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Berberine Sulfate Blocks Adherence of *Streptococcus pyogenes* to Epithelial Cells, Fibronectin, and Hexadecane

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Berberine sulfate is an alkaloid extracted from the roots and bark of various plants and possesses antibacterial, antifungal, and antiprotozoal activities. Most studies have focused on the bacteriostatic or bactericidal activities of this compound. In this study, we report that berberine sulfate is bacteriostatic for streptococci and that sub-MICs of berberine blocked the adherence of streptococci to host cells, immobilized fibronectin, and hexadecane. Concentrations of berberine below its MIC caused an eightfold increase in release of lipoteichoic acid from the streptococci. Higher concentrations of berberine directly interfered with the adherence of streptococci to host cells either by preventing the complexing of lipoteichoic acid with fibronectin or by dissolution of such complexes once they were formed. Thus, berberine sulfate interferes with the adherence of group A streptococci by two distinct mechanisms: one by releasing the adhesin lipoteichoic acid from the streptococcal cell surface and another by directly preventing or dissolving lipoteichoic acid-fibronectin complexes.

Berberine sulfate is an extract of plants that has been used either as a crude extract or in pure form to treat trachoma (19), diarrhea (9, 14, 18, 20), amoebiasis (12, 24), and pharyngeal pyogenic infections (25). The drug thus appears to have a broad antimicrobial activity (2) and seems to be relatively nontoxic to humans (9, 18), even though it and its derivatives intercalate themselves into DNA and phospholipid bilayers (5, 13) and inhibit enzymes such as alcohol dehydrogenase and xanthine oxidase (22). These latter activities probably account for the ability of berberine sulfate to inhibit reverse transcriptase activity and thereby to inhibit the replication of certain retroviruses (22).

The efficacy of berberine sulfate in the treatment of certain bacterial diseases appears to be primarily dependent upon its direct antibacterial effects. In some cases, however, berberine appears to be efficacious even though high concentrations of the drug have no effect on bacterial growth. The therapeutic effect of berberine sulfate against enterotoxigenic *Escherichia coli* was suggested to be due to its antisecretory effects on the host rather than any effect on the invading organisms (18, 20).

In most studies of the anti-infectious activities of berberine sulfate, the focus has been on its antimicrobial effects; little or no attention has been paid to the effects of berberine on the adherence of bacteria to host cells. Since adherence of an organism to mucosal surfaces usually precedes invasion, some of the beneficial properties of berberine could be due to interference with the adherence process. The present study was undertaken to determine the effects of berberine sulfate on the adhesive properties of *Streptococcus pyogenes*. These microorganisms adhere to epithelial cells (3, 4), fibronectin (6, 23), and hexadecane (7, 17); in each case, lipoteichoic acid (LTA) has been found to be the major streptococcal surface molecule involved in adherence. In this report, we present data to show that berberine sulfate inhibits the attachment of streptococci to host cells, fibronectin, and hexadecane by causing release of LTA from the streptococcal cells or by interfering directly with the formation of complexes between streptococcal surface LTA and host cell surface fibronectin.

MATERIALS AND METHODS

Reagents. Berberine sulfate was obtained from Shanghai Chemical Co., Shanghai, China. Hyaluronidase was purchased from Sigma Chemical Co., St. Louis, Mo. Peroxidase-labeled goat anti-rabbit immunoglobulin G was obtained from Cooper Biomedical Inc., Malvern, Pa. Fibronectin was purified from human plasma by affinity chromatography over gelatin-Sepharose as described by Engvall and Ruoslahti (11).

Preparation of antisera. Rabbit antifibronectin was prepared as previously described (8). Rabbit anti-LTA was prepared as described elsewhere (6). Antibodies to *S. pyogenes* type M5 were prepared in New Zealand White rabbits by intracutaneous injection of 10^9 heat-killed organisms in Freund incomplete adjuvant behind the neck. A second injection of 10^9 heat-killed organisms in phosphate-buffered saline (PBS) was given 4 weeks later intraperitoneally. Sera were collected every 2 weeks and stored at 4°C.

