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Mitogenic Activity of Staphylococcal Exfoliative Toxin

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Exfoliative toxin from *Staphylococcus aureus* was mitogenic for murine spleen cells. It was primarily active on T lymphocytes but also significantly stimulated B cells from the spleens of nude mice. The mitogenicity was not affected by simple sugar or α-methylpyranosides. *N*-acetylglucosamine and *N*-acetylgalactosamine inhibited its effect. Exfoliatin was as powerful a mitogen as the enterotoxins of *S. aureus*. Some significant differences between the mitogenic activity of the two toxins were demonstrated.

A variety of materials isolated from plant and animal sources have been shown to be mitogens for mouse and human lymphoid cells. These mitogens are not antigen specific; they produce polyclonal activation of subsets of T or B cells or the entire lymphocyte population. Certain mitogens, e.g., the lectin phytohemagglutinin, are active only on T cells (2), whereas some (e.g., bacterial lipopolysaccharide [LPS]) stimulate B cells exclusively and some (as pokeweed lectin) can activate both types of cells.

Some bacterial exoproteins have also been shown to possess mitogenic properties. Ling et al. (6) found that culture filtrates of *Staphylococcus aureus* contained nonspecific mitogens. More specifically, Peavy et al. (8) found that staphylococcal enterotoxin B (SEB) was mitogenic for murine spleen cells but also noted that the SEB was active on a different population than was bacterial LPS. Shambaugh and Blumenschein (11) demonstrated that SEB was mitogenic for human lymphocytes but did not determine whether the SEB was active on T or B lymphocytes specifically. Later, Warren et al. (14) found that staphylococcal enterotoxins type A (SEA) and C_1 (SEC_1) were as mitogenic as SEB. Greaves et al. (3) demonstrated that SEB was mitogenic for T but not for B lymphocytes. Smith and Johnson (12) showed that SEA was also a T-cell mitogen.

This investigation was undertaken to determine whether exfoliative toxin, a purified extracellular product from *S. aureus*, was mitogenic for murine lymphocytes and to compare its effect with that observed with staphylococcal enterotoxins and concanavalin A (ConA).

MATERIALS AND METHODS

Mitogens and toxin. ConA was purchased from Pharmacia Fine Chemicals Inc., (Piscataway, N.J.). LPS W of *Escherichia coli* O127:B8 was obtained from Difco Laboratories (Detroit, Mich.). Both were reconstituted in 10 mM phosphate-buffered saline (PBS), pH 7.2.

Staphylococcal exfoliative toxins were purified by the method of Johnson et al. (4) from strains TA and DI of *S. aureus*; SEA was purified from strain 13N-2909 by the method of Schantz et al. (9). Toxins were stored as lyophilized powders and were reconstituted and dialyzed against PBS before use.

Cell cultures. Cell suspensions were prepared from spleens of 4- to 6-week-old, inbred, male BALB/c mice obtained from Microbiological Associates (Bethesda, Md.). Data presented in this paper were obtained with BALB/c mice, but similar results were obtained with C57BL/6 mice. In experiments using athymic nude mice, lymphocytes were obtained from the spleens of BALB/c background, congenitally athymic homozygous nu/nu mice, obtained from Sprague-Dawley (Madison, Wis.). All animals were sacrificed by cervical dislocation.

Media used were RPMI 1640 with glutamine added, Hanks balanced salt solution, and Eagle minimal essential medium with nonessential amino acids, all obtained from GIBCO Laboratories (Grand Island, N.Y.). Fetal bovine serum (FBS), obtained from Reheis Chemical Co. (Phoenix, Ariz.), was heated at 56°C for 30 min before use. Complete medium contained 5% heat-inactivated FBS, 100 U of penicillin per ml and 100 μg of streptomycin per ml. Spleen cell suspensions were prepared by straining scissor-minced spleens through a 60-gauge stainless-steel mesh screen into complete medium. This preparation was then forced through a 25-gauge needle to obtain a single cell suspension. Cells were centrifuged and washed in complete medium; concentration and viability were determined by using a hemacytometer before plating and suspension into 96-well flat-bottomed microtiter plates obtained from Falcon Labware (Oxnard, Calif.).

