REDUCTION OF THE CYTOLYTIC ACTION OF STAPHYLOCOCCAL ALPHA TOXIN
BY PROGESTERONE†

Staphylococci are efficient chemical factories producing a variety of toxic products. Of the many virulence factors elaborated by these microorganisms, the alpha toxin appears to be most closely associated with tissue damage. It has been known that staphylococcal alpha toxin exerts a cytopathic effect on rabbit smooth muscle and kidney cells,† mouse kidney cells,* chick embryo cells,† guinea pig skin,* human amnion, kidney and skin cells,†,5,6,7 as well as Ehrlich ascites carcinoma cells.* The study now to be reported demonstrates that progesterone in pharmacological concentrations is capable of diminishing the cytolytic action of staphylococcal alpha toxin for primary rabbit kidney cells and represents an approach to the elucidation of the previously reported suppression of furunculosis by steroids.*

MATERIALS AND METHODS

Tissue cultures. Primary rabbit kidney tissue cultures were prepared by removing aseptically the kidneys of rabbits 1-2 weeks old and processing them according to the method of Youngner.† The cells were grown in Eagle's medium M-199 (Grand Island Biological Company) supplemented with 10% gamma globulin free calf serum, 1% lactalbumin hydrolysate, 100 i.u. of penicillin and 100 µg of streptomycin sulfate per milliliter. The pH of Eagle's medium was adjusted to 7.2 with 7.5% solution of sodium bicarbonate and 1 ml. was dispensed in tissue culture tubes 18 × 150 mm. A volume of 1 ml. of an 18,000-21,000 cells per milliliter suspension 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, brought separately to the desired temperature of incubation and containing either 1-20 µg/ml of crystalline progesterone (Sigma Chemical Company, St. Louis, Mo.) dissolved in absolute ethyl alcohol (0.5-1% in the final medium), or in the case of the controls, the hormone vehicle alone. The cultures were incubated again for 3-4 days, the nutrient medium was replaced with maintenance medium, consisting of 97% Eagle's medium, 2% gamma globulin free calf serum, 1% lactalbumin hydrolysate, 100 i.u. of penicillin and 100 µg of streptomycin sulfate per milliliter, and then one tissue cytopathic dose of staphylococcal alpha toxin was added and the fate of tissue cultures was followed.

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

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

Cell counts. Cell viability was determined by the trypan blue exclusion method. One milliliter of cell suspension was added to 0.05 ml. of a 1% water solution of trypan blue. After 10 minutes 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. 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 Eagle's tissue culture medium were determined by the anthrone-sulfuric acid method. The protein content of the rabbit kidney tissue cells was assayed by the Lowry method as modified for tissue culture by Oyama and Eagle, using crystalline bovine albumin as standard.

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

RESULTS

Morphological changes

The characteristic cytopathic effect produced by alpha toxin in rabbit kidney cells is injury to cellular membrane resulting in the release of intracellular substances and cross morphologic changes. Using the hematoxylin-eosin staining technique, the susceptibility of rabbit kidney cells to alpha toxin in the presence and absence of progesterone was determined. Figure 1 is a photomicrograph of rabbit kidney cells seven days after inoculation into tissue culture tubes containing the hormone vehicle and 24 hours following 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 that did not cause diminution of growth. Figure 2 is a photomicrograph of rabbit kidney cells after seven days of inoculation into tissue culture tubes containing the
Fig. 1. Photomicrograph of rabbit kidney cells after seven days of growth and 24 hr. following treatment with heat inactivated or antibody neutralized staphylococcal alpha toxin, from × 100.

Fig. 2. Photomicrograph of rabbit kidney cells after seven days of growth and 24 hr. following treatment with one tissue culture dose of staphylococcal alpha toxin, from × 100.
FIG. 3. Photomicrograph of rabbit kidney cells grown in medium containing progesterone after 7 days of growth and 24 hr. following treatment with one tissue culture dose of staphylococcal alpha toxin, from × 100.
hormone vehicle and 24 hours following 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 3 demonstrates the effect of progesterone added on the third day of growth and at a concentration of 20 μg/ml. It can be seen that a significant reduction in the cytolytic action of alpha toxin was obtained by prior exposure of tissue culture cells to 20 μg/ml of progesterone. Concentrations of 10 μg/ml. yielded a still noticeable reduction in

![Graph showing the effect of progesterone on total cell counts of rabbit kidney cells treated with alpha toxin.](image)

**Fig. 4.** Effect of progesterone on total cell counts of rabbit kidney cells treated with alpha toxin. One ml. samples in tissue culture tubes were incubated statically at 37°C. After 72 hr. the medium was replaced by fresh medium containing increasing concentrations of progesterone. After further incubation for 72 hr. the nutrient medium was replaced with maintenance medium containing one tissue cytotoxic dose of alpha toxin, incubation continued and the total number of cells per ml. was determined. The results represent the mean of five independent experiments.
the cytolysis of alpha toxin, while concentrations lower than 10 μg/ml were without any noticeable effect. Similarly, cultivation of rabbit kidney cells with 20 μg/ml of progesterone for periods shorter than 48 hours failed to exert any detectable effect on the cytotoxic action of alpha toxin. In tissue cultures without progesterone a clear cytopathic effect was visible in 30 minutes following exposure to alpha toxin, while in tissue cultures containing progesterone evidence of cytopathology did not appear until the 5th to 8th hour of exposure to alpha toxin.

