Action of Diethylstilbestrol on Staphylococci: Further Observations on the Effect of Resting Cells

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Diethylstilbestrol (DS) has been shown to be active against staphylococci and other gram-positive bacteria but not against gram-negative microorganisms. The present study extends these findings. Standardized suspensions of 14C-labeled Staphylococcus aureus serotypes III and IV and Shigella flexneri were prepared and exposed to pharmacological concentrations of DS (1 to 20 µg/ml) under diverse environmental conditions; the cells were removed by membrane filtration and the presence of radioactive substances in release to the supernatant fraction was followed by standard radioisotopic techniques. Controls were exposed similarly to the hormone vehicle alone (buffer containing 2% ethyl alcohol). DS at bactericidal concentrations above 6 µg/ml caused significant leakage of cellular radioactivity of S. aureus labeled with 14C-glucose and 14C-glutamic acid within 1 to 4 hr after exposure to DS. Maximum leakage of radioactivity occurred under anaerobic conditions at 37 C. Absorption studies of 14C-labeled DS indicated that the affinity of S. flexneri for DS is markedly less than that of S. aureus. This might be one reason for the resistance of gram-negative bacteria to DS.

The synthetic crystalline compound diethylstilbestrol (4,4'-dihydroxy-alpha, beta-diethylstilbene; DS) employed for replacement therapy in estrogen deficiency and other disorders is toxic toward a variety of microorganisms. Previous work has demonstrated that diethylstilbestrol in pharmacological concentrations exerted an inhibitory effect on the growth of all of the nine gram-positive microorganisms but did not influence any of the seven gram-negative bacteria tested. Metabolic experiments have indicated that diethylstilbestrol inhibited the utilization of tryptic soy broth, pyruvate, glucose, and succinate by staphylococci (10). In addition, in vivo studies have shown that a single cutaneous injection of 15 µg of diethylstilbestrol into rabbits was sufficient to suppress the growth of staphylococci in the sera of these animals. Staphylococcal skin lesions were also retarded by diethylstilbestrol in the treated rabbits as compared to control rabbits (11).

The present paper attempts to determine whether the inhibitory action of DS on staphylococci involves the function of the staphylococcal membrane. Accordingly, special attention was given to the leakage and uptake of compounds from resting cell suspensions of staphylococci exposed to DS.

MATERIALS AND METHODS

Bacteria. Staphylococcus aureus serotypes III and IV were obtained from the American Type Culture Collection (ATCC 12600 and 12601). They were tested for glucose and mannitol utilization under anaerobic conditions by the method of Baird-Parker (1) and for gelatin liquefaction, pigmentation, hemolysis, and both free and bound coagulase by accepted microbiological methods (8). These strains were kept in stock at 4 C on Tryptic soy agar (Difco) slants in screw-capped tubes and transferred every 4 weeks. Each transfer was followed by coagulase titration and tests for anaerobic glucose and mannitol utilization. Shigella flexneri was obtained from stock culture collections maintained at the Stritch School of Medicine and had been originally purchased from the American Type Culture Collection.

Preparation of 14C-labeled cells and determination of cellular leakage. The microorganisms were grown in Brain Heart Infusion broth on a rotary shaker for 6 hr at 37 C, harvested by centrifugation at 3,500 × g, and suspended in a synthetic medium composed of 20 ml of Eagle's nonessential amino acid solution, 20 ml of Eagle's essential amino acid solution, 10 ml of a 10% glucose solution, and 950 ml of salt solution which contained 3% NaCl, 0.02% KCl, 0.01% MgSO4·7H2O, 0.15% KH2PO4, 0.4% Na2HPO4, 0.001% thiamine hydrochloride, and 0.001% nicotinamide. To 100 ml of this medium, 2.2 × 106 counts/min of uniformly labeled 14C-glucose or 14C-glutamic acid was added and the cells were incubated for 1 hr at 37 C on a rotary shaker. The 14C-labeled cells were har-
vested by centrifugation, washed three times with 0.2 M phosphate buffer (pH 7.0), and resuspended in the same buffer at a concentration of 0.3 to 0.4 mg (dry weight) of cells per ml. A 49-ml amount of the 14C-labeled standardized cell suspension was added to each sterile flask under study. A 1-ml amount of 95% ethyl alcohol containing 0 to 20 μg/ml of DS was added in the appropriate flasks, and the flasks were incubated under various environmental conditions. Duplicate 5-ml samples were removed at various time intervals. The cells were harvested by centrifugation at 5,000 × g for 15 min, and the supernatant fluid was assayed for radioactivity. Controls included cells exposed to 2% ethyl alcohol. A 1-ml amount of the supernatant fluid was added to 9 ml of scintillation fluid in a low-potassium vial. The scintillation medium contained 900 ml of p-dioxane, 150 ml of anisole, 150 ml of 1,2-dimethylethane, 18 g of 2,5-diphenyl-oxazole, and 60 mg of 1,4-bis-(5-phenyloxazolyl)-benzene (3). Radioactivity was determined in a model 3320 Tri-Carb scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). The efficiency of counting of the liquid scintillation spectrometer for 14C was approximately 85%. Each experiment was repeated six to nine times; the mean value of these trials was recorded and the reproducibility of the values was in a range between ± 5 and 12% of the mean.

