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Activation of Murine T-Suppressor Lymphocytes by Group A Streptococcal and Staphylococcal Pyrogenic Exotoxins

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The effect of group A streptococcal pyrogenic exotoxin (PE) type C and staphylococcal PE on the in vitro antibody response to sheep erythrocytes was studied in cultures of mouse spleen cells. Both exotoxins suppressed the day 4 direct plaque-forming cell response when added to the cultures. The maximum suppression was obtained with 1.0 or 0.1 ng of toxin per culture, and the suppressive effect was reversed by addition of gangliosides to the cultures at the same time as the exotoxins. Preincubation of T lymphocytes for 4 days with either exotoxin resulted in the generation of a suppressor cell population, which produced dose-dependent suppression of the direct plaque-forming cell response when added to fresh sheep erythrocyte-activated splenocytes. The suppression obtained was not reversed by gangliosides indicating toxin carry-over was not responsible for the effect. B cells, preincubated with exotoxin, failed to suppress the direct plaque-forming cell response of fresh erythrocyte-activated spleen cells.

Pyrogenic exotoxins (PE) from group A streptococci and *Staphylococcus aureus* have many effects on the host, including the defining properties, the capacity to produce fever (1, 9, 16, 19, 29) and enhance susceptibility to lethal shock and myocardial damage by other agents (9, 16, 19, 22, 23, 29). In addition, the exotoxins alter the host immune system. The group A streptococcal PE are potent nonspecific T-lymphocyte mitogens, yet are capable of provoking a specific immune response (2, 12, 20). This family of toxins also enhances Arthus-type and delayed hypersensitivity skin reactions (17). Furthermore, the toxins alter reticuloendothelial clearance function (3, 18), a property which may depend on the ability of the exotoxins to inhibit ribonucleic acid synthesis in Kupffer cells and thereby prevent detoxification of agents such as endotoxin (18). The streptococcal PE also suppress immunoglobulin M (IgM) and enhance IgG synthesis to a second antigen (4, 6, 7). Both properties depend on toxin effects on T lymphocytes (4, 6).

The staphylococcal PE, recently described, is also a potent nonspecific T-lymphocyte mitogen (20), but has not been tested for the other immune effects typical of the group A streptococcal PE.

This investigation was undertaken to assess whether or not the staphylococcal PE is capable of suppressing IgM synthesis to a second antigen and to elucidate more fully the lymphocyte population responsible for the observed immunosuppression.

MATERIALS AND METHODS

All reagents and glassware were maintained pyrogen-free.

Bacteria. Group A streptococcal strain T18P (M-type 18) was isolated at Naval Medical Research Unit IV, Great Lakes, Ill. The *Staphylococcus aureus* strain was isolated by David Schlossberg, Polyclinic Medical Center, Harrisburg, Pa. The organisms were maintained in the lyophilized state in the presence of whole defibrinated fresh rabbit blood.

Animals. Male BALB/c mice, 20 to 25 g, were obtained from Simonsen Laboratories, Inc., Gilroy, Calif.

Gangliosides. Gangliosides were purchased from Calbiochem-Behring Corp., La Jolla, Calif., and were added at a concentration of 5 μg/culture.

Production and purification of exotoxins. Group A streptococcal PE type C was obtained after growth of strain T18P in a dialyzable beef-heart medium (9, 29). The toxin was purified from cell-free culture supernatant fluids by differential precipitation with ethanol and resolubilization in acetate-buffered saline (9), followed by preparative thin-layer isoelectric focusing (16). Staphylococcal PE was purified comparably, except the toxin was redissolved in pyrogen-free distilled water after ethanol precipitation rather than in acetate-buffered saline (19).

In vitro antibody response system. The antibody response system of Mishell and Dutton was used (11). Culture medium reagents were obtained from GIBCO Laboratories, Grand Island, N.Y. The nutritional mixture was prepared as described elsewhere (11) and combined with 33% fetal calf serum for daily feedings of 100 μl/culture.

Spleen cell suspensions were prepared as described by Cunningham and Watson (4). The viability of the spleen cells was greater than 90% as determined by trypan blue exclusion.

Cell suspensions were made and incubated with sheep erythrocytes (SRBC) with or without exotoxin (4). Cells from triplicate dishes were pooled, centrifuged, and resuspended in 1 ml of Hanks balanced salt solution (GIBCO Laboratories) for use in the hemolytic plaque assay.

Direct plaque-forming cells (PFC) making IgM against SRBC were determined by the slide modification of the Jerne plaque assay as described by Mishell and Dutton (11). Triplicate slides were made with 10, 50, or 100 μl of cells harvested from the cultures. Results were expressed as PFC per culture ± standard error of the mean. The difference in mean PFC response between experimental and control groups was evaluated with a Student's t test analysis of normally distributed unpaired data.

