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Biogenic Amine Involvement in Pyrogenicity and Enhancement of Lethal Endotoxin Shock by Group A Streptococcal Pyrogenic Exotoxin (40663)

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Group A streptococcal pyrogenic exotoxin (SPE) type C produces fever in rabbits by direct stimulation of the hypothalamic temperature control center, rather than indirectly through leukocyte endogenous pyrogen (1). As reported for other pyrogens (2–4), fever production by SPE C was inhibited (5) by the prostaglandin synthetase inhibitor salicylate (6), suggesting toxin pyrogenicity depends upon release of a prostaglandin-like molecule. Other mediators of SPE fever have not been defined.

The biogenic amines norepinephrine and serotonin previously were shown to modulate the fever response of rabbits to pyrogens (7–12). Whereas rabbits pretreated with α-methyl tyrosine to deplete norepinephrine stores showed depressed fever responses after challenge with pyrogens (8, 10), intraventricular administration of norepinephrine resulted in fever (7, 11). These studies indicated that norepinephrine had a positive effect on fever production in the rabbit. It was shown further that α-adrenergic stimulation by norepinephrine was necessary for pyrogenicity (9, 11, 12).

Depletion of serotonin stores using *p*-chlorophenylalanine in rabbits resulted in accentuated fever responses after challenge with pyrogens (8), and intraventricular injection of serotonin depressed fever responses (7). The data suggest serotonin exerted a negative effect on pyrogenicity.

In addition to production of fever, group A streptococcal pyrogenic exotoxins have many other biological properties (13–21), including capacity to enhance host susceptibility to lethal shock by endotoxin (13) or streptolysin O (14). The enhancement phenomenon was not prevented by treatment of rabbits with the antipyretics salicylate, indomethacin, or cortisone (5). Later it was shown that unlike pyrogenicity, enhancement was not a property requiring an SPE effect on the brain (1).

This study was designed to test the involvement of biogenic amines and α- versus β-receptors in the pyrogenicity of SPE C. Also, the effects of the same agents on the capacity of SPE C to enhance lethal endotoxin shock were examined.

Materials and methods. Purified preparations of SPE C, containing 25% pyrogen-free hyaluronic acid as a stabilizing agent, were made as described previously (1, 13, 22). The minimum dose of toxin given i.v. to produce an average fever response of 0.5° at 4-hr postinjection (MPD-4) was approximately 1 μg/kg rabbit body wt; the dose of toxin used was 20 MPD-4/kg i.v. Toxin concentrations were determined using the microbiuret protein assay (23) with bovine serum albumin (Pentex, Miles Laboratories, Inc., Kankakee, Ill.) serving as the standard.

Biological assays. Solutions of SPE were prepared in sterile pyrogen-free phosphate-buffered saline (PBS 0.005 *M* phosphate buffer, pH 7.0, plus 0.15 *M* NaCl) for all biological assays. American–Dutch belted rabbits 1.0–1.5 kg were used for assays of SPE pyrogenicity and capacity of SPE to enhance lethal endotoxin shock (13). Depending on the day, starting temperatures of the rabbits ranged from 37 to 38.5° but differed by less than 0.5° for individual experiments. Unless otherwise stated, enhanced susceptibility to lethal endotoxin shock was measured by giving an i.v. injection of either 25 or 1 μg/kg of endotoxin (*Salmonella typhimurium*) to each rabbit 4 hr after i.v. SPE injection. The 1 μg/kg dose of endotoxin represented sufficient toxin to kill four or five of five rabbits pretreated with SPE C. The higher dose of endotoxin (25 μg/kg) was employed previously (1, 5) as the standard dose for tests of enhancement of susceptibility to lethal shock by SPE C. The LD_{50} of endotoxin for normal rabbits was 535 μg/kg. The LD_{50} of endotoxin for rabbits pretreated with 20 MPD-4/kg SPE C was 0.028 μg/kg.

