Antimicrobial Mechanisms against Acinetobacter calcoaceticus of Rat Polymorphonuclear Leukocyte Granule Extract

MICHAEL J. LOEFFELHOLZ* AND MALCOLM C. MODRZAKOWSKI1,2

Program in Microbiology, Department of Zoological and Biomedical Sciences, and College of Osteopathic Medicine, Ohio University, Athens, Ohio 45701

Received 8 September 1987/Accepted 20 November 1987

The antimicrobial mechanisms of rat polymorphonuclear leukocyte granule extract and isolated extract fractions against Acinetobacter calcoaceticus were examined. Crude granule extract and a fraction containing low-molecular-weight cationic peptides (peak D) reduced the viability of A. calcoaceticus and inhibited the uptake of radiolabeled macromolecule precursors by cells. The inhibitory activity observed with peak D was not as great as that of crude granule extract containing equivalent amounts of peak D protein. Crude extract also inhibited incorporation of uracil into trichloroacetic acid-precipitable material, while no isolated fraction, including peak D, had any substantial effect on incorporation. The antimicrobial activities of crude granule extract were more sensitive to boiling than those of isolated peak D. Preincubation of A. calcoaceticus with either crude granule extract or a fraction (peak B) possessing proteolytic activity but lacking any antimicrobial activity caused cells to become sensitive to a subinhibitory concentration of actinomycin D, suggesting that granule extract and peak B increase the outer membrane permeability of A. calcoaceticus. The antimicrobial granule extract fraction, peak D, did not affect outer membrane permeability. These results suggest that rat polymorphonuclear leukocyte granule extract reduces the viability of A. calcoaceticus by inhibiting the transport and incorporation of macromolecule precursors and that either whole granule extract is required for complete antimicrobial activity or an unidentified component is responsible for antimicrobial activity in addition to peak D. The granule extract activity that increases outer membrane permeability does not appear to be directly responsible for the observed decrease in viability.

The contents of polymorphonuclear leukocyte (PMN) granules possess nonoxidative antimicrobial activity which provides defense against a broad spectrum of potentially harmful microorganisms (4, 15). Likely responsible for the majority of this antimicrobial activity are cationic proteins—highly positively charged proteins ranging widely in molecular weight and possessing potent nonenzymatic antimicrobial activity (4, 9, 11, 14–19). A number of studies have characterized the mechanisms of nonoxidative antimicrobial action of contents isolated from human (2, 14, 18) and rabbit (3, 17, 19) PMN granules against gram-negative bacteria. Buck and Rest (2) showed that unfractionated granule extract from human PMNs reduced the viability of Neisseria gonorrhoeae without affecting macromolecular synthesis. Odeberg and Olsson (14), working with purified cationic proteins from human PMN granules, showed these granule components to inhibit both macromolecular synthesis and viability of Escherichia coli. They also observed a disruptive effect on the cytoplasmic membrane caused by the cationic proteins, resulting in the inhibition of energy-dependent transport. Hovde and Gray (8) proposed that killing of Pseudomonas aeruginosa by an isolated human PMN granule protein was due to the depolarization of the cytoplasmic membrane of the organism. Weiss et al. (18, 19) have characterized cationic proteins from rabbit (19) and human (18) PMN granules which display potent antimicrobial activity, while having little or no effect on macromolecular synthesis. Several investigations have shown granule contents from rabbit (17, 19) and human (2) PMNs to increase outer membrane permeability of target gram-negative bacteria without destroying the structural integrity of the cell.

We have previously demonstrated that rat PMN granules possess cationic peptides which reduce the viability of the nosocomial pathogen Acinetobacter calcoaceticus (1, 5) in nonoxidative in vitro assays (11). In this study, we examined possible mechanisms by which rat PMN granule contents (both isolated and unfractionated) reduce the viability of A. calcoaceticus.

