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Variables Which Affect Suppression of the Immune Response Induced by *Pseudomonas aeruginosa* Exotoxin A

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***Pseudomonas* exotoxin A has been shown previously to induce suppression of the murine immune response. In the present study, various parameters were examined which may have an effect on immunosuppression. The addition of 10^{-4} ng of exotoxin A induced suppression of the immune response to trinitrophenylated Ficoll from days 3 to 10, while 10 ng of toxin exerted no suppressive effect over the same examination periods. When the toxin was administered 1 or 2 days before antigen stimulation, suppression of the response was observed with both 10 and 10^{-4} ng. Priming splenocytes with toxin either in vivo or in vitro for 1 or 2 days suppressed the response of fresh cultured splenocytes to antigenic stimulation. Heated toxin, photoaffinity-labeled toxin, or preincubation of the toxin with rabbit anti-exotoxin A antiserum eliminated the toxin-induced suppression. These results suggest that *Pseudomonas* exotoxin A can generate multiple biological effects.**

Exotoxin A is a protein toxin produced by the gram-negative bacterium *Pseudomonas aeruginosa*. The toxin has been shown to be directly cytotoxic for a number of mammalian cells primarily through the ADP ribosylation of protein elongation factor 2, thereby inhibiting cellular polypeptide synthesis (18, 20, 21). We have been studying the effects of exotoxin A on the murine immune response and have found that the toxin can induce suppression in euthymic *nu*/+ mice to thymus-dependent and thymus-independent antigens both in vitro and in vivo (11). The suppression was observed over a toxin range of 1 to 10^{-6} ng, while 10 and 100 ng had no apparent effect (11). A number of parameters can influence the overall action of immunomodifiers. Tubercle bacilli have been observed to elicit an immunoenhancing effect when administered with the antigen (7) but to induce suppression of the response when given before the antigen (31). A similar effect has also been observed for cholera toxin (13, 16) and *Bordetella pertussis* vaccine (6). We now report that timing of exotoxin A administration relative to antigenic stimulation can have a dramatic effect on the ability of the toxin to modify the immune response. Given concomitantly with the antigen, 10 ng of exotoxin A is not immunosuppressive. However, this same dose of toxin induced suppression when administered 1 or 2 days before the antigen stimulation. Modification of the toxin by heating, photoaffinity labeling, or preincubation with rabbit anti-exotoxin A antibodies eliminated the capacity of exotoxin A to induce suppression. These studies support the concept that *Pseudomonas* exotoxin A is a potent biological response modifier.

MATERIALS AND METHODS

Mice. NFR/N (*H-2*^{q}) *nu*/+ mice were derived from brother-and-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, Bethesda, Md. Mice were maintained in autoclaved plastic cages containing autoclaved bedding and were fed autoclaved mouse chow and acidified water ad libitum. In all experiments 6- to 12-week-old mice of the same sex were used.

Reagents. Highly purified exotoxin A was a generous gift of O. Pavlovskis, Department of Microbiology, Naval Medical Research, Bethesda, Md., and was produced by the method of Leppla (14). The toxin contained <0.1% lipopolysaccharide (LPS) as determined by the Limulus amebocyte assay (23), had a 50% lethal dose for mice at 100 to 200 ng, and exhibited cytotoxic effects for Chinese hamster ovary (CHO) cells at 10 ng.

Immunogens. Ficoll containing trinitrophenol (TNP) was purchased from Biosearch, San Rafael, Calif. Sheep erythrocytes (SRBCs) from a single sheep were purchased from Hazelton Dutchland, Inc., Denver, Pa.

Spleen cell cultures. Spleen cells were cultured in medium consisting of RPMI 1640 supplemented with glutamine, sodium pyruvate, nonessential amino acids, 15% fetal bovine serum, and 2-mercaptoethanol (5×10^{-5} M). Gentamicin (100 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml) were added as the antibiotics. Spleen cells at a density of 10^7 plus 0.1 ml of the immunogen and 0.1 ml of various concentrations of exotoxin A were seeded into the inner chamber of a small single-chamber Marbrook vessel. Ten milliliters of culture medium was placed in the outer reservoir chamber, and the cultures were incubated at 37°C in 5% CO_2 and 100% relative humidity for 5 days. The cells were then harvested and assayed for direct plaque-forming cells (PFC). The kinetics of the toxin-induced modulation was followed over 10 days. Three cultures per group on days 3, 4, 5, 6, 8, and 10 were harvested and assayed for an anti-TNP response.