Each well of the microtiter plate received 10^6 cells in 200 μl of complete medium, plus an additional 50 μl of medium (control) or medium containing mitogen or toxin. All experimental groups were run in triplicate. The cell cultures were incubated for 42 to 46 h at 37°C in a humidified atmosphere of 5% CO_2–95% air and then pulsed for 4 h with 1 μCi of [^{3}H]thymidine (specific activity 40 Ci/mmol), obtained from Amersham Corp. (Arlington Heights, Ill.). The 42- to 46-h incu-

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bation with mitogen has previously been shown to be maximal for SEB (8). Similar results were obtained for exfoliative toxin (data not shown); therefore these conditions were chosen as standard. Cultures were harvested by using a multiple automated sample harvester (MASH) from Microbiological Associates. Cells were collected on filter paper 934 AH from Reeve Angel, Whatman, Inc. (Clifton, N.J.), and the filters were placed in small scintillation vials with 5 ml of Liquifluor-toluene (42 ml/liter) added before analyzing the level of radioactivity with a Searle Mark III liquid scintillation counter. Results are expressed as mean counts per minute ± standard error of the mean.

Separation of T-cells. T cells were obtained by a modification of the technique described by Julius et al. (5). Large nylon wool columns were prepared by packing 3.5 g of detoxified nylon wool in a 50-ml syringe. Cell suspensions were incubated on the column at 37°C for 45 min. T cells were eluted by using Hanks balanced salt solution containing FBS at a flow rate of 2 to 3 ml/min. Recovery was 15 to 20%, and viability was >90%. Less than 10% of these separated cell cultures were B cells as determined by immunofluorescence staining.

Chemicals. D-Glucose was obtained from Fisher Scientific Co. (Fairlawn, N.J.), *N*-acetyl-D-glucosamine (GlcNAc) and α-methyl-D-glucoside from J. T. Baker (Phillipsburg, N.J.), *N*-acetyl-D-galactosamine (GalNAc) from Schwartz/Mann (Orangeburg, N.Y.), and α-methyl-D-mannoside from Calbiochem (San Diego, Calif.). Liquifluor scintillation fluid was purchased from New England Nuclear Corp. (Boston, Mass.).

RESULTS

Mitogenic effects of exfoliative toxins. Figure 1 demonstrates the comparative responses of spleen cells to TA exfoliative toxin, SEA, ConA, and LPS. ConA evoked the greatest level of stimulation, and exfoliatin was more effective than either SEA or LPS. The exfoliatin also exhibited a sharp inhibition of deoxyribonucleic acid synthesis at high concentrations in contrast to SEA, where a broad plateau was usually observed. The TA and DI types of exfoliatin exhibited similar levels of mitogenicity in spleen cell cultures. Due to limited availability and variable stability, the DI type toxin was not used in any further experiments.

Mitogenicity in separated cell cultures. In cultures enriched for T cells by passage through nylon wool columns, type TA exfoliative toxin retained its mitogenic activity (Fig. 2). In agreement with previous findings, the mitogenic responses of ConA and SEA were also retained, whereas the LPS response was almost eliminated. An overall depression in total stimulation was evident in all mitogens active in these separated cell cultures. The SEA response was most markedly diminished and exhibited a stimulatory dose response from 5 to 500 μg per culture.

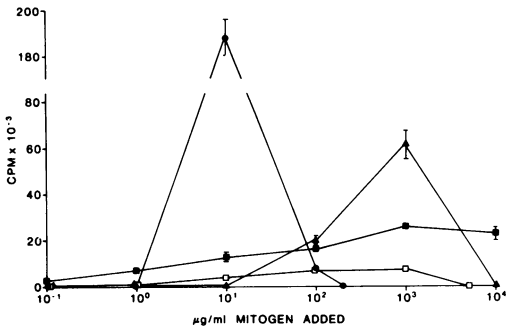

FIG. 1. *Dose-response curves of various mitogens on unseparated BALB/c spleen cells. Symbols:* (●) *ConA;* (■) *SEA;* (▲) *exfoliative toxin type TA;* (□) *LPS.*

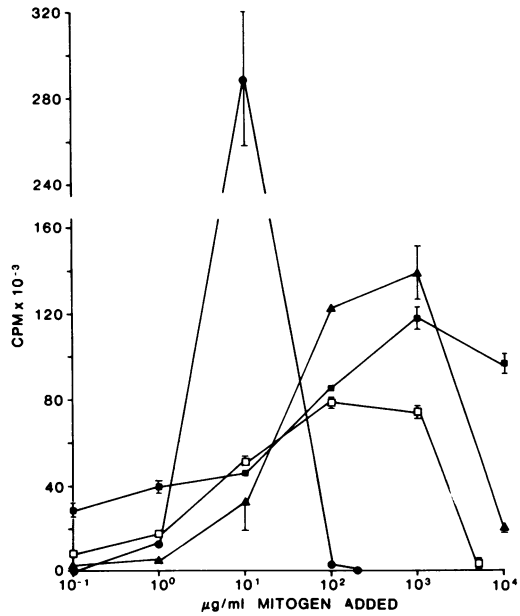

FIG. 2. *Dose-response curves of mitogens on nylon wool column-passed BALB/c spleen cells. Symbols are as in Fig. 1.*