Determination of the MIC of berberine sulfate. *S. pyogenes* type M5 was subcultured 1:100 and grown at 37°C in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) containing various concentrations of berberine sulfate. At indicated times the A_{530} was recorded. The lowest dilution of berberine that completely inhibited growth was considered to be the MIC.

Assays for the effect of sub-MICs of berberine sulfate on the interaction of streptococci with epithelial cells, immobilized fibronectin, and hexadecane. *S. pyogenes* type M5 was grown for 18 h at 37°C in Todd-Hewitt broth containing 0 to 20 μg of berberine per ml. The bacteria grown in each concentration of berberine were washed and suspended in PBS (0.02 M PO_4, 0.15 M NaCl [pH 7.3]) to an identical A_{530}. Buccal epithelial cells were obtained by scraping the buccal mucosa with a cotton swab and vigorously stirring the swab in PBS. The cells were washed and suspended in PBS to obtain 10^5 cells per ml. Streptococci were added to obtain an A_{530} of 0.4. The mixtures were rotated for 30 min at the ambient

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temperature. Nonadherent bacteria were removed by differential centrifugation, and adherent bacteria were counted under light microscopy. Adherence of streptococci to hexadecane was determined as previously described (7, 17).

Streptococci grown in sub-MICs of berberine were tested for their ability to interact with immobilized fibronectin by utilizing an enzyme-linked immunoassay. Purified human plasma fibronectin was immobilized onto microdilution plates (U16, high binding, microwell module; Nunc, Roskilde, Denmark) by adding 100 μl of fibronectin (100 μg/ml in 0.15 M NaCl [pH 9.5]) to each well and incubating for 1 h at 37°C. The plates were washed with PBS, and unoccupied plastic surfaces were blocked by adding bovine serum albumin (50 mg/ml in PBS) to each well and incubating for 1 h at 37°C. Streptococci were grown in sub-MICs of berberine and prepared as described above and adjusted to an A_{530} of 0.4. The streptococci (100 μl) were added to wells containing immobilized fibronectin and rotated horizontally for 30 min at ambient temperature. The wells were then washed five times with PBS, and a 1:400 dilution of rabbit anti-*S. pyogenes* type M5 serum was added to the wells and incubated for 30 min at 37°C. The wells were washed with PBS, and a 1:1,000 dilution of peroxidase-labeled goat anti-rabbit immunoglobulin G (Cooper Biomedical Inc., Malvern, Pa.) was added to the wells. The plates were incubated for 30 min at 37°C and then washed with PBS. 5-Aminosalicylic acid (100 μl) was added to all wells, and after 15 to 30 min A_{450} was measured with a Dynatech microtiter plate reader. The percentage of adherence was calculated as follows: percent adherence = (A_{450} of test well/A_{450} of control well) × 100. Controls consisted of wells without immobilized fibronectin or without the first antibody. These values were essentially the same as blank wells.

Assays for the effect of berberine sulfate in the reaction mixture on the interaction of streptococci with epithelial cells, hexadecane, and insoluble fibronectin. Type M5 *S. pyogenes* were grown in Todd-Hewitt broth for 18 h at 37°C. The bacteria were washed, suspended in PBS, and adjusted to an A_{530} of 0.4 in the indicated amounts of berberine sulfate. Assays for the adherence to epithelial cells, hexadecane, and insoluble fibronectin were performed as described above.

Assays for the binding of soluble fibronectin to streptococci. The effect of berberine on the binding of soluble fibronectin to streptococci was determined by using an enzyme-linked immunoassay. Streptococci were grown in Todd-Hewitt broth for 18 h at 37°C and washed in PBS. Streptococci (250 μl, A_{530} of 1.0) were added to 250 μl of fibronectin (10 μg/ml) and 500 μl of dilutions of berberine sulfate. The mixtures were rotated for 30 min at the ambient temperature and washed three times with PBS. A 1:400 dilution of rabbit antifibronectin was added, and the tubes were incubated for 30 min at 37°C. The bacteria were washed three times with PBS, and a 1:1,000 dilution of peroxidase-labeled goat anti-rabbit immunoglobulin G was added. The mixtures were incubated for 30 min at 37°C and washed in PBS. The bacterial pellet was suspended in 100 μl of water and transferred to new tubes, and 1 ml of 5-aminosalicylic acid solution was added to each tube. After 15 to 30 min the A_{450} of each tube was recorded. The percentage of binding was determined as follows: percent binding = (A_{450} of test substance/A_{450} of PBS control) × 100. Controls consisted of tubes without bacteria and of tubes with normal rabbit serum instead of antifibronectin.

