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Wide-Spectrum Antibiotic Activity of Bovine Granulocyte Polypeptides

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The antibiotic activity of a polypeptide fraction purified from bovine granulocyte granules was tested against *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Bacillus subtilis*, *Bacillus stearothermophilus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus faecalis*, *Streptococcus pyogenes*, and clinical isolates of *Staphylococcus* and *Enterobacter* spp. All of these bacterial species were susceptible to the antibiotic polypeptide(s), with MICs ranging from 3 to 100 μg of protein per ml. The antimicrobial activity was resistant to boiling and abolished by proteinase treatment. *Saccharomyces cerevisiae* and human fibroblasts grew normally in the presence of 100 and 50 μg of antibiotic polypeptide(s) per ml, respectively. [^{3}H]thymidine incorporation into bacterial, but not fibroblast, DNA was efficiently and promptly inhibited by the antimicrobial polypeptide preparation. This suggests that its main target is a component of the system, which catalyzes and regulates the biosynthesis of bacterial DNA.

Substances with antibiotic activity are produced by a variety of living organisms. "Classical" antibiotics are generated by the secondary metabolisms of actinomycetes, fungi, and bacteria. However, small or large molecules with diverse chemical structures and inhibitory activity against various microorganisms can be isolated from lichens, algae, higher plants, insects, and vertebrates (1, 3, 8).

Blood granulocytes of mammals contain complexes of antibiotic polypeptides, which are presumably employed to arrest the growth or kill endocytosed bacteria (4, 10). In a few instances, these complexes have been resolved into components with various levels of effectiveness against and specificity for several bacterial species (10, 11). We have recently reported (4) the purification from bovine blood granulocytes of polypeptides with potent antibacterial activity against one strain each of *Staphylococcus aureus* and *Escherichia coli*. In the present study, we expanded our investigation of the effects of one of the purified fractions of granulocyte antibiotic polypeptide(s) (GAP) on several bacterial species, including clinical isolates. Furthermore, we analyzed whether inhibition of macromolecular synthesis by bovine GAP might provide a common basis for its wide spectrum of activity.

MATERIALS AND METHODS

Preparation of GAP. GAP was purified from granules of bovine granulocytes as previously reported (4). Briefly, granule pellets were extracted with 0.2 M sodium acetate (pH 4); after concentration by ultrafiltration and dialysis against 0.2 M NaCl in 10 mM sodium phosphate (pH 7), the extract was applied to a column of carboxymethyl cellulose equilibrated with the same buffer. Two pools of proteins, including bactericidal polypeptides, were eluted from the resin with 0.3 and 1 M NaCl. These pools were then resolved into various fractions by gel permeation through a column of Sephadex G-50, equilibrated, and eluted with 0.1 M NaCl in 5 mM sodium acetate (pH 4). The polypeptide preparation employed in this study corresponded to fraction I and contained one major component and one minor component of ca. 4,000 and 11,000 daltons, respectively, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions (9).

Bacteria and culture conditions. Test bacteria were either strains from the American Type Culture Collection (see below) or clinical isolates kindly provided by C. Monti Bragadin, Laboratory of Microbiology, Health Services of Trieste, and by E. Crevatin, Institute of Hygiene, University of Trieste, Italy. The organisms were grown overnight in Iso-Sensitest broth (Oxoid Ltd.) and diluted in fresh medium before each experiment.

Yeast and fibroblasts. *Saccharomyces cerevisiae* and human fibroblasts were grown in L broth (Difco Laboratories) and RPMI 1640 medium (Flow Laboratories) with 10% fetal calf serum (Flow Laboratories), respectively.

Determination of MIC and CFU. The MIC of each antibiotic was assayed by the microdilution susceptibility test. Serial dilutions of either GAP or other antibiotics in 50 μl of Iso-Sensitest medium were pipetted into the wells of microtiter plates. Bacteria (5×10^4 CFU) in 50 μl of the same medium were then added, and the plates were incubated at 37°C for 16 h (*Bacillus stearothermophilus* was incubated at 60°C). For each antibiotic, the MIC was considered to be that concentration at which the organism tested did not exhibit visible growth. Occasionally, the Iso-Sensitest medium was replaced by L broth. CFU were counted on nutrient agar plates as previously described (4).

Heat denaturation and proteinase treatment of GAP. MIC assays were also run with GAP samples pretreated by boiling for 5 min or with proteinase K (type XI [Sigma Chemical Co.], 50 μg/ml) for 30 min at 37°C.

