INFECTION AND IMMUNITY, Aug. 1974, p. 347-351

Vol. 10, No. 2
Printed in U.S.A.

Pyrogenic Specificity of Streptococcal Exotoxins, Staphylococcal Enterotoxin, and Gram-Negative Endotoxin

K. W. BRUNSON AND DENNIS W. WATSON

Department of Microbiology, University of Minnesota Medical School, Minneapolis, Minnesota 55455

Received for publication 29 April 1974

Streptococcal exotoxin and staphylococcal enterotoxin share several biological properties, including pyrogenicity, lymphocyte mitogenicity, and enhancement of gram-negative endotoxin lethality. These analogies of the toxins prompted comparative pyrogenic studies. When American Dutch rabbits were immunized by repeated intravenous injections of small amounts of staphylococcal enterotoxin, they exhibited a decreased febrile response upon challenge when compared with control animals not previously injected. The same animals responded similarly to control groups when challenged with streptococcal exotoxin A, B, or C. No cross-reactivity was observed in reciprocal cross-tests, using animals immune to the streptococcal toxins and challenging with staphylococcal enterotoxin. No cross-reactivity between either the streptococcal or staphylococcal toxins and bacterial endotoxin was observed.

Streptococcal pyrogenic exotoxin (SPE) (Synonyms: Dick toxin, erythrogenic toxin, scarlet fever toxin, streptococcal exotoxin) and staphylococcal enterotoxin (SET) are interestingly similar in several respects, sharing certain chemical and biological properties. In contrast to the more ubiquitous pyrogen gram-negative bacterial endotoxin, both SPE and SET are extracellular products obtained from gram-positive microorganisms. Both SPE and SET are rather small molecules; the sedimentation coefficients were determined as 1.8 and 3.0, respectively (1, 10). Enterotoxin A was found to have a molecular weight near 28,000; the toxin was determined to be a single polypeptide chain (16). Purified exotoxin A yielded a molecular weight value of approximately 29,000 (10, 14; K. V. Kushko, Fifth International Congress of Biochemistry, vol. 9, abstr., p. 47, 1963). The exotoxin was found to be made up of a single polypeptide chain with glutamic acid as the N-terminal residue (13), although some purified preparations also contained associated hyaluronic acid (10).

Pyrogenicity of SPE in rabbits has been well established (10, 21). The pyrogenic response of cats to SET has been documented (2, 3), and this paper will show that the toxin is pyrogenic upon intravenous administration to rabbits.

One of the most striking biological features of SPE is its dramatic potentiating effect of gram-negative endotoxin lethality, as demonstrated in rabbits, monkeys, and mice (10, 11). The probable clinical implications for human disease have been pointed out (11). SET also enhances bacterial endotoxin lethality, as demonstrated in either the mouse or rabbit (20).

Further analogy of the toxins is provided in that both are mitogenic for lymphocytes. SPE is a potent agent for the transformation of human peripheral blood lymphocytes, requiring only 0.00005 μg/ml of culture for 50% transformation (11). The toxin is also active on mouse, guinea pig, and rabbit lymphocytes (12). SET is also a potent mitogen, as determined by using lymphocytes from the guinea pig (6), mouse (15, 18), or human (15).

The above analogies prompted comparative pyrogenic studies of the staphylococcal and streptococcal toxins. The specificity of the pyrogenic response to each toxin was also distinguished from that resulting from the administration of gram-negative endotoxin.

MATERIALS AND METHODS

Glassware and equipment. All glassware used was freed of pyrogen contamination by heating at 190 C for 5 h. For injections, sterile, pyrogen-free disposable syringes and needles were used.

Water and buffers. Water used for solutions and buffers was first deionized and then made pyrogen-free by distilling in a glass still. Freshly distilled water was kept for short periods of time at 80 C or stored briefly at 4 C. Care was taken that any buffers prepared were free of pyrogen contamination.

Bacterial strains. Group A streptococcal strains were used to produce streptococcal pyrogenic exotoxin. Strain NY5 (type 10) was used for the production of exotoxin A. The NY5 strain, originally from

Rebecca Lancefield of the Rockefeller University, came from NMRU-4, Great Lakes, Ill. Strain Cook B, received from the late Aaron H. Stock, Department of Microbiology, University of Pittsburgh, was used for the production of exotoxin B. T18P strain, isolated at NMRU-4, Great Lakes, Ill., was used for exotoxin C production.