**Determination of binding of DS to bacterial cells.**

Binding of 14C-labeled DS by *S. aureus* and *S. flexneri* was determined by adding 3.0 × 10^8 counts per min of labeled DS per ml to bacterial suspensions containing 3.8 × 10^10 to 5 × 10^10 colony-forming units per ml in 0.2 M phosphate buffer (pH 7.0) at 37°C. Immediately after mixing and at subsequent 15-min intervals, 5-ml portions were obtained, the cells were removed, either by centrifugation at 5,000 × g for 10 min or by filtration through a type HA membrane (Millipore Corp., Bedford, Mass.), and the supernatants or filtrates were assayed for radioactivity in a Packard model 3320 Tri-Carb scintillation spectrometer.

**Chemicals.** Uniformly labeled D-14C-glucose, uniformly labeled L-14C-glutamic acid, and 14C-DS were obtained from the New England Nuclear Corp., Boston, Mass. DS was dissolved in absolute ethyl alcohol and was added to bacterial cells at a final concentration of 2%. Controls received 2% ethyl alcohol only. In the concentrations used (2%), ethyl alcohol produced a slight stimulation of growth and had no effect on other processes tested.

**RESULTS**

**Dose and time responses.** The possibility that the inhibition of the growth of staphylococci by DS involved changes in cellular permeability was considered, and, to this end, the effect of concentration and time of exposure of staphylococci to DS upon the leakage of radioactive substances from cells labeled with 14C-glucose was assayed by the technique previously described. Figure 1 shows a plot of the mean values, calculated from seven consecutive experiments, of radioactivity released from staphylococci exposed aerobically at 37°C to various concentrations of DS. Concentrations of 5 to 20 μg/ml of DS caused progressively increased leakage of radioactive substances from labeled staphylococci. At DS concentrations ranging from 0.5 to 2.5 μg/ml cell, release of radioactivity remained unaffected.

**FIG. 1. Course of release of 14C-labeled cell constituents from Staphylococcus aureus [0.3 to 0.4 mg (dry weight) of cells per ml] tagged with uniformly labeled 14C-glucose and incubated aerobically at 37°C. The amount of radioactivity in counts per minute appearing in the supernatant fluid is depicted for 5, 10, and 20 μg/ml of diethylstilbestrol.**

**FIG. 2. Leakage of 14C-labeled materials from Staphylococcus aureus suspensions treated with DS and tagged with uniformly labeled 14C-L-glutamic acid. Conditions as in Fig. 1.**
The release of radioactive materials from staphylococcal cells progressed linearly for 2 to 3 hr and then it reached a plateau. Since glucose is readily metabolized and staphylococci do not accumulate large amounts of this compound in their intracellular pools, an assessment of the leakage of radioactivity from staphylococci labeled with \(^{14}\text{C}\)-glutamic acid exposed aerobically at 37 C to various concentrations of DS was made. The results (Fig. 2) showed, for the first 30 min, an immediate and rapid increase in the rate of release of radioactivity from staphylococci labeled with \(^{14}\text{C}\)-glutamic acid after addition of DS. This was followed by a much slower increase from 30 to 60 min and, thereafter, it remained constant. The rapid initial release of radioactivity from labeled staphylococci was also found to depend on the concentration of DS added to the suspension medium, the saturating concentration for the system being approximately 20 \(\mu\text{g}\) of DS per ml. When \(S.\) \textit{flexneri} was labeled with \(^{14}\text{C}\)-glutamic acid or \(^{14}\text{C}\)-glucose and then exposed to DS, the leakage of radioactivity from the cells was similar to the controls.

**Fig. 3.** Effect of anaerobiosis on the release of \(^{14}\text{C}\)-labeled cell constituents from \(S.\) \textit{aureus} \([0.3 \text{ to } 0.4 \text{ mg (dry weight) of cells per ml}]\) tagged with uniformly labeled \(^{14}\text{C}\)-glucose and incubated statically on a shaker and in a Thelco anaerobic incubator at 37 C. The amount of radioactivity appearing in the supernatant fraction is plotted against time for cells exposed to 20 \(\mu\text{g/ml}\) of diethylstilbestrol.

