Interactions Between Neisseria sicca and Viridin B, a Bacteriocin Produced by Streptococcus mitis

DAVID J. LAW AND ADNAN S. DAJANI
Division of Infectious Diseases, Children’s Hospital of Michigan, and Department of Pediatrics, Wayne State University School of Medicine, Detroit, Michigan 48201

Received for publication 8 September 1977

Viridin B, a bacteriocin produced by Streptococcus mitis (mitior), is bactericidal to Neisseria sicca. Oxygen consumption by actively growing N. sicca cultures ceased immediately upon exposure to viridin B. Adenosine triphosphate production was slightly enhanced within 1 h of exposure to the bacteriocin but was subsequently repressed. The uptake and incorporation of glucose was prevented in the presence of viridin B. The bacteriocin also blocked uptake of an amino acid mixture in chloramphenicol-pretreated cells. Pretreatment or concomitant treatment with a variety of antibiotics known to inhibit specific synthetic pathways did not alter the inhibition of macromolecular synthesis produced by the bacteriocin. Although viridin B blocks protein and nucleic acid syntheses, no degradation of such macromolecules was observed. The inhibitory effects of viridin B on macromolecular synthesis and on viability required the presence of sufficient nutrients to allow active metabolism of N. sicca. The bacteriocin did not inhibit viability or macromolecular synthesis in anaerobically incubated N. sicca. Thus, active, oxidative metabolism by N. sicca cells is essential for viridin B action. A model for viridin B action is proposed.

Bacteriocin production is one of several mechanisms of interaction among microbial populations. Viridins, bacteriocins produced by alpha-hemolytic streptococci, were previously isolated and characterized in this laboratory and were shown to possess several unusual properties (5). Among these are heat lability, narrow range of pH stability, and wide spectrum of activity (5). In addition, the frequency of bacteriocin production among alpha-hemolytic streptococci is high. Of 120 such isolates tested, 78% demonstrated bacteriocin-like inhibition against one or more indicators (5). Viridin B, produced by Streptococcus mitis (mitior) strain 42885, is bactericidal to Neisseria sicca but is only bacteriostatic to a coagulase-negative staphylococcus (5). The bactericidal effect of viridin B on N. sicca has been demonstrated by conventional survival techniques, by cessation of macromolecular synthesis, and by progressive deterioration of ultrastructure in treated cells (4, 5).

Nutrient medium was required to demonstrate the killing of N. sicca by viridin B (5). This suggested that the bacteriocin killed only actively metabolizing cells. The studies reported here provide additional evidence to this effect and describe other effects of viridin B on metabolic pathways in N. sicca.

MATERIALS AND METHODS

Bacterial strains. S. mitis (mitior) strain 42885, which produces viridin B, and N. sicca strain 15362, which is killed by viridin B, have been described in detail previously (5).

Media. N. sicca was usually grown in TSB (tryptic soy broth, Difco). For some experiments, a dilute medium was employed which did not support growth or affect viability of the organism. A dilution of 1 part TSB in 127 parts 0.067 M Sorensen’s phosphate buffer, pH 7.2 (TSB/128), was determined to be optimal.

Bacteriocin preparation. Viridin B was prepared as previously described (4, 5). The final concentration of viridin B used in most experiments ranged from 20 to 80 arbitrary units (4, 5).

Respiration. Oxygen uptake by N. sicca was determined by standard manometric techniques (16), with the use of a Warburg apparatus (Gilson Medical Electronics). Cells were grown to logarithmic phase in TSB, harvested by centrifugation, washed, and resuspended in phosphate buffer.

ATP production. Adenosine triphosphate (ATP) production was assayed by the luciferin-luciferase technique of Stanley and Williams (13).

Radioisotopes. Radioactive precursors used in uptake and incorporation experiments were 2H-labeled L-amino acid mixture (generally labeled), [5,6-3H]uridine, [methyl-3H]thymidine, and D-[14C]glucose (New England Nuclear Corp.). Since viridin B comparably inhibited ribonucleic acid (RNA), deoxyribonucleic
acid (DNA), and protein syntheses in actively growing _N. sicca_ (4), tritiated uridine was frequently used as a general indicator of the bacteriocin's effect on macro-

molecular synthesis.

Uptake and incorporation of glucose. Total uptake of D-[^3]C]glucose was assayed in viridin B-treated and control cultures of _N. sicca_ by membrane filtration (0.45 μm, Millipore Corp.) (15). Incorporation of [^3]C glucose into glycogen-like polymer was measured by the method of Abraham and Hassid (1). Incorporation of glucose into trichloroacetic acid-insoluble material was assayed as previously described (4).