Hemagglutination assay for the release of LTA. The ability of berberine to induce the release of LTA was determined by a passive hemagglutination assay as previously described (4). Streptococci were grown in Todd-Hewitt broth containing 0 to 10 μg of berberine per ml. The bacteria were sedimented by centrifugation, and the supernatants were removed and tested for hemagglutinating activity.

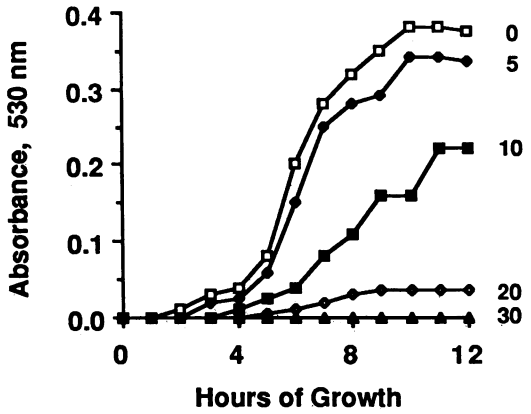

FIG. 1. MIC of berberine. *S. pyogenes* was grown in Todd-Hewitt broth containing the indicated concentrations of berberine (right side of figure, in micrograms per milliliter). At timed intervals the A_{530} was measured.

RESULTS

MIC of berberine sulfate. The MIC of berberine for *S. pyogenes* was 30 μg/ml (Fig. 1). Berberine was found to be bacteriostatic for streptococci; after removal of berberine the bacteria resumed normal growth. Amin et al. (2) found a slightly lower MIC (12.5 μg/ml) for group A streptococci.

Effect of berberine on adherence of streptococci to epithelial cells, fibronectin, and hexadecane. Streptococci grown in sub-MICs of berberine lost their ability to adhere to epithelial cells, immobilized fibronectin, and hexadecane (Fig. 2). In each case, the decrease in adherence was dependent on the concentration of berberine.

Since the hyaluronic acid capsule of group A streptococci is produced during the log phase of growth and prevents adherence (17), it is possible that the effects of berberine on adherence might be due to a prolonged expression of the capsule. To examine this possibility, the bacteria were treated with hyaluronidase and then assayed for their adherence to immobilized fibronectin. Hyaluronidase treatment only partially restored the adhesive capabilities of streptococci (Fig. 3), suggesting that berberine, in addition to prolonging the presence of capsule on the organism, was affecting adherence by another mechanism as well.

Berberine-induced release of LTA from streptococci. Previous work has shown that sub-MICs of penicillin can induce an increase in release of LTA from streptococci (1, 16). Therefore, berberine sulfate was assayed for its ability to cause an increase in the release of LTA from streptococci. Supernatants from bacteria grown in sub-MICs of berberine caused at least an eightfold increase in the release of LTA. LTA hemagglutination titers in supernatants from cultures grown in the following concentrations of berberine sulfate were as follows: no berberine, 1:40; 5 μg/ml, 1:80; 10 μg/ml, 1:160; 20 μg/ml, 1:320. However, in contrast to penicillin, berberine did not cause any increase in the release of LTA from organisms in the resting phase (data not shown), suggesting that the mode of action of berberine sulfate may be different than that of penicillin. Since LTA is the major adhesin involved in the adherence of streptococci to epithe-

