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Toxicity of *Pseudomonas aeruginosa* Exotoxin A for Human Macrophages

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Pseudomonas aeruginosa exotoxin A was toxic in vitro for human peripheral blood macrophages. Cytotoxicity, manifested by morphological evidence of cell death and inhibition of [^{3}H]thymidine uptake, followed exposure to as little as 10 ng of exotoxin per ml for 1 h. In addition, phagocytosis of heat-killed *Candida albicans* by macrophages exposed to sublethal concentrations of exotoxin was impaired. This cytotoxicity was neutralizable with antiexotoxin serum.

Exotoxin A is produced by a majority of *Pseudomonas aeruginosa* strains in vitro (14), and there is serological evidence for its in vivo release during most clinical infections (12, 13). Toxin produced in experimental mouse infections inhibits protein synthesis (10) and interferes with bacterial clearance (11). A possible mechanism for the latter is toxin-induced damage to host phagocytes. Diphtheria toxin, which is very similar to pseudomonas toxin in its mechanism of action, has been shown to inhibit protein synthesis in vitro and to impair the phagocytic function of human and animal mononuclear and polymorphonuclear leukocytes (18). Because macrophages probably play an important protective role in pseudomonas infections (7, 15-17), we examined the effect of purified exotoxin A on human macrophages in vitro.

Healthy volunteers with no prior history of *P. aeruginosa* infection were studied. Serum hemagglutination assays for pseudomonas exotoxin antibody (13) were negative in all cases. Mononuclear cells were obtained from heparinized peripheral blood by means of a Ficoll-Hypaque gradient (19). The cells were suspended in tissue culture medium 199 (M 199, Grand Island Biological Co., Grand Island, N.Y.) containing penicillin (100 U/ml), streptomycin (100 μg/ml), and 20% (vol/vol) autologous serum (AS). Macrophage monolayers were derived from in vitro culture of monocytes as previously described (1), with these minor modifications. A 0.2-ml volume of M 199 with 20% AS containing 2×10^5 mononuclear cells was dispensed into flat-bottomed wells in plastic microculture plates (Linbro Scientific, Inc., Hamden, Conn.). After 4 h of incubation at 37°C in an atmosphere of air and 5% CO_2 (5% CO_2-air), culture supernatants were aspirated, and monolayers were washed with warm Hanks balanced salt solution to remove nonadherent cells. The cell cultures were reincubated with 0.2 ml of fresh M 199 with 10% AS, and the medium was changed again on day 5. The viability of monolayers was considered satisfactory if >90% of the macrophages excluded trypan blue dye. After 5 days in culture, adherent monocytes had developed the morphological characteristics of macrophages (4), and ≥75% of the macrophages phagocytosed heat-killed *Candida albicans* (2×10^5 yeast per culture) after an incubation period of 60 min.

To assess cytotoxicity, *P. aeruginosa* exotoxin A purified by the method of Callahan (2, 3) was diluted in 0.2 ml of M 199 with 10% heat-inactivated fetal calf serum and added to 5- to 7-day-old macrophage cultures. The fetal calf serum was negative for hemagglutination antibody to exotoxin. M 199 with 10% fetal calf serum without toxin was added to controls. Cultures were then incubated at 37°C in 5% CO_2-air for 48 h (or shorter intervals in some experiments, as indicated). A 1-μCi amount of [^{3}H]thymidine (Schwarz/Mann, Orangeburg, N.J.; specific activity, 60 Ci/mol) in 0.05 ml of medium was then added to each microculture well. The cultures were incubated for an additional 24 h, and the monolayers then were graded on the basis of morphology, harvested, and counted (6, 12). All cultures were prepared in quadruplicate except where otherwise indicated. The microscopic appearance of monolayers was graded from 0 to +++ as follows: no intact cells, 0; 1 to 5 cells per high-power field, almost all with abnormal rounded up appearance, +; 5 to 20 cells per high-power field, >50% with normal stretched out macrophage morphology, but some cells with abnormal rounded-up appearance, ++; and >20 cells per high-power field with characteristic macrophage morphology, +++.

