INFECTION AND IMMUNITY, Apr. 1982, p. 123–128
0019-9567/82/040123-06$02.00/0

Vol. 36, No. 1

Enhancement of Host Susceptibility to Lethal Endotoxin Shock by Staphylococcal Pyrogenic Exotoxin Type C

PATRICK M. SCHLIEVERT

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

Received 28 September 1981/Accepted 18 November 1981

Staphylococcal pyrogenic exotoxin (PE) type C enhanced the susceptibility of rabbits to lethal shock by endotoxin by as much as 50,000-fold. A graph of log PE type C dose used for pretreatment versus log 50% lethal dose of endotoxin gave a straight line with a slope of approximately −1. Rabbits that received PE type C alone showed fevers only, but those given both PE type C and endotoxin showed initial fever followed by hypothermia, labored breathing, diarrhea, evidence of vascular collapse, and finally death. When a PE type C dose of 3 μg/kg was used, pretreatment of the animals with PE for 2 h before giving the endotoxin was required to obtain maximal susceptibility. However, when 15 μg of PE type C per kg was utilized, the endotoxin could be given before, concurrently, or after PE type C. The capacity of PE type C to prepare rabbits for enhanced susceptibility to endotoxin was lost after 24 to 48 h. Animals could be protected from enhanced susceptibility to endotoxin by prior immunization with either PE type C or endotoxin. However, 30% of the rabbits which were immunized with PE type C failed to develop immunity, and after three injections of PE type C, these animals developed gram-negative bacteremia and succumbed. In addition, rabbits with diarrhea initially, possibly caused by *Pasteurella* infection, died less than 24 h after a single injection of PE type C.

Staphylococcal pyrogenic exotoxin (PE) type C recently was shown to be produced by strains of *Staphylococcus aureus* from patients with toxic-shock syndrome (TSS) (19). This low-molecular-weight protein toxin is defined by its capacity to induce fever and to enhance host susceptibility to lethal shock by endotoxin. Furthermore, PE type C has profound effects on the host immune system, including nonspecific stimulation of T lymphocytes (19), suppression of immunoglobulin M synthesis (19), and enhancement of acquired hypersensitivity to induce a skin rash. These properties are shared by all PEs thus far described (2, 4, 5, 8, 12, 17, 18).

Although it is now clear that *S. aureus* is associated with the production of TSS, it was recently proposed that staphylococcal PE type C in concert with host-derived endotoxin may trigger the onset of the syndrome in susceptible individuals (19). TSS is an acute scarlet fever-like illness most often characterized by fever, a rash with subsequent desquamation, and a collection of symptoms which resemble gram-negative sepsis (6, 21, 23). Cases have been described which lack components that are listed in the defining criteria.

The present investigation was undertaken to study the kinetics of the enhancement of susceptibility to lethal endotoxin shock by PE type C. Furthermore, models for the development of TSS were investigated.

MATERIALS AND METHODS

All reagents and glassware utilized in preparation of staphylococcal PE type C and in biological assays were maintained pyrogen-free. All injection fluids were free of bacterial contamination.

Bacteria. The Harrisburg (18) and 587 (19) strains of *S. aureus* served as sources of PE type C. The organisms were stored lyophilized in the presence of whole, defibrinated fresh rabbit blood.

Animals. The American Dutch belted rabbits weighed 1.0 to 1.5 kg. Animals which were used for studies of TSS production by exotoxin alone were tested for bacteremia before use. For this purpose, blood taken from the marginal ear veins was spread onto two blood agar plates (0.1 ml per plate) for each rabbit. None of the animals had positive blood cultures.

Protein assay. Protein concentration was measured by the method of protein-dye binding (3). Bovine serum albumin (Pentex Biochemicals, Kankakee, Ill.) was the standard.

