described ultrasensitive antimicrobial assays (25).

(i) **Gel overlay assay.** Samples were subjected to PAGE with 12.5% acid–urea polyacrylamide gels (acid-urea PAGE). Next the gels were washed in 10 mM NAPB (pH 7.4) and incubated at 37°C on top of 1-mm-thick underlay agar containing 10⁵ mid-logarithmic-phase bacteria per ml. The underlay agar (pH 6.5) consisted of 9 mM NAPB, 1 mM citrate, 1% (vol/vol) TSB (for bacteria) or Sabouraud broth
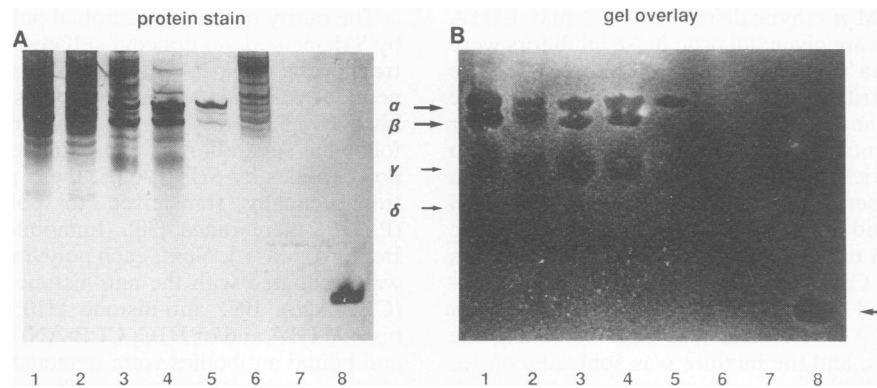
FIG. 1. Gel overlay analysis of extracts from IFN-γ-activated RAW264.7 cells, resident macrophages, and nonadherent spleen cells from BALB/c mice. Whole-cell extract (lane 1), cytosol extract (lane 2), and granule extract (lane 3) from 5×10^6 RAW264.7 cells; whole-cell extract from 5×10^6 resident peritoneal macrophages of BALB/c mice (lane 4); whole-cell extract (lane 5), cytosol extract (lane 6), and granule extract (lane 7) from 5×10^6 nonadherent spleen cells; and 1 μg of rabbit defensin NP-1 (lane 8) were electrophoresed on acid-urea PAGE, and stained with Coomassie blue (A) or analyzed by the gel overlay assay with S. typhimurium 14028S as the target organism (B). The major bacterial clearance zones are indicated by the Greek letters α, β, γ, and δ. The clearance zone caused by NP-1 is indicated with an arrow on the right of panel B.

(for fungi), and 1% (wt/vol) agarose. The underlay agar for *M. fortuitum* also contained 0.05% Tween 80. After allowing 3 h for diffusion of its proteins into the underlayer, we removed the PAGE gel and added a nutrient-rich top agar on top of the underlay agar; we then incubated the plate for 18 h at 37°C to allow growth of the microorganisms. The location of antimicrobial polypeptides in the gels was indicated by a clear, microorganism-free zone in the underlay agar. In some experiments agar was removed from these clearance zones, homogenized, and incubated in TSB for 6 h at 37°C to investigate whether the compounds causing these clearance zones did so through a bacteriostatic or bactericidal effect on the target microorganisms. As a control, agar was also removed from the area adjacent to the clearance zones and incubated in TSB. In these samples, bacterial growth was always noted.

(ii) **Radial diffusion assay.** Samples (5 μl in 0.01% [vol/vol] acetic acid) were added directly to 3-mm-diameter wells that were made in an underlay agar (as described above). After incubation for 3 h at 37°C, the underlay agar was covered with a nutrient-rich top agar overlay and incubated for 18 h at 37°C to allow growth of the microorganisms. The presence of antimicrobial activity in the samples was indicated by the presence of a zone of bacterial clearance around the wells. The diameter of this clearance zone was measured and expressed in arbitrary units (AU; 0.1 mm = 1 AU) after subtraction of the diameter of the well (3 mm). The rabbit defensin NP-1, isolated as previously described (44), was used as a control in the assays for antimicrobial activity.