Seven-day-old macrophage cultures obtained

from four different subjects were exposed to graded concentrations of exotoxin with similar results. As indicated in a representative dose-response curve from a single subject (Fig. 1), inhibition of [^{3}H]thymidine uptake occurred at toxin concentrations of 10 ng/ml or less and was almost complete at concentrations ≥100 ng/ml. Inhibition of thymidine uptake was accompanied by disappearance of some macrophages and rounding up of others at lower toxin concentrations and by virtual destruction of monolayers at concentrations ≥100 ng/ml. The morphological effects of toxin on macrophage monolayers cultured in Lab Tek slides are shown in Fig. 2. Examination of media aspirated from these cultures revealed cellular debris and a rare intact cell that showed cytoplasmic uptake of trypan blue dye.

In another experiment, 7-day-old macrophage cultures were exposed to toxin in a concentration of 120 ng/ml for time intervals of 1 to 48 h. After the removal of toxin-containing medium, the monolayers were washed three times with Hanks balanced salt solution and fresh, toxin-free incubation medium added. The cultures were simultaneously pulsed with [^{3}H]thymidine 24 h before harvesting and counting as in previous experiments. As shown in Fig. 3, there was significant inhibition of thymidine uptake by macrophages exposed to toxin for as little as 1 h. Some further inhibition occurred after longer exposures but reached a maximum by 6 h, after which counts remained constant. [^{3}H]thymidine uptake at 24 and 48 h, not shown in Fig. 3, was essentially the same as at 12 h.

To evaluate neutralization of cytotoxicity by toxin-specific antibody, exotoxin was incubated with serial 10-fold dilutions of rabbit antitoxin serum or rabbit anti-bovine serum albumin (anti-BSA) control serum in M 199 with 10% fetal calf serum for 1 h at 37°C before addition to 5- to 7-day-old monolayers in microculture wells. The final toxin concentration was 280 ng/ml, or approximately three times that necessary for complete inhibition of [^{3}H]thymidine uptake. Additional controls consisted of culture wells to which were added medium containing antitoxin or anti-BSA serum in the absence of toxin, medium containing toxin in the absence of antiserum, or medium alone. Monolayers were then incubated for 48 h before pulsing for 24 h with [^{3}H]thymidine, microscopic grading, harvesting, and counting.

Prior incubation of exotoxin with antitoxin blocked cytotoxicity, as indicated by thymidine uptake and relatively intact monolayers with normal macrophage morphology and viability (Fig. 4). That the protective effect observed represented specific neutralization of toxin was

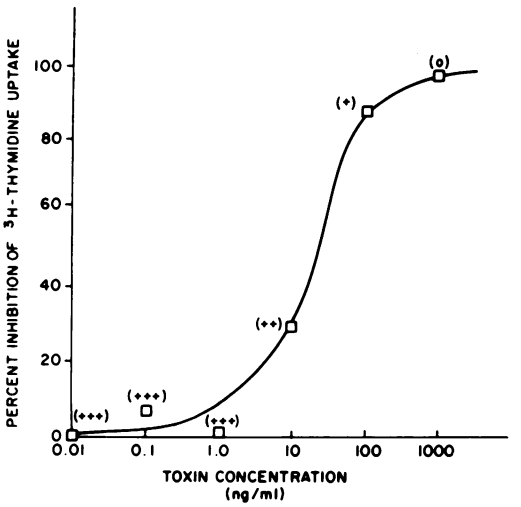

FIG. 1. *Effect of P. aeruginosa exotoxin concentration on cytotoxicity for human macrophages as measured by inhibition of [^{3}H]thymidine uptake. Macrophage cultures were exposed to toxin in the concentrations shown for 48 h, pulsed with [^{3}H]thymidine, and harvested for counting 24 h later. Each point represents the average of counts from quadruplicate cultures. Monolayer scores appear in parentheses above each point (see text for explanation).*

indicated by the absence of protection by anti-BSA control serum (Fig. 4) or by antitoxin serum previously absorbed with purified toxin and then heated at 56°C for 1 h to inactivate residual toxin (data not shown).

