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# Alteration of Murine Immune Response by *Pseudomonas aeruginosa* Exotoxin A

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***Pseudomonas* exotoxin A has been implicated as a possible virulence factor in *Pseudomonas* infections. This toxin has a direct cytotoxic effect on a number of cell types, including macrophages and their precursors, and therefore may affect other cells of the immune system. NFR/N(H-2$^q$) (+/nu or nu/nu) mice were immunized with either T-dependent or T-independent antigens along with various doses of exotoxin A. The immune response was then assayed by a modification of the Jerne plaque assay. Exotoxin A induced a dose-dependent suppression of the in vitro and in vivo immune responses to T-dependent and T-independent antigens in immunocompetent +/nu mice. However, in NFR/N nu/nu mice, suppression of the immune response to the T-independent antigen trinitrophenylated-Ficoll was not observed. Instead, a marked enhancement of the response was observed at doses of 100 and 10 ng of exotoxin A. Removal of T-cells with anti-Thy 1.2 antiserum plus complement before antigen and exotoxin A stimulation in +/nu mice results in abrogation of the suppression. These data suggest that *Pseudomonas* exotoxin A exerts an effect on both B- and T-lymphocyte populations to modulate the immune response and that this activity may be one facet of the pathogenic effects of this toxin.**

*Pseudomonas aeruginosa* produces a number of factors, many of which may affect its ability to cause infection. Exotoxin A is a protein toxin which, like diphtheria toxin, inhibits polypeptide synthesis via ADP ribosylation of elongation factor 2 (6, 7, 16, 17, 27). It is the most toxic substance produced by this organism and as such may have a significant role in its pathogenicity (21). Exotoxin A has been shown to be cytotoxic for a wide range of mammalian cells, including human macrophages, and inhibits human granulocyte and macrophage progenitor cell proliferation (24, 28, 37). Patients with bacteremic infections by exotoxin A-producing strains of *P. aeruginosa* have been shown to have a better prognosis if their antibody titer to the toxin was high (8, 29, 39). The role of exotoxin A as a virulence factor has been reported for several model systems (1, 33, 36), whereas other studies have been less conclusive (3, 9).

Exotoxins produced by various bacterial species have been shown to exert a modulatory effect on the immune response (15, 25). Cholera toxin has been shown to enhance or impair the immune response to sheep erythrocytes, depending on the time of toxin administration relative to sheep erythrocyte injection (18, 22). Staphylococcal enterotoxin B and pyrogenic exotoxin as well as streptococcal pyrogenic exotoxin have been shown to modulate the immune response in mice. The effects of these toxins appear to be generated through T-cells (10, 11, 25, 34). Preliminary studies with *Pseudomonas* exotoxin A indicated that this toxin may also have a modulatory effect on the murine immune response (O. R. Pavlovskis, B. Wretlind, and M. L. Hale, Toxicon **17S**:139, 1979). The present study was undertaken to assess whether exotoxin A was capable of modulating the murine immune response to a second antigen and to determine the lymphocyte population through which exotoxin A exerts its activity.

## MATERIALS AND METHODS

**Mice.** NFR/N(H-2$^q$) nude (nu/nu) mice and their littermates NFR/N (+/nu) mice were derived from brother × sister breeding pairs maintained in the animal care facilities at the University of Missouri-Columbia, School of Medicine, from breeding stock originally obtained from Carl Hansen, National Institutes of Health.

**Immunogens.** Dinitrophenylated keyhole limpet hemocyanin (DNP-KLH) was purchased from Calbiochem-Behring, La Jolla, Calif. Trinitrophenylated-Ficoll (TNP-Ficoll) was obtained from Biosearch, San Rafael, Calif.

**Generation of in vivo immune response.** In vivo immune responses were generated by intravenous injection of 10 μg of TNP-Ficoll followed 1 h later by intravenous injection of various doses of exotoxin A. The spleen cells were harvested 5 days post-immunization, and single-cell suspensions were obtained and assayed for direct plaque-forming cells (PFC).