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Drugs. *p*-Chlorophenylalanine (PCPA, Aldrich Chemical Co., Inc., Milwaukee, Wis., 300 mg/kg) and α-methyl tyrosine (αMT, Sigma Chemical Co., St. Louis, Mo., 100 mg/kg) were administered i.p. to rabbits as suspensions in PBS 12 hr before toxin treatment. Norepinephrine (Levophed bitartrate, Winthrop Laboratories, N.Y.) and propranolol (Inderal, Ayerst Laboratories, N.Y.) were diluted according to the manufacturers' specifications to 50 μg/0.1 ml. Serotonin (Sigma, 50 μg/0.1 ml) and isoproterenol (Sigma, 50 μg/0.1 ml) were prepared in PBS. Intracisternal (i.c.) injections of these drugs into rabbits were made into the cisterna magna with syringes equipped with 27 gauge by 0.5-in. needles; the volume injected i.c. was 0.1 ml into each rabbit. Phenoxybenzamine (Dibenzyline, Smith, Kline, and French Laboratories, Philadelphia, Pa.) was prepared in PBS and 1 mg/kg injected intravenously. To test the ability of phenoxybenzamine (PBA) alone to suppress fever and prevent enhanced susceptibility to lethal shock, 1 mg/kg was given to rabbits i.v. 1 hr before SPE C and 5 hr before endotoxin. Capacity of PBA and fluid replacement to alter enhancement was assessed using the following procedures: Rabbits given SPE C were treated with PBA (2 mg/kg) 1 hr before receiving endotoxin (1 μg/kg). Immediately after administration of endotoxin and every 15 min for a total of seven injections, each rabbit received i.v. 10 ml of 5% dextrose in pyrogen-free distilled water. Controls consisted of: Rabbits treated with SPE and endotoxin; SPE, PBA, and endotoxin; and SPE, endotoxin, and fluid.

Statistical analyses. The difference in mean fever responses between two groups was evaluated at the 4-hr time point or 1 hr after drug injection, using Student's *t* test analysis. The variability in fever responses is indicated by ± SEM. Statistical analyses were performed only at the 4-hr time point or 1 hr after injection of drug.

Results. Rabbits pretreated with the tyrosine hydroxylase inhibitor αMT showed depressed 4-hr fever responses after challenge with SPE C (Fig. 1) compared to control animals which received PBS and toxin ($P < 0.01$). However, control and experimental rabbits showed comparable enhanced susceptibility to lethal endotoxin shock (enhancement) at the doses of toxin used. There was no apparent difference in timing of deaths between groups.

α-Adrenergic blockage (Figs. 1 and 2) using PBA also inhibited the pyrogenicity of SPE C compared to control animals receiving toxin only ($P < 0.01$). However, alone PBA did not prevent enhancement; 5 of 5 animals died in either group after receiving 25 μg/kg endotoxin (Fig. 1). PBA alone did not inhibit enhancement when animals received a lower dose of endotoxin (1 μg/kg); 9 of 10 rabbits died in each group (Fig. 2). Again, there was no apparent difference in timing of deaths between groups for both doses of endotoxin.

It is important to note that separate PBS controls were run for each group of experimental animals presented in Fig. 1. The variability in average fever response between experiments when animals were given PBS and toxin may have resulted for at least two reasons: Different batches of toxin were used for the experiments, and whereas batches of toxin have comparable MPD-4/kg, differences in pyrogenic activity exist. Also, be-