Preparation of T- and B-lymphocyte populations. Anti-thymocyte serum was prepared in rabbits by administering thymocytes from BALB/c mice intravenously every other week for two injections (10). To remove T lymphocytes, 5×10^8 splenocytes were incubated 1 h at 37°C in a 5% CO_2 atmosphere with 5 ml of 1:100 diluted anti-thymocyte serum. Subsequently, 45 ml of Hanks balanced salt solution was added, and the cells were centrifuged for 10 min at 250 × g. The cells were resuspended in 2 ml of 10% guinea pig complement previously absorbed with mouse spleen cells and incubated for 1 h at 37°C in a 5% CO_2 atmosphere. The treated cells were washed three times with Hanks balanced salt solution and resuspended in culture medium and were considered B cells. Viability by trypan blue dye exclusion was approximately 80% for the cells remaining. After treatment, the remaining cells failed to respond to concanavalin A in a 3-day assay for mitogen-induced lymphocyte proliferation (2). The anti-thymocyte serum did not significantly affect responsiveness of treated cells to lipopolysaccharide (*Salmonella minnesota* Re 595), 1 μg/well, in a 3- to 4-day assay for mitogen-induced lymphocyte proliferation (2). T lymphocytes were prepared comparably, except the splenocytes were treated with 1:100 diluted anti-mouse immunoglobulin (Miles Laboratories, Inc., Elkhart, Ind.). The antiserum was used unabsorbed. Treated cells failed to respond significantly above background to lipopolysaccharide in 3- and 4-day assays for mitogen-induced lymphocyte proliferation (2). The cells also failed to give a positive response when tested in the in vitro antibody system.

Toxin activation of T and B lymphocytes. Preparations of spleen cells or T or B lymphocytes (10^7) were incubated with 1 ng of exotoxin per ml at 37°C in a 5% CO_2 atmosphere. Control lymphocytes were incubated without toxin. After 4 days, the cells were harvested, washed 3 times with Hanks balanced salt solution, and then added in graded numbers to fresh SRBC-stimulated spleen cells.

RESULTS

Suppression of direct PFC by group A streptococcal and staphylococcal PE. Previously, it was reported that streptococcal PEs suppressed the IgM response to SRBC (4). Staphylococcal PE also suppressed the direct PFC response to SRBC (Table 1). The suppression was dose dependent, comparable to that observed for group A streptococcal PE type C (Table 1) used as a positive control, with the maximum reduction in PFC at 1 and 0.1 ng/culture ($P < 0.001$ at both doses when compared to cultures not treated with toxin). For either toxin, low or high doses did not produce immunosuppression. The suppression of direct PFC by the exotoxins was effectively reversed by addition of gangliosides (Table 2) to the cultures ($P < 0.001$).

Generation of suppressor cells. After incubation of spleen cells for 4 days with either streptococcal PE type C or staphylococcal PE, the cells were harvested, washed, and resuspended in fresh culture medium. Graded numbers of control or toxin-treated splenocytes were then added to fresh SRBC-stimulated spleen cell cultures. As few as 10^4 toxin-activated spleen cells markedly suppressed ($P < 0.07$) the day 4 direct PFC response (Table 3). Larger numbers of toxin-activated splenocytes augmented the suppression ($P < 0.001$). Control cells did not reduce significantly the direct PFC response.

The immunosuppression was not reversed by

TABLE 1. *Effect of group A streptococcal PE type C and staphylococcal PE on in vitro PFC response to SRBC*

Toxin dose (ng)	IgM PFC/culture ± SEM[a]	
	Streptococcal PE type C	Staphylococcal PE
1,000	1,123 ± 28	1,203 ± 37
100	960 ± 39	970 ± 8
10	560 ± 27	747 ± 22
1	320 ± 21	420 ± 18
0.1	363 ± 8	400 ± 35
0.01	673 ± 46	650 ± 20
0.001	987 ± 13	950 ± 20
0	953 ± 13	927 ± 13

[a] SEM, Standard error of the mean.

TABLE 2. *Ganglioside reversal of the suppression of direct PFC response to SRBC by exotoxins*

Toxin dose (ng)	Gangliosides[a]	IgM PFC/culture ± SEM[b]	
		Streptococcal PE type C	Staphylococcal PE
1	−	363 ± 17	290 ± 15
1	+	950 ± 23	973 ± 46
0.1	−	310 ± 15	283 ± 35
0.1	+	903 ± 18	1,001 ± 50
0	−	957 ± 28	957 ± 8
0	+	960 ± 30	960 ± 30

[a] Gangliosides added at 5 μg/culture.
[b] SEM, Standard error of the mean.