**Spleen cell cultures.** Spleen cells were cultured in media consisting of RPMI 1640 containing glutamine and supplemented with 15% fetal calf serum and 2-mercaptoethanol. Gentamicin at a concentration of 100 μg/ml was added as antibiotic. Spleen cells at a density of $10^7$ cells plus 10 μg of DNP-KLH or 10 ng of TNP-Ficoll and various concentrations of exotoxin A were seeded in a volume of 0.7 ml into the inner chamber of a small single-chamber Marbrook vessel. Cultures were incubated at 37°C in 5% $CO_2$ and 100% relative humidity for 5 days, at which time the cells were harvested and assayed for direct PFC.

**Depletion of B- and T-cell populations.** T-cells were eliminated by incubating splenocytes with a mouse monoclonal anti-Thy 1.2 (HO-13-4) antiserum (23) for 30 min on ice followed by a 45-min incubation at 37°C with baby rabbit complement that had been prescreened for low toxicity.

B-cells were eliminated by incubating splenocytes with a goat anti-mouse immunoglobulin antiserum for 30 min on ice followed by a 45-min incubation at 37°C with complement. Ascites fluid from the SP2/O myeloma cell line was used as an antibody control in these experiments.

The effectiveness of the treatments was analyzed by mitogenic studies with [$^3$H]thymidine. Anti-Thy 1.2 plus complement eliminated >90% of the response to the T-cell mitogens concanavalin A and phytohemagglutinin as compared with the untreated spleen cells. Yet the response to lipopolysaccharide was unaffected. Anti-mouse immuno-

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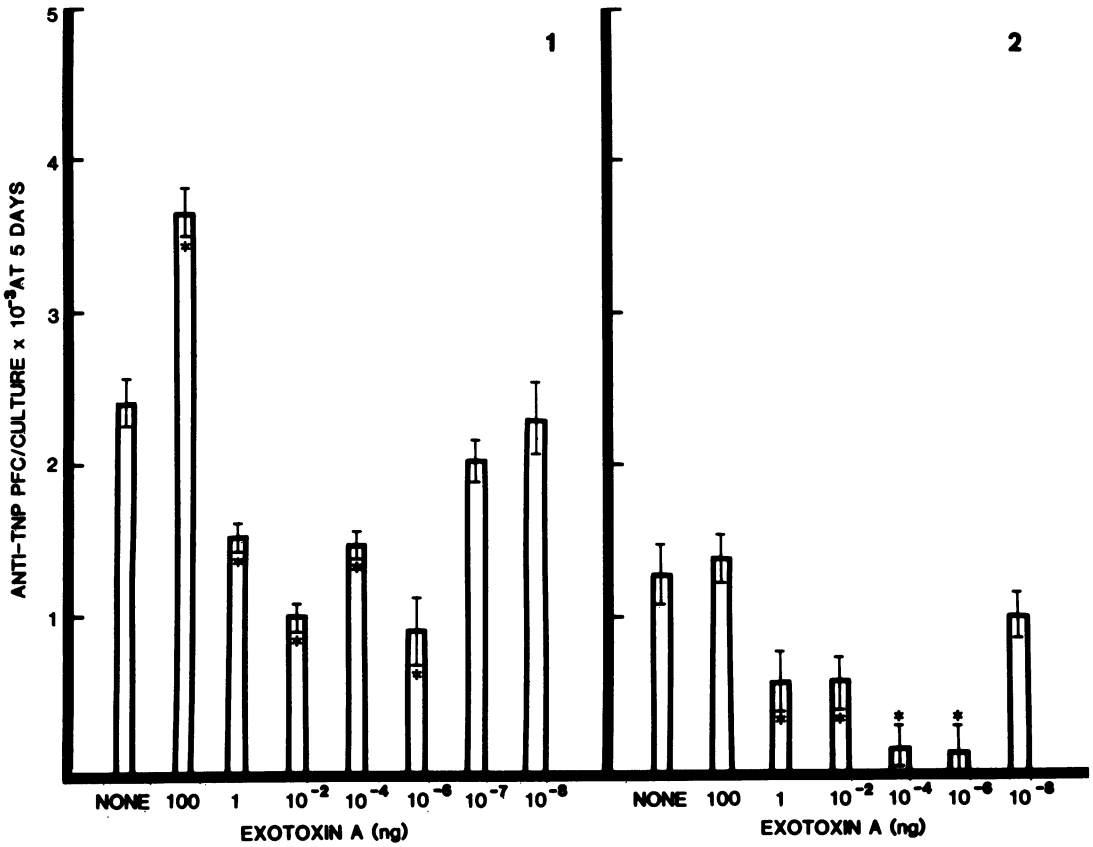