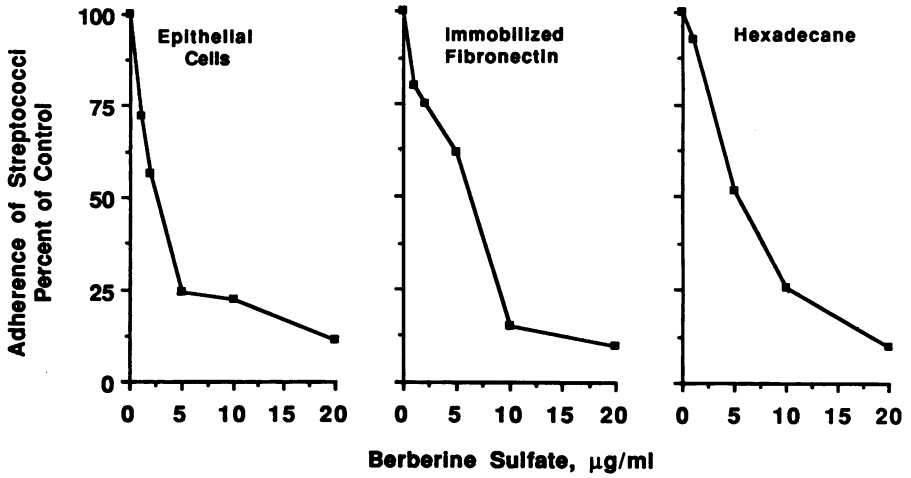

FIG. 2. Effect of sub-MICs of berberine on the adherence of streptococci to epithelial cells, fibronectin, and hexadecane. *S. pyogenes* was grown in sub-MICs of berberine, washed in PBS, and assayed for the ability to adhere to the above surfaces as described in Materials and Methods.

lial cells (3, 4), fibronectin (6, 23), and hexadecane (7, 15), these data indicate that berberine can also affect the adherence of streptococci by causing loss of LTA from the cell surface.

Direct effects of berberine on the adherence of streptococci to epithelial cells. The adherence of streptococci to epithelial cells is considered to be essentially a hydrophobic one mediated by the interaction of the lipid moiety of LTA with fibronectin on host cells (3). Since berberine is a hydrophobic molecule, the possibility that berberine might directly interfere with the adherence of streptococci to epithelial cells was investigated. Berberine inhibited the adherence of streptococci in a dose-dependent fashion (Fig. 4). Since berberine did not cause release of LTA from bacteria in the resting phase, these data suggest that berberine directly interferes with the interaction of LTA on the surface of streptococci with fibronectin exposed on epithelial cells.

Inhibition of fibronectin-streptococcal interactions by berberine. To determine whether berberine directly interferes with the interaction of streptococci with fibronectin, streptococci were incubated with fibronectin in the presence of increasing concentrations of berberine. Berberine inhibited the binding in a concentration-dependent manner (Fig. 5). Furthermore, berberine was able to elute fibronectin already bound to the streptococci. The data suggest that high concentrations of berberine can directly prevent complexes of LTA and fibronectin from forming, and that berberine can dissolve such complexes once they are formed.

DISCUSSION

Recent studies have shown that certain antimicrobial agents can block the adherence of microorganisms to host cells at doses much lower than those needed to kill cells or to inhibit cell growth. In some cases, this is due to an alteration or suppression of the bacterial adhesin (ligand on the bacterial surface that mediates adherence) (10, 21). In other cases, it is due to a loss of the adhesin from the cell surface (1). Thus, strategies that interrupt the adhesive functions of bacteria before host tissue invasion occurs may be an effective prophylactic approach against bacterial infectious diseases.

In this study, group A streptococci were chosen as a paradigm for studying the effects of berberine sulfate on the

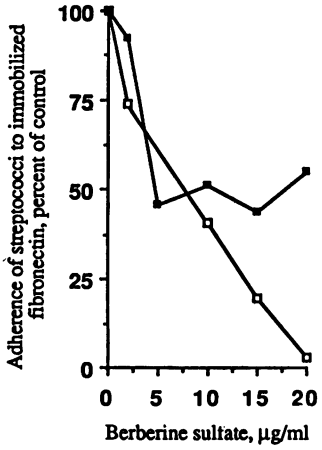

FIG. 3. Effect of hyaluronidase on the adherence of streptococci grown in sub-MICs of berberine to immobilized fibronectin. Symbols: □, PBS control; ■, hyaluronidase-treated bacteria.