MATERIALS AND METHODS

Organism and culture conditions. The organism used in this study, A. calcoaceticus HO1-N, is a laboratory-maintained strain. Bacteria were grown to the mid-log phase on Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) in a 37°C shaking water bath before use in all antimicrobial assays.

Isolation of PMN granule extract and extract fractions. PMNs were obtained from rat peritoneal exudates, and subcellular PMN granules were isolated as previously described (6). Granule contents were extracted overnight in 0.2 M sodium acetate buffer (pH 4.0) containing 10 mM calcium chloride (6) and either dialyzed against phosphate-buffered saline for use in assays as crude granule extract or concentrated by ultrafiltration (UM-05 filter; Amicon Corp., Lexington, Mass,) to 20 to 30 mg of protein per ml before fractionation by fast protein liquid chromatography (Pharmacia, Uppsala, Sweden) as previously described (10).

Viability assays. The ability of granule extract and isolated fractions to reduce viability (defined as the ability to inhibit CFU formation) was determined as previously described (6). Assay mixtures contained either granule extract or a particular extract fraction, tryptone-NaCl, and either 10⁵ or 10⁶ bacteria per ml. We found it necessary to add a greater amount of granule extract material to assay mixtures containing 10⁵ bacteria per ml to observe antimicrobial activity comparable to that observed in mixtures containing 10⁶.
bacteria per ml. Viability was expressed as the percentage of CFU on Trypticase soy agar plates (BBL Microbiology Systems) plates compared with controls.

Radioisotope uptake and incorporation. The effect of granule extract and isolated fractions on transport of DNA, RNA, and protein precursors was measured by following the uptake of 2.5 μCi of [8-3H]deoxyadenosine per ml (specific activity, 16 Ci/mmol; ICN Radiochemicals, Irvine, Calif.), 1.25 μCi of [5,6-3H]uracil per ml (specific activity, 40 μCi/mmol; ICN), and 1.0 μCi of 3H-amino acid mix per ml (specific activity, 240 mCi/mg; ICN), respectively. Assay mixtures contained 108 bacteria per ml, tryptone-NaCl, and PMN granule contents in addition to radiolabeled precursors, except for controls which lacked any granule material. After various periods of incubation at 37°C, 100-μl samples were removed (in duplicate) from the assay mixture, filtered (0.45-μm pore size; Gelman Sciences, Inc., Ann Arbor, Mich.), mixed with 6 ml of ScintiVerse II scintillation fluid (Fisher Scientific Co., Fair Lawn, N.J.), and counted in a liquid scintillation system, model LS 8000 (Beckman Instruments, Inc., Fullerton, Calif.). Results were expressed as the percentage of counts compared with control mixtures containing no granule contents, yet otherwise treated identically.

To measure the effect of granule extract and isolated fractions on biosynthetic activity, we followed the incorporation of 1.25 μCi of [5,6-3H]uracil per ml of assay mixture (see uptake experiment) into trichloroacetic acid-precipitable material. After incubation, 150-μl samples were removed, added to 75 μl of lysis buffer containing 3% sodium dodecyl sulfate and 50 mM Tris (pH 12.6), vortexed, and allowed to stand at room temperature for 10 min. After lysis, 100-μl samples were transferred (in duplicate) to glass filters. The filters were washed three times with cold 10% trichloroacetic acid, once with 95% ethanol, and once with ether. Counting of the trichloroacetic acid precipitates was done as described above for uptake experiments. Counts at zero time incubation were subtracted from experimental values. Controls (containing no granule contents) were treated identically to test mixtures.