Toxins. Production and purification of group A streptococcal pyrogenic exotoxins has been previously described (10, 19, 21; K. W. Brunson, Ph.D. thesis, University of Minnesota, Minneapolis, 1973). Briefly, a heavily inoculated dialyzable medium (10) was incubated for 6 to 8 h at 37 C, and the chilled supernatant fluid was precipitated with cold ethanol. The crude precipitant was dissolved in acetate-buffered physiological saline, pH 4.0, and 0.005 M acetate. Five to ten repeated precipitation steps yielded a highly purified toxin preparation which retained potent biological activity (10; K. W. Brunson, Ph.D. thesis, University of Minnesota, Minneapolis, 1973).

Staphylococcal enterotoxin was obtained from Edward Schantz of Fort Detrick, Md. It was supplied in the lyophilyzed form as purified staphylococcal enterotoxin A, lot CA-2-E.

Gram-negative endotoxin was prepared from *Salmonella minnesota*, wild-type smooth, or from *Salmonella typhosa* as described by Kim and Watson (9).

The concept of the minimal pyrogenic dose at 3 h (MPD-3) has been thoroughly described and discussed elsewhere (22).

Experimental animals. Young adult American Dutch rabbits, about 3 months old and weighing 1.0 to 1.5 kg each, were used. They were obtained from a single local supplier, kept in air-conditioned rooms in cages which were regularly cleaned, and fed a diet of Purina rabbit chow checkers (Ralston Purina Co., St. Louis, Mo.).

Pyrogen test. For the pyrogen test, rabbits were retained in a rack having 15 separate stalls (Chemical and Pharmaceutical Industry Co., New York, N.Y.). A wooden collar secured the neck of the animal rather loosely, but served to immobilize the head while injections were made.

Individual rabbit temperatures were measured by an electric universal thermometer, type TR3 (Elektrolaboratoriet, Copenhagen, Denmark). Fifteen separate leads and thermocouple probes were used; a probe was inserted into the rectum of each rabbit and secured with a soft lead solder wire wrapped around the tail.

Before use in the pyrogen test, incoming rabbits were routinely conditioned in the rack for 3 to 5 h, a day or more before the test. On the day of the pyrogen test, the rabbits were contained in the rack for 1 h before injections to allow temperature equilibrium after handling. All injections were made into the marginal ear vein.

A group of at least five rabbits was injected with a substance being tested for the febrile response. Individual rabbit temperatures were then recorded subsequently at 30-min intervals for 5 h. Temperatures of the five animals were then averaged, and changes from the 0-h reading were plotted to graphically depict the fever curve. An average temperature change greater than 1 F (-17.2 C) was considered significant based upon extensive experimentation in this laboratory (22, 23).

Immunization. Rabbits were injected intravenously every other day for a total of seven injections, with doses of ten MPD-3 of SPE per kg (10). For immunization with staphylococcal enterotoxin, the dose was 1.0 μg/kg. If rabbits were not immune upon challenge, this series was repeated. Immunization with endotoxin was accomplished by increasing the daily intravenous administration (22).

RESULTS

Normal unimmunized rabbits respond to SET with a febrile response (Fig. 1). These same animals could be immunized to yield a depressed response when challenged with the same dose of SET. There was no pyrogenic cross-reactivity with SPE A, B, or C, respectively (Fig. 1). A homologous challenge (Fig. 1) shows that the experimental animals retained pyrogenic immunity to the challenging dose of SET. Furthermore, the SET material employed exhibited no pyrogenic cross-reaction with gram-negative endotoxin (Fig. 2).