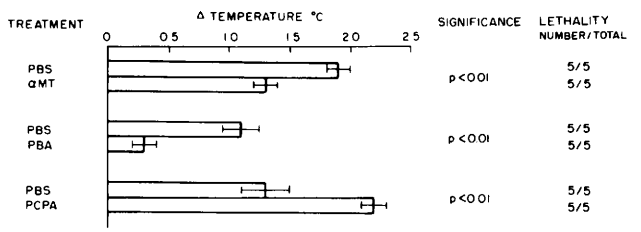

FIG. 1. The effect of pretreatment of rabbits with α-methyl tyrosine (αMT), phenoxybenzamine (PBA), and P-chlorophenylalanine (PCPA) on SPE C fever production and capacity to enhance lethal endotoxin shock. Control animals received phosphate-buffered saline (PBS) followed by SPE C. Shown are the average fever responses of five rabbits in each group 4 hr after SPE C administration i.v. (20 μg/kg). Bars indicate ± 1 SE. The numbers of rabbits in each group that showed enhanced susceptibility to lethal endotoxin shock are shown. Significant differences in the mean 4-hr fever responses between control and experimental groups were assessed using Student's *t* test.

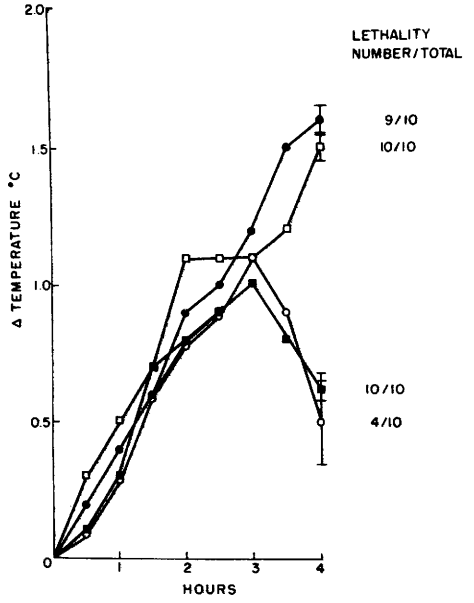

FIG. 2. The effect of phenoxybenzamine (2 mg/kg) on SPE-C-induced fever and together with fluid replacement on capacity to enhance lethal endotoxin shock. Animals (10/group) given: SPE C and endotoxin (●); SPE C, phenoxybenzamine, endotoxin, and fluid replacement (○); SPE C, phenoxybenzamine, and endotoxin (■); SPE C, endotoxin, and fluid replacement (□). Bars indicate ± 1 SE.

cause the experiments were not performed on the same day, differences in animals may have contributed to the variability.

PBA, given in combination with fluid replacement, was partially effective in reversing the course of the enhancement phenomenon as shown in Fig. 2 (endotoxin dose used was 1 μg/kg). Comparable treatment was effective previously in altering the course of lethal endotoxin shock (24–26). Unlike control animals, 6 of 10 rabbits survived challenge with SPE and endotoxin (1 μg/kg) when treated with PBA and fluid.

Rabbits pretreated with PCPA to deplete serotonin stores showed accentuated fever responses (Fig. 1) after challenge with SPE C ($P < 0.01$), but control and experimental animals exhibited enhanced susceptibility to lethal shock.

Administration of norepinephrine i.c. to rabbits with fevers due to SPE C (Fig. 3) resulted in further heightened responses compared to controls receiving diluent ($P < 0.01$) 1 hr after injection. Again, enhancement of lethal shock was not prevented. Unlike norepinephrine, serotonin administered i.c. (Fig. 4) to rabbits exhibiting fevers due to SPE C resulted in a significant reduction of the average fever response ($P < 0.001$) 1 hr after injection. All animals showed enhanced susceptibility to lethal endotoxin shock; endotoxin was given at the 5-hr time point.

The β-receptor stimulating and blocking compounds, isoproterenol and propranolol, did not affect SPE-induced fevers in rabbits and alone did not prevent enhancement.