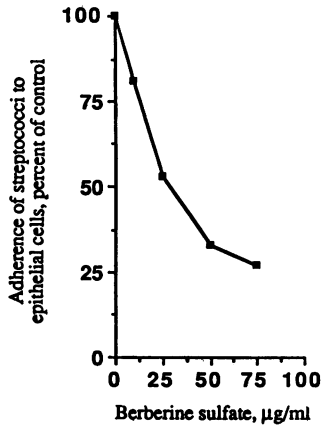

FIG. 4. Direct effects of berberine on streptococcal adherence to epithelial cells. Various concentrations of berberine were added to suspensions of streptococci and epithelial cells, and adherence was assayed as described in Materials and Methods.

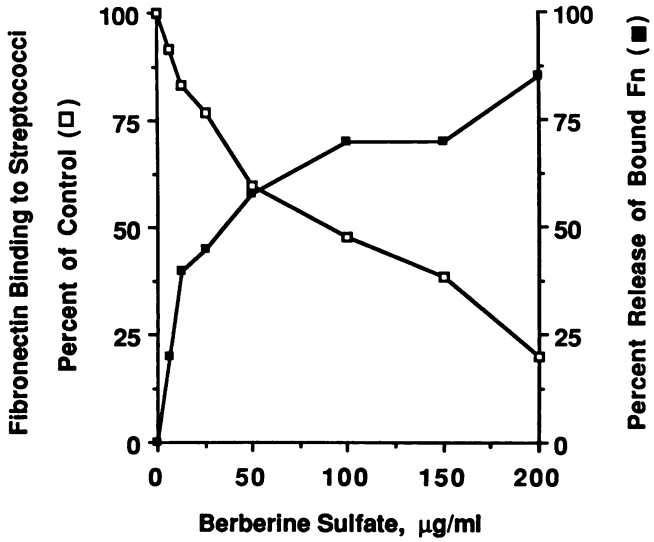

FIG. 5. Effect of berberine on the interaction of soluble fibronectin (Fn) with streptococci. Berberine was tested for its effect on binding of fibronectin to streptococci (□) and its ability to elute fibronectin bound to streptococci (■).

adherence of bacteria, since much is known about the adherence of streptococci to host cells. LTA has been identified as an adhesin, and fibronectin has been identified as a host cell receptor involved in the adherence of streptococci to host cells (reviewed in reference 3). In addition, the hydrophobic surface properties (as measured by adherence to hexadecane) of group A streptococci are due to LTA. Therefore, the effect of berberine sulfate on adherence of streptococci to epithelial cells, fibronectin, and hexadecane was examined. The results suggest that berberine might interfere with the adhesive properties of group A streptococci by several modes of action.

Berberine sulfate at concentrations of ≥30 μg/ml completely inhibited growth of streptococci. However, berberine was not bactericidal, since the streptococci resumed normal growth upon removal of berberine. The bacteriostatic effect of berberine is probably due to its ability to interact with nucleic acids. Amin et al. (2) found that bacteriostatic concentrations of berberine rapidly suppressed synthesis of RNA and proteins in *Staphylococcus aureus* but had little or no effect on DNA synthesis.

Sub-MICs of berberine sulfate prevented the adherence of streptococci to host cells, fibronectin, and hexadecane. An explanation for this effect comes from the observation that berberine caused an eightfold increase in release of LTA. Since LTA is the major ligand responsible for the adherence of streptococci to epithelial cells, fibronectin, and hexadecane, loss of LTA would reduce the capacity of streptococci to adhere to these surfaces.

In addition, berberine sulfate directly interferes with the adhesive function of group A streptococci. This conclusion is supported by the observation that, although berberine does not cause release of LTA from stationary-phase streptococci, it can block adherence of these organisms when added to the assay mixture. In addition, berberine blocked the binding of fibronectin to stationary-phase streptococci and was able to elute fibronectin already bound to these organisms. Thus, berberine sulfate may interfere with infections due to group A streptococci not only by inhibiting streptococcal growth but also by blocking adherence of these organisms to host cells. Although berberine sulfate has been used to treat pharyngeal pyogenic infections, further clinical studies are needed to determine the efficacy of treatment with berberine sulfate.

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