Amino acid composition and N-terminal sequence analysis. The amino acid composition of the purified polypeptides was determined by quantitating amino acids as phenylthiocarbamyl derivatives on RP-HPLC by using a Novapak C18 column (Millipore) as described previously (2, 14). The N-terminal amino acid sequence of the purified peptides was determined on a model 475A sequencing system (Applied Biosystems Inc., Foster City, Calif.) as previously described (6, 30). Sequence data were analyzed by using the Swiss-Prot 19 data base and the FASTA algorithm (36).

Other methods. The enzymatic activity of the granule marker β-glucuronidase in Triton X-100 (0.1%, vol/vol)-treated subcellular fractions was determined by standard

procedures (31). Lysozyme activity in lyophilized column fractions or in Triton X-100-treated subcellular fractions was determined by radial diffusion in agar containing *Micrococcus lysodeikticus* (42). Protein was measured by using the bicinchoninic acid method (Pierce, Rockford, Ill.) with bovine serum albumin standards. NO_2^- in the culture supernatant of RAW264.7 cells as a measure for NO production by these cells was determined by using the Griess reagent (40).

Purified fractions of calf thymus histones were obtained from R. J. DeLange, Department of Biological Chemistry, University of California Los Angeles School of Medicine.

RESULTS

Detection of antimicrobial activity in subcellular fractions of murine macrophage cell lines and peritoneal macrophages. RAW264.7 cells incubated with IFN-γ were activated according to their increased NO_2^- secretion in the supernatant (IFN-γ treated, 4.9 ± 1.2 nmol of NO_2^- per 10^6 cells; control treated, <0.2 nmol of NO_2^- per 10^6 cells). IFN-γ-activated RAW264.7 cells were subjected to subcellular fractionation, and a cytosol fraction and granule fraction were obtained. The activities of the granule markers β-glucuronidase and lysozyme in both fractions were determined; the granule fraction contained 76% of the total β-glucuronidase and 77% of the total lysozyme activity.

A gel overlay procedure was used to analyze the samples for the presence of antimicrobial polypeptides. With *S. typhimurium* 14028S as the indicator (Fig. 1B), we obtained sharply demarcated bands of clearing, designated zones α and β, from extracts prepared from unfractionated (whole) IFN-γ-activated RAW264.7 cells (lane 1) or their granules (lane 3). When agar was removed from the area of zones α and β and suspended in TSB, no bacterial growth was noted, indicating that the compounds were bactericidal. Zones α and β were also present, but less marked, when we tested the extract from the cytosol fraction of an equivalent number (5×10^6) of activated RAW264.7 cells (lane 2). Extracts of resident peritoneal macrophages of BALB/c mice caused sharp bands of antimicrobial activity in zones α and β (lane 4), but an extract of whole, nonadherent splenocytes (lane 5)
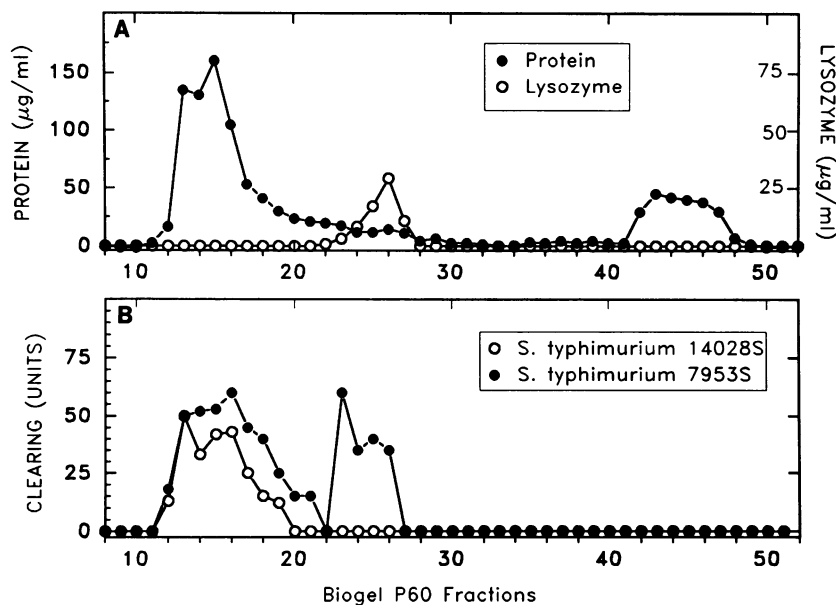
FIG. 2. Gel filtration of granule extract from IFN-γ-activated RAW264.7 cells on Bio-Gel P-60 (1). An extract prepared from 2 × 10⁹ cells was fractionated on a Bio-Gel P-60 column. The fractions were assessed for protein content and lysozyme activity (A) and for antimicrobial activity against *S. typhimurium* 14028S and 7953S (B).