Another manifestation of exotoxin-induced cytotoxicity was the impairment of phagocytosis of heat-killed *C. albicans* by macrophages exposed to low concentrations of toxin. For phagocytosis studies, macrophage monolayers were prepared as described above except that four-chambered culture slides (Lab-Tek Products, Div. of Miles Laboratories, Inc., Westmont, Ill.) were used instead of microculture plates, and each slide chamber was seeded with 4×10^5 mononuclear cells in 0.4 ml of M 199 with 10% AS. After 5 to 7 days of incubation, the medium was replaced with that containing 30 ng of toxin/ml, a concentration that produced ≤50% inhibition of [^{3}H]thymidine uptake after 72 h of exposure. After 72 h, the monolayers were challenged with 2.5×10^5 heat-killed *C. albicans* per chamber. After 30 min of incubation at 37°C in 5% CO_2-air, the slides were rinsed with warm HBSS, methanol fixed, and stained with Giemsa stain. Slides were examined by light microscopy, and the percentage of cells phagocytosing yeast and the number of yeast particles per cell was determined by counting a minimum of 400 macrophages.

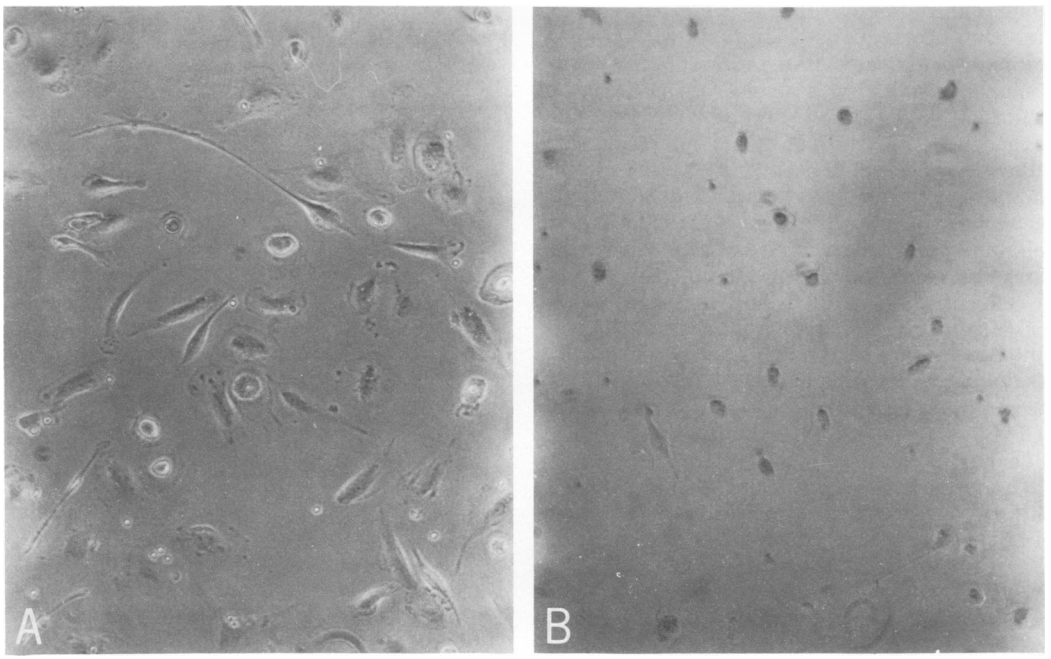

FIG. 2. *Cytopathic effect of P. aeruginosa exotoxin on human macrophages. Seven-day-old macrophage monolayers cultured on Lab-Tek slides (1.7 × 10^6 mononuclear cells in 0.4 ml of M 199 with 10% AS per chamber) were incubated in the presence or absence of 120 ng/ml of toxin for 72 h and stained with trypan blue. (A) Untreated control cells with typical macrophage morphology and intact monolayer. Greater than 95% of such cells excluded trypan blue dye (×150). (B) Macrophages exposed to toxin. There is almost complete destruction of the monolayer. Remaining adherent cells have an abnormal rounded-up appearance and >98% stain with trypan blue dye. One or two cells in the field are apparently still viable (×150).*

Exposure of macrophages to 30 ng/ml of toxin for 72 h produced less than 50% inhibition of [^{3}H]thymidine uptake and left approximately one half of the cells in the monolayers intact, with 80 to 90% still viable on the basis of dye exclusion. Yet, after 30 min of incubation with 2 × 10^5 heat-killed *C. albicans,* only 20% of 400 toxin-exposed macrophages ingested yeast, compared with 56% of 400 unexposed control cells ($P < 0.0001$). In addition, of the ingesting macrophages, a significantly smaller proportion contained more than 5 yeast per cell following toxin exposure (25% of 80 cells) than the unexposed controls (39% of 222 cells, $P = 0.039$).