Discussion. The data presented in this study suggest SPE C-induced fever in rabbits was positively affected by norepinephrine and negatively affected by serotonin. Furthermore, pyrogenicity depended in part upon stimulation of α-adrenergic receptors. Presumably, these observations could be extended to include the rat and sheep, which have been shown to respond comparably to norepinephrine and serotonin (27, 28). However, the reverse would be expected when studying cats, dogs, or monkeys (27, 28); that

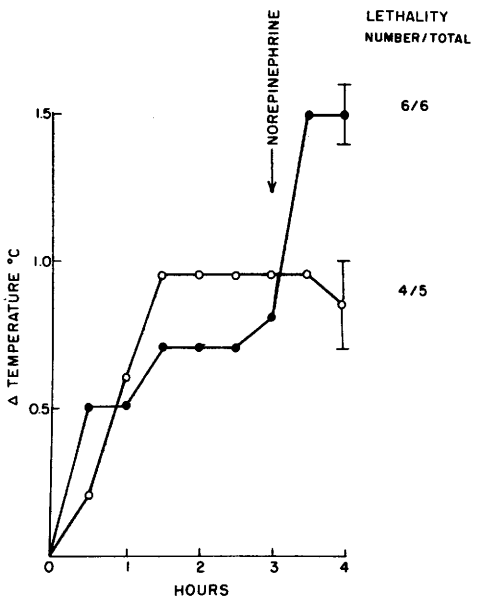

FIG. 3. The effect of intracisternal administration of norepinephrine (50 μg/0.1 ml) on SPE fever production and capacity to enhance lethal endotoxin shock. SPE C (20 μg/kg) was administered i.v. at 0 hr to all rabbits in each group. Norepinephrine (●) or buffer (○) was given 3 hr after SPE C. *Salmonella typhimurium* endotoxin (25 μg/kg) was given i.v. to all animals at the 4-hr time point. Bars indicate ± 1 SE.

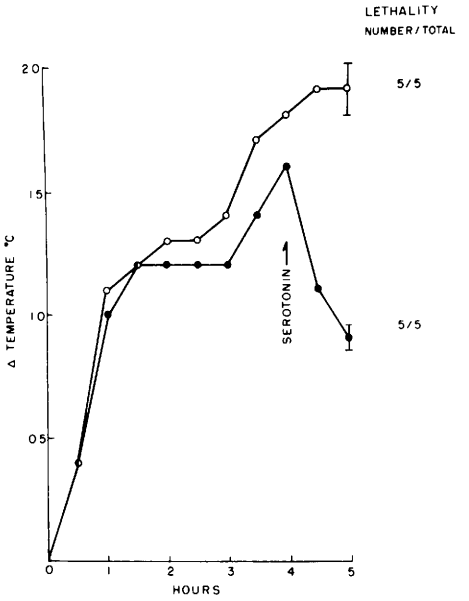

FIG. 4. The effect of intracisternally administered serotonin (50 μg/0.1 ml) on SPE C-induced fever and capacity to enhance lethal endotoxin shock. All animals (five/group) received SPE C (20 μg/kg) at 0 hr followed by serotonin (●) or phosphate-buffered saline (○) at 4 hr. *Salmonella typhimurium* endotoxin (25 μg/kg) was administered i.v. 5 hr after SPE C. Bars indicate ± 1 SE.

is, fever after challenge with serotonin and body cooling after challenge with norepinephrine. The reason for the species variation is not clear.

Researchers have shown that norepinephrine produced fever in rabbits when injected into the hypothalamic area (7, 11). Also, it was suggested that fever production by norepinephrine depended upon α-adrenergic stimulation, since agents such as PBA inhibited fever (9–12). The high concentration of norepinephrine contained in the hypothalamus is consistent with the amine mediating pyrogenicity (29).

Other investigators have implicated norepinephrine as a mediator of endotoxin-induced fever. Intracisternal injection of PBA inhibited endotoxin pyrogenicity (12). Administration of αMT to deplete norepinephrine stores, reduced leukocyte pyrogen fever (8); monoamine oxidase inhibitors potentiated fever (30). The results presented in this study indicate group A streptococcal pyrogenic exotoxin type C produces fever also dependent upon norepinephrine and stimulation of α-adrenergic receptors, although not dependent upon endogenous pyrogen release (1).