gave only the α band. Both zones α and β were absent when the extracts were made from their cytosol or granule fraction (lanes 6 and 7, respectively).

Another, more diffuse zone of antimicrobial activity was evident when extracts prepared from resident peritoneal macrophages or intact activated RAW264.7 cells or their cytosolic or granule fractions were overlaid on *S. typhimurium* 14028S (Fig. 1, lanes 1 to 4). This zone was designated zone γ on the basis of its electrophoretic mobility, which corresponded closely to that of murine lysozyme (data not shown). One additional band of antimicrobial activity, designated zone δ, was evident only with extracts prepared from the whole cells or cytosol fractions of the IFN-γ-activated RAW264.7 cells (lanes 1 and 2).

We also performed gel overlay assays with several other indicator microorganisms, including *L. monocytogenes*, *S. aureus* 502A and 42D, *M. fortuitum*, *E. coli*, and *C. neoformans*. The results with *L. monocytogenes* closely resembled those illustrated in Fig. 1, except that zone γ was considerably more marked (data not shown). In contrast, we observed only zones α and β when both *S. aureus* strains, *M. fortuitum*, *E. coli*, and *C. neoformans* were used as the indicator.

Since *S. typhimurium* 14028S appeared to be a suitable indicator strain for detecting murine microbicidal proteins (MUMPs), these bacteria were used in the gel overlay procedure to screen the antimicrobial activity of nonactivated RAW264.7 cells; cells from a different murine macrophage cell line (J774A.1); resident peritoneal macrophages from C57BL/10J, CBA/J, and outbred Swiss/Webster mice; and peritoneal exudate macrophages from Swiss Webster mice. All reproduced the findings illustrated in Fig. 1 with respect to zones α, β, and γ, indicating that the polypeptides responsible for this antimicrobial activity were not mouse strain specific, macrophage cell line specific, or specific for IFN-γ-activated cells. Zone δ, however, was present in the cytosol or whole-cell extract of IFN-γ-activated RAW264.7

cells only but not in nonactivated RAW264.7 cells or in any of the other cells listed above.

Purification of MUMPs from granules of IFN-γ-activated RAW264.7 cells. An acetic acid extract of RAW264.7 cell granules was selected for further purification of the molecules responsible for the antimicrobial activity in zones α and β, since most known phagocyte antimicrobial polypeptides are granule associated. The extract was chromatographed on a Bio-Gel P-60 column, and the fractions were tested for activity against *S. typhimurium* 14028S and its isogenic, mouse-avirulent *phoP* derivative *S. typhimurium* 7953S. Fractions 12 to 19 were active against both strains (Fig. 2), and fractions 23 to 26 were active only against *S. typhimurium* 7953S. Lysozyme activity was present in fractions 23 to 27.

The activity of the Bio-Gel P-60 fractions against *L. monocytogenes* and *E. coli* is shown in Fig. 3. Fractions 12 to 19 were active against both organisms, and fractions 22 to 27 were active only against *L. monocytogenes*. Two additional peaks, comprising fractions 38 to 42 and fractions 44 to 46, were active only against *E. coli*.