FIG. 1. Effect of exotoxin A on the in vitro response to DNP-KLN. Spleen cells ($10^7$) were coincubated for 5 days with 10 μg of DNP-KLH plus various concentrations of exotoxin A. Each bar represents the mean value (± standard error) of the anti-TNP PFC minus background for three to four cultures per group. * , Significantly different from antigen control response ($P < 0.05$). Background count for experiment 1 was 993 PFC and for experiment 2 was 940 PFC.

globulin plus complement reduced the response to the B-cell mitogen, lipopolysaccharide, by >80% when compared with control spleen cells. However, no reduction in the response to concanavalin A was observed.

**PFC assays.** Estimations of hapten-specific anti-TNP PFC responses were assessed by the slide modification of the Jerne plaque assay (26). Briefly, molten agarose in Eagle minimal essential medium was mixed with washed TNP sheep erythrocytes (32), target cells, and the appropriate spleen cell suspension. The mixture was poured onto microscope slides, allowed to gel, and incubated in a humidified chamber at 37°C for 1.5 h. The slides were flooded with guinea pig complement that had been absorbed with sheep erythrocytes and incubated at 37°C for an additional 1.5 h at which time direct PFC were ascertained.

***P. aeruginosa* exotoxin A.** Highly purified exotoxin A was a generous gift of O. Pavlovskis, Department of Microbiology, Naval Medical Research Institute, Bethesda, Md., and was produced by the method of Leppla (20). The toxin contained <0.1% lipopolysaccharide as determined by the Limulus amebocyte assay (31), had a mouse 50% lethal dose of between 100 and 200 ng, and exhibited cytotoxic effects for CHO cells at 10 ng.

**Statistical analysis.** Statistical analysis of the data was performed on the Amdahl 470/V7 computer at the University of Missouri Computing and Information Center. The Fischer least significant difference test at a 5% confidence level was the method used for data analysis (35). Student's *t* test was also performed and correlated with the findings of the Fischer least significant difference test.

## RESULTS

**Effect of exotoxin A on DNP-KLH response.** Figure 1 shows the effects of exotoxin A on the in vitro immune response of spleen cells from +/nu mice to DNP-KLH. Suppression of the immune response by exotoxin A was seen over a wide range of doses with 1 to $10^{-6}$ ng giving significant suppression. On the other hand, 100 ng had no suppressive effect but rather induced significant enhancement of the immune response (153%). A second experiment corroborated these results with 1 to $10^{-6}$ ng inducing suppression of the response, whereas 100 ng seemed to boost the response of +/nu mice slightly although nonsignificantly.

**Effect of exotoxin A on TNP-Ficoll response.** An examination of the in vitro effects of exotoxin A on the immune response of spleen cells from +/nu mice to TNP-Ficoll, a T-independent antigen, was conducted (Fig. 2). The results of these experiments mirror those of the T-dependent antigen DNP-KLH in that concentrations of 1 to $10^{-6}$ ng induced suppression of the immune response, whereas 100 ng had no suppressive effect. Ten nanograms was also found not to have a suppressive effect on the immune response (see Table 1 and Fig. 4).

An analysis of the effects of exotoxin A on the in vivo immune response of +/nu mice to TNP-Ficoll (Fig. 3) showed that the same doses of exotoxin A induced suppres-

sion of the in vivo response as was observed in the in vitro immune response. A 10- to $10^{-6}$-ng concentration of the toxin induced significant suppression, whereas 100 ng had a somewhat stimulatory effect on the immune response. A second experiment showed similar results with a concentration of 10 to $10^{-6}$ ng of toxin exerting suppression, whereas 100 ng exerted no significant suppression. This reflects the tendency shown previously of high doses of toxin to have less of a suppressive effect on the immune response than low doses.