Similar results were obtained in reciprocal pyrogenic cross-tests. Figure 3 depicts the establishment of pyrogenic immunity in three

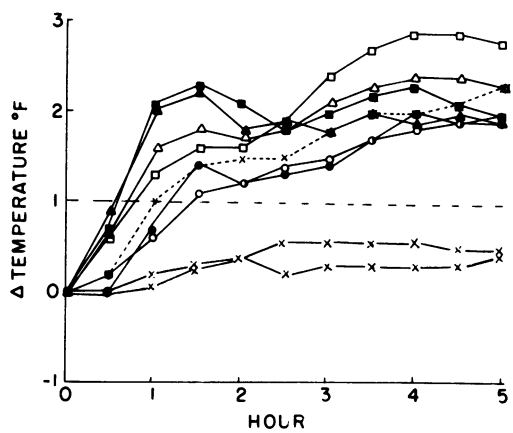

FIG. 1. *Test for pyrogenic immunity. For heterologous challenge, closed figures represent rabbits immune to SET-A, and open figures represent control rabbits (not immunized). For homologous challenge with SET-A, ×——× represents immune rabbits and ×----× represents controls. Challenge with SET-A, 1.0 μg/kg, compares immune animals (×——×) with controls (×----×). When rabbits immune to SET-A were challenged with 10 MPD-3 of either SPE-A (●, ○), SPE-B (■, □), or SPE-C (▲, △), no cross-immunity was noted. After cross-testing, rabbits immune to SET-A (×——×) were again challenged with the homologous toxin and were found still immune when compared with controls (×----×). Each curve represents the average febrile response of five rabbits when injected with the toxin indicated.*

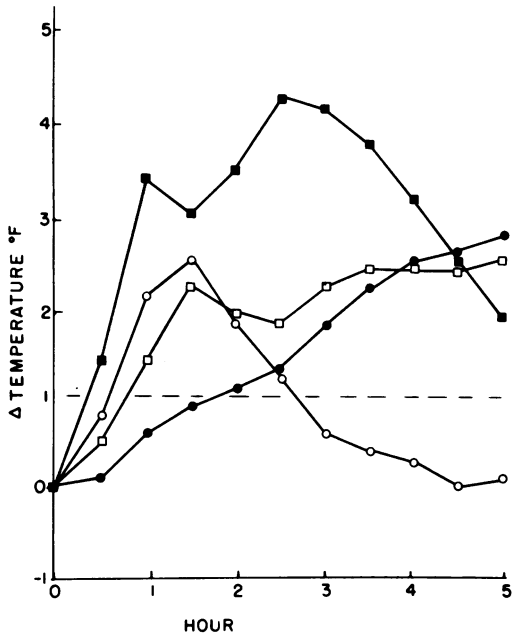

FIG. 2. *Pyrogenic cross-test. Endotoxin-immunized rabbits, when challenged with 10 MPD-3 of endotoxin, were found to be immune (O) compared with nonimmunized controls (■). When challenged with SET-A, 1.0 μg/kg, the endotoxin-immune animals (□) exhibited a febrile response similar to challenged controls (●). Each curve represents the average febrile response of five rabbits.*

groups of rabbits when immunized with either SPE-A, SPE-B, or SPE-C. Immune animals were compared with normal, unimmunized controls when challenged with the respective homologous toxin preparation. When SPE-immune rabbits were challenged with SET, the response was essentially the same as in control animals (Fig. 4). Further pyrogenic tests showed that rabbits immune to SPE-A, SPE-B, or SPE-C responded the same as control animals to a challenge with gram-negative endotoxin, indicating no cross-reactivity with this commonly encountered pyrogen (Fig. 5). Figure 6 depicts retention of immunity to the respective homologous toxin challenge of SPE-A, SPE-B, or SPE-C. Pyrogenic immunity was complete in the groups of rabbits immunized with either SPE-A or SPE-B; a good partial immunity was retained in the group immunized with SPE-C.

DISCUSSION

All bacterial toxins used in this study typically elicited a biphasic febrile response when appropriate doses were administered intravenously to American Dutch rabbits. The time interval between fever peaks was generally about 2 h. The peaks usually occurred at 1 and 3 h after injection with bacterial endotoxin; for either of the exotoxin preparations, febrile peaks at 2 and 4 h postinjection were more common.