Production of staphylococcal PE type C. Staphylococci were cultured in a dialyzable beef heart medium until stationary-phase growth was achieved (19). The bacteria were removed by centrifugation, and the toxin was precipitated from culture supernatant fluids by ethanol (19). Ethanol-precipitated exotoxin was then subjected to thin-layer isoelectric focusing either

in commercial ampholytes (19, 27) or in the natural buffer system described by Prestidge and Hearn (9). All electrofocusing experiments were performed using a Multiphor 2117 apparatus and an LKB 2197 power supply (LKB-Produkter, Stockholm, Sweden). The conditions for electrofocusing in commercial ampholines were those described by the manufacturer (27). Electrofocusing in the natural buffer system was done using the standard plus a basic buffer system with minor modifications of the methods of Prestidge and Hearn. The modifications were: (i) glycyl glycine and lactic acid were omitted from the system, and glycine was added; (ii) the anode and cathode solutions were 1 M H_3PO_4 and 1 M NaOH, respectively; (iii) the maximum settings on the power supply were 10 W, 750 V, and 20 mA; and (iv) the buffer reagents were 0.05 M.

The time until completion of electrofocusing was 6 to 8 h in commercial ampholines and 24 to 30 h in the natural buffer system. Toxin was located by the zymogram print method when commercial ampholines were used (27) or by direct visualization when the natural buffer system was employed. Ampholytes were removed by dialysis against normal saline (0.15 M NaCl). Purified toxin thus obtained was filtered (0.22-μm pore size, Millipore Corp., Freehold, N.J.) and stored for less than 1 week at 4°C. Exotoxin was administered to rabbits intravenously in normal saline (1 ml/kg).

Endotoxin. Endotoxin, derived from *Salmonella typhimurium* (26), was diluted in phosphate-buffered saline (0.005 M phosphate, 0.15 M NaCl, pH 7.0) for intravenous injection (1 ml/kg). The 50% lethal dose (LD_{50}) of endotoxin alone, determined by the method of Reed and Muench (10), was approximately 500 μg/kg.

Immunizations. Rabbits were immunized against staphylococcal PE type C by giving them 100 minimum pyrogenic doses of exotoxin (MPD-4) per kg intravenously on every other day for five injections. The rabbits were then rested for 1 day before use. One MPD-4 per kilogram was defined as the dose of exotoxin which was required to produce an average fever response of 0.5°C after 4 h in rabbits (three per group). The MPD-4 per kilogram was typically 0.15 μg of exotoxin.

Rabbits were immunized against endotoxin by single intravenous daily injections of increasing doses of 200, 200, 400, 400, 600, and 600 MPD-3/kg (25). The MPD-3 of endotoxin per kilogram was 0.01 μg (dry weight). The animals were then rested for 1 day before use.

RESULTS

Healthy rabbits which received single injections of PE type C 4 h before the endotoxin showed enhanced susceptibility to shock which depended upon both the dose of PE used for pretreatment and the dose of endotoxin given (Table 1). For each dose of PE type C, the LD_{50} of endotoxin and the degree of enhancement of susceptibility to shock could be determined. For example, when rabbits were given 100 μg of PE type C per kg, the LD_{50} dose of endotoxin was 0.01 μg/kg. This represented a 50,000-fold enhanced susceptibility to endotoxin when compared with the LD_{50} of endotoxin alone (LD_{50} of endotoxin alone, 500 μg/kg). Similarly, at a given dose of endotoxin, the pretreatment LD_{50} of PE type C could be determined. Thus, at a fixed endotoxin concentration of 1.0 μg/kg, the LD_{50} dose of PE type C was 0.3 μg/kg. In contrast, none of the animals treated with exotoxin alone, even at doses as high as 100 μg/kg, died. In the range of doses utilized, a plot of the log PE type C pretreatment dose versus the log LD_{50} of endotoxin gave a straight line with a slope of approximately −1 (Fig. 1).

The rabbits which showed enhanced susceptibility to endotoxin died in less than 24 h, and when high doses of PE type C and endotoxin were given, the animals succumbed 1 to 3 h after receiving the endotoxin. Toxin-treated animals showed the typical fever response due to PE for the first 4 h. Then, after the endotoxin was given, the animals first showed fever, then became hypothermic, showed evidence of vascular collapse, developed diarrhea, and had labored breathing. In contrast, the animals which received exotoxin alone showed only the fever responses typical of PEs.