**Effect of exotoxin A on TNP-Ficoll response in athymic nude mice.** Since the suppression of the immune response by exotoxin A was observed in immunocompetent +/nu mice, a study was conducted to see what effect the toxin would have on the immune response to TNP-Ficoll in athymic nude mice which lack functional T-cells. A comparison of the effects of exotoxin A on the in vitro immune response to TNP-Ficoll in athymic nude mice versus euthymic +/nu littermates is shown in Fig. 4. Samples of toxin (100 ng and 10 ng) markedly enhanced the immune response of nude mouse splenocytes to TNP-Ficoll, with 100 ng increasing the anti-TNP PFC 2-fold in the first experiment and better than 6-fold in the second experiment, whereas 10 ng stimulated the response 3-fold and 2.5-fold in the first and second experiment, respectively. Other concentrations of toxin had no significant effect on the immune response by the cells. This is contrasted with the response of the +/nu littermates to exotoxin A, showing that 100 and 10 ng of exotoxin A did not induce a significant enhancement of the immune response, whereas lower toxin concentrations significantly suppressed the response.

**Effect of T-cell depletion on the suppression induced by toxin.** Experiments were conducted to determine the role of T-cells in the exotoxin A-induced suppression. Splenocytes were depleted of T-cells and then exposed to antigen plus 10 or $10^{-5}$ ng of exotoxin A. The results of a representative experiment are shown in Table 1. Significant suppression was induced by $10^{-5}$ but not 10 ng of toxin (group A). Removal of T-cells with anti-Thy 1.2 plus complement treatment resulted in abrogation of the suppression at $10^{-5}$ ng and an enhancement at 10 ng (group B). Adding a T-cell-enriched population (group C) to the T-cell-depleted population (group B) reinstated the suppression induced by $10^{-5}$ ng and eliminated the enhanced response induced by 10 ng of exotoxin A (group D).

## DISCUSSION

Exotoxins have been shown to be capable of experimentally modulating the immune response. Some exotoxins, such as staphylococcal and streptococcal pyrogenic exotoxins and staphylococcal enterotoxin B, induce a suppression of the immune response (10, 11, 34). Other exotoxins such as cholera toxin have been shown to induce suppression or enhancement of the immune response, depending on when the toxin is administered during the immunization schedule (18, 22). The data presented in this study indicate that *P.*