Since fractions 13 to 18 possessed the widest spectrum of antimicrobial activity, they were subjected to further analysis, beginning with SDS-PAGE, acid-urea PAGE, and the gel overlay assay with *S. typhimurium* 14028S (Fig. 4). Fraction 13 contained a protein band that colocalized with a prominent protein in the RAW264.7 cell granule extract and appeared to be almost homogeneous by acid-urea PAGE. When analyzed by the gel overlay assay, its antimicrobial activity was found to reproduce the α zone of the unfractionated RAW264.7 cell granule extract. On SDS-PAGE, fraction 13 was found to contain two major components, MUMP-1 (M_r, 36,000) and MUMP-2 (M_r, 31,000), and various minor components. Fraction 16 contained a single major component with an apparent M_r of 16,000 as determined by SDS-PAGE. It migrated identically to a principal component of the RAW264.7 cell granule extract on acid-urea PAGE,
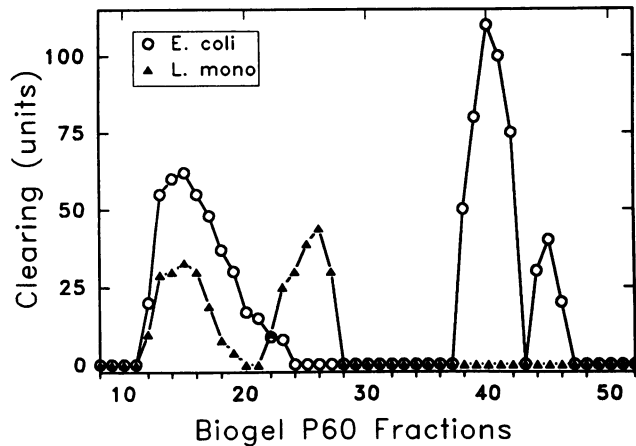
FIG. 3. Gel filtration of granule extract from IFN-γ-activated RAW264.7 cells on Bio-Gel P-60 (2). The antimicrobial activity of the Bio-Gel P-60 fractions shown in Fig. 2 was also tested against *E. coli* and *L. monocytogenes* (L. mono).

and its antimicrobial activity corresponded to zone β in the gel overlay assay.

Both fractions 13 and 16 were subjected to several modes of RP-HPLC with various gradients and ion-pairing agents. Ultimately, we obtained substantially purified preparations of three proteins (Fig. 5): MUMP-1 (apparent M_r, 36,000), MUMP-2 (apparent M_r, 31,000), and MUMP-3 (apparent M_r, 16,000).

Characterization of purified antimicrobial polypeptides. When subjected to amino acid analysis, all three MUMPs proved to be unusually lysine rich (Table 1). All MUMPs were subjected to N-terminal gas phase amino acid sequencing. MUMP-1 proved to be extremely interesting. The fact that we obtained the N-terminal sequence data shown in Table 2 provided prima facie evidence that its N terminus was unblocked. However, its sequence suggested that it was a member of the highly diverse H1 histone family, all of whose other known members are blocked by posttranslational N-terminal acetylation (Fig. 6). N-terminal sequencing of MUMP-2 gave us a very low yield, suggesting that it was N terminally blocked. At some steps, two residues were noted. Although the sequence data were not adequate to identify MUMP-2, its high lysine/arginine ratio (Table 1) and the fact that it was N terminally blocked suggested that it was an H1 histone. This possibility was confirmed by showing that MUMP-2 reacted strongly, by Western immunoblot analysis, with an antibody to mouse H1A and/or H1C histones. This antibody reacted only minimally with MUMP-1 (Fig. 7). Although the actual molecular weight of H1 molecules is approximately 21,000 to 22,000, their high lysine content makes them appear to be larger in SDS-PAGE molecular weight estimations. Indeed, when we electrophoresed purified calf thymus H1 histone on SDS-PAGE, two major components with estimated M_rs of 37,100 and 33,900 and a minor component with an apparent M_r of 31,500 were seen. These apparent M_r values closely resemble those observed for MUMP-1 and MUMP-2.