TABLE 3. *Effect of exotoxin-activated splenocytes on the direct PFC response after co-culture with fresh SRBC-stimulated spleen cells*

Suppressor cells[a]		IgM PFC/culture ± SEM[b]
Treatment	Cell dose (×10⁴)	
	Medium[c]	1,060 ± 37
	Streptococcal PE[c]	317 ± 19
	Staphylococcal PE[c]	413 ± 34
None	1	1,068 ± 61
None	5	1,057 ± 51
None	10	1,002 ± 67
None	50	967 ± 19
Streptococcal PE	1	833 ± 32
Streptococcal PE	5	573 ± 27
Streptococcal PE	10	357 ± 17
Streptococcal PE	50	237 ± 37
Staphylococcal PE	1	700 ± 21
Staphylococcal PE	5	590 ± 50
Staphylococcal PE	10	200 ± 17
Staphylococcal PE	50	163 ± 16

[a] Suppressor cells were obtained after culture of 10^7 spleen cells per ml with 1 ng of exotoxin per ml for 4 days.
[b] SEM, Standard error of the mean.
[c] Medium, streptococcal PE type C (1 ng), or staphylococcal PE (1 ng) was added to fresh SRBC-stimulated spleen cells.

gangliosides, which were added with the toxin-treated splenocytes to SRBC-activated cells (Table 4). These data suggest that the suppressive effect did not result from carry-over of either toxin type.

Characterization of the suppressor cell population. Toxin-activated T lymphocytes suppressed the PFC response of SRBC-stimulated fresh splenocytes (Table 5). The suppressive effect was dependent on the number of toxin-activated T lymphocytes added. The PFC responses obtained were significantly different from controls at the $P < 0.001$ level. In contrast comparable numbers of PE-activated B lymphocytes failed to suppress the direct PFC response of fresh spleen cells (Table 6).

DISCUSSION

Group A streptococcal PEs previously were shown to suppress the direct PFC response to heterologous erythrocytes (4, 7). The suppressive effect resulted from direct effect of the toxins on T lymphocytes rather than on macrophages or B lymphocytes (4). The studies of Cunningham and Watson (4) were extended in this investigation, in which it was shown that the recently described staphylococcal PE (19) was also capable of suppressing IgM synthesis. Furthermore, it was demonstrated that both families of exotoxins produced the effect by activation of a T-suppressor lymphocyte population. The observation that T lymphocytes were the target cells for suppression of IgM synthesis is consistent with other immunological effects of the toxins (2, 6, 20) in which T cells were the targets.

The dose-response curves for suppression by

TABLE 4. *Effect of gangliosides on suppression of the direct PFC response of fresh SRBC-stimulated splenocytes co-cultured with PE-activated spleen cells*

Suppressor cells[a]		Gangliosides (5 μg/culture)	IgM PFC/culture ± SEM[b]
Treatment	Cell dose (×10⁴)		
	Medium[c]	−	1,060 ± 37
	Medium[c]	+	1,023 ± 73
None	10	−	1,002 ± 67
None	10	+	977 ± 33
None	50	−	967 ± 19
None	50	+	983 ± 14
Streptococcal PE	10	−	357 ± 17
Streptococcal PE	10	+	343 ± 20
Streptococcal PE	50	−	237 ± 37
Streptococcal PE	50	+	186 ± 30
Staphylococcal PE	10	−	200 ± 17
Staphylococcal PE	10	+	207 ± 35
Staphylococcal PE	50	−	163 ± 16
Staphylococcal PE	50	+	140 ± 13

[a] Suppressor cells were obtained after culture of 10^7 spleen cells per ml with 1 ng of exotoxin per ml for 4 days.
[b] SEM, Standard error of the mean.
[c] Medium with or without gangliosides was added to fresh SRBC-stimulated spleen cells.

TABLE 5. *Effect of exotoxin-activated T lymphocytes on the direct PFC response after co-culture with fresh SRBC-stimulated spleen cells*

Suppressor cells[a]		IgM PFC/culture ± SEM[b]
Treatment	Cell dose (×10⁴)	
	Medium[c]	977 ± 60
	Streptococcal PE[c]	346 ± 25
	Staphylococcal PE[c]	285 ± 38
None	1	980 ± 60
None	5	965 ± 23
None	10	1,007 ± 42
None	50	1,110 ± 51
Streptococcal PE	1	483 ± 29
Streptococcal PE	5	360 ± 45
Streptococcal PE	10	202 ± 17
Streptococcal PE	50	143 ± 23
Staphylococcal PE	1	509 ± 27
Staphylococcal PE	5	347 ± 33
Staphylococcal PE	10	200 ± 50
Staphylococcal PE	50	125 ± 16

[a] Suppressor cells were obtained after culture of 10^7 T cells per ml with 1 ng of exotoxin per ml for 4 days.
[b] SEM, Standard error of the mean.
[c] T lymphocytes were not added to the cultures. Medium, streptococcal PE type C (1 ng), or staphylococcal PE (1 ng) was added to fresh SRBC-activated spleen cells.