Priming with exotoxin A. Splenocytes were cultured in no. 3406 six-well tissue culture trays (Costar, Cambridge, Mass.) at a toxin concentration of 10 or 10^{-4} ng/10^7 cells for 1 or 2 days; the cells were then harvested, washed two times, and suspended to a level of 2×10^7 viable cells per ml in culture medium. Primed cells were either cultured directly with TNP-Ficoll for 5 days and assayed for an anti-TNP response

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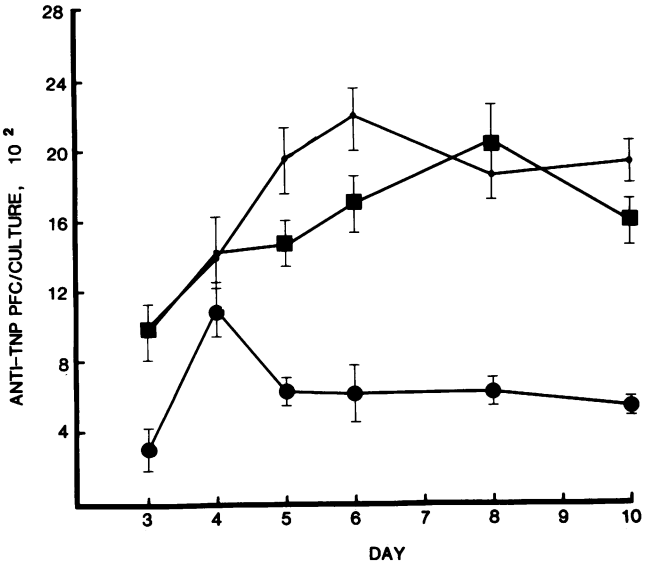

FIG. 1. Kinetics of toxin-induced suppression. Spleen cells (10^7) were coincubated with TNP-Ficoll plus 10 (—) or 10^{-4} (●) ng of exotoxin A or the dilution buffer HBSS (■). cultures were harvested on days 3, 4, 5, 6, 8, and 10 and assayed for a direct anti-TNP response with a Jerne plaque assay. Each point represents the mean value (± standard error) of the anti-TNP PFC minus background for three cultures per group.

or cultured with fresh cells (at various primed-to-fresh-cell ratios) plus TNP-Ficoll for 5 days to ascertain the effect of primed cells on the anti-TNP response elicited by fresh cells.

Effect of anti-exotoxin A antibody. The immunoglobulin G (IgG) fraction of rabbit anti-exotoxin A antibody was the kind gift of David Fitzgerald, National Institutes of Health, Bethesda, Md., and was used at a 10^{-1} dilution in RPMI 1640. Exotoxin A was incubated at 37°C with antibodies for 45 min before addition to the culture. Incubation of exotoxin A with antibodies abrogates cytotoxicity for the CHO indicator cell line (data not shown).

Exotoxin A toxoid. Exotoxin A toxoid was generated by photoaffinity labeling as described by Marburg et al. (17). Briefly, 150 μg of exotoxin A in 1 ml of phosphate-buffered saline was mixed with 1 ml of 2.6 mM 8-azido adenosine. The mixture was placed on ice under an N_2 atmosphere and irradiated in a quartz reactor for 6 min with a high-pressure quartz mercury-vapor lamp. Unbound 8-azido adenosine was removed by dialysis against saline. Toxicity of the toxoided material in the CHO cell assay was decreased 100- to 1,000-fold compared with toxin exposed to the light in the absence of 8-azido adenosine (data not shown).

Effect of heating exotoxin A. Exotoxin A was heat inactivated as described by Liu et al. (15) by incubating the toxin at 70°C for 30 min. Such treatment abrogates cytotoxicity for the CHO indicator cell line (data not shown).