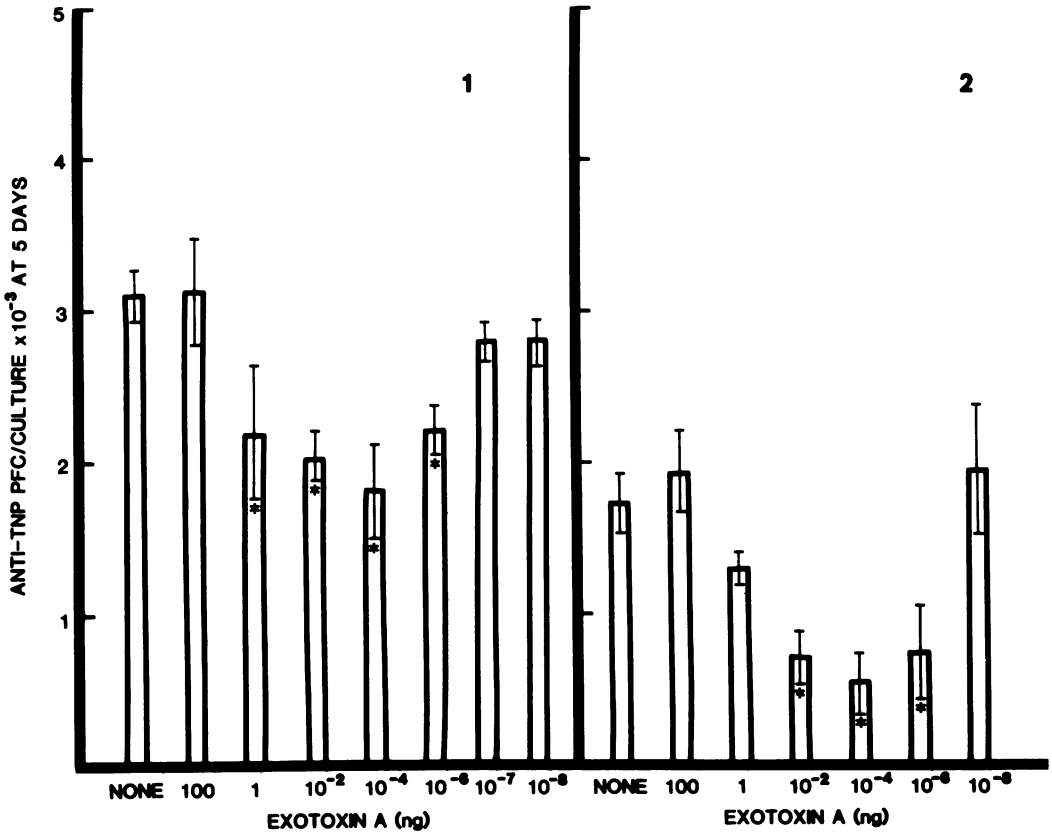


FIG. 2. Effect of exotoxin A on the in vitro response to TNP-Ficoll. Spleen cells ($10^7$) were coincubated for 5 days with 10 ng of TNP-Ficoll plus various concentrations of exotoxin A. Each bar represents the mean value (± standard error) of the anti-TNP PFC minus background for three to four cultures per group. *, Significantly different from antigen control response ($P < 0.05$). Background count for experiment 1 was 940 PFC and for experiment 2 was 464 PFC.