Complete pyrogenic immunity could be obtained with SPE or with SET; that is, suppression of both of the febrile peaks below the "normal" 1 F (−17.2 C) limit of change was exhibited in immune animals. In rabbits ren-

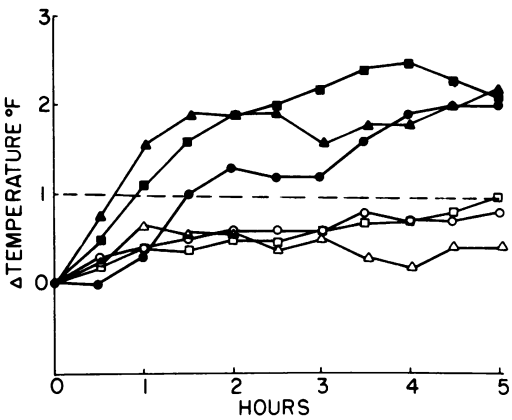

FIG. 3. *Test for pyrogenic immunity to homologous challenge with 10 MPD-3 of SPE. Open figures depict immune rabbits; closed figures indicate controls (not immunized). A depressed febrile response is exhibited by either of three immunized groups when compared with nonimmunized controls. The challenging toxins are SPE-A (O, ●), SPE-B (□, ■), and SPE-C (Δ, ▲). Each curve represents the average febrile response of five rabbits.*

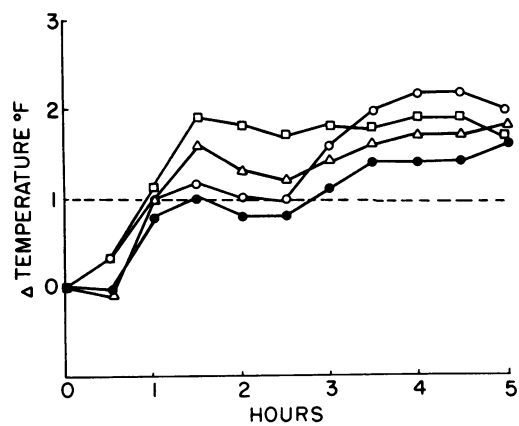

FIG. 4. *Pyrogenic cross-test of rabbits immune to either SPE-A (O), SPE-B (Δ), or SPE-C (□) when challenged with SET-A, as compared with SET-A challenged control (normal, unimmunized) rabbits (●). Each curve represents the average febrile response of five rabbits.*

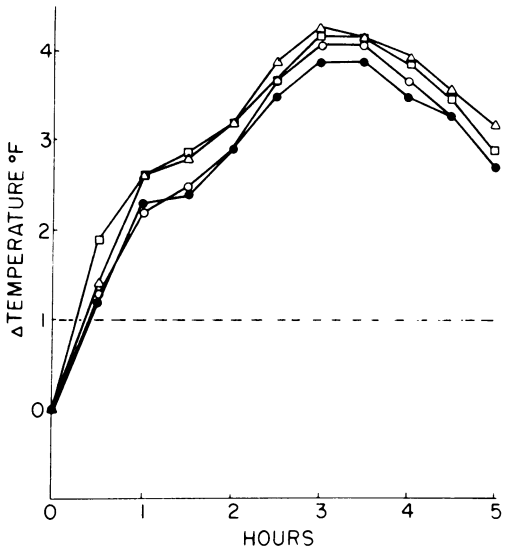

FIG. 5. *Pyrogenic cross-test of rabbits immune to either SPE-A (○), SPE-B (△), or SPE-C (□) when challenged with gram-negative bacterial endotoxin, as compared with the endotoxin response of normal, unimmunized rabbits (●). Each curve represents the average febrile response of five rabbits.*

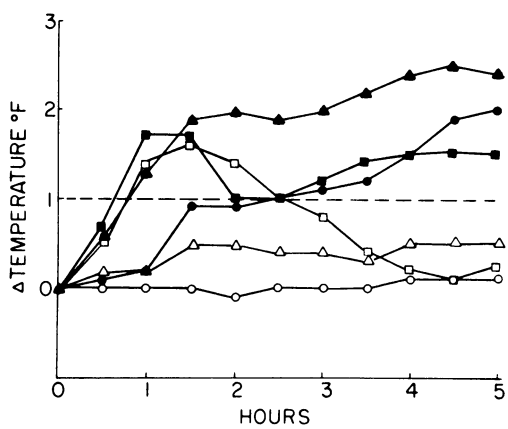

FIG. 6. *Test for retention of pyrogenic immunity in immune rabbits (open figures) when challenged with the homologous toxin, as compared with unimmunized controls (closed figures). The challenges are SPE-A (○, ●), SPE-B (△, ▲), or SPE-C (□, ■). Complete pyrogenic immunity has been maintained in rabbits immunized with either SPE-A or SPE-B; partial immunity is exhibited by that group of animals immunized with SPE-C. Each curve represents the average febrile response of five rabbits.*