PFC assay. Estimation of direct erythrocyte or hapten-specific PFC responses was assessed by the slide modification of the Jerne plaque assay (11, 12, 19). Molten agarose in Eagle minimal essential medium was mixed with washed SRBCs or TNP-coupled SRBC target cells (24) 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 previously absorbed with SRBCs and incubated at 37°C for an additional 1.5 h, after which direct PFCs were ascertained.

Statistical analysis. Our statistical analysis was performed on the Amdahl 470V7 computer at the University of Missouri Computing and Information Center. The Fischer least-significant-difference test at a 5% confidence level was used for data analysis (29).

RESULTS

Kinetics of the toxin-induced suppression. We had previously examined the dose response of the exotoxin A-induced suppression and had found that the suppression occurred over a wide range of toxin doses (11). To analyze whether this was an actual suppression of the response or merely a delay in the responsiveness due to toxicity caused by exotoxin A, we performed a study to examine the effects of exotoxin A on the immune response to TNP-Ficoll over 10 days. Exotoxin A at a dose (10^{-4} ng) previously shown to induce suppression had a suppressive effect, which began at day 3 and continued to day 10 (Fig. 1). Ten nanograms of toxin, however, had no suppressive effect over the test period and appeared to enhance the response on days 5 and 6.

Effect of prior toxin exposure on the immune response. Splenocytes were exposed to 10 or 10^{-4} ng of toxin for 1, 2, or 3 days and then washed and put into cultures with or without the antigen TNP-Ficoll to determine if priming the cells with exotoxin A would induce suppression. Suppression of the immune response was observed in cells primed with 10^{-4} ng for 1 to 3 days (Fig. 2). In contrast to the data in Fig. 1 and previous data (11), in which no suppression was observed when 10 ng of exotoxin A and the antigen were added concomitantly, priming the splenocytes with 10 ng of toxin for 1 or 2 days induced suppression of the response to the antigen.

Effect of toxin-primed cells on the response by fresh cells. We investigated whether exotoxin A-primed cells could suppress the response of fresh cells to antigenic stimulation. Cells exposed in vitro to 10 or 10^{-4} ng of exotoxin A for 48 h and then washed and added to fresh splenocytes at a primed-to-fresh-cell ratio of 1:3 suppressed the response of the fresh splenocytes to TNP-Ficoll. Cells primed with 10 ng of toxin generally were the most suppressive (Table 1). Other experiments showed that cultures with a primed-to-fresh-cell ratio of 1:1 also were suppressed (data not shown). Cells from toxin-primed mice were also able to suppress the response of fresh cells (Table 2), indicating that toxin-induced suppressor cells could be generated both in vitro and in vivo.

Effect of specific antibody and toxin heating on toxin-induced suppression. To determine whether the immune suppression was specifically due to the toxin, we next analyzed whether exotoxin A would still induce suppression when exposed to specific anti-exotoxin-A antibodies or when it was heated at 70°C for 30 min. Both treatments were found to eliminate the cytotoxic effects of exotoxin A on CHO indicator cells (data not shown). Prior incubation of exotoxin A with specific antibodies abrogated the ability of this toxin to induce suppression, indicating that the suppression was due specifically to exotoxin A (Table 3). Heating of the toxin to 70°C also eliminated the toxin-induced suppression (Table 4), which indicated that the inducer of suppression was heat labile and that LPS, a product stable at 100°C (32), was not the immunosuppressive component.

Effect of toxoid on the immune response. Since exotoxin A exerts its cytotoxic action via its capacity to induce ADP-