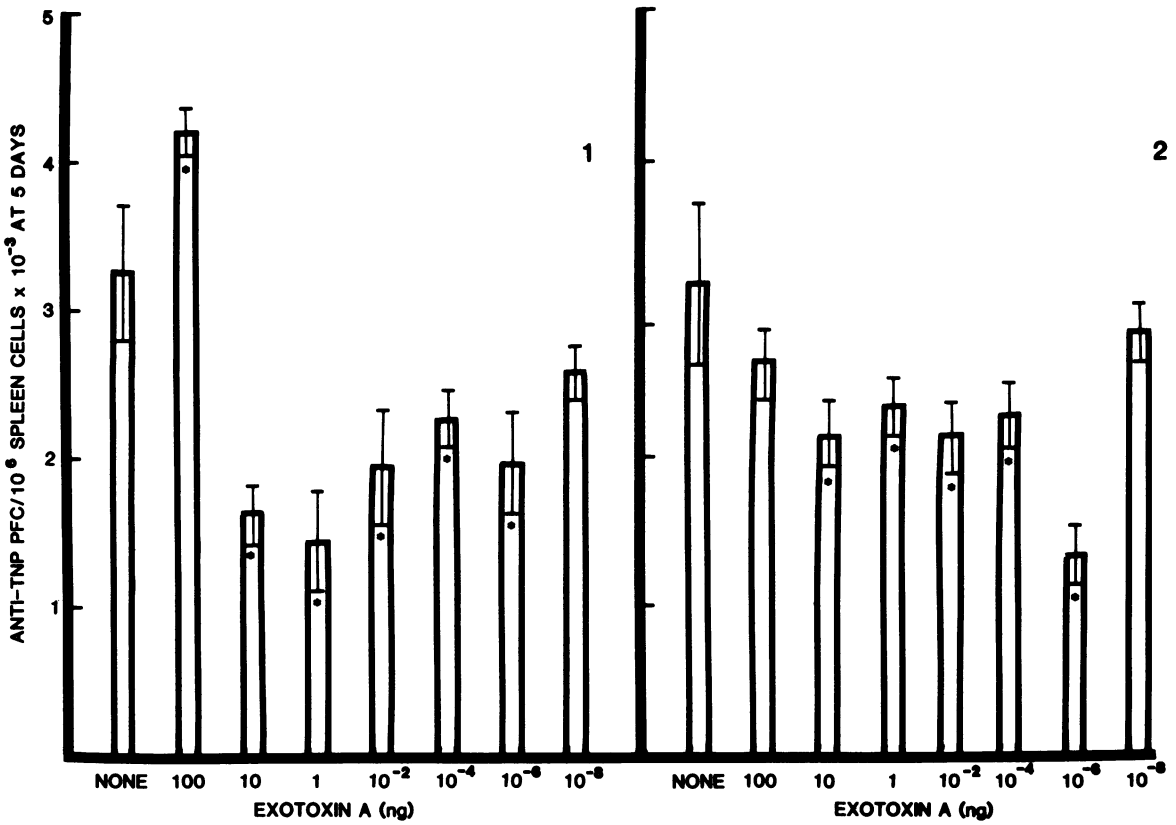


FIG. 3. Effect of exotoxin A on the in vivo response to TNP-Ficoll. Mice were immunized with 10 μg of TNP-Ficoll intravenously, followed 1 h later with various concentrations of exotoxin A. Each bar represents the mean value (± standard error) of the anti-TNP PFC per $10^6$ spleen cells. Each group consisted of three to four mice per group. Background responses for both experiments were <50 PFC per $10^6$ spleen cells. * , Significantly different from antigen control response ($P < 0.05$).

*aeruginosa* exotoxin A exerts a modulatory effect on the in vitro and in vivo immune responses in NFR/N mice. The type of modulation by the toxin appears to be a reflection of the immunocompetency of the host since exotoxin A induces enhancement of the response in athymic nude mice and suppression of the response in their euthymic littermates.

The suppression induced by exotoxin A on the immune response in euthymic +/nu mice was shown for both the T-dependent antigen DNP-KLH and the T-independent antigen TNP-Ficoll. Despite the different cellular interactions required for T-dependent and T-independent antigens, the concentrations of exotoxin A required to induce suppression of the immune response were similar for both antigen types. Concentrations down to $10^{-6}$ ng of the toxin could suppress the immune response, which would indicate that exotoxin A is a potent modulator of the immune response. Little or no suppression was induced by high toxin concentrations, which indicates that the suppression observed may not be due merely to a toxic effect on the spleen cells. Indeed, viability studies were performed, by trypan blue exclusion after 5 days of incubation with exotoxin A, which showed that there were few, if any, differences in viabilities of spleen cells incubated with various concentrations of exotoxin A (100, 10, 1, and 0.1 ng showed 90, 105, 100, and 95% of the control viable count, respectively). This indicates that exotoxin A may activate a regulatory population of cells capable of exerting a suppressive effect on the immune response. Schlievert previously showed similar effects in experiments with staphylococcal and streptococcal exotoxins in which he observed a higher degree of suppression when the concentration of exotoxin was decreased. He attributed this to the activation of a suppressor T-cell population. Higher doses of toxin reduced this effect, and it was thought to be due to either a saturation of toxin receptors or nonspecific cell proliferation induced by the toxin (34).

T-cell mitogens such as concanavalin A have been shown to induce a population of T-cells which can suppress the immune response (12, 30). Several investigators have noted that the staphylococcal enterotoxins (11, 19) and streptococcal pyrogenic exotoxin (2) are potent T-cell mitogens, and Donnelly and Rogers (11) found that staphylococcal enterotoxin B induced a population of cells with a Thy $1^+$ Lyt $1^-2^+3^+$ phenotype which could act to suppress the immune response when mixed with normal, unprimed splenocytes. Our experiments with monoclonal anti-Thy 1.2 antiserum plus complement to eliminate the T-cell population before antigen and exotoxin A stimulation resulted in the abrogation of the toxin-induced suppression. This suppression could be reinstated by adding T-cells back to this T-cell-depleted population. This may indicate that a T-cell is involved in the suppression, although one cannot rule out the involvement of other cell populations. The role of these other cell populations and the determination of the specific T-cell subset responsible for the exotoxin A-induced suppression are currently being investigated.