The time course for the ability of staphylococ-

TABLE 1. Enhanced susceptibility of healthy rabbits to endotoxin shock by staphylococcal PE type C

Pretreatment with PE type C (μg/kg)	Results (dead/total) of different endotoxin doses (μg/kg) given 4 h after exotoxin							Endotoxin LD_{50} (μg/kg)
	0	0.001	0.01	0.1	1.0	10.0	100.0	
0				0/3	0/3	0/3	0/3	
0.1				0/3	1/3[a]	2/3	2/3	7.5
1.0	0/3	0/3	0/3	1/3	2/3	3/3		0.4
5.0	0/3	0/3	1/3	1/3	2/3	2/3		0.1
10.0	0/3	0/3	1/3	3/3	3/3	3/3		0.025
100.0	0/3	0/3	2/3	2/3	3/3			0.01
LD_{50} dose of PE type C (μg/kg)	ND[b]	ND	17.8	5.9	0.3	ND	ND	

[a] Animals succumbed in less than 24 h.
[b] ND, Not determined.

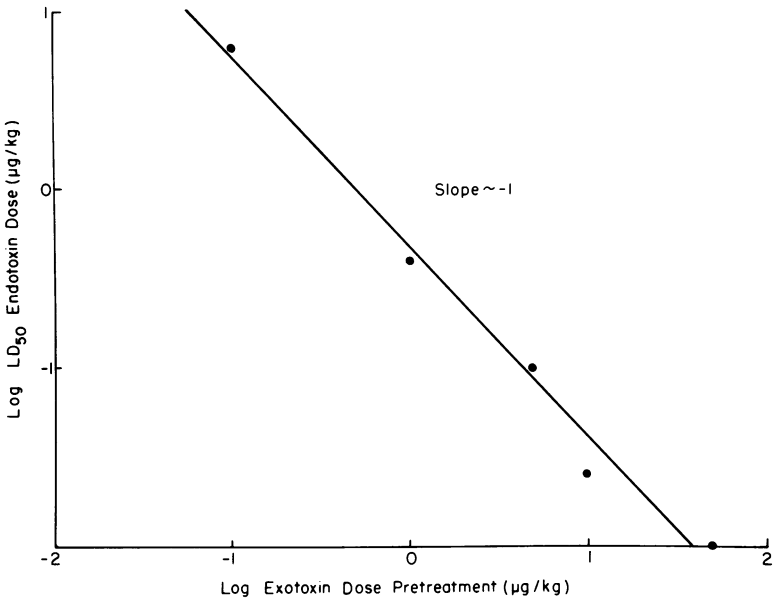

FIG. 1. Quantitative relationship between staphylococcal PE type C and endotoxin in the enhancement of susceptibility to lethal shock in rabbits. Rabbits were pretreated with PE type C 4 h before administration of endotoxin (from *S. typhimurium*).

cal PE type C to prepare animals for enhanced susceptibility to endotoxin was then examined (Fig. 2). Two doses of PE type C (20 MPD-4/kg, or 3 µg/kg; and 100 MPD-4/kg, or 15 µg/kg) and one dose of endotoxin (10 µg/kg) were used in these studies.

Rabbits that were given endotoxin before or simultaneously with the low dose of exotoxin did not show enhanced susceptibility to shock by endotoxin. However, after 2 h of pretreatment with PE type C, all of the animals died. The capacity of the lower dose of PE type C to enhance susceptibility to endotoxin lasted for 12 h without a significant loss in effectiveness, but by 24 h to 48 h the effect was lost.

Animals given endotoxin either 1 h before,

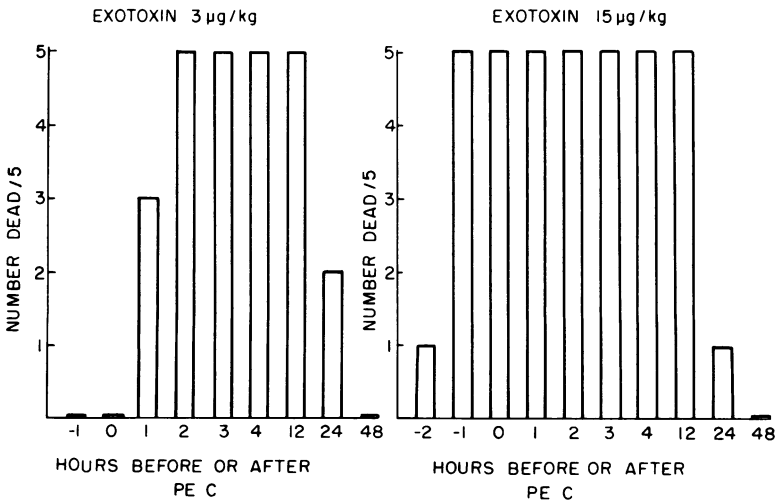

FIG. 2. Time courses for the capacity of a low dose (20 MPD-4/kg, 3 µg/kg) and a high dose (100 MPD-4/kg, 15 µg/kg) of staphylococcal PE type C to prepare rabbits for enhanced susceptibility to endotoxin shock; the endotoxin (from *S. typhimurium*) concentration was 10 µg/kg.