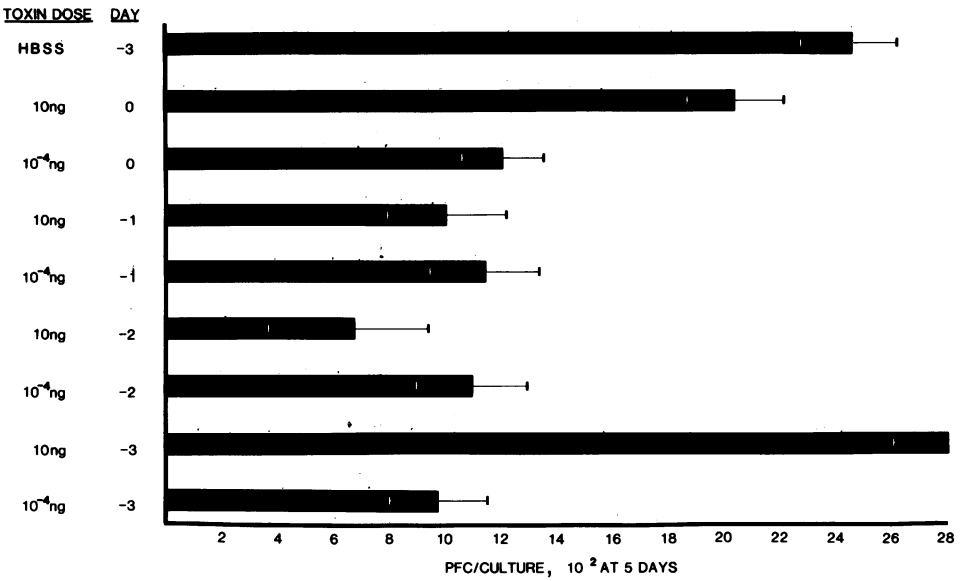

FIG. 2. Effect of toxin priming on in vitro immune response. Cells were cultured in vitro for 3 days in six-well microtiter trays, when exotoxin A at the concentrations shown per 10^7 cells was added to the cells at the times noted. After the 3-day culture (day 0), the cells were harvested, washed two times, and suspended to 2×10^7 cells per ml, and 0.5 ml of the suspension was transferred to Marbrook vessels and stimulated with 10 ng of TNP-Ficoll or dilution buffer. The cultures were incubated for 5 days; cells were then harvested and assayed for a direct anti-TNP response with a Jerne plaque assay. Results represent the mean PFC standard error for three cultures per group. PFC response of fresh cells: 3,710 ± 221 (HBSS control), 3,810 ± 446 (+ 10 ng), and 2,187 ± 227 (+ 10^{-4} ng).

ribosylation of protein elongation factor 2, which inhibits protein synthesis within the cell, we next asked if blocking of the ADP-ribosylating moiety of exotoxin A by photoaffinity labeling affected the ability of the toxin to suppress the immune response. The suppressive capacity of exotoxin A was eliminated after photoaffinity labeling, indicating that the ADP-ribosylating moiety may be involved in the toxin-induced suppression (Table 5).

DISCUSSION

The ability of bacterial products to alter the immune response is well documented. A number of bacterial cell wall products as well as material isolated from culture supernatants have been shown to have an immunoenhancing effect (1, 7, 27), and conversely, other products isolated from bacteria have been shown to suppress the response of immune cells to antigenic stimulation (6, 31). Bacterial toxins have been observed to modulate the immune response. The enterotoxins from *Staphylococcus aureus* and the pyrogenic exotoxins produced by *S. aureus* and *Streptococcus pyogenes* have been shown to be T-cell mitogens, capable of activating T cells which can suppress the immune response (3, 9, 10, 25, 26, 28). The enterotoxin from *Vibrio cholerae* can also enhance or suppress the immune response which is dependent on the timing of toxin administration relative to antigen stimulation (13, 16). *P. aeruginosa*, a gram-negative nonfermentative bacterium, produces a protein toxin, exotoxin A, which is also capable of altering the immune response. In euthymic *nu/+* mice, exotoxin A induces suppression of the in vitro and in vivo immune response to both thymus-dependent and thymus-independent antigens (11), while in athymic *nu/nu* mice, exotoxin A induces an enhanced response to thymus-independent antigens (11) and thymus-dependent antigens (12). This study attempted to establish certain parameters which affect the ability of exotoxin A to induce suppression.