The suppression that was induced by exotoxin A was observed in immunocompetent +/nu mice and appeared to be induced through a T-cell. However, when athymic nude

splenocytes were exposed to 100 and 10 ng of the toxin, an enhancement rather than suppression of the immune response was observed. This same effect was observed in cultures depleted of T-cells and stimulated with 10 ng of exotoxin A (Table 1, group B). This gives further credence to the idea that a T-cell may be involved in the induction of suppression and also that the mode of action of the toxin is possibly through a mitogenic stimulation of the splenocytes similar to the effect observed by Donnelly and Rogers (11) for staphylococcal enterotoxin B. The data also indicate that exotoxin A may exert a stimulatory effect for multiple lymphocyte populations (i.e., B-cells as well as T-cells) (Fig. 4). These results differ with previous studies with staphylococcal and streptococcal exotoxins which showed the target cells to be primarily T-cells (2, 11, 19). The possibility that exotoxin A stimulates both T-cells and B-cells is currently under investigation in this laboratory.

A number of studies have cited exotoxin A as a possible virulence factor for a number of disease states. Although it is difficult to demonstrate the in vivo presence of exotoxin A directly, the rise in titer of antibodies to exotoxin A in patients infected with *P. aeruginosa* has provided an indirect means to show that the host is being exposed to the toxin (8, 29). Exotoxin A has been shown to exert a direct toxic effect on different cell populations, and this alone could aid in the pathogenicity of the organism. The immune suppression by exotoxin A shown in the present study could also enhance the ability of the organism to cause disease. *P. aeruginosa* produces a number of potential virulence factors, including the proteases, elastase and alkaline protease (3, 9, 36). Suppression of the immune response may cause a delay in the formation of antibodies to the organism as well as antibodies which neutralize these products. This could result in a prolonged exposure of the host cells to the destructive action of *Pseudomonas* extracellular products and therefore provide a more optimum environment for invasion.

Previous studies have shown that infections by *P. aeruginosa* or exposure of the immune system to *P. aeruginosa* products can have a suppressive effect on the immune response. In one study of renal transplant patients, Woodruff et al. (38) described a patient with a chronic *P. aeruginosa* infection. Whenever antibiotics were administered to treat the infection, the patient exhibited signs of kidney rejection. One explanation offered by the authors was that *P. aeruginosa* was producing a factor which depressed the immune system of the patient. Elimination of the organism also eliminated this factor, which allowed the immune system to respond to the transplanted kidney. Floersheim et al. (13, 14) showed that injection of lyophilized extracts of *P. aeruginosa* depressed both cell-mediated and humoral response in guinea pigs and mice. Campa and co-workers (4) also have shown that in experimental infections of mice with *P. aeruginosa*, there was a depressed delayed-type hypersensitive response to oxazolone. Colizzi et al. (5) showed that this