MUMP-3 was sequenced for 30 residues, and the sequence PEPAK SAPAP KKGSK KAVTK AQKKD GK KRH was obtained. This sequence proved to be identical to that of mouse histone H2B (29). This conclusion was confirmed by immunoblot analysis, showing that MUMP-3
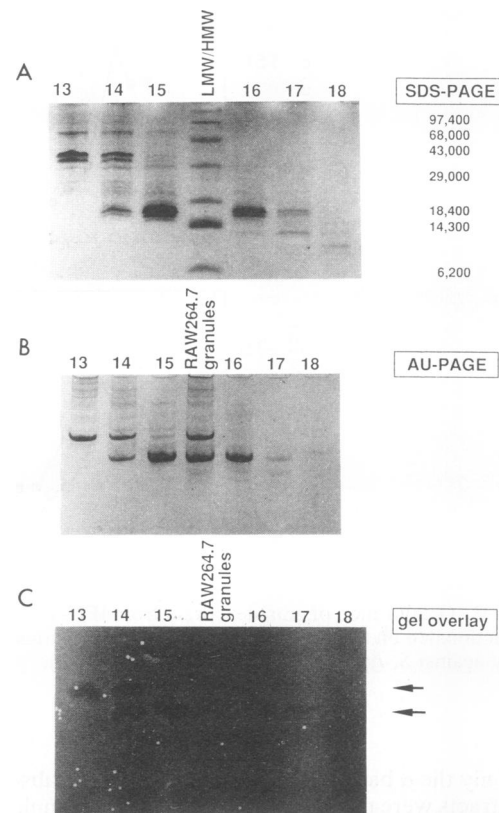

FIG. 4. Analysis of selected Bio-Gel P-60 fractions from IFN-γ-activated RAW264.7 cells. Fractions were subjected to SDS-PAGE and stained with Coomassie blue (A), subjected to acid-urea PAGE (AU-PAGE) (B), or subjected to acid-urea PAGE followed by the gel overlay assay with *S. typhimurium* 14028S as the target organism (C). The center lane contained an extract of granules from 5×10^6 IFN-γ-activated RAW264.7 cells. The two major clearance zones observed in the gel overlay assay are indicated with arrows.

reacts strongly with an anti-mouse histone H2B monoclonal antibody (data not shown). In addition, SDS-PAGE analysis of calf thymus histone H2B demonstrated that it ran almost identically to MUMP-3 as a single component with an estimated M_r of approximately 15,500 (data not shown).

DISCUSSION

The present study demonstrates that mouse macrophages contain, besides lysozyme, three lysine-rich, broad-spectrum MUMPs. These MUMPs were found to be present in cells from the mouse macrophage cell lines RAW264.7 and J774A.1 and in peritoneal macrophages from various mouse strains. Because we used protease inhibitors in isolating MUMPs, our methods were inherently biased against finding precursor molecules that require proteolysis to exert antimicrobial activity. Although Zanetti et al. have reported that precursor forms of the antimicrobial polypeptides called bactenecins exist in bovine neutrophils (55), it is not known whether counterparts exist in macrophages. Since the protease inhibitors would also have inactivated any endogenous proteases, their use might preclude the detection of any MUMPs whose activity depended on intrinsic protease activity. Antimicrobial proteases, whose antimicrobial activity is usually independent of their enzymatic activity, are
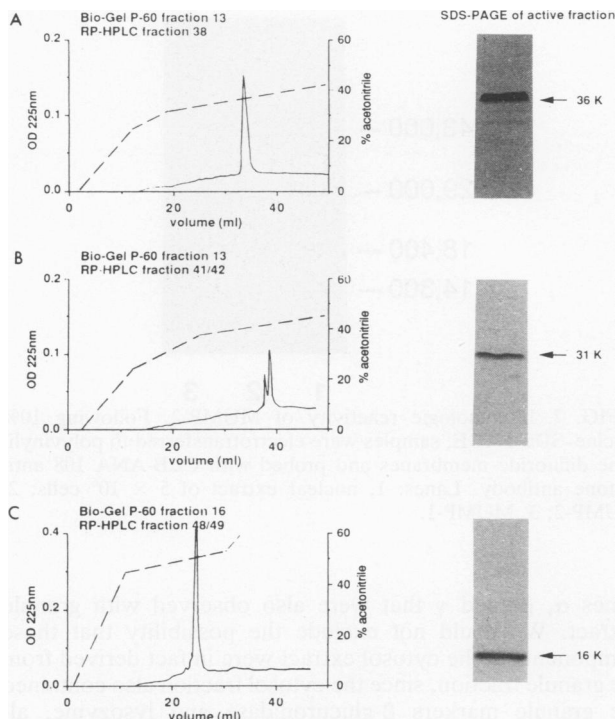
FIG. 5. Final RP-HPLC purification of MUMP-1 to MUMP-3. The chromatograms of the final repurification cycle of MUMP-1 (A), MUMP-2 (B), and MUMP-3 (C) and SDS-PAGE analysis of the active fractions are shown. The two peaks shown in the MUMP-2 tracing were collected separately. The first component had an apparent M_r of 35,000 and was not antimicrobial. The second peak component (MUMP-2) is shown on the gel. All SDS-PAGE gels were silver stained. Abbreviation: OD 225nm, optical density at 225 nm.