We investigated the toxin-induced suppression of the immune response and observed that the depressed immune response was due to suppression rather than delay of the response (Fig. 1). A toxin dose of 10^{-4} ng suppressed the

TABLE 1. Transfer of suppression with exotoxin A-primed spleen cells

Toxin dose (ng)[a]	Response	
	Mean PFC ± SEM[b]	% of control[c]
None (HBSS control)	1,253 ± 113	100
10	547 ± 92	44
10^{-4}	823 ± 135	66

[a] Spleen cells were incubated in six-well microtiter trays with exotoxin or HBSS at listed doses per 10^7 cells for 48 h; cells were then washed two times and suspended to 2×10^7 cells per ml. Toxin-primed cells (2.5×10^6) were mixed with 7.5×10^6 fresh spleen cells and incubated with 10 ng of TNP-Ficoll for 5 days; cells were then harvested and assayed for anti-TNP PFC.

[b] For three cultures per group.

[c] Mean response of each toxin group divided by the mean antigen control response.

TABLE 2. Transfer of suppression with spleen cells from exotoxin A-primed mice

Prime (h)[a]	Response	
	Mean PFC ± SEM[b]	% of control[c]
HBSS	1,043 ± 82	100
Toxin (24)	630 ± 105	60
Toxin (48)	543 ± 68	52

[a] Mice received 100 ng of exotoxin A i.v. at the specified hours before sacrifice. Cells 2.5×10^6 from primed mice were mixed with 7.5×10^6 fresh cells and incubated with 10 ng of TNP-Ficoll for 5 days; cells were then harvested and assayed for anti-TNP PFC.

[b] For three cultures per group.

[c] Mean response of each toxin group divided by the mean antigen control response.

TABLE 3. Elimination of toxin-induced suppression by antibodies to exotoxin A

Exotoxin A dose (ng)[a]	Ab[b]	Response	
		Mean PFC ± SEM[c]	% of control[d]
—	−	1,050 ± 165	100
10^{-1}	−	530 ± 243	51
10^{-2}	−	395 ± 303	38
10^{-1}	+	1,045 ± 291	100
10^{-2}	+	1,150 ± 367	110
—	+	1,008 ± 253	96

[a] Exotoxin A was exposed for 45 min at 37°C to a 1:10 dilution of rabbit anti-exotoxin A antibodies or dilution buffer; Toxin preparations were then added to 10^7 spleen cells plus SRBC antigen. The various groups were allowed to incubate at 37°C for 5 days; cultures were then harvested and assayed for anti-SRBC responses.

[b] Ab, antibody is the IgG fraction of rabbit anti-exotoxin A.

[c] For three cultures per group.

[d] Mean response of each toxin group divided by the mean antigen control response.

response to TNP-Ficoll out to day +10. Ten nanograms of exotoxin A did not suppress the immune response during the same test period. These results suggest that exotoxin A does not have a toxic effect on spleen cells. Indeed, we had previously shown that cultures treated with 100 ng of the toxin showed little difference in viability versus cultures treated with 0.1 ng of the toxin (11).

The effects induced by certain immunomodulatory agents can be changed by altering the time of their administration relative to antigenic stimulation. Concanavalin A, a plant lectin and T-cell mitogen, has been shown to result in immunosuppression when administered before or during antigenic stimulation but results in enhancement when administered after the antigen (5, 30). Similar effects have been observed with interferon (2), *Bordetella pertussis* vaccine (6), *Mycobacterium bovis* BCG (31), and cholera enterotoxin (13, 16). This study suggests that timing of administration of exotoxin A determines the immunomodulatory effect of the toxin. Prior exposure of splenocytes to 10 ng of exotoxin A for 48 or 24 h induced suppression of the immune response, while concomitant administration of the toxin with the antigen had no effect (Fig. 2).