Mitogenicity in congenital athymic nude mice. Type TA exfoliative toxin evoked a strong response from spleen cells from nude mice (Fig. 3). Levels of toxin up to 500 μg per culture were significantly mitogenic; the inhibition observed at this concentration with the mixed spleen cell population and the enriched T-cell preparations from the BALB/c mice did not occur. As expected, LPS showed a strong mitogenic response and ConA gave no response. Only a limited response to SEA was observed.

Effects of saccharides on mitogenic stimulation. Glucose was ineffective in modifying the mitogenic stimulation brought about by

either SEA or TA type exfoliatin. α-Methyl-D-mannoside and α-methyl-D-glucoside were also without effect, in marked contrast to the classical inhibition observed with ConA.

The effect of two acetylated amino sugars, GlcNAc and GalNAc, upon the mitogenicity of type TA exfoliatin and SEA is shown in Table 1. Both compounds markedly reduced the mitogenic stimulation of the toxins. These results are presented as stimulation ratios because of the variability of the effect of the carbohydrates upon the incorporation of radiolabel into unstimulated cultures. Nevertheless, the differences in the stimulation ratios were statistically significant at the $P < 0.001$ level (Student's t test).

These results differ from those of Smith and Johnson (12) for SEA and Warren et al. (14) for SEB, in that they did not detect interference by GlcNAc. This apparent discrepancy may perhaps be attributed to differing methodologies (Smith and Johnson measured plaque-forming cell inhibition, and Warren et al. used tube cultures).

DISCUSSION

Staphylococcal exfoliative toxin proved to be an active mitogen for murine splenocytes. It is primarily a T-cell mitogen; the shape of the dose-response curve and the concentration yielding a maximal response were very similar in unfractionated cells and in preparations enriched in T cells by passage through a nylon wool column. SEA is also a T-cell mitogen (12), but in our hands the stimulation it elicited in T-cell preparations was markedly reduced compared with the response of the total spleen cell population. It is suggested that the exfoliative toxin stimulates a different, less nylon wool-adherent subset of T lymphocytes.

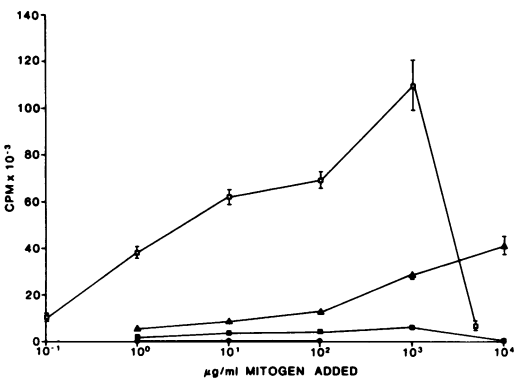

FIG. 3. *Dose-response curves of mitogens on congenitally athymic nu/nu BALB/c spleen cells. Symbols are as in Fig. 1.*

The response of B cells obtained from nude mice provides another aspect in which the exfoliatin and the enterotoxins differ. Only a minimal response was brought about by SEA, but type TA exfoliatin was significantly mitogenic. It has been suggested that for stimulation B cells require a large number of stimulant molecules bound simultaneously to receptor sites on the cell surface (7). It will be of interest to learn whether the exfoliatin does have a measurable association constant with these cells.

The inhibition by GlcNAc and GalNAc of the mitogenic stimulation elicited by both type TA exfoliatin and SEA suggests that these molecules possess a structurally similar feature which is involved in their interaction with the cells in this system. The existence of a receptor containing an acetylated amino sugar may also be inferred, but it would be premature to assume that this receptor is located on splenic T cells. All attempts to demonstrate significant binding of enterotoxin to lymphocytes have failed (J. L. Middlebrook, personal communication). Thus, the putative receptor-toxin complex has a very low association constant or the toxin affects the lipid membrane per se. Both the spleen- and T-cell-enriched preparations contain macrophages which have been shown to release mediators that affect the function of lymphoid cells. The mitogens phytohemagglutinin and LPS, for example, stimulate monocytes to produce a lymphocyte-activating factor which not only is directly mitogenic but acts synergistically with them in the induction of T-lymphocyte prolif-