In a previous report PCPA (8) was used to deplete stores of serotonin in rabbits (31). Subsequent challenge with leukocyte endogenous pyrogen resulted in accentuated fever responses, indicating the presence of serotonin depressed pyrogenicity. In the present study comparable results were obtained using SPE C to induce fever. Also, administration of serotonin i.c. to rabbits showing fevers resulted in a significant decline in temperatures. As proposed earlier (9), norepinephrine and serotonin may act reciprocally to regulate body temperature. Elevation of norepinephrine relative to serotonin would result in fever whereas the reciprocal would result in body cooling.

It is significant that PBA given i.v. in this study depressed SPE fever production. Previous researchers using a lower dose observed that the drug had to be injected into the hypothalamic area to reduce fever (12). It is possible the higher dose utilized in the present study permitted sufficient PBA to penetrate the blood–brain barrier. Alternatively, SPE C which was shown to alter the permeability of the blood–brain barrier in rabbits (1) may have allowed sufficient PBA to cross.

The treatment of endotoxin shock has been highly controversial (24–26, 32–34). While one approach has been to use steroids and vasopressors (32), a number of investigators have shown endotoxin shock in rabbits responded favorably to PBA plus fluid replacement (24–26). In this study comparable techniques employing PBA plus fluid replacement were partially successful in altering the lethal effect. The partial success in reversing shock may have resulted from a combination of increased cardiac output (26) and blocked catecholamine-induced arteriolar vasoconstriction by PBA (25, 26) together with increased blood pressure after fluid replacement (26).

Based on the observation that PBA and fluid partially reversed the enhancement phenomenon, it would be tempting to speculate that enhancement of lethal endotoxin shock indirectly results from SPE inactivation of the endotoxin detoxification system. Such an effect would allow endotoxin to persist in the

circulation to produce a typical shock syndrome. Other studies are consistent with this proposal. It was shown that SPE alters reticuloendothelial system (RES) clearance function (15, 16). Also, a very low dose of endotoxin (0.028 μg/kg LD_{50}) is required for lethality after SPE treatment compared to 535 μg/kg LD_{50} without SPE. A much higher dose of endotoxin may be required to produce shock in the normal animal by overwhelming the host detoxification system, thus allowing a small amount of endotoxin to persist. An alternative hypothesis is that SPE plus endotoxin may act synergistically on the same cell type to produce lethality, whereas much higher doses of endotoxin alone may be required to produce the effect. The use of SPE in combination with endotoxin may serve as a probe for the elucidation of the mechanism of enhancement of lethal shock.

Summary. Group A streptococcal pyrogenic exotoxin type C (SPE C) was shown to produce fever which in part depended upon norepinephrine and stimulation of α-adrenergic receptors. Intracisternal injection of norepinephrine into rabbits already showing fevers due to SPE C resulted in further heightened fevers. Pretreatment of animals with either α-methyl tyrosine to deplete norepinephrine stores or phenoxybenzamine to block α-receptors depressed SPE-induced pyrogenicity. Pretreatment of animals with P-chlorophenylalanine to deplete serotonin stores accentuated fevers due to SPE and giving serotonin intracisternally to rabbits with fevers resulted in a significant drop in body temperature. This indicated serotonin exerted a negative effect on SPE pyrogenicity. Isoproterenol and propranolol did not affect SPE C fever production. When used alone, none of the drugs prevented the capacity of SPE C to enhance lethal endotoxin shock. However, phenoxybenzamine in combination with fluid replacement increased the survival rate of rabbits. It is proposed that SPE C may alter the endotoxin detoxification system, thus allowing endotoxin to persist in the circulation producing shock.