Proof of the presence of a suppressor cell is the ability to transfer suppression via primed cells without the activating agent. Rich and Pierce showed that splenocytes activated by concanavalin A and transferred to fresh splenocytes suppress the response of the fresh cells to subsequent antigenic stimulation (22). The same effect was demonstrated by Schlievert with staphylococcal and streptococcal pyrogenic exotoxins (25) and by Donnelly and Rogers with staphylococcal enterotoxin B (3). The suppression which is induced by exotoxin A can also be transferred by toxin-primed cells. Fresh splenocytes mixed with splenocytes primed either in vitro (Table 1) or in vivo (Table 2) with exotoxin A exhibited a suppressed response to antigens as compared with that of the control. This suppression does not appear to be due to toxin carry-over, since addition of anti-exotoxin-A antibodies along with primed cells had no effect on the suppression exerted by the primed cells on the response of fresh cells (data not shown). The suppression observed in these experiments is also not a reflection of the cytotoxic effects of exotoxin A on the splenocytes, since splenocytes from exotoxin A-primed mice were stimulated by both concanavalin A and LPS to the same extent as splenocytes from mice treated with Hanks balanced salt solution (HBBS) (data not shown). Therefore, the suppression is due to the presence of a suppressor cell. The phenotype of the primed suppressor cell has not been determined, although a T cell has been implicated in the toxin-induced suppression (11). The ability to induce suppressor cells through toxin priming is an important requirement for the identification of the cell(s) involved in the immune suppression. It allows one to determine whether one cell or a cell circuit composed of a number of cells (4, 8) is responsible for the suppression of the immune response. In addition, it allows one to determine whether the cell acts on the afferent or efferent arm of the immune response as well as to explore the mechanism(s) by which the cell exerts its suppressive effect. Experiments are under way to answer these questions.

The suppression of the immune response is due specifically to exotoxin A, since preincubation of exotoxin A with rabbit anti-exotoxin-A antibodies eliminated the toxin-induced suppression. Although LPS is a minor contaminant of the exotoxin A preparation, it is not the moiety responsible for the suppression, since heating the toxin preparation

TABLE 4. Elimination of toxin-induced suppression by heating exotoxin

Treatment of spleen cells[a]	Response	
	PFC ± SEM[b]	% of control[c]
HBSS control	1,935 ± 99	100
Normal exotoxin A		
10	2,458 ± 106	127
10^{-4}	1,113 ± 122	58
Heated exotoxin A[d]		
10	2,058 ± 142	106
10^{-4}	2,107 ± 189	109

[a] Spleen cells (10^7) were mixed with specified doses of normal or heated toxin and incubated with 10 ng of the TNP-Ficoll antigen for 5 days at 37°C; cultures were then harvested and assayed for anti-SRBC responses.

[b] For three or four cultures per group.

[c] Mean response of each toxin group divided by the mean antigen control response.

[d] Heat inactivation of exotoxin A at 70°C for 30 min.

TABLE 5. Elimination of toxin-induced suppression by photoaffinity labeling of exotoxin A

Treatment of spleen cells[a]	Response	
	PFC ± SEM[b]	% of control[c]
HBSS control	1,650 ± 141	100
Normal exotoxin A		
10^{-2}	1,162 ± 86	70
10^{-4}	1,038 ± 151	63
Photoaffinity-labeled toxoid[d]		
10^{-2}	1,747 ± 151	106
10^{-4}	1,865 ± 119	113

[a] Spleen cells (10^7) were mixed with specific doses of exotoxin A or its toxoid and incubated with the SRBC antigen for 5 days at 37°C; cultures were then harvested and assayed for anti-SRBC responses.

[b] For three cultures per group.

[c] Mean response of each toxin group divided by the mean antigen control response.

[d] Exotoxin A was converted to toxoids via photoaffinity labeling by mixing the toxin with the light-sensitive substrate 8-azido adenosine and exposing the mixture to high-intensity light.

at 70°C for 30 min eliminated the toxin-induced suppression. Since LPS is stable at 100°C (32), it is not the immunosuppressive component.

Toxin-induced suppression was also eliminated by photoaffinity labeling the toxin. These observations suggest that the ADP-ribosylating moiety may be involved in the toxin-induced suppression. However, the photoaffinity-labeled toxin retained its capacity to induce athymic nude splenocytes to respond to the thymus-dependent antigen, SRBCs (12; unpublished data). In addition, the photoaffinity-labeled toxin could also activate athymic nude splenocytes to proliferate (unpublished data). Therefore, exotoxin A may contain additional moieties which enable the toxin to exert its multiple biological effects. Future studies should enable us to elucidate the mechanism(s) by which *P. aeruginosa* exotoxin A perturbs the intrinsic immune regulation of the host.

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