Amino acid uptake and incorporation. The ef-

effect of viridin B on amino acid accumulation was measured in _N. sicca_ cells that had been pretreated with chloramphenicol to block protein synthesis. Tritiated amino acid mixture was added to a logarithmic-phase culture of _N. sicca_ to a final concentration of 3 μCi/ml, and 30 min later chloramphenicol was added to a concentration of 200 μg/ml (three times the minimal bactericidal concentration). After an additional 30 min, the culture was split, one portion receiving an equal volume of viridin B and the other receiving heat-inactivated bacteriocin. Sampling was continued for an additional 3 h. Total intracellular label was measured by liquid scintillation spectroscopy of samples collected on membrane filters (0.45 μm, Schleicher & Schuell Co.). In addition, the effects of chloramphenicol and viridin B on incorporation of the amino acid mixture into macromolecules were compared by analysis of trichloroacetic acid-precipitable counts per minute as described previously (4).

Effects of antibiotics on viridin B activity. Anti-

biotics tested were actinomycin D (Cosmegen, Merck & Co., Inc.), chloramphenicol (Chloromycetin, Parke, Davis & Co.), nalidixic acid (Neg Gram, Winthrop Laboratories), potassium penicillin G (E. R. Squibb & Sons), puromycin dihydrochloride, and rifampin (Sigma Chemical Co.). Minimal inhibitory and/or bac-
tericidal concentrations of each antibiotic against _N. sicca_ were determined by standard double-dilution techniques. An antibiotic concentration of two to three times the minimal inhibitory and/or bactericidal con-

centration was used in the experiments. _N. sicca_ was grown in the presence of radioactive uridine or amino acid mixture for 2 h, at which time the culture was divided and added to equal volumes of the following reaction mixtures: viridin B alone, antibiotic alone, antibiotic and viridin B, antibiotic followed 30 min later by viridin B, and inactivated viridin B as a control. Sampling was continued for 2 h, and macro-
molecular synthesis was assayed as described previ-

ously (4).

Degradation of macromolecules. _N. sicca_ cul-
tures were grown for 2 h in the presence of [3H]-labeled amino acid mixture, [3H]uridine, or [3H]thymidine, and incorporation of precursor into macromolecules was determined by trichloroacetic acid precipitation as previously described (4). The cultures were then split and treated with viridin B or inactivated viridin B, and sampling was continued for 2 to 3 h. Degradation of macromolecules was also assayed by measuring absorbancy at 260 and 280 nm in supernatants of viridin B-treated and control cell suspensions.

Susceptibility of _N. sicca_ to viridin B under conditions allowing active metabolism. Logarithmic- and stationary-phase _N. sicca_ cultures were exposed to viridin B in the presence and absence of fresh growth medium, and macromolecular synthesis was assayed. Tritiated uridine was added to each culture to a final concentration of 5 μCi/ml. After mixing, a culture was divided, and portions were added to equal volumes of viridin B in TSB, viridin B in phosphate buffer, inactivated bacteriocin in TSB, or inactivated bacteriocin in phosphate buffer. Incubation was continued with agitation, and samples were assayed for trichloroacetic acid-insoluble material as previously described (4). Viable-cell counts of identically treated _N. sicca_ cultures were performed.

In similar experiments, logarithmic- and stationary-

phase cells were harvested by centrifugation and re-
suspended in TSB/128. After addition of tritiated uridine, the cells were treated and assayed for macro-
molecular synthesis, as above.

Effects of viridin B on anaerobically incubated _N. sicca_. Viridin B or inactivated viridin B was added to an unvented anaerobic blood culture bottle containing Peptone II broth under CO₂ (Vacutainer, Becton, Dickinson & Co.). The bottles were inoculated with an overnight culture of _N. sicca_ at a ratio of 1:20. The inoculum also contained sufficient tritiated uridine to produce a final concentration of 5 μCi/ml. Controls consisted of similarly treated _N. sicca_, with the excep-
tion that the culture bottles were vented and vigor-
ously aerated. Samples were taken hourly for 5 h and assayed for trichloroacetic acid-insoluble material. Vi-

able-cell counts of similarly treated cultures lacking radioactive uridine to were performed as previously described (5).

RESULTS

Respiration. When _N. sicca_ cells were sus-

pended in phosphate buffer with 0.03 M glucose as substrate, oxygen consumption was minimal. After 2 h of incubation, viridin B-treated and control cells consumed only 49 μl and 32 μl of O₂, respectively. Under these conditions, when growth of _N. sicca_ did not occur, viridin B did not inhibit oxygen consumption. However, when TSB was added to the reaction mixtures, allowing active metabolism of _N. sicca_, exposure to viridin B caused immediate cessation of oxygen consumption (Fig. 1). Control cells consumed 700 μl of O₂ within the 1-h incubation period.