**Effect of progesterone 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 Figure 4 indicate that 24 hours 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 $3.8 \times 10^2$ cells per milliliter while the total number of cells in tissue culture tubes containing 20 μg/ml progesterone reached a level of $1.3 \times 10^4$ cells per ml. A $p$ value of 0.001 was ob-

![Graph](image_url)

**Fig. 5.** Effect of progesterone on viable cell counts of rabbit kidney cells treated with alpha toxin under conditions described in legend to Fig. 4.
tained indicating that the results were statistically significant. When a concentration of 10 μg/ml. progesterone was employed, the total number of cells in the tissue tubes was $6.3 \times 10^3$ per ml. and yielded $p$ values of 0.005.

Figure 5 indicates the percentage of viable cells in control, alpha-toxin treated, and progesterone and alpha-toxin treated tissue cultures. Twenty-four hours after the addition of alpha toxin there were 5% viable cells in the tissue cultures while 10, 20, and 45% of the cells remained viable in tissue cultures that had been treated with 5, 10, and 20 μg/ml. of progesterone. The viability of control tissue cultures was 80%, indicating that more than 50% of kidney cells treated with 20 μg/ml. of progesterone remained viable following exposure for 24 hours to staphylococcal alpha toxin.

![Figure 6](image_url)

**Fig. 6.** Effect of progesterone on glucose utilization by rabbit kidney cells treated with alpha toxin under conditions described in legend to Fig. 4.
Assays of glucose and protein

Since metabolic activity can be used as a criterion of cellular function, the utilization of glucose from the maintenance medium was determined under conditions described in MATERIALS AND METHODS. The results in Figure 6 indicate that 24 hours after the addition of alpha toxin the level of glucose in the supernatant of the maintenance medium was approximately 850 μg/ml. for the toxin treated, 680 μg/ml. for cells treated with 20 μg/ml. progesterone and 600 μg/ml. for the control tissue culture cells. This difference is significant, for p values smaller than 0.01 were obtained.

Finally an attempt was made to perform protein analysis of the cells remaining in the monolayer of tissue cultures treated with alpha toxin and progesterone to ascertain whether the protein content of the kidney cells treated with progesterone and alpha toxin was correlated with the cell number. Figure 7 illustrates the protein profile of tissue culture cells exposed to various concentrations of progesterone and one tissue cytotoxic dose of alpha toxin. If for correlation purposes the 24 hour period after the addition of alpha toxin is employed, it can be seen that the level of cellular protein was approximately 150 μg/ml. for the alpha-toxin treated cells,
DISCUSSION

The nature of the progesterone-induced reduction in the cytolytic action of staphylococcal alpha toxin as revealed by the present study is not clear. Experimental evidence indicates that the site of action of alpha toxin is the cytoplasmic membrane.1 Rabbit kidney cells treated with alpha toxin have been shown to undergo profound alterations in cell morphology with eventual lysis of the cell. It may be that the cell which has been allowed sufficient time of association with progesterone or has been able to incorporate the hormone into its membrane is not as easily amenable to the cytolytic action of alpha toxin as the nontreated cell.

An alternate possibility would place the primary site of hormone action in the intracellular metabolic reactions. There is ample experimental proof that steroids influence the uptake of precursors in tissues and cells in vivo and in vitro and recent experiments suggest that the increased amounts of RNA and protein of the rat uterus after estrogen treatment are initiated by the production of messenger RNA.25

The results presented in this study, which show that progesterone can reduce the cytotoxic action of staphylococcal alpha toxin for primary rabbit kidney cells, represent, as far as the authors know, the first demonstration of amelioration of the cytolytic action of staphylococcal alpha toxin by this hormone for rabbit kidney cells. In previous experiments it was found that treatment of adult rabbits with six preparatory doses of 5 μg testosterone and subsequent challenge with staphylococcal alpha toxin could result in significant protection of rabbits from the dermonecrotic action of the alpha toxin. Furthermore, incubation of the alpha toxin with testosterone and subsequent intradermal injection into rabbits that had not received any preparatory doses of testosterone did not alter the potency of alpha toxin, indicating that prior conditioning of the rabbits with testosterone was necessary for the suppression of the dermonecrotic action of alpha toxin.26 In this study, addition of progesterone to rabbit kidney cells 2-3 days prior to exposure to alpha toxin was also necessary for the reduction of the cytolytic action of alpha toxin. As interesting as these findings may be, no attempt is being made here to relate the reduction of the cytolytic action of progesterone to the suppression of induced furunculosis by testosterone due to the interference with the necrotic action of the alpha toxin.

Toxins of bacterial origin differ in their effects on cell cultures. Staphylococcal alpha toxin is cytolytic for human amnion, kidney and skin

220 μg/ml. for cells treated with toxin plus 10 μg/ml. progesterone, 310 μg/ml. for cells treated with alpha toxin plus 20 μg/ml. progesterone, and 350 μg/ml. for the controls.
However, the significance of the reduction of the cytolysis action of alpha toxin by progesterone in relation to staphylococcal infection is uncertain. Staphylococcal alpha toxin is formed \textit{in vivo} as shown by the development of antibodies to it during many staphylococcal infections. Experimentally, at least, large amounts may be produced. Cohn\textsuperscript{6} has 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.\textsuperscript{7} Thus it would be logical to assume that any amelioration of the cytolysis action of alpha toxin by progesterone would be of value to the host.

**SUMMARY**

This work represents an approach to the elucidation of the previously reported suppression of furunculosis by steroids. The cytolysis 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 progesterone. To 3-day-old primary rabbit baby kidney tissue cultures, 20 \( \mu \text{g/m}\)l. of progesterone was added; growth of tissue cultures in Eagle's medium was continued until the 6th day, and then one tissue cytopathic dose/ml. 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 did the control cultures. In control cultures, CPE was observed on the third hour after the addition of alpha toxin and it was complete in 24 hours, whereas in tissue cultures treated with hormones the CPE was significantly reduced.

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