Outer membrane permeability. Sensitivity to actinomycin D (ActD; Sigma Chemical Co., St. Louis, Mo.) was used to measure granule extract-mediated changes in outer membrane permeability since resistance of gram-negative bacteria to ActD is due to the inability of the drug to cross the outer membrane permeability barrier (13). To determine changes in outer membrane permeability, we incubated bacteria in the presence of granule extract or isolated fractions at 37°C for 1 h before adding a subinhibitory concentration (1 μg/ml) of ActD. The assay mixtures were then incubated for an additional hour. The detergent Triton X-100 (0.2%; Sigma) served as the positive control for this experiment. After specified periods of incubation, samples were removed (in duplicate) from all assay mixtures, plated on Trypticase soy agar plates, and incubated overnight. Results were expressed as the percentage of CFU compared with controls containing no PMN granule contents. All antimicrobial assays, including bactericidal assays, were repeated at least twice on different days.

RESULTS

Effect of granule contents on viability. An earlier study in this laboratory showed that crude rat PMN granule extract and an isolated fraction containing low-molecular-weight cationic peptides (peak D) reduced the viability of A. calcoaceticus in nonoxidative in vitro assay systems, while fractions containing myeloperoxidase, neutral proteases, and lysozyme, designated peaks A, B, and C, respectively, had no antimicrobial activity (11). To reduce the viability of 103 bacteria per ml to approximately 65% of controls, 50 μg of granule extract protein per 0.4 ml (assay volume used) or a concentration of 125 μg/ml was required. In this study and in a similar study by Buck and Rest (2), assay mixtures contained 108 bacteria per ml to detect uptake and incorporation of radiolabeled precursors. A granule extract concentration of 550 μg/ml resulted in a viability (55%) roughly similar to that caused by the lower concentration used against 105 bacteria per ml (Fig. 1). A granule extract concentration of 125 μg/ml, antimicrobial for 105 bacteria per ml, had no activity against 106 bacteria per ml and actually promoted growth (data not shown). It was also previously stated that peak D represents approximately 15% of the total crude granule extract protein (12). Therefore, in assays containing 106 bacteria per ml, 80 μg of peak D protein per ml was often used to accurately assess its contribution to the antimicrobial activities of 550 μg of crude granule extract per ml. Both 550 μg of granule extract per ml and 80 μg of peak D per ml reduced the viability of A. calcoaceticus, although viability in the presence of peak D was substantially higher than viability in the presence of granule extract after 60 min (Fig. 1).

Effect of granule contents on transport. The effect of granule extract and isolated fractions on membrane transport was followed by measuring the uptake of macromolecule precursors by A. calcoaceticus (108 bacterial per ml) over 2 h. To ensure that uptake was not due to adsorption of radiolabeled precursors to the bacterial cell surface, we incubated radiolabeled precursors in the presence of heat-killed (65°C, 15 min) cells in amounts and under conditions identical to controls described above. No adsorption of [3H]deoxyadenosine, [3H]uracil, or 3H-amino acids to heat-killed cells was observed over 2 h. Not only did granule extract (550 μg/ml) inhibit uptake of [3H]uracil substantially more than peak D (80 μg/ml), but the inhibition by granule extract was observed after 30 min of incubation, while peak

![FIG. 1. Viability-reducing activity of 550 μg of crude granule extract protein per ml (C) and 80 μg of isolated peak D protein per ml (C) against A. calcoaceticus. Assay mixtures contained 106 bacteria per ml. Data represent means ± standard errors of the mean (SEM).](http://iai.asm.org/)
D had no effect on [3H]uracil uptake after 30 min (Fig. 2). Concentrations of peaks A (myeloperoxidase), B (proteases), and C (lysozyme) comparable to those present in 550 μg of crude granule extract per ml (12) had no substantial inhibitory effect on [3H]uracil uptake. Peak B protein actually caused an increase in uptake after 60 min. Crude granule extract also inhibited the uptake of [3H]deoxyxyadenosine and [3H]-amino acids by A. calcoaceticus. There was essentially no uptake of these precursors after 1 h in the presence of 550 μg of granule extract per ml (data not shown). The inhibition of [3H]uracil uptake by crude extract and peak D was dose dependent (Fig. 3), with 625 μg of granule extract per ml completely inhibiting the uptake. The inhibitory activity of 100 μg of peak D per ml was nearly equal to that of 550 μg of granule extract per ml after 120 min, yet this amount of peak D protein is estimated to be present in approximately 700 μg of granule extract per ml, a concentration which would likely inhibit [3H]uracil uptake completely. It should be noted that 550 μg of crude extract per ml inhibited uptake of [3H]uracil by ca. 50% after 2 h of incubation, while it reduced viability by ca. 95%.