together with, or after the higher dose of PE type C showed enhanced susceptibility to shock (Fig. 2). As with the lower dose of PE, the ability to enhance susceptibility to shock was lost by 24 to 48 h.

Rabbits could be protected from enhanced susceptibility to endotoxin by prior immunization with PE type C (Table 2). Of the 10 animals which began the immunization protocol, 7 developed immunity to the pyrogenicity of PE type C, and these were used in this experiment. All of the seven animals were protected from challenge with 20 MPD-4 of PE type C per kg (3 μg/kg) at 0 h and then 10 μg of endotoxin per kg at 4 h by prior immunization against PE type C.

The remaining three animals could not be immunized against exotoxin after the third injection, since they developed gram-negative bacteremia and died. The animals did not have detectable gram-negative bacteria in their blood before the initiation of the immunization scheme. Thus, the exotoxin appeared to enhance susceptibility to endogenous gram-negative bacteria (*Pasteurella* sp.) in these animals. Before death, the rabbits showed labored breathing, evidence of vascular collapse, renal shutdown (urine obtained was clear brown), diarrhea, and numerous gram-negative bacteria in the blood, liver, and spleen.

Rabbits could also be protected from the enhancement effect if they were preimmunized against endotoxin (Table 2). None of the 10 animals succumbed to endotoxin shock during the immunization period, but 1 animal died when given 20 MPD-4 of PE type C per kg (3 μg/kg) at 0 h followed by endotoxin (10 μg/kg) at 4 h.

In a less well-defined system, it was observed that rabbits which had mild diarrhea, possibly due to *Pasteurella* infection although they did not have gram-negative bacteremia, could be given a TSS-like disease with exotoxin alone (nine of nine animals died when given 15 μg of exotoxin per kg). The animals died in less than 24 h and had small numbers of *Pasteurella* sp. in the blood by then.

TABLE 2. Ability of prior immunization with either staphylococcal PE type C or endotoxin to protect against enhanced susceptibility to lethal shock

Prior immunization[a]	No. of rabbits challenged[b]	No. of animals which showed enhancement[c] (dead/total)
None	10	10/10
PE type C	7	0/7
Endotoxin	10	1/10

[a] Animals were immunized against PE type C by giving them injections of 15 μg/kg intravenously every other day for five injections. Rabbits were immunized against endotoxin by giving single daily injections of progressively increasing doses (2, 2, 4, 4, 6, and 6 μg/kg). All animals were then rested 1 day before use.

[b] Rabbits were challenged with 3 μg of PE type C per kg at 0 h and 10 μg of endotoxin per kg was given at 4 h.

[c] Animals died in less than 24 h.

DISCUSSION

TSS is an acute multisystem illness resembling scarlet fever which is caused by group A streptococci. Indeed, the earliest probable cases of TSS which were reported were called syndromes resembling scarlet fever (1, 7, 22). However, *S. aureus* was isolated from the patients, but not group A streptococci. The two illnesses share many clinical features, including high fever, the presence of a scarlatiniform rash with subsequent desquamation, hypotension progressing to shock in the most severe cases, and a variable multisystem component which often includes vomiting and diarrhea (6, 21, 23, 24). Cases of each disease which lack certain of the defining criteria such as the rash or the hypotension have been described.

Streptococcal scarlet fever results from infection with strains which produce PEs in susceptible hosts. Since TSS and scarlet fever share many features, it has been suggested that staphylococcal PEs may trigger the onset of TSS in susceptible hosts (18, 19). PEs are defined by a capacity to induce high fever and to enhance host susceptibility to lethal shock by endotoxin (8, 14, 18, 20). Other properties of the toxins include the alteration of immunoglobulin synthesis and the enhancement of delayed and Arthus hypersensitivity skin reactions (5, 12, 13, 15). The latter property led to the observation that erythrogenic toxin may be a combination of preexistent hypersensitivity in the host plus PE, and therefore, disease in the absence of rash may occur (13, 15).

