Interaction of Staphylococcal Alpha Toxin and the Estrogenic Hormone Diethylstilbestrol

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Previous work in this laboratory showed that diethylstilbestrol was capable of suppressing induced furunculosis in rabbits. The present study indicates that the synthetic estrogenic hormone diethylstilbestrol which is used for acne, estrogen deficiency, cancer, and other disorders, can reduce the cytolytic action of staphylococcal alpha toxin. The cytotoxic action of purified alpha toxin for tissue cultures was evaluated by use of such parameters as total and viable cell counts, glucose, and protein determination, and cytopathic effects (CPE) in the presence and absence of steroids. To 3-day-old primary rabbit baby kidney tissue cultures, 1 to 5 μg of diethylstilbestrol per ml was added; growth of tissue cultures in Eagles medium was continued till the 6th day, and then one tissue cytotoxic dose per milliliter of alpha toxin was added, and the subsequent fate of tissue cultures was assayed. Such cultures yielded higher total and viable cell counts, utilized more glucose, and contained more protein than the control cultures. In control cultures, CPE was observed on the 3rd hr after the addition of alpha toxin, and it was complete in 24 hr, whereas in tissue cultures treated with diethylstilbestrol, the CPE was significantly reduced. The data presented in this study made possible the availability of a suppressor of the cytolytic action of alpha toxin and might be useful in assaying the action of alpha toxin in an in vitro inexpensive test system.

In the rabbit, staphylococcal alpha toxin produces extensive dermal necrosis when given intradermally, renal cortical necrosis when given intravenously in sublethal doses, and rapidly fatal circulatory collapse when given intravenously in a dose of approximately 5 to 50 μg (5, 23). The local and general changes appear to be mediated both by the cytolytic action of staphylococcal alpha toxin on the cell membrane (1, 10) and by the liberation of serotonin and histamine from blood and tissue stores (21). In the course of experiments on the effect of gonadal steroids on staphylococcal infection, it was noted that these hormones, in pharmacological concentrations, were capable of suppressing induced staphylococcal infection in rabbits and mice (26–30). This observation raised the question whether the synthetic estrogen diethylstilbestrol, employed in estrogen deficiency, has any influence on the cytotoxic action of alpha toxin. Therefore, the ability of diethylstilbestrol to suppress induced furunculosis (26) was investigated further by comparing the cytotoxic action of staphylococcal alpha toxin in the presence and absence of diethylstilbestrol.

MATERIALS AND METHODS

Cell counts. Cell viability was determined by the trypan blue exclusion method (18, 19). Cell suspension (1 ml) was added to 0.05 ml of a 1% water solution of trypan blue. After 10 min, the viable (unstained) and dead (stained blue) cells were counted in a standard hemacytometer chamber. The percentage of viability was determined by dividing the total number of viable cells by the total number of viable plus dead cells, times 100. In view of the findings of Berkson et al. (3) and others concerning the errors inherent in the hemacytometer method of cell counting, the chambers were always loaded with identical bore pipettes and at a reasonable approximation of the same speed. Moreover, multiple repeat counts were made to establish good reproducibility of the results.

Estimation of glucose and protein. Glucose levels of the Eagles tissue culture medium were determined by the anthrone-sulfuric acid method (19). The protein content of the rabbit kidney tissue cells were assayed by the Lowry method (13) as modified for tissue culture by Oyama and Eagle (17), by using crystalline bovine albumin as standard.

Tissue cultures. Primary rabbit kidney tissue cultures were prepared by removing aseptically the kidneys of 1- to 2-week-old rabbits and processing them according to the method of Youngner (31). The cells were
grown in Eagles medium M 199 (Grand Island Biological Co.) supplemented with 10% gamma globulin-free calf serum, 1% lactalbumin hydrolysate, 100 immunizing unit (IU) of penicillin, and 100 μg of streptomycin sulfate per ml. The pH of Eagles medium was adjusted to 7.2 with 7.5% solution of sodium bicarbonate and 1 ml was dispensed in tissue culture tubes (18 by 150 mm). A 1-ml volume of suspension (18,000 to 21,000 cells per ml) was used to seed each tube. The tubes were incubated statically at 37°C. On the third day when the cells were in the logarithmic phase of growth, the old tissue culture medium was replaced with fresh medium, which was brought separately to the desired temperature of incubation and contained either 1 to 5 μg/ml of crystalline diethylstilbestrol (Sigma Chemical Company, St. Louis, Mo.) dissolved in absolute ethyl alcohol (0.5 to 1% in the final medium) or, in the case of the controls, the hormone vehicle alone. The cultures were incubated again for 3 to 4 days; the nutrient medium was replaced with maintenance medium, consisting of 97% Eagles medium, 2% gamma globulin-free calf serum, 1% lactalbumin hydrolysate, 100 IU of penicillin, and 100 μg of streptomycin sulfate per ml; one tissue cytotoxic dose of staphylococcal alpha toxin was added, and then the fate of tissue cultures was followed.

**Photography.** Photomicrographs were taken with a Zeiss photomicroscope with an automatic exposure device. Kodak, high-speed, type M-135-20 panchromatic film was used at 100 × magnification.