dered immune to the pyrogenic effect of endotoxin, only the second peak was depressed.

Evidence is strong for the involvement of antibody in the protection against pyrogenicity of endotoxin. Passive transfer of immune serum was found in serum fractions containing 19*S* immunoglobulins (7, 8). Antiserum to streptococcal toxin, when incubated with toxin in vitro before injection, was found to neutralize the toxin's pyrogenic effect. Activity of the antiserum was found to be type specific for the toxin involved (21). Specificity of acquired pyrogenic immunity to each of the SPEs A, B, or C can also be demonstrated by pyrogenic immunity cross-tests (21).

That the pyrogenic response to the various toxins used is specific can be demonstrated by multiple cross-tests. Thus, even though a general stimulation of the reticuloendothelial system may aid the host upon encountering a pyrogenic toxin, this cannot account for complete protection, as shown by cross-testing.

The pyrogen test using American Dutch rabbits has distinct advantages as a biological assay system for pyrogenic bacterial toxins. With immunized animals it is specific, distinguishing febrile responses to toxins derived from three different genera of bacteria. Further specificity is possible in that three different immunological types of SPE can be separated on the basis of the pyrogen test. Preliminary work indicates that a fourth SPE may also be distinguished by this method (17). Specificity has been observed for at least two different types of SET when injected into cats (3). With SET-A, a depressed pyrogenic response was obtained after repeated administration by the intravenous but not by the intraventricular (cerebral) route. Immunization of rabbits with bacterial endotoxin extracted from one gram-negative species usually yields pyrogenic cross-reactivity with other endotoxins. This cross-reaction is apparently due to similar molecular construction of the "lipid A" portion of endotoxin, which has been amply demonstrated to contain the pyrogenically active site (5).

Fairly small amounts of pyrogenic toxins are needed for the pyrogen test. The MPD-3 of SPE-A is 0.07 μg/kg, using an intravenous dose in the American Dutch rabbit (10). MPD-3 values of SPE-B and SPE-C are somewhat larger, being 1.2 and 4.5 μg/kg, respectively (K. W. Brunson, Ph.D. thesis, University of Minnesota, Minneapolis, 1973). For SET-A, amounts of 1.0 μg/kg administered intravenously to the rabbit produce fever consistently. Smaller doses are effective in cats, and the measure of pyrogenicity has been suggested as a more sensitive alternative to the emetic dose response in such animals (3). The MPD-3 for endotoxins is generally about 1 to 10 ng (9); this compares favorably with amounts needed for complete coagulation in the limulus lysate test (4).

Several in vitro tests have been designed for monitoring bacterial toxins both qualitatively and quantitatively; these are important in routine assays. When one wants to ascertain biological activity of the toxins used in this study, the pyrogen test is probably the method of choice.

ACKNOWLEDGMENTS

We gratefully acknowledge the use of staphylococcal enterotoxin preparations sent to us by Edward Schantz, and the use of streptococcal exotoxin A prepared by Y. B. Kim.