The capacity to enhance susceptibility to endotoxin is the most striking and perhaps the most significant biological property of PEs. In a recent study, it was postulated that the combination of staphylococcal PE type C and host-derived endotoxin may induce many of the features of TSS (19). This proposal is consistent with reports made to me of 17 TSS patients who were thought to have infections due to opportunistic gram-negative bacteria, such as *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *Escherichia coli*, and with data presented in this study in which the kinetics of enhancement of susceptibility to endotoxin by PE type C were studied. It is particularly interesting to note that 3 of the 10 healthy animals given PE type C every other day for 3 injections developed TSS-like signs and correspondingly developed gram-negative bacteremia. The mechanism behind the

appearance of gram-negative bacteria in the bloodstream of these rabbits is unclear, but may be due in part to immunosuppression and a blockade of reticuloendothelial function. A similar inability to develop immunity was reported previously in studies of staphylococcal PE type A (13), and other PE types have been shown to block reticuloendothelial clearance function (16). Further studies are under way to characterize this effect more fully.

In this study, the kinetics of the development of increased susceptibility to endotoxin by PE type C were investigated. Depending on the doses of exotoxin and endotoxin given, rabbits may show a greater than 50,000-fold enhanced susceptibility to either exotoxin or endotoxin. Furthermore, the log LD_{50} of endotoxin was directly proportional to the dose of PE type C pretreatment. Both of these observations are consistent with previous work involving studies of group A streptococcal PEs (8). The data are significant since a sublethal dose of PE type C made animals susceptible to shock by extremely small quantities of endotoxin, yet neither toxin is highly lethal alone. This effect may be even more important in humans, who apparently are more sensitive than rabbits to endotoxin (11).

The time course for the preparation for enhanced susceptibility to endotoxin also resembled that obtained previously when low doses of streptococcal PE type C were used (16). However, in that study a time-course was not obtained when the higher dose of exotoxin was used. The data in the present study indicated that whereas pretreatment of animals with low PE doses was required to achieve enhanced susceptibility to endotoxin, when a higher dose of PE type C was used, endotoxin could be coadministered or could be given before PE. The persistence of enhanced susceptibility to endotoxin by single doses of staphylococcal PE type C parallels that of streptococcal PE type C (16).

Since TSS was described, investigators have worked to develop models for the study of the disease. In this investigation, potential models based upon an enhanced susceptibility to endotoxin in rabbits have been described. Like other staphylococcal and streptococcal PEs, the staphylococcal PE type C used in this study induced only fever in rabbits when given alone. Furthermore, no sign of rash was evident, consistent with previous reports which suggest that hypersensitivity is required for rash production (13, 15). In addition, the PE by itself was not highly lethal. However, with the exception of rash, staphylococcal PE type C in conjunction with endotoxin or host-derived gram-negative bacteria, like other PE types, induced TSS-like symptoms in animals which were not immune to either PE or endotoxin. The symptoms included fever, diarrhea, evidence of vascular collapse, labored breathing, shock, and finally death. Thus, the combination of PE and endotoxin may provide a model suitable for the study of TSS.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI 18359 from the National Institute of Allergy and Infectious Diseases and by research grant AO-83-81 from the Minnesota Medical Foundation.

Julia A. Kelly is gratefully acknowledged for her technical assistance.