**Statistical analysis.** The determination of the probability of significant change in the means (P) was determined by the method described by Batson (2).

**Alpha toxin.** The method of production, purification, and assay of alpha toxin from *Staphylococcus aureus* strain Wood 46 was that of Bernheimer and Schwartz (4). The specific activities of stage 5 toxin were approximately 20 minimal lethal doses, 200 derrmonecrotic doses, 20 tissue cytotoxic doses, and 300 hemolytic units per mg of protein. The above mentioned biological activities of alpha toxin were destroyed by heating the alpha toxin at 60°C for 30 min at pH 7.0. Staphylococcus alpha toxin of approximately 800 units per ml was kindly supplied by M. Sterne of the Wellcome Research Laboratories, Beckenham, England. Two units of antitoxin neutralized 1 μg of alpha toxin.

**RESULTS**

**Effect of diethylstilbestrol on viable and total cell counts.** Studies on cytopathology are useful in following changes in cell morphology under diverse conditions, however, they do not provide an accurate description of the total number of cells or do not indicate the proportion of cells in a tissue culture that are viable. To this end, determinations of cell viability and total cell counts were made by the method previously described. The results in Fig. 1 indicate that, 24 hr after the addition of alpha toxin, the total cell count in the tissue culture tubes treated with alpha toxin dropped quickly to a level of 6.1 × 10^6 cells per ml, whereas the total number of cells in tissue culture tubes containing 5 μg of diethylstilbestrol per ml reached a level of 2.0 × 10^6 cells per ml. A P value of 0.001 was obtained, indicating that the results were statistically significant. When a concentration of 2.5 μg of diethylstilbestrol per ml was employed, the total number of cells in the tissue culture tubes was 2.3 × 10^6 per ml and yielded P values of 0.05.

Figure 2 indicates the percentage of viable cells in control, alpha toxin treated, and tissue cultures treated with diethylstilbestrol and alpha toxin. Twenty-four hour after the addition of alpha toxin, there were 4% viable cells in the tissue cultures, whereas 18, 27, and 66% of the cells remained viable in tissue cultures which had been treated with 1, 2.5, and 5 μg of diethylstilbestrol per ml. The viability of control tissue cultures was 80%, indicating that 82.5% of kidney cells treated with 5 μg of diethylstilbestrol per ml re-
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Fig. 2. Effect of diethylstilbestrol (DS) on viable cell counts of rabbit kidney cells treated with alpha toxin under conditions described in the legend to Fig. 1.

Fig. 3. Effect of diethylstilbestrol (DS) on the protein content of rabbit kidney cells treated with alpha toxin under conditions described in the legend to Fig. 1.

remained viable after 24 hr of exposure to staphylococcal alpha toxin.

Assays of glucose and protein. Since metabolic activity can be used as a criterion of cellular function, an attempt was made to perform protein analysis of the cells remaining in the monolayer diethylstilbestrol-alpha toxin-treated tissue cultures to ascertain whether the protein content of the diethylstilbestrol-alpha toxin-treated kidney cells was correlated with the cell number. Figure 3 illustrates the protein profile of tissue culture cells exposed to various concentrations of diethylstilbestrol and one tissue cytotoxic dose of alpha toxin. If for correlation purposes the 24 hr period after the addition of alpha toxin is employed, it can be seen that the level of cellular protein was approximately 200 µg/ml for the alpha toxin-treated cells, 245 µg/ml for cells treated with toxin plus 2.5 µg of diethylstilbestrol per ml, 305 µg/ml for cells treated with alpha toxin plus 5 µg of diethylstilbestrol per ml and 340 µg/ml for the controls. Finally, the utilization of glucose from the maintenance medium was determined under conditions described in Materials and Methods. The results in Figure 4 indicate that, 24 hr after the addition of alpha toxin, the level of glucose in the supernatant fraction of the maintenance medium was approximately 710 µg/ml for the toxin treated, 600 µg/ml for cells treated with 5 µg of diethylstilbestrol per ml, and 550 µg/ml for the control tissue culture cells. This difference is significant since $P$ values smaller than 0.01 were obtained. Progesterone at a concentration of 20 µg/ml yielded similar results to those obtained with 5 µg of diethylstilbestrol per ml, and its activity was not significantly affected by the addition of diethylstilbestrol.

Morphological changes. The characteristic cytotoxic effect produced by alpha toxin in rabbit kidney cells is injury to cellular membrane, resulting in the release of intracellular substances and gross morphologic changes (14). By using the hematoxylin-eosin staining technique (18), the susceptibility of rabbit kidney cells to alpha toxin in the presence and absence of diethylstilbestrol was determined. Figure 5 is a photomicrograph of rabbit kidney cells after 7 days of inoculation into tissue culture tubes containing the hormone vehicle and after 24 hr of exposure to...
one tissue culture dose of alpha toxin. The cytopathic effect progresses until no evidence of the normal cell remains. Large irregular cytoplasmic masses with cytoplasmic strands are present, within which are large areas of vacuolation and numerous small nuclei. Eventually many of the cytoplasmic masses lyse, leaving only cellular debris.