**Fig. 4.** Absorption of \(^{14}\text{C}\)-diethylstilbestrol by \(S.\) \textit{aureus} and \(S.\) \textit{flexneri} plotted as a function of radioactivity of \(^{14}\text{C}\)-diethylstilbestrol in the supernatant fluid of cell suspensions \((3.8 \times 10^8 \text{ to } 3 \times 10^8 \text{ colony-forming units per ml})\) treated aerobically at 37 C with \(^{14}\text{C}\)-diethylstilbestrol and then separated by either membrane filtration or centrifugation.
Effect of oxygen. Aerobically and in tryptic soy broth, a concentration of DS of 6 to 7.2 \( \mu g/ml \) was required to produce complete inhibition of the growth of \( S. aureus \), whereas anaerobically a concentration of DS of 1.6 to 2.8 \( \mu g/ml \) was needed to achieve the same effect (10). It was of interest to determine whether an enhancement in the leakage of radioactivity from cells exposed to DS could be obtained by anaerobiosis. Cells labeled with uniformly labeled \( \alpha \)-glucose were prepared for exposure to 10 \( \mu g/ml \) of DS in the usual manner; however, incubation at \( 37 \) C was performed under anaerobic conditions in a precision Thelco anaerobic incubator, as well as aerobically on a rotary shaker and statically. The data obtained from a series of 10 determinations employing \( S. aureus \) serotypes III and IV (Fig. 3) demonstrate that leakage of \( ^{14}C \)-labeled materials from staphylococci after exposure to DS under anaerobic conditions was three to four times higher and proceeded more quickly than under aerobic conditions. The finding that cell suspensions of \( ^{14}C \)-labeled staphylococci incubated on a rotary shaker and exposed to DS released more radioactivity than the ones incubated statically was unexpected, and it may be due to the prevention of cells from sedimentation during incubation and, thus, from efficient contact with DS.

Absorption of DS by DS-sensitive and DS-resistant cells. Previous work in this laboratory showed that DS in pharmacological concentrations inhibited the growth of staphylococci and other gram-positive bacteria but failed to inhibit any gram-negative microorganisms tested. Since it is reasonable to assume that part of the antimicrobial activity of DS might involve its uptake by the bacterial cell, experiments were performed to test this possibility by the technique previously described. With the membrane filtration technique, the absorption increased for the first 30 min and then gradually decreased and remained constant after 60 min (Fig. 4). The absorption pattern was similar whether the staphylococci were suspended in 0.2 M phosphate buffer (pH 7.0) or in the synthetic culture medium previously described. When binding of \( ^{14}C \)-labeled DS was studied by the centrifugation assay procedure, absorption of DS was greatly reduced; it increased during the first 30 min and then remained constant. It is concluded by this experiment that DS was adsorbed onto \( S. aureus \) but was loosely bound on the staphylococcal cell surface and thus easily detached by centrifugation; for this purpose, membrane filtration technique is recommended. In comparison to \( S. aureus \), with \( S. flexneri \), which is known to be resistant to the action of DS, the uptake of \( ^{14}C \)-DS was extremely low regardless of the assay method employed.

DISCUSSION

The data presented above provide evidence that the antibacterial action of DS on \( S. aureus \) involves alteration in cellular permeability, resulting in the release of substances from the staphylococcal cell. There are reasons for supporting the view that permeability effects are in some way connected with the influence on growth and viability of staphylococci. They occur at as low concentrations of DS as do bacteriostatic and bactericidal effects. Equivalent responses are easily produced in other gram-positive bacteria, such as \( Staphylococcus epidermidis \), but no influence is observed upon the nonsusceptible organisms such as \( S. flexneri \) or \( Escherichia coli \) in similar experiments. The initial and final rates of absorption of DS by \( S. flexneri \) were approximately similar. It is clear then from the relative rates of absorption that the affinity of \( S. flexneri \) for DS is markedly less than that of \( S. aureus \). This might be one reason for the resistance of \( S. flexneri \) and possibly other gram-negative bacteria to DS. This finding is consistent with the action of other antimicrobial agents such as polymyxins (5), detergents (2), phenols (4), synthetic azasteroids (9), and quaternary ammonium compounds (6), where it has been shown that an important feature of the antimicrobial action of these compounds was their absorption only onto the sensitive microorganisms.

The results on cellular leakage are in accord with the experiments of Smith and Shay (7). They found that synthetic steroids caused lysis of \( Sarcina lutea \) protoplasts by direct damage to the cell membrane. Because the \( Sarcina lutea \) cells were not changed morphologically, they suggested that loss of cell viability might be due to membrane damage, followed by loss of cytoplasmic constituents. Be that as it may, it is quite possible that the overall effectiveness of DS may well be the result of several parameters which cause various effects detrimental to bacterial cells. Inhibition of staphylococcal growth may not be due entirely to leakage of cellular components. The fact that leakage does occur demonstrates that DS can affect the staphylococcal membrane. The inhibition of other metabolic processes such as the suppression of the oxidation of glucose and other substrates (10) may well contribute to the growth inhibition and loss of viability.
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