TABLE 1. *Effect of acetylated 100 mM amino sugars[a] on the stimulation of murine spleen cells by 100 μg of SEA or type TA exfoliative toxin per ml*

Expt	Stimulation ratio[a]					
	SEA			Exfoliative toxin		
	None	+GlcNAc	+GalNAc	None	+GlcNAc	+GalNAc
1	14	4	4	42	16	8
2	15	3	1	27	7	2
3	21	1	5	36	14	7
4	20	7	3	22	13	9

[a] Mean cpm in the presence of mitogen/cpm in its absence.

eration (1). Analogous observations have been made with SEB and with exfoliatin; T-cell preparations cleared of macrophages do not show a mitogenic response to either toxin, and both toxins cause the release of a soluble factor from macrophages which enables T cells to respond mitogenically (W. H. Adler, personal communication). Further work is needed to characterize the activating factor and to determine the exact mechanism of toxin stimulation.

Several different mitogens have now been described in cultures of *S. aureus*, including the enterotoxins (14), two forms of pyrogenic exotoxins (10, 13), and our description herein of exfoliative toxin. These families of toxins are biologically and immunologically distinct, yet all are lymphocyte mitogens. The possibility exists that these molecules share a common mitogenic determinant. Further work on protein structure may provide an insight into the active site.

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LITERATURE CITED

1. **Gery, I., R. K. Gershon, and B. H. Waksman.** 1972. Potentiation of the T-lymphocyte response to mitogens. I. The responding cell. J. Exp. Med. **136**:128-142.
2. **Greaves, M., and G. Janossy.** 1972. Elicitation of selective T and B lymphocyte responses by cell surface binding ligands. Transplant. Rev. **11**:87-130.
3. **Greaves, M., G. Janossy, and M. Doenhoff.** 1974. Selective triggering of human T and B lymphocytes in vitro by polyclonal mitogens. J. Exp. Med. **140**:1-18.
4. **Johnson, A. D., L. Spero, J. S. Cades, and B. T. DeCicco.** 1979. Purification and characterization of different types of exfoliative toxins from *Staphylococcus aureus*. Infect. Immun. **24**:679-684.
5. **Julius, M. H., E. Simpson, and L. A. Herzenberg.** 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur. J. Immunol. **3**:645-649.
6. **Ling, N. R., E. Spicer, K. James, and N. Williamson.** 1965. The activation of human peripheral lymphocytes by products of staphylococci. Br. J. Haematol. **11**:421-431.
7. **Lis, H., and N. Sharon.** 1973. The biochemistry of plant lectins (phytohemagglutinins). Annu. Rev. Biochem. **42**:541-574.
8. **Peavy, D. L., W. H. Adler, and R. T. Smith.** 1970. The mitogenic effects of endotoxin and staphylococcal enterotoxin B on mouse spleen cells and human peripheral lymphocytes. J. Immunol. **105**:1453-1458.
9. **Schantz, E. J., W. G. Roessler, M. J. Woodburn, J. M. Lynch, H. M. Jacoby, S. J. Silverman, J. C. Gorman, and L. Spero.** 1972. Purification and some chemical and physical properties of staphylococcal enterotoxin A. Biochemistry **11**:360-366.
10. **Schlievert, P. M., D. J. Schoettle, and D. W. Watson.** 1979. Nonspecific T-lymphocyte mitogenesis by pyrogenic exotoxins from group A streptococci and *Staphylococcus aureus*. Infect. Immun. **25**:1075-1077.
11. **Shambaugh, G. E., III, and G. R. Blumenschein.** 1974. Combined effects of phytohemagglutinin and staphylococcal enterotoxin B or deoxyribonucleic acid synthesis during blast transformation in human lymphocytes. Infect. Immun. **9**:384-390.
12. **Smith, B. G., and H. M. Johnson.** 1975. The effect of staphylococcal enterotoxins on the primary in vitro immune response. J. Immunol. **115**:575-578.
13. **Taranta, A.** 1974. Lymphocyte mitogens of staphylococcal origin. Ann. N.Y. Acad. Sci. **236**:362-375.
14. **Warren, J. R., D. L. Leatherman, and J. F. Metzger.** 1975. Evidence for cell-receptor activity in lymphocyte stimulation by staphylococcal enterotoxin. J. Immunol. **115**:49-53.