Effect of granule contents on biosynthetic activity. The effect of granule extract and peak D cationic peptides on macromolecular synthesis was followed by measuring incorporation of [3H]uracil into trichloroacetic acid-precipitable material over 2 h. Before examination of incorporation of [3H]uracil by A. calcoaceticus, we conducted experiments to determine whether acid-precipitable PMN granule contents bound uracil, giving false-positive results. [3H]uracil was incubated in the presence of various concentrations of granule extract, precipitated onto glass filters, and washed as described in Materials and Methods. In an additional experiment, granule extract (625 μg/ml) and [3H]uracil were incubated together and then bound to nitrocellulose filters which were subsequently washed to remove any unbound uracil. Filters were dried, and their radioactivity was counted in a scintillation counter. Counts were compared with those of controls containing no granule extract, yet otherwise treated identically. These control experiments showed that granule extract did not bind [3H]uracil. Results of incorporation studies were expressed as a percentage of the total [3H]uracil taken up by cells rather than as a percentage of incorporation in controls, which does not take into account changes in uptake caused by granule contents. For example, when compared directly with controls containing no granule contents, incorporation of [3H]uracil in the presence of 80 μg of peak D per ml was substantially inhibited after 120 min (data not shown). When expressed in this form, however, the data fail to reveal the likelihood that a reduced uptake of [3H]uracil was responsible for the subsequently reduced incorporation. On the other hand, when incorporation was expressed as a percentage of [3H]uracil taken up over the same period, peak D had essentially no effect on incorporation (Table 1). In control mixtures, approximately 36 to 41% of [3H]uracil taken up by A. calcoaceticus was incorporated during 2 h. In mixtures containing 80 μg of peak D per ml, approximately 35 to 39% of [3H]uracil was incorporated. A higher concentration of peak D (100 μg/ml) also had no effect on incorporation after 120 min. However, crude granule extract (550 μg/ml) did inhibit incorporation of [3H]uracil. After 120 min, incorporation in the presence of granule extract was less than half of that in controls.

Effect of heat denaturation on antimicrobial mechanisms of granule contents. Crude granule extract retained partial viability-reducing activity after boiling for 5 min, and the

![FIG. 2. Effect of crude granule extract and isolated granule extract fractions on uptake of [3H]uracil by A. calcoaceticus (10⁶ bacteria per ml). Symbols: O, 190 μg of peak A per ml; ■, 165 μg of peak B per ml; ▲, 110 μg of peak C per ml; ○, 80 μg of peak D per ml; ●, 550 μg of crude granule extract per ml. SEM were less than ±5%.](image)

![FIG. 3. Effect of various concentrations of crude granule extract and isolated peak D on uptake of [3H]uracil by A. calcoaceticus. Concentrations of 350 (○), 550 (■), and 625 (△) μg of granule extract per ml and 80 (●) and 100 (■) μg of peak D per ml were used. SEM were less than ±5%.](image)

TABLE 1. Percentage of [3H]uracil taken up by A. calcoaceticus which is incorporated into acid-precipitable material

<table>
<thead>
<tr>
<th>Addition (μg/ml)</th>
<th>% Incorporation after an incubation time (min) of:</th>
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<tbody>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Control</td>
<td>37.9</td>
</tr>
<tr>
<td>Granule extract (550)</td>
<td>32.2</td>
</tr>
<tr>
<td>Peak D</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>35.2</td>
</tr>
<tr>
<td>100</td>
<td>ND*</td>
</tr>
</tbody>
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* Controls contained no PMN granule material.
* ND, Not determined.
activity of peak D was virtually unaffected (Fig. 4), indicating that peak D has a nonenzymatic nature, as does some, if not all, of the viability-reducing components of crude extract. The heat sensitivity of crude extract activity was also observed with a higher dose. Viability of A. calcoaceticus in the presence of 250 μg of untreated granule extract per ml after 1 h was ca. 5% of controls, while viability in the presence of the same concentration of heat-denatured granule extract was not reduced at all (data not shown).