also known to exist in neutrophils (reviewed in reference 23). Despite these caveats, we did manage to characterize MUMP-1 through MUMP-3 reasonably well and obtained evidence that additional MUMPs exist. MUMP-1 through MUMP-3 proved to be histones or histone-like cationic proteins, molecules whose potent antimicrobial activity has long been recognized (15). MUMP-1 and MUMP-2 have partial homology with H1 histones in their N-terminal amino acid sequence and amino acid composition, and MUMP-2 is recognized by an antibody directed against histone H1A and/or H1C. MUMP-3 is identical to mouse histone H2B.

Histones have been divided into two functional types: core histones (histones H2A, H2B, H3, and H4) which form an octameric complex with about 146 bp of DNA to create the nucleosome, and H1 linker histones, which seal loops of DNA that enter and leave nucleosomes and condense these structures into compact, higher-order fibers (5, 46). The structure of the core histones does not differ much among species: the H4 histone of pea plants differs from that of calf thymus by two amino acid substitutions; the H3 molecule differs by only five (53).

H1 histones are much more varied. At least five principal H1 subtypes (H1a to H1e) exist in most mouse and rat tissues, and their relative rates of synthesis and degradation can differ with the dynamics of cell division, developmental stage, and differentiation (5, 26, 27). The expression of certain H1 variants is even tissue specific, e.g., the testicular histone H1t (54). All of the H1 molecules whose sequence

TABLE 1. Amino acid compositions of MUMPs from IFN-γ-activated RAW264.7 cells[a]

Amino acid	Amt of amino acid (mol%) in			
	MUMP-1	MUMP-2	Mouse H1[0]	Rabbit H1.3
Aspartic acid	3.71	3.52	3.62	2.24
Glutamic acid	5.34	4.99	5.18	3.57
Serine	10.21	9.98	10.36	5.80
Glycine	8.30	7.24	4.14	6.70
Histidine	0.23	0.42	1.04	0.00
Arginine	2.80	3.14	3.62	1.34
Threonine	6.74	4.90	5.70	5.80
Alanine	18.32	22.61	14.50	25.90
Proline	9.30	8.83	7.77	11.61
Tyrosine	0.73	1.02	1.55	0.45
Valine	5.21	5.11	7.25	3.57
Methionine	0.46	0.34	0.52	0.00
Isoleucine	1.23	1.43	3.10	0.89
Leucine	3.05	3.37	2.07	4.46
Phenylalanine	0.81	0.73	1.04	0.45
Tryptophan	ND[b]	ND	0.00	0.00
Lysine	23.58	22.36	28.50	29.46

[a] MUMP-1 and MUMP-2 were purified from granule extract of IFN-γ activated RAW264.7 cells. The expected compositions of mouse H1[0] (1) and rabbit H1.3 (5) were calculated from their published sequences.
[b] ND, not detectable.