Macromolecule biosynthesis. The effect of GAP on the biosynthesis of bacterial DNA and protein was evaluated by incubating 0.5×10^6 to 3.0×10^6 CFU of *S. aureus* or *E. coli* per ml of Iso-Sensitest medium at 37°C in the presence of the appropriate precursor. [*methyl*-^{3}H]thymidine (15 μCi/ml) or L-[4,5-^{3}H]leucine (30 μCi/ml) (both from Amersham Corp.) were added to the bacterial cultures simultaneously with GAP (100 μg/ml). Subsequently, at regular time intervals,

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samples of the bacterial suspensions were withdrawn for CFU counting and evaluation of acid-precipitable radioactivity (6, 7).

The potential DNase activity of GAP was assayed by incubating 5 μg of GAP with 1 μg of plasmid pBR322 (2) at 37°C in 50 μl of 10 mM Tris-hydrochloride (pH 7.5)–50 mM NaCl–10 mM $MgCl_2$. After 15 min, the plasmid was extracted with phenol and ether, precipitated with ethanol, and electrophoresed on a 1% agarose gel (7).

RESULTS

A number of gram-negative and gram-positive bacteria were susceptible to the antibiotic action of purified bovine GAP (Table 1). The MIC values, which ranged from 3 to 50 μg/ml (except for *Klebsiella pneumoniae*), were very reproducible, with a variation of at most one dilution value in repeated assays.

The growth-inhibitory activity of GAP was preserved after heating in boiling water for 5 min, whereas it was completely abolished by proteolytic treatment.

Clinical isolates of *Staphylococcus* and *Enterobacter* spp., which were resistant to one or more of the commonly used commercial antibiotics (Table 2), were all susceptible to GAP, with MIC values ranging from 3 to 100 μg/ml. *Staphylococcus epidermidis* and two isolates of *Staphylococcus saprophyticus* were the most susceptible to the antibacterial action of GAP.

The inhibitory activity of GAP is likely to be very specific for bacteria. In fact, *S. cerevisiae* and human fibroblasts displayed normal growth in the presence of 100 and 50 μg of GAP/ml of medium, respectively. Occasionally, after the evaluation of MIC values, we plated the contents of the microtiter wells on nutrient agar and incubated them at 37°C for 18 h. These assays showed that the concentration of GAP immediately preceding that of the MIC caused >99% killing of bacteria. The bactericidal activity of GAP was confirmed by monitoring the reduction in CFU at short intervals after the exposure of *S. aureus* or *E. coli* to GAP (Fig. 1).

In consideration of the wide spectrum of inhibitory and bactericidal activities of GAP, we thought that it could directly affect a vital process common to all the microorganisms tested, such as the biosynthesis of DNA. [^{3}H]thymidine incorporation into the DNAs of *S. aureus* and *E. coli* was fully and promptly inhibited by GAP (Fig. 1). When *S. aureus* suspensions denser than 10^7 cells per ml were used, an inhibitory effect on thymidine incorporation was detected as early as 4 min after the addition of GAP to bacteria.

Unlike DNA synthesis, bacterial protein synthesis was not inhibited by GAP. In fact, the rate of incorporation of [^{3}H]leucine was comparable in control and treated cultures of *S. aureus* and *E. coli*, at least within 1 h after addition of GAP to the bacterial suspensions. Analogous to the observed lack of inhibition of growth, no inhibition by GAP of DNA and protein biosynthesis in human fibroblasts was detected.

Finally, potential DNase activity of GAP could be ruled out since treatment of plasmid pBR322 with GAP did not

TABLE 1. Susceptibility of bacterial species to bovine GAP[a]

Organism and strain	MIC (μg/ml)
E. coli ATCC 25922	50
K. pneumoniae ATCC 13883	>100
Pseudomonas aeruginosa ATCC 27853	50
Salmonella typhimurium ATCC 14028	50
Bacillus subtilis ATCC 9372	50
B. stearothermophilus ATCC 7953	3
S. aureus ATCC 25923	50
S. epidermidis ATCC 12228	12
Streptococcus faecalis ATCC 19433	12
Streptococcus pyogenes ATCC 19615	12

[a] Purified protein fraction I (4) was diluted serially from 200 to 1.5 μg/ml in 50 μl of Iso-Sensitest medium in microtiter plates. Bacteria (5×10^4 CFU in 50 μl of Iso-Sensitest medium) were then added, and the plates were incubated at 37°C for 16 h (*B. stearothermophilus* was incubated at 60°C).

TABLE 2. Response of clinical isolates to antibiotics

Organism	MIC[a] (μg/ml)					
	Trimethoprim/Sulfamethoxazol	Oxacillin	Ampicillin	Cefamandole	Gentamicin	GAP
S. aureus	≤0.062/1.2	>16	>16	8	>8	50
	≤0.062/1.2	0.25	8	≤0.5	≤0.25	50
	≤0.062/1.2	≤0.125	0.25	≤0.5	>8	50
	0.25/4.75	4	>16	2	>8	100
S. saprophyticus	>8/152	>16	>16	32	8	3
	2/38	2	16	1	4	12
	>8/152	>16	>16	8	0.25	100
	2/38	8	16	2	0.25	100
	>8/152	>16	>16	>16	>8	100
S. epidermidis	2/38	2	16	1	4	12
Enterobacter spp.	16/304		256	256	16	100

[a] MICs of all antibiotics were determined as described in Table 1; footnote *a*. Bacteria were considered susceptible to the antibiotics when MIC values were as follows: trimethoprim/sulfamethoxazol, ≤2/38; oxacillin, ≤0.6; ampicillin, ≤2 (for *Enterobacter* spp., ≤16); cefamandole, ≤16; gentamicin, ≤8.