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1. Schlievert, P. M., and Watson, D. W., Infect. Immun. **21,** 753 (1978).
2. Lin, M. T., and Chai, C. Y., J. Pharmacol. Exp. Ther. **180,** 603 (1972).
3. Cranston, W. I., and Rawlins, M. D., J. Physiol. **222,** 257 (1972).
4. Woolf, C. J., Willies, G. H., Laburn, H., and Rosendorff, C., Neuropharmacology **14,** 397 (1975).
5. Schlievert, P. M., Bettin, K. M., and Watson, D. W., Proc. Soc. Exp. Biol. Med. **157,** 472 (1978).
6. Vane, J. R., Nature (New Biol.) **231,** 232 (1971).
7. Cooper, K. E., Cranston, W. I., and Honour, A. J., J. Physiol. **181,** 852 (1965).
8. Giarman, N. J., Tanaka, C., Mooney, J., and Atkins, E., Advan. Pharmacol. **6A,** 307 (1968).
9. Feldberg, W., and Saxena, P. N., J. Physiol. **212,** 23P (1971).
10. Cranston, W. I., Hellon, R. F., Luff, R. H., and Rawlins, M. D., J. Physiol. **212,** 24P (1971).
11. Dhawan, B. N., and Dua, P. R., Brit. J. Pharmacol. **43,** 497 (1971).
12. Laburn, H., Woolf, C. J., Willies, G. H., and Rosendorff, C., Neuropharmacology **14,** 405 (1975).
13. Kim, Y. B., and Watson, D. W., J. Exp. Med. **131,** 611 (1970).
14. Schwab, J. H., Watson, D. W., and Cromartie, W. J., J. Infect. Dis. **96,** 14 (1955).
15. Hanna, E. E., and Watson, D. W., J. Bacteriol. **89,** 154 (1965).
16. Cunningham, C. M., and Watson, D. W., Infect. Immun. **19,** 51 (1978).
17. Cunningham, C. M., and Watson, D. W., Infect. Immun. **19,** 470 (1978).
18. Hanna, E. E., and Watson, D. W., J. Bacteriol. **95,** 14 (1968).
19. Hanna, E. E., and Hale, M., Infect. Immun. **11,** 265 (1975).
20. Watson, D. W., and Kim, Y. B., *in* "Microbial Toxins" (T. C. Montie, S. Kadis, and S. J. Ajl, eds.), Vol. 3, p. 173. Academic Press, New York (1970).
21. Kim, Y. B., and Watson, D. W., *in* "Streptococci and Streptococcal Diseases" (L. W. Wannamaker and J. M. Matsen, eds), p. 33. Academic Press, New York (1972).
22. Schlievert, P. M., Bettin, K. M., and Watson, D. W., Infect. Immun. **16,** 673 (1977).
23. Zamenhof, S., Methods Enzymol. **3,** 696 (1957).
24. Lillehei, R. C., Longerbeam, J. K., Bloch, J. H., and Manax, W. G., Ann. Surg. **160,** 682 (1964).
25. Vick J. A., Ciuchta, H. P., and Manthei, J. H., J. Pharmacol. Exp. Ther. **150,** 382 (1965).
26. Kawarada, Y., Wolferth, C. C., Jr., and Matsumoto, T., Intern. Surg. **57,** 141 (1972).
27. Myers, R. D., Advan. Pharmacol. **6A,** 318 (1968).
28. Bligh, J., Cottle, W. H., and Maskrey, M., J. Physiol.

212, 377 (1971).
29. Adam, H. M., *in* "Metabolism of Amines in the Brain" (G. Hooper, ed.), p. 5. MacMillan, London (1969).
30. Cooper, K. E., and Cranston, W. I., Nature (London) **210,** 203 (1966).
31. Koe, B. K., and Weissman, A., J. Pharmacol. Exp. Ther. **154,** 499 (1966).
32. Spink, W. W., and Vick, J., Circ. Res. **9,** 184 (1961).
33. Weil, M. H., Sudrann, R. B., and Shubin, H., Calif. Med. **96,** 86 (1962).
34. Hinshaw, L. B., Solomon, L. A., Freeny, P. C., and Reins, D. A., Arch. Surg. **94,** 61 (1967).

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