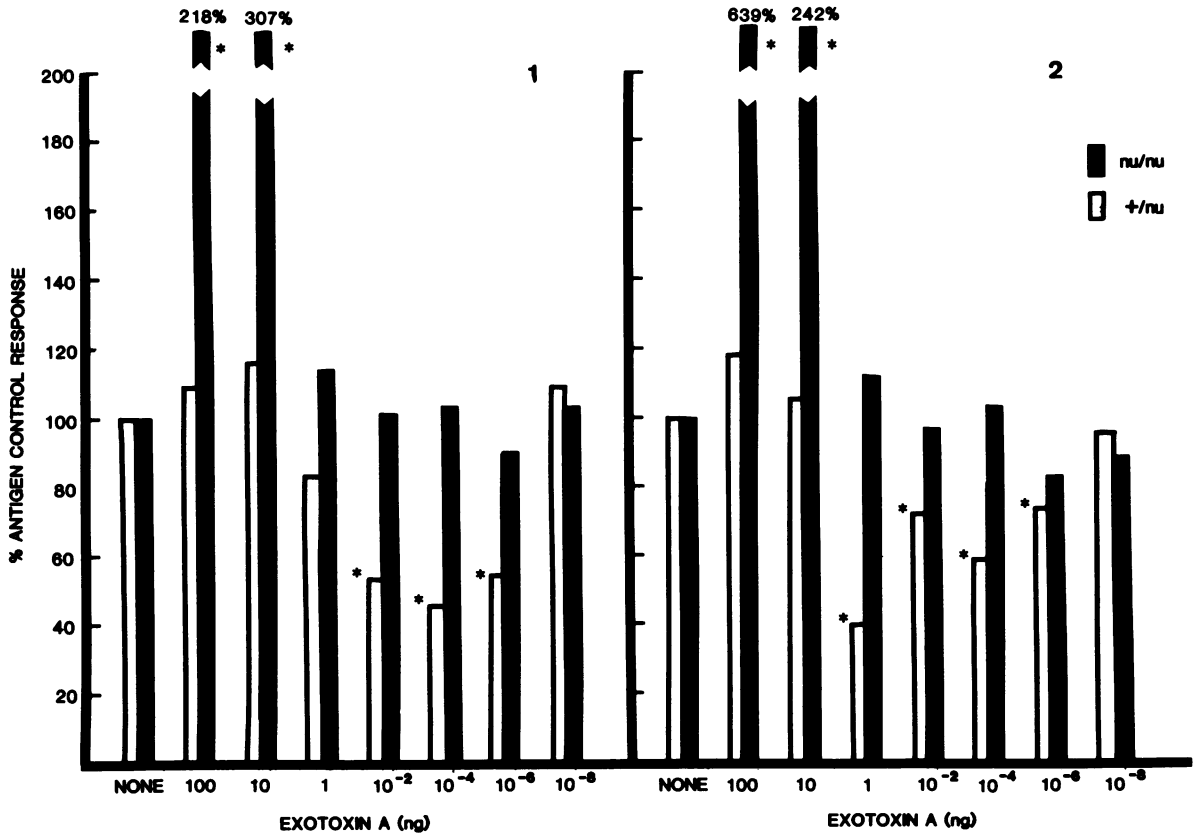


FIG. 4. Comparison of the in vitro effects of exotoxin A on the immune response by NFR/N nu/nu versus +/nu splenocytes. Spleen cells ($10^7$) were coincubated for 5 days with 10 ng of TNP-Ficoll plus various concentrations of exotoxin A. Each bar represents the percentage of the anti-TNP response of the antigen control group. Each group consists of three to four cultures per group for separate experiments. Antigen control PFC responses: experiment 1, +/nu 2,139 and nu/nu 954; experiment 2, +/nu 3,237 and nu/nu 745. * , Significantly different from antigen control response ($P < 0.05$).

TABLE 1. Effect of T- and B-cell depletion on the in vitro immune suppression induced by exotoxin A

| Group | Treatment of spleen cells[a] | Mean anti-TNP PFC ± SE per culture (% Ag control)[b] | | |
|---|---|---|---|---|
| | | Ag[c] | Ag + 10 ng of exotoxin A | Ag + $10^{-5}$ ng of exotoxin A |
| A | Normal | 1,490 ± 181 | 1,500 ± 144 (101) | 787 ± 87 (53)[d] |
| B | Anti-Thy 1.2 | 1,120 ± 95 | 1,623 ± 298 (145)[d] | 1,070 ± 50 (96) |
| C | Anti-mouse immunoglobulin | 220 ± 39 | ND[e] | 200 ± 40 (91) |
| D | Groups B + C[f] | 1,283 ± 81 | 1,127 ± 132 (88) | 590 ± 99 (46)[d] |

[a] Cells were treated with the appropriate antibody for 0.5 h on ice, washed, and then treated with infant rabbit complement for 0.5 h at 37°C.
[b] Ag, Antigen.
[c] Antigen is 10 ng of TNP-Ficoll.
[d] Values significantly different from the antigen control ($P < 0.05$).
[e] ND, Not determined.
[f] Group mixes reflect $5 \times 10^6$ cells of each treatment group per vessel.

suppression could be transferred into oxazolone-sensitized recipients with lymph node cells or splenocytes from *P. aeruginosa*-infected, oxazolone-sensitized donors. These experiments indicated that a suppressor cell population was activated in the spleens and lymph nodes of these mice during *P. aeruginosa* infection. The role of exotoxin A in these studies, although not examined, could be responsible for these effects.

The present study shows that exotoxin A can act on host cells through a mechanism other than direct cytotoxicity. Exotoxin A can induce suppression of the immune response which appears to be mediated through a T-cell population. In the absence of regulatory T-cells, as in athymic nude mice, the presence of exotoxin A served to enhance the immune response. The immunomodulation induced by exotoxin A could have important implications in the compromised patient which could result in increased pathogenicity of *P. aeruginosa*.

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