Inhibition of [3H]uracil uptake by crude granule extract (550 μg/ml) appeared to be more sensitive to heat denaturation than the viability-reducing activity. Boiling of granule extract resulted in nearly a doubling of [3H]uracil uptake after 2 h (Table 2). Boiling had little effect (much less than proportional) on the ability of peak D to inhibit [3H]uracil uptake, offering further evidence that an additional PMN granule component(s) more sensitive to heat denaturation than peak D peptides was responsible for inhibiting transport of macromolecular precursors by A. calcoaceticus.

Effect of granule contents on outer membrane permeability. Viability of A. calcoaceticus in the presence of a subinhibitory concentration of ActD and crude granule extract, isolated peak B (the granule extract fraction possessing proteolytic activity), or the positive control Triton X-100 was significantly (P < 0.05) lower after 60 min of incubation than the viability of similar mixtures lacking ActD (Table 3), indicating that crude granule extract and peak B increased the outer membrane permeability of A. calcoaceticus. Antimicrobial concentrations of peak D did not affect sensitivity to ActD (data not shown). With these results in mind, an experiment was conducted to determine whether the permeability-increasing activity of peak B increased the sensitivity of A. calcoaceticus to peak D. The experimental design was essentially identical to that of outer membrane permeability assays, except that peak D replaced ActD. A. calcoaceticus (105 bacteria per ml) was preincubated in the presence of peak B (40 μg/ml) before adding peak D (13 to 40 μg/ml) and incubating further. Viable plate counts showed that preincubation with peak B did not increase sensitivity to peak D over levels observed with peak D alone (data not shown).

**DISCUSSION**

The nonoxidative antimicrobial potential of crude, unfractionated extract from PMN granules (2, 3, 7, 11) and isolated cationic granule proteins (4, 9, 11, 14–19) has been extensively studied. Although cationic proteins are considered to be largely responsible for the nonoxidative antimicrobial activity of granule contents, the question of whether their activity accurately represents the total nonoxidative antimicrobial potential of PMN granule contents has been raised (2, 7). Buck and Rest (2) showed that crude granule extract from human PMNs reduced the viability of N. gonorrhoeae without affecting macromolecular synthesis. Other investigators isolated cationic proteins from human PMN granules, Odeberg and Olsson (14) demonstrating that a fraction which contained several 25,000-molecular-weight proteins inhibited macromolecular synthesis by *Staphylococcus aureus* and *E. coli* and Weiss et al. (18) showing that a protein with a molecular weight of 58,000 to 60,000 had little effect on macromolecular synthesis by several gram-positive and gram-negative bacteria. Earlier results in this laboratory indicated that while low-molecular-weight cationic peptides were the only components of rat PMN granules isolated to date which possessed antimicrobial activity against *A. calcoaceticus*, this activity was much lower than the activity of a dose of crude granule extract containing a similar amount of cationic peptides (11). The results from this study are consistent with these earlier observations. According to our results, after 2 h of incubation, isolated peak D cationic peptides possess approximately 50 to 75% of the antimicrobial (CFU-reducing) activity of crude granule extract containing a similar amount of peak D protein (assuming that peak D represents approximately 15% of the total extract protein). The remaining antimicrobial activity (25 to 50%) has not been accounted for. It is possible that there is an additional component(s) of rat PMN granules possessing antimicrobial activity which, because of various characteristics (e.g., extremely low molecular weight, present in low concentration) avoids detection or is lost during isolation by