As judged by its close correlation with morphological evidence of cell damage and alteration of phagocytic function, [^{3}H]thymidine uptake by human macrophages is a good index of toxicity produced by *P. aeruginosa* exotoxin. In addition, it is conveniently measured and quantifiable. Although not examined, in the case of other tissue cultures exposed to similar concentrations of exotoxin, decreases in thymidine uptake closely parallel reductions in amino acid incorporation (8, 9). The latter probably reflects the primary mechanism of action of exotoxin that is the inhibition of protein synthesis by the enzymatic transfer of the adenosine 5'-diphosphate–ribosyl moiety from nicotinamide adenine dinucleotide to elongation factor 2 (5). The impairment of phagocytosis by macrophages after exposure to low doses of toxin presumably reflects the same defect in protein synthesis responsible for impaired thymidine uptake and the observed cytopathic effects.

These in vitro observations suggest the possibility of an in vivo correlate during actual infections. It has been demonstrated serologically during human pseudomonas infections (12, 13) and biochemically during experimental infections in mice (10) that exotoxin is released systemically. In the latter case, circulating toxin impairs clearance of bacteria from the blood (11). This observed in vivo effect of toxin may be due in part to its demonstrated toxicity for macrophages. This possibility appears more likely in view of the low concentrations of exotoxin that produce toxicity in vitro.

Although it has been suggested that polymorphonuclear leukocytes represent the main cellular defense against pseudomonas infections (20), there is growing evidence that macrophages

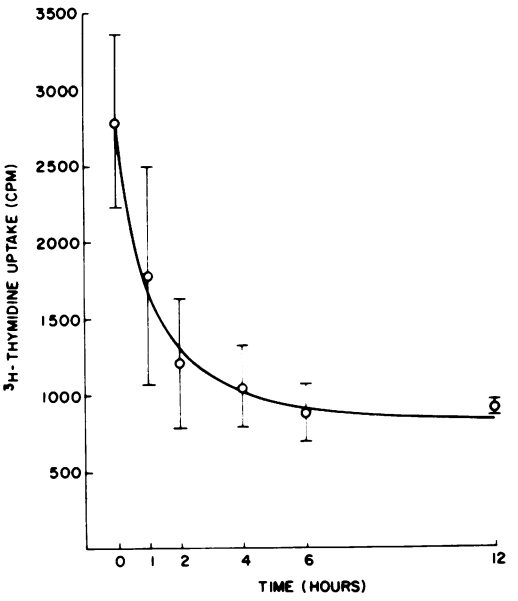

FIG. 3. *Effect of length of P. aeruginosa exotoxin exposure on cytotoxicity for human macrophages, as measured by inhibition of [^{3}H]thymidine uptake. Each point represents the average of counts from quadruplicate macrophage cultures incubated with 120 ng/ml of exotoxin for the time periods shown. The brackets indicate ± standard error of the mean.*

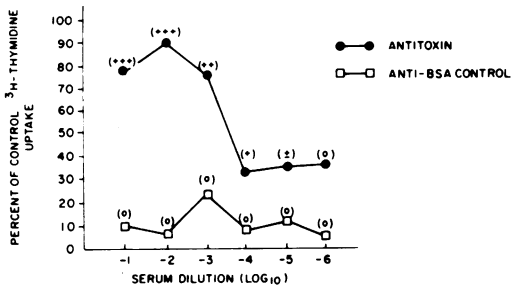

FIG. 4. *Neutralization of macrophage toxicity of P. aeruginosa exotoxin as measured by inhibition of [^{3}H]thymidine uptake. Toxin was preincubated with heat-inactivated rabbit antitoxin serum (●——●) or anti-BSA control serum (□——□) in the dilutions shown before being added to cell cultures in a final concentration of 280 ng/ml. Each point represents the average of counts from 12 replicate toxin-treated cultures as compared with the average of counts from 12 replicate control cultures incubated with serum alone. Monolayer scores appear in parentheses above each point (see text for explanation.)*

also play a protective role (7), especially in pulmonary infections (15–17). If this is the case, the cytopathic effect of exotoxin on these cells might be a significant factor in pathogenesis.

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