has been determined by classical protein methods have been found to be blocked (4, 16, 28) as a result of N-terminal acetylation. This precluded their direct sequencing without prior proteolytic cleavage. H1 molecules generally contain 210 to 225 residues organized in three domains. The amino terminus (residues 1 to 42) is cationic and variable, the midsection (approximately residues 43 to 120) is hydrophobic and highly conserved, and the carboxyl terminus is also cationic. H1 histones are synthesized on cytoplasmic polyribosomes, and their transport to the nucleus appears to be mediated by cytoplasmic carriers (3). Large cytoplasmic pools of H1 histones, but not of core histones, were found in nondividing mouse liver cells and in exponentially growing

TABLE 2. N-terminal amino acid sequence of antimicrobial polypeptides from IFN-γ-activated RAW264.7 cells[a]

Cycle	MUMP-1		MUMP-2	
	Residue	Amt (pmol)	Residue	Amt (pmol)
1	Serine	50	Tyrosine	7
2	Glutamic acid	14	Glutamic acid	6
3	Threonine	16	Proline	6
4	Alanine	25	Alanine	10
5	Proline	20	Proline	10
6	Alanine	32	Threonine, lysine	5, 5
7	Glutamic acid, lysine	7, 5	Alanine	4
8	Threonine, lysine	8, 7	Proline	4
9	Proline	6	ND[b]	
10	Alanine	10	Alanine	4
11	Proline	7	Alanine	2
12	Alanine	3		
13	Lysine	18		
14	Alanine	20		
15	Glutamic acid	2		

[a] Approximately 180 pmol of MUMP-1 and 220 pmol of MUMP-2 were sequenced directly, without prior polyvinylidene difluoride transfer. The residue(s) and recovered amount(s) are indicated for each cycle.
[b] ND, none detected.

```
MUMP-1        S E T A P A E K P A P A K A E

Rabbit H1   Ac S E - A P A E T A A P A P A E

Bovine H1.1 Ac S E T A P A A P A A A P P A E

Mouse H1       S E A A P A A P A A A P P A E
```
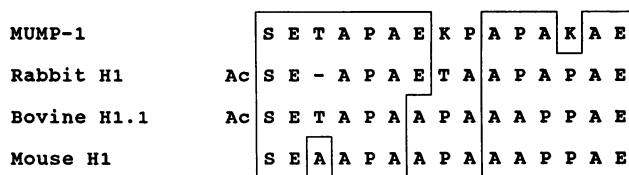
FIG. 6. Homology of MUMP-1 to H1 histone. The N-terminal amino acid sequence of MUMP-1 is aligned with selected H1 histones of rabbit (16), bovine (28), and murine (54) origin. Fully conserved residues are boxed. The rabbit and bovine sequences were determined directly from the respective proteins, which were N terminally acetylated. The murine H1 sequence was translated from the nucleotide sequence of its gene, and its initial residue (methionine) is not shown in the figure.
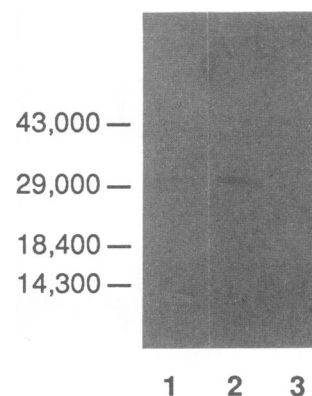

FIG. 7. Immunologic reactivity of MUMP-2. Following 10% Tricine–SDS-PAGE, samples were electrotransferred to polyvinylidene difluoride membranes and probed with CLB-ANA 108 antihistone antibody. Lanes: 1, nuclear extract of 5×10^6 cells; 2, MUMP-2; 3, MUMP-1.