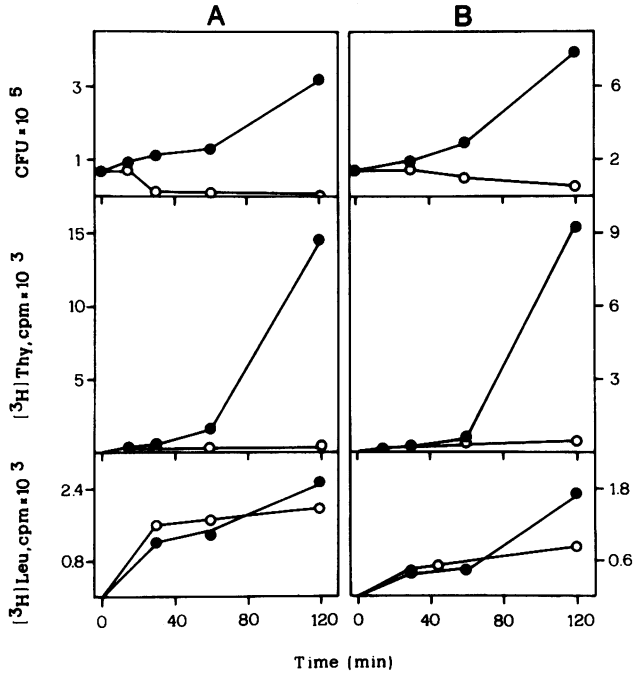

FIG. 1. Effects of GAP on bacterial DNA and protein biosynthesis. Representative experiments carried out with *S. aureus* ATCC 25923 (A) and *E. coli* ATCC 25922 (B) are shown. [^{3}H]thymidine and [^{3}H]leucine were added to parallel cultures of bacteria (0.5×10^6 to 3×10^6 CFU/ml) at zero time without (●) or with (○) GAP (100 μg/ml). CFU and counts per minute (cpm) refer to a total volume of 50 μl of bacterial suspension.

cause any alterations of the supercoiled and relaxed forms of the plasmid DNA.

DISCUSSION

The main feature of the GAP preparation employed here was its inhibitory activity on several gram-negative and gram-positive organisms, including some clinical isolates that were resistant to classical antibiotics. Pretreatment of GAP with a proteinase abolished its antibacterial activity but boiling did not. This confirms that the active substance(s) in the GAP preparation is a polypeptide and suggests that it does not function by catalyzing an enzymatic reaction.

A rather early effect of GAP was prevention of [^{3}H]thymidine incorporation into bacterial, but not fibroblast, DNA. This was not caused by a derangement of the energy supply to the metabolic pathways of bacteria because incorporation of [^{3}H]leucine into proteins was preserved in GAP-treated cells for at least 1 h. Actually, considering that treated bacteria did not grow, their rate of protein biosynthesis per cell appeared to be stimulated by GAP.

The full inhibition of precursor incorporation into DNA is unlikely to be caused by denaturation or enzymatic splitting of DNA. In fact, GAP did not relax or degrade plasmid pBR322, and its inhibitory activity was resistant to boiling. Furthermore, direct inhibition of DNA polymerase by GAP is also unlikely. In fact, preliminary experiments performed with the *E. coli* mutant strain AKEC28 transformed with plasmid pBR322 have indicated that GAP inhibits the biosynthesis of chromosomal but not of plasmid DNA.

The assembly of enzymes responsible for initiation and elongation of DNA chains in bacteria is presumably under some control by membrane proteins (5). The bovine GAP here tested may thus function by perturbing the normal topology of bacterial membranes, thereby preventing correct activity of proteins regulating or catalyzing the synthesis of the bacterial chromosomes. It is interesting that other bactericidal polypeptides purified from *Enterobacteriaceae* or granulocytes are thought to enter into a hydrophobic interaction with bacterial membranes upon initial electrostatic binding (11).

In conclusion, the GAP preparation we used in this study may become a powerful tool for the understanding not only of the mechanism of bactericidal action of mammalian granulocytes but also of the control of bacterial DNA synthesis. Work is now in progress to improve the purification of the polypeptide components of the GAP complex. This will allow the characterization of the GAP structure and identification of polypeptide domains which are responsible for the antibiotic activity.

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