ATP production. perchloric acid extracts from viridin B-treated and control _N. sicca_ cells were assayed for ATP content. The results (Fig. 2) show that viridin B slightly enhanced ATP production for about 1 h after exposure, but subsequently repressed production compared to control cells.

Uptake and incorporation of glucose. Glucose metabolism was assayed by total uptake, incorporation into glycogen-like polymer,
INTERACTIONS OF VIRIDIN B WITH N. SICCA

and incorporation into trichloroacetic acid-insoluble material. Logarithmic-phase N. sicca cultures exposed to viridin B showed immediate cessation of total glucose uptake and of glucose incorporation into glycogen-like polymer (Fig. 3). Results with trichloroacetic acid-insoluble material were comparable.

Amino acid uptake and incorporation. It was previously shown that viridin B treatment of actively growing N. sicca prevented incorporation of an amino acid mixture into trichloroacetic acid-insoluble material (4). Such an inhibitory effect could be at the transport or macromolecular biosynthetic level. Therefore, viridin B was compared with chloramphenicol, an antibiotic known to block protein synthesis. Exposure of logarithmic-phase N. sicca cultures to chloramphenicol prevented the incorporation of a tritiated amino acid mixture into trichloroacetic acid-insoluble material, as did exposure to viridin B. Viridin B and chloramphenicol were compared also as to their capacity to inhibit total accumulation of an amino acid mixture in N. sicca. Cells were pretreated with chloramphenicol and subsequently exposed to active or inactivated viridin B (Fig. 4). Continued increase in total intracellular label occurred in the control cells (treated with chloramphenicol only), whereas addition of viridin B to chlorampheni-
E. coli-pretreated cells caused immediate cessation of such accumulation.

Effects of antibiotics on viridin B activity. When a variety of antibiotics was tested for its ability to influence viridin B-mediated inhibition, no synergistic or antagonistic effects were observed. Viridin B caused immediate cessation of macromolecular synthesis when added before, with, or after the various antibiotics. In contrast, the inhibitory effects of these antibiotics were delayed 15 to 30 min.

Degradation of macromolecules. Inhibition of macromolecular synthesis was previously demonstrated by adding viridin B to logarithmic-phase *N. sicca* cultures 15 min after adding a tritiated precursor (4). To detect any macromolecular degradation, *N. sicca* cultures were prelabeled in the presence of tritiated precursors of RNA, DNA, or protein for 2 h, and macromolecular incorporation was assayed in viridin B-treated and control cells. Viridin B did not cause significant degradation of previously formed macromolecules. In some instances there was a gradual reduction (5 to 15%) in the amount of trichloroacetic acid-insoluble material during 2 h of exposure to viridin B. However, a similar decline was seen in cells treated with antibiotics known to specifically inhibit DNA, RNA, or protein synthesis. This decline likely represents normal activity of endogenous proteases and nucleases. Failure of viridin B to cause specific degradation of macromolecules was confirmed by testing for efflux of ultraviolet-absorbing material from treated cells. No significant change in absorbancy at 260 or 280 nm occurred in supernatants of viridin B-treated cultures throughout a 3-h test period.

Susceptibility of *N. sicca* to viridin B under conditions allowing active metabolism. Previous studies in this laboratory have shown that nutrient medium must be present to demonstrate the bactericidal effect of viridin B against *N. sicca* by kill curve techniques (5). This suggested that viridin B killed only actively metabolizing *N. sicca* cultures. In the studies reported here, the effects of viridin B on uridine incorporation were compared in actively growing and nongrowing *N. sicca* cultures. Viridin B rapidly inhibited uridine incorporation by logarithmic-phase *N. sicca* cultures, whether or not fresh TSB was included in the mixtures. In contrast, uridine incorporation by stationary-phase *N. sicca* cultures was not inhibited by viridin B unless fresh medium was added to the mixtures (Fig. 5). Cells treated in this manner ceased to incorporate uridine after 15 min of exposure to the bacteriocin and were killed within 2 h. Stationary-phase cells exposed to viridin B without fresh medium remained viable and incorporated uridine throughout the test period.

In similar experiments, logarithmic- and stationary-phase *N. sicca* cultures were harvested by centrifugation, washed, and resuspended in TSB/128 before exposure to viridin B. Viridin B did not inhibit uridine incorporation or viability in cell suspensions derived from logarithmic- or stationary-phase *N. sicca* cultures, unless fresh, undiluted medium was added.

Effects of viridin B on anaerobically incubated *N. sicca*. Having observed that only actively metabolizing *N. sicca* cells were susceptible to viridin B, we decided to compare the effects of aerobic and anaerobic incubation on viridin B activity. As noted in Fig. 6, the bactericidal effect of viridin B was demonstrable only under aerobic conditions.