Figure 6 demonstrates the effect of diethylstilbestrol added on the third day of growth and at a concentration of 5 μg/ml. It can be seen that an approximately 65% reduction in the cytolytic action of alpha toxin was obtained by prior exposure of tissue culture cells to 5 μg of diethylstilbestrol. Concentrations of 2.5 μg/ml yielded a 20% reduction in the cytolytic action of alpha toxin, whereas concentrations lower than 2.5 μg/ml were without any noticeable effect. Similarly, cultivation of rabbit kidney cells with 5 μg of diethylstilbestrol per ml for periods shorter than 48 hr failed to exert any detectable effect on the cytotoxic action of alpha toxin. In tissue cultures without diethylstilbestrol, a clear cytopathic effect was visible 30 min after exposure to alpha toxin, whereas in tissue cultures containing diethylstilbestrol, evidence of cytopathology did not appear till the 3rd to 6th hr of exposure to alpha toxin.

Figure 7 is a photomicrograph of rabbit kidney cells 7 days after inoculation into tissue culture tubes containing the hormone vehicle and 24 hr after exposure to one tissue culture dose of heat inactivated or antibody neutralized alpha toxin. It shows the typical heavy growth of normal cells. The shape of the cells and interlocking of processes are typical of normal fibroblasts, as well as of those cells with inactivated alpha toxin in combination with the hormone vehicle at concentrations at which did not cause diminution of growth.

DISCUSSION

The data in this investigation made possible the availability of a fairly potent inhibitor of staphylococcal alpha toxin and proved useful in studying the inhibition of the cytolytic action of the alpha toxin in an in vitro inexpensive assay system. Furthermore, knowledge of the nature of the interaction between staphylococcal alpha toxin and inhibitory steroids may lead to some understanding of the active site of the staphylococcal alpha toxin.
A survey of literature indicates that the primary action of staphylococcal alpha toxin on cells is direct damage to their membrane, which leads to loss of cellular integrity and results in lysis (1, 10). Since diethylstilbestrol protected rabbit kidney cells from the cytolytic action of alpha toxin, it seems likely that diethylstilbestrol acts by preventing the interaction of alpha toxin with its substrate or membrane receptor. Thus, it may be that the cell which was allowed sufficient time of association with diethylstilbestrol or was able to adsorb the hormone on its membrane is not as easily amendable to the cytolytic action of alpha toxin as the nontreated cell. The hormone insulin was shown to act by being bound to or inserted into the membranes (25).

It is noteworthy that uptake of insulin was shown to increase viability of mammary tissues in vitro (7). The mechanism of increased cell viability induced by insulin or other hormones is still obscure. Perhaps the function or structure of cell membrane is altered by the hormonal treatment. Steroids can influence the uptake of precursors in cells (11, 15), and recent data suggest the increased amount of ribonucleic acid (RNA) and protein of the rat uterus after estrogen treatment are initiated by the production of messenger RNA (24.) Hydrocortisone, among other hormones, influences the uptake of precursors in tissues and cells in vivo and in vitro. Repeated experiments demonstrated that hormones increase the intracellular concentration of amino acids (11, 15). Cortisone reduces permeation of fructose into red blood cells (20). Regulation of the membrane transport of precursors could be the site of action of hormones, resulting in an increased or decreased size of the intracellular precursor pool, which could then determine the structure or function of cell membrane and its response to the cytolytic action of the staphylococcal alpha toxin.

What is microbiological significance of the described amelioration of the cytolytic action of staphylococcal alpha toxin by the synthetic estrogenic hormone diethylstilbestrol? The results presented in this study represent, as far as the authors know, the first demonstration of reduction of the cytotoxic action of staphylococcal alpha toxin by this hormone for primary baby rabbit kidney cells and may extend earlier work in which it was shown that pretreatment of rabbits with diethylstilbestrol suppressed induced furun-
culsis in rabbits (26). Staphylococcal alpha toxin is cytolytic for human and rabbit kidney, skin, and amnion cells (1, 8, 12, 16), and is formed in vivo as shown by the development of antibodies to it during many staphylococcal infections. Experimentally at least, large amounts may be produced. Cohn (6) demonstrated free alpha toxin in the peritoneal cavity of mice dying from experimental staphylococcal infection. Thus, it is possible that this toxin is produced in sufficient quantities in certain human infections and affects healthy tissues, including human polymorphonuclear leucocytes (9). Thus it would be logical to assume that any amelioration of the cytolytic action of alpha toxin by diethylstilbestrol would be of value to the host. Finally, the response of diethylstilbestrol-treated cells to alpha toxin may not have any relation to the increased susceptibility of the kidney to staphylococcal infection (21, 22), however, the question might be raised whether the excretion and conjugation of hormones by the kidney contributes to its marked vulnerability to staphylococcal infection.

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Fig. 7. Photomicrograph of rabbit kidney cells after 7 days of growth and 24 hr after treatment with heat-inactivated or antibody-neutralized staphylococcal alpha toxin. ×100.
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