TABLE 6. *Effect of exotoxin-activated B lymphocytes on the direct PFC response after co-culture with fresh SRBC-stimulated spleen cells*

B lymphocytes[a]		IgM PFC/ culture ± SEM[b]
Treatment	Cell dose (×10⁴)	
	Medium[c]	1,110 ± 25
	Streptococcal PE[c]	320 ± 18
	Staphylococcal PE[c]	285 ± 20
None	1	1,119 ± 17
None	5	1,210 ± 18
None	10	1,225 ± 37
None	50	1,250 ± 28
Streptococcal PE	1	1,098 ± 60
Streptococcal PE	5	1,101 ± 37
Streptococcal PE	10	1,200 ± 33
Streptococcal PE	50	1,217 ± 41
Staphylococcal PE	1	1,115 ± 29
Staphylococcal PE	5	1,201 ± 18
Staphylococcal PE	10	1,202 ± 35
Staphylococcal PE	50	1,247 ± 27

[a] B lymphocytes were activated by treatment of 10^7 cells per ml with 1 ng of exotoxin per ml for 4 days.

[b] SEM, Standard error of the mean.

[c] B lymphocytes were not added to the cultures. Medium, streptococcal PE type C (1 ng), or staphylococcal PE (1 ng) was added to fresh SRBC-activated spleen cells.

streptococcal PE type C and staphylococcal PE were comparable, with low and high doses of toxin failing to reduce direct PFC numbers. The optimal suppressive doses were approximately 1000 toxin molecules per lymphocyte in the culture system. The loss of suppression at doses below 0.01 ng/culture is probably a consequence of not having enough toxin molecules to effectively activate the target cells. The loss of the suppressive effect at higher toxin concentrations, as reported earlier for group A streptococcal PE (4), may have resulted from oversaturation of cell receptors. Alternatively, the loss of suppression may have resulted from activation of an overriding population of cells, thus masking the suppressive effect. In support of the latter hypothesis are recent studies in our laboratory (unpublished data) which indicate that low doses of exotoxins suppress lymphocyte proliferation in response to specific antigen stimulation. However, if higher doses of exotoxin are used (1 μg) the suppressive effect, if present, is masked by the nonspecific proliferation induced by the toxins.

The experiments in which T lymphocytes were activated with toxins and then added to fresh SRBC-activated spleen cells suggested the toxins exerted their effect on a suppressor cell population rather than on another T-cell population. Toxin-activated T cells induced more pronounced suppression of the direct PFC response of fresh spleen cells than did toxin added to the cultures. This may have resulted from recruitment of more suppressor cells during the longer incubation period.

The immunosuppression by the exotoxins was reversed by the addition of a pool of gangliosides to the culture system. It is not known which ganglioside type was responsible for the effect, but suggests that gangliosides are a part of the cell receptor for the toxins. These results are consistent with data reported previously in which gangliosides were capable of blocking the nonspecific T-lymphocyte mitogenicity of the group A streptococcal PE (21). It should be pointed out, however, that the PE immunosuppressive and nonspecific mitogenic properties may be independent of each other since both streptococcal and staphylococcal PEs suppress IgM synthesis, but only the staphylococcal PE is a nonspecific mitogen for mouse splenocytes (2; unpublished data). Gangliosides have been implicated as receptors for other toxins (5, 27, 28). It was demonstrated that lymphocyte membrane gangliosides can be redistributed by cholera toxin (13), and antibodies specific for ganglioside GM_1 induced mitogenic activity in rat thymocytes (24).

Other agents have been shown to suppress IgM synthesis against SRBC. Rich and Pierce described the activation of a nonspecific T-suppressor cell population after incubation of mouse spleen cells with either concanavalin A (15) or phytohemagglutinin (14). The authors suggested that suppressor cells activated by concanavalin A inhibited the clonal expansion or terminal differentiation of previously activated B cells (15). Spleen cells from mice infected with the nematode *Trichinella spiralis* showed suppressed direct PFC responses to SRBC (8). The observed suppressive effect was abolished by lysis with anti-thy 1 antisera, indicating the involvement of T-suppressor cells. Staphylococcal enterotoxins A and B, which share some biological properties with the PE, also suppress IgM synthesis (25). The suppression induced by the enterotoxins resulted from a toxin effect on T cells.

The ability of the group A streptococcal and staphylococcal PEs to suppress IgM synthesis may facilitate establishment of the microorganisms in the host and in this way may contribute to development of disease. Studies are underway to evaluate whether the exotoxins suppress the host response to the microorganisms producing the toxins.

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