In similar experiments (Fig. 7), viridin B had no effect on uridine incorporation in anaerobically incubated *N. sicca*. Incorporation was similar to that in anaerobically incubated control cells. Maximal incorporation was seen in aerobically incubated cells exposed to inactivated bacteriocin. Cells aerobically exposed to viridin B showed typical inhibition of macromolecular synthesis.

**DISCUSSION**

The data presented show that optimal conditions for viridin B activity are those which allow active, oxidative metabolism by *N. sicca*. Under these conditions, cells rapidly ceased macromolecular synthesis and died within 2 h of exposure to the bacteriocin. However, stationary-phase cells, cells suspended in dilute medium, and cells
metabolizing anaerobically were unaffected by viridin B unless they resumed active, oxidative metabolism. When fresh medium was added to metabolically inactive cells, uridine incorporation was inhibited by viridin B after 15 min, suggesting that active metabolism was resumed during this interval. Other bacteriocins reported to maximally inhibit only actively metabolizing cells include bacteriocin X-14 (hemolysin) of Streptococcus faecalis subsp. zymogenes (2), megacin C (8), the bacteriocin of Clostridium perfringens strain 28 (11), streptococcin A-FF22 (15), and staphylococcin 1580 (10).

Since viridin B inhibition depended on active, oxidative metabolism, we examined the bacteriocin’s effect on respiration and ATP production. Viridin B caused immediate cessation of oxygen consumption in actively metabolizing N. sicca; however, the inhibitory effect on ATP production was delayed 1 h. It is possible that viridin B rapidly inhibits respiration by interference with membrane components (plasma and/or mesosomal) of the aerobic electron transport system (6, 7). ATP synthesis by substrate-level phosphorylation, which is localized in the cytoplasm (7), may not be immediately affected by viridin B. This possibility is in accord with the widely accepted model that many bacteriocins initiate their inhibitory effects at the plasma membrane (12, 14).

Since viridin B rapidly inhibited respiration in N. sicca, similar inhibition of any uptake mechanism coupled to aerobic electron transport would be expected. Viridin B was compared, therefore, with chloramphenicol to determine whether the bacteriocin prevents amino acid transport or blocks amino acid incorporation into protein. Chloramphenicol does not alter transport immediately, but inhibits protein synthesis by preventing peptidyl transfer on 50S ribosomal subunits. Since accumulation of amino acids continued in chloramphenicol-pre-treated cells, but was totally blocked upon exposure to viridin B, we conclude that the bacteriocin’s inhibition of protein synthesis is at the transport level. This is unlike colicin E3 which inactivates ribosomal subunits directly (3).

A number of antibiotics were compared with viridin B in their inhibition of macromolecular synthesis in actively growing N. sicca. Inhibitors of protein, nucleic acid, and cell wall syntheses did not affect incorporation as rapidly as did viridin B. Moreover, pretreatment of cells with antibiotics for 30 min did not prevent the immediate cessation of incorporation when viridin B was added. These findings suggest that viridin B inhibits RNA, DNA, and protein syntheses independently of one another. In contrast to viridin B, megacin C (8) exerted its lethal effect by inducing a potent deoxyribonuclease in sensitive cells. Prior inhibition of protein synthesis by antibiotic treatment prevented megacin C-induced degradation of DNA. In addition, our data indicate that 30-min pretreatment with the antibiotics tested does not inhibit overall cellular
metabolism to the degree that viridin B no longer affects the cells. This rapid, independent inhibition of macromolecular synthesis caused by viridin B further suggests a primary effect of the bacteriocin at the plasma membrane level. Viridin B rapidly prevented the uptake of glucose and its incorporation into glycogen-like polymer and trichloroacetic acid-insoluble material. Similar effects have been reported for staphylococcin 1580 (9) and streptococcin A-FF22 (15). This immediate effect on glucose metabolism may also be due to a primary effect on transport and/or energy transduction.

On the basis of these findings, we propose that viridin B inhibits actively growing *N. sicca* by disruption of membrane-bound components responsible for oxygen-dependent electron transport, which in turn uncouples energy transduction necessary for accumulation of precursors. Cells which are not actively metabolizing or are metabolizing anaerobically utilize other methods of energy transduction which are unaffected by viridin B.

ACKNOWLEDGMENTS

We are grateful to Peter S. Ecklund and Robert O. Bollinger for constructive suggestions throughout the conduct of this investigation and during preparation of the manuscript. The secretarial assistance of Noralee Cyplik is also gratefully acknowledged.

This investigation was supported by a grant from the Matilda Wilson Fund, Detroit, Mich.

LITERATURE CITED