Friend erythroleukemia cells (56). Fluoresceinated calf thymus H1 that was microinjected into renal epithelial cells entered the nucleus but also appeared to accumulate in punctate cytoplasmic organelles when high concentrations were injected or its active transport was inhibited by low temperature or by metabolic inhibitors (3). Recently, a protein involved in the stabilization of axonemal microtubules of sea urchin sperm flagella was identified as histone H1 (32). The N terminus of the protein was unblocked, and it migrated on SDS-PAGE as a protein with an M_r of 34,000, thus resembling MUMP-1 and MUMP-2. There is considerable evidence for transport of nonhistone chromosomal proteins from the lysosomal compartment to the nucleus, but whether this is bidirectional has not been established (37, 38). Therefore, although the presence of several histone-like proteins in nonnuclear subcellular fractions prepared from murine macrophages initially surprised us, it has several precedents. In preliminary immunofluorescence studies with anti-histone H2B monoclonal antibodies, we observed strong cytoplasmic staining and weak nuclear staining. These data indicate that MUMP-3/histone H2B does indeed have a cytoplasmic localization in cells from the RAW264.7 cell line.

Before this work, the only other antimicrobial polypeptides identified in mononuclear phagocytes were lysozyme and defensins. Lysozyme is present in macrophages from most species, but the only mononuclear phagocytes in which defensins have so far been described are rabbit alveolar macrophages (9, 23). Defensin-related peptides also appear to be present in murine intestinal Paneth cells, since these cells contain mRNA encoding cryptdin, a defensin-related polypeptide (35). Recently several cryptdin polypeptides with antimicrobial activity have been purified from murine small intestine (7, 43). Mouse neutrophils, in contrast to other rodent and human neutrophils, do not contain defensins (8). The polypeptides described in the present study are clearly distinct from lysozyme, defensins, and cryptdin. Although it has previously been demonstrated that mononuclear phagocytes may contain additional antimicrobial polypeptides that are distinct from lysozyme and defensins, these compounds have only been partially characterized (20, 52). Mouse macrophages secrete compounds with M_rs of 15,000 and 30,000 that display static activity against C. neoformans (12). Because these secreted components were only partially purified and characterized, it is not clear whether the MUMPs described in the present study are related to them.

Analysis of the antimicrobial polypeptides present in the cytosol extract from IFN-γ-activated RAW264.7 cells by the gel overlay procedure showed the presence of the clearance zones α, β, and γ that were also observed with granule extract. We could not exclude the possibility that these components in the cytosol extract were in fact derived from the granule fraction, since the cytosol fraction also contained the granule markers β-glucuronidase and lysozyme, although they contained less of these markers than the granule fraction did. In addition to these clearance zones, a fourth clearance zone with a higher electrophoretic mobility was observed, which was not present in granule extract from IFN-γ-activated RAW264.7 cells (zone δ). Interestingly this component was also absent in the cytosol extract from nonactivated RAW264.7 cells, suggesting that the presence of this component is related to IFN-γ activation of the cells. The RAW264.7 cells were activated with IFN-γ, which enhances their antimicrobial activity (19), because it was thought that this would provide a better chance to isolate antimicrobial polypeptides from these cells. It has to be noted that, on the basis of the gel overlay analysis, the MUMPs purified from the granule fraction were also present in RAW264.7 and J774A.1 cells that were not treated with IFN-γ and in resident peritoneal macrophages from various mouse strains.

Little is known about the physicochemical milieu—such as pH, ionicity, concentration of divalent cations—in the phagolysosome, where most antimicrobial polypeptides are thought to be active. The pH of the phagolysosome of mouse macrophages has been reported to reach values of 5.8 to 6.1 (18), although values lower than 4.0 have also been reported (52). In the present study, antimicrobial activity was studied under such mildly acidic conditions (pH 6.5), which had been defined as optimal in preliminary experiments. These preliminary experiments also indicated that the presence of citrate enhances the antimicrobial activity of the polypeptides from the mouse peritoneal macrophages. Citrate most probably enhances the sensitivity of the assay by chelating Ca^{2+} and Mg^{2+}, divalent cations that have been shown to inhibit the activity of defensins against Candida albicans (24) and E. coli (21). The Ca^{2+} concentration in the phagolysosome probably favors optimal antimicrobial activity of antimicrobial polypeptides, since this concentration has been reported to be very low in human macrophages (<100 μM [39]).

Although the present report demonstrates that mouse macrophages contain several antimicrobial polypeptides,

these studies do not reveal whether the polypeptides are delivered to the phagolysosome and thus equip macrophages to inhibit the growth of or kill ingested microorganisms.

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