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**TABLE 2.** Effect of heat denaturation on the ability of granule extract and peak D to inhibit [3H]uracil uptake by A. calcoaceticus

<table>
<thead>
<tr>
<th>Addition (μg/ml)</th>
<th>Treatment</th>
<th>Uracil uptake (% of controls)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granule extract (550)</td>
<td>Untreated</td>
<td>42.9</td>
</tr>
<tr>
<td></td>
<td>100°C, 5 min</td>
<td>78.3</td>
</tr>
<tr>
<td>Peak D (80)</td>
<td>Untreated</td>
<td>71.6</td>
</tr>
<tr>
<td></td>
<td>100°C, 5 min</td>
<td>79.2</td>
</tr>
</tbody>
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* Uptake after 2-h incubation period. SEM were less than ±5%.
methods currently used or, alternatively, that certain granule extract components act synergistically against A. calcoaceticus. An earlier study showed that all possible combinations of the four fast protein liquid chromatography-separated granule protein fractions possessed no antimicrobial activity against A. calcoaceticus (12). It is also possible that peak D cationic peptides actually represent a greater percentage of the total granule extract protein than we estimated, although analyses of chromatograms and electropherograms of granule extract do not support this. Further indications that an additional granule component(s) other than peak D is involved in the nonoxidative activity of crude granule extract are the observations in this study that (i) crude granule extract inhibits incorporation of [3H]uracil while peak D does not and (ii) the antiproteolytic activities of crude granule extract (i.e., viability reduction, inhibition of [3H]uracil uptake) are more sensitive to heat denaturation than the same activities of peak D. It should be noted, however, that the observed differences in heat sensitivity of crude extract and isolated peak D may be due also to the different complexities of the two protein mixtures.

The outer membrane permeability-increasing activity observed with peak B of granule extract cannot be considered an antimicrobial mechanism by itself, as peak B has been shown to lack any antimicrobial activity against A. calcoaceticus (11). Yet this does not rule out the possibility that peak B is involved in a synergistic mode of action which potentiates antimicrobial activity. We have previously demonstrated that a combination of peaks B and D lacked any antimicrobial activity against A. calcoaceticus (12), and in this study we showed that preincubation of A. calcoaceticus with peak B did not increase sensitivity to peak D. These results indicate that the outer membrane permeability-increasing activity associated with peak B is not involved in the antimicrobial activity of peak D cationic peptides, although it may possibly be involved in the total antimicrobial activity of crude granule extract.

As discussed by Odeberg and Olsson (14), the reported inhibition of macromolecular synthesis by human PMN cationic proteins may have actually been the result of an inhibition of transport of the radiolabeled precursors across the cytoplasmic membrane. Inhibited transport, by decreasing the intracellular pool of available precursors, would indirectly cause a decrease in incorporation. In the absence of any uptake data, what appeared to be an inhibition of incorporation may have actually been an inhibition of transport. Our results suggest that transport is indeed affected and that crude granule extract reduces the viability (i.e., colony-forming ability) of A. calcoaceticus by inhibiting the transport of precursor molecules and the subsequent synthesis of macromolecules. Peak D cationic peptides are at least partially responsible for the inhibition of transport but do not appear to be involved in the inhibition of macromolecular synthesis. An additional, unidentified granule component or perhaps complete, unfractionated granule extract may be required for inhibition of macromolecular synthesis. The following mechanism of nonoxidative action of rat PMN granule contents against gram-negative bacteria is suggested. Certain granule contents, including peak D cationic peptides, penetrate the outer membrane with or without the aid of outer membrane permeability-increasing granule components of peak B; the granule contents then act on the cytoplasmic membrane, disrupting functions essential to cell survival and growth, including transport, biosynthesis, and possibly energy generation and the permeability barrier, among others.