This research was supported by Public Health Service grant AI-06487 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Bergdoll, M. S. 1967. The staphylococcal enterotoxins. Jap. J. Microbiol. **11**:358-368.
2. Clark, W. G., and H. L. Borison. 1963. Pyrogenic effect of purified staphylococcal enterotoxin. J. Pharmacol. Exp. Ther. **142**:237-241.
3. Clark, W. G., and J. S. Page. 1968. Pyrogenic responses to staphylococcal enterotoxins A and B in cats. J. Bacteriol. **96**:1940-1946.
4. Elin, R. J., and S. M. Wolff. 1973. Nonspecificity of the *Limulus* amebocyte lysate test: positive reactions with polynucleotides and proteins. J. Infect. Dis. **128**:349-352.
5. Galanos, C., E. T. Rietschel, O. Luderitz, O. Westphal, Y. B. Kim, and D. W. Watson. 1972. Biological activities of lipid A complexed with bovine-serum albumin. Eur. J. Biochem. **31**:230-233.
6. Kaplan, J. 1972. Staphylococcal enterotoxin B induced release of macrophage migration inhibition factor from normal lymphocytes. Cell. Immunol. **3**:245-252.
7. Kim, Y. B., and D. W. Watson. 1965. Modification of host response to bacterial endotoxins. II. Passive transfer of immunity to bacterial endotoxin with fractions containing 19S antibodies. J. Exp. Med. **121**:751-759.
8. Kim, Y. B., and D. W. Watson. 1966. Role of antibodies in reactions to gram-negative bacterial endotoxins. Ann. N.Y. Acad. Sci. **133**:727-745.
9. Kim, Y. B., and D. W. Watson. 1967. Biologically active endotoxins from *Salmonella* mutants deficient in O- and R-polysaccharides and heptose. J. Bacteriol. **94**:1320-1326.
10. Kim, Y. B., and D. W. Watson. 1970. A purified group A streptococcal pyrogenic exotoxin. Physicochemical and biological properties including the enhancement of susceptibility to endotoxin lethal shock. J. Exp. Med. **131**:611-628.
11. Kim, Y. B., and D. W. Watson. 1972. Streptococcal exotoxins. Biological and pathological properties, p. 33-50. *In*. L. W. Wannamaker, and J. M. Matsen (ed.), Streptococci and streptococcal diseases. Academic Press Inc., New York.
12. Nauciel, C. 1973. Mitogenic activity of purified streptococcal erythrogenic toxin on lymphocytes. Ann. Immunol. (Inst. Pasteur Paris) **124C**:383-390.
13. Nauciel, C., J. Blass, R. Mangalo, and M. Raynaud. 1969. Evidence for two molecular forms of streptococcal erythrogenic toxin. Conversion to a single form by 2-mercaptoethanol. Eur. J. Biochem. **11**:160-164.
14. Nauciel, C., M. Raynaud, and B. Bizzini. 1968. Purification et proprietes de la toxine erythrogene du streptocoque. Ann. Inst. Pasteur Paris **114**:796-811.
15. 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.
16. 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.
17. Schuh, V., V. Hribalova, and E. Atkins. 1970. The pyrogenic effect of scarlet fever toxin. IV. Pyrogenicity of strain C203U filtrate: comparison with some basic characteristics of the known types of scarlet fever toxin. Yale J. Biol. Med. **43**:31-42.
18. Shands, J. W., Jr., D. L. Peavy, and R. T. Smith. 1973. Differential morphology of mouse spleen cells stimulated in vitro by endotoxin, phytohemagglutinin, pokeweed mitogen and staphylococcal enterotoxin B. Amer. J. Pathol. **70**:1-12.
19. Stock, A. H., and E. Verney. 1952. Properties of scarlet fever toxin of the NY5 strain. J. Immunol. **69**:373-378.
20. Sugiyama, H., E. M. McKissic, Jr., M. S. Bergdoll, and B. Heller. 1964. Enhancement of bacterial endotoxin lethality by staphylococcal enterotoxin. J. Infect. Dis. **114**:111-118.
21. Watson, D. W. 1960. Host-parasite factors in group A streptococcal infections. Pyrogenic and other effects of immunologic distinct exotoxins related to scarlet fever toxins. J. Exp. Med. **111**:255-284.
22. Watson, D. W., and Y. B. Kim. 1963. Modification of host responses to bacterial endotoxins. I. Specificity of pyrogenic tolerance and the role of hypersensitivity in pyrogenicity, lethality and skin reactivity. J. Exp. Med. **118**:425-446.
23. Watson, D. W., and Y. B. Kim. 1964. Immunological aspects of pyrogenic tolerance, p. 522-536. *In* M. Landy, and W. Braun (ed.), Bacterial endotoxins. Rutgers University Press, New Brunswick, N.J.