LITERATURE CITED

1. **Aranow, H., Jr., and W. B. Wood, Jr.** 1942. Staphylococcal infection simulating scarlet fever. J. Am. Med. Assoc. **119**:1491–1495.
2. **Barsumian, E. L., C. M. Cunningham, P. M. Schlievert, and D. W. Watson.** 1978. Heterogeneity of group A streptococcal pyrogenic exotoxin type B. Infect. Immun. **20**:512–518.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Cunningham, C. M., E. L. Barsumian, and D. W. Watson.** 1976. Further purification of group A streptococcal pyrogenic exotoxin and characterization of the purified toxin. Infect. Immun. **14**:767–775.
5. **Cunningham, C. M., and D. W. Watson.** 1978. Suppression of antibody response by group A streptococcal pyrogenic exotoxin and characterization of the cells involved. Infect. Immun. **19**:470–476.
6. **Davis, J. P., P. J. Chesney, P. J. Wand, M. LaVenture, and Investigation and Laboratory Team.** 1980. Toxic-shock syndrome: epidemiologic features, recurrence, risk factors, and prevention. N. Engl. J. Med. **303**:1429–1435.
7. **Feldman, C. A.** 1962. Staphylococcal scarlet fever. N. Engl. J. Med. **267**:877–878.
8. **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.
9. **Prestidge, R. L., and M. T. W. Hearn.** 1979. Preparative flatbed electrofocusing in granulated gels with natural pH gradients generated with simple buffers. Anal. Biochem. **97**:95–102.
10. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent end points. Am. J. Hyg. **27**:493–497.
11. **Sauter, C., and C. Wolfensberger.** 1980. Interferon in human serum after injection of endotoxin. Lancet **ii**:852–853.
12. **Schlievert, P. M.** 1980. Activation of murine T-suppressor lymphocytes by group A streptococcal and staphylococcal pyrogenic exotoxins. Infect. Immun. **28**:876–880.
13. **Schlievert, P. M.** 1981. Staphylococcal scarlet fever: role of pyrogenic exotoxins. Infect. Immun. **31**:732–736.
14. **Schlievert, P. M., K. M. Bettin, and D. W. Watson.** 1977. Purification and characterization of group A streptococcal pyrogenic exotoxin type C. Infect. Immun. **16**:673–679.
15. **Schlievert, P. M., K. M. Bettin, and D. W. Watson.** 1979. Reinterpretation of the Dick test: role of group A streptococcal pyrogenic exotoxin. Infect. Immun. **26**:467–472.
16. **Schlievert, P. M., K. M. Bettin, and D. W. Watson.** 1980. Inhibition of ribonucleic acid synthesis by group A streptococcal pyrogenic exotoxin. Infect. Immun. **27**:542–548.
17. **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.

18. **Schlievert, P. M., D. J. Schoettle, and D. W. Watson.** 1979. Purification and physicochemical and biological characterization of a staphylococcal pyrogenic exotoxin. Infect. Immun. **23**:609–617.
19. **Schlievert, P. M., K. N. Shands, B. B. Dan, G. P. Schmid, and R. D. Nishimura.** 1981. Identification and characterization of an exotoxin from *Staphylococcus aureus* associated with toxic-shock syndrome. J. Infect. Dis. **143**:509–516.
20. **Schlievert, P. M., and D. W. Watson.** 1978. Group A streptococcal pyrogenic exotoxin: pyrogenicity, alteration of blood-brain barrier, and separation of sites for pyrogenicity and enhancement of lethal endotoxin shock. Infect. Immun. **21**:753–763.
21. **Shands, K. N., G. P. Schmid, B. B. Dan, D. Blum, R. J. Guidotti, N. T. Hargrett, R. L. Anderson, D. L. Hill, C. V. Broome, J. D. Band, and D. W. Fraser.** 1980. Toxic-shock syndrome in menstruating women: its association with tampon use and *Staphylococcus aureus* and the clinical features in 52 cases. N. Engl. J. Med. **303**:1436–1442.
22. **Stevens, F. A.** 1927. Occurrence of *Staphylococcus aureus* infection with scarlatiniform rash. J. Am. Med. Assoc. **88**:1957.
23. **Todd, J., M. Fishaut, F. Kapral, and T. Welch.** 1978. Toxic-shock syndrome associated with phage-group I staphylococci. Lancet **ii**:1116–1118.
24. **Trousseau, A.** 1979. Scarlatina. Rev. Infect. Dis. **1**:1016–1026.
25. **Watson, D. W., and Y. B. Kim.** 1963. Modification of the host response 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.
26. **Westphal, O., O. Lüderitz, and F. Bister.** 1952. Uber die Extraktion von Bacterien mit Phenol/Wasser. Z. Naturforsch. **7b**:148–155.
27. **Winter, A., H. Perlmutter, and H. Davis.** 1975. Preparative flat-bed electrofocusing in a granulated gel with the LKB 2117 Multiphor. LKB Instruments, Stockholm, Sweden.