Endotoxic Properties of an Extract of *S. aureus*.* (29148)

R. D. HIGGINBOTHAM AND J. A. BASS
Department of Microbiology, University of Texas Medical Branch, Galveston

The pathogenic effects of *Staphylococcus aureus* are usually attributed to the action of one or more of the various *exotoxic* substances elaborated by this group of organism (1). A few reports in the literature, however, indicate that certain strains of *S. aureus* also contain *endotoxic* substances. The Fritchie strain of *S. aureus*, for example, possesses a toxic somatic material which appears to be largely responsible for the acutely fatal type of infection characteristically produced by this organism in mice(2). Moreover, the lethal effects of this material are similar to those induced by a commercially available Boivin-type extract obtained from cells of a different strain of *S. aureus*(3). Other reports indicate that similar extracts obtained from *S. aureus* cells have pharmacologic properties which resemble those of endotoxins derived from gram negative bacteria (*e.g.*, tumor necrosis(4), and the epinephrine-enhanced Shwartzman reaction(5)).

Inasmuch as administered endotoxins can markedly influence resistance of animals to various infectious agents(6), including *S. aureus*(6,7), it has been of interest to determine similarities and differences in the pharmacologic effects of *S. aureus* extract and a gram negative endotoxin. For this purpose Boivin-type extracts of *S. aureus* and *E. coli* have been compared on a quantitative basis with regard to: 1) lethality for intact and for adrenalectomized mice, 2) tumor necrosis, and 3) lethality for 10-day-old chick embryos.

Materials and methods. Animals: Male mice of the Fairfax-Webster strain obtained from Euers Farms, Austin, Texas, weighing 19 to 21 g, were used for determining the lethal activity of the respective bacterial extracts. After receipt in the laboratory, the animals were rested for one week in an air-conditioned environment before use. When employed, bilateral adrenalectomies were performed by the usual dorsal approach 18 hours prior to challenge. The operated animals were fed *ad libitum* and maintained on a 0.9% saline solution as drinking water throughout the experimental period. Swiss mice of the "Johnson strain," obtained from a local supplier, were utilized for testing the tumor necrotizing activities of the bacterial extracts. Two to three days after receipt in the laboratory these animals, weighing 14 to 16 g, were inoculated in the thigh with a heavy suspension of S-180 mouse sarcoma cells and were ready for testing 5 to 7 days later. This tumor, originally obtained from Drs. Ludwig and Dorothy Anigstein, was maintained in this laboratory by frequent intramuscular (thigh) passage in this strain of mouse. The inoculum for both passage and testing was prepared by grinding 5- to 7-day-old tumor implants in a loose-fitting Ten Broeck glass tissue grinder containing approximately 5 volumes of a saline-phosphate (M/15) buffer with a pH of 7.2 and containing both penicillin (100 μ/ml) and streptomycin (100 μg/ml). Embryonated eggs, employed to determine the lethal hemorrhagic necrotizing effects of the test substances, were obtained from the hatcheries at Texas A & M. They were incubated at a temperature of 37°C for 10 days in an egg incubator and candled for viability before use.

Drugs. All drugs were prepared for injection in an isotonic, pyrogen-free, sterile saline before use. Cortisol (hydrocortisone acetate, Merck Sharp & Dohme) was administered to the chick embryo in mixture with the active endotoxic agent. The bacterial extracts of *S. aureus* A and *E. coli* 055 B were obtained from Difco Laboratories and the respective potencies of these materials compared in each system by titration. A minimum of 5 animals (mice or embryos) was employed for each dose level of the substances tested. Each titration was repeated 2 or more times to give a minimum of 15 animals per dosage group. Data from the

* Work supported in part by USPHS grant.

TABLE I. Comparison of Activities of *S. aureus* and *E. coli* Extracts.

Test system	*S. aureus*	*E. coli*	Relative potency
A. Mouse lethality			(%)
a. intact	480. ±59.3	420. ±276.	88.
b. adrex	1.05 ±.35	.0222 ±.023	2.1
B. Tumor necrosis	2.0 ±1.4	.054 ±.12	2.7
C. Chick embryo lethality	18. ±9.8	14. ±14.4	78.

various experiments were routinely accumulated on the basis of results present 24 hours after challenge. Results are presented in terms of the 50% effective doses as determined by the method of Miller and Tainter (8).

Results. The dose response curves of the *S. aureus* material were roughly parallel to those of the *E. coli* extract in each of the systems tested, and the results have been presented in terms of the respective ED_{50} doses (Table I). For convenience, the ratio of the ED_{50} of the *E. coli* extract to that of the *S. aureus* material, multiplied by 100, has been used as a means to express the "Relative Potency" of this latter agent.

Lethal effects. The marked resistance of the experimental animals to the lethal effects of both the *E. coli* endotoxin and the *S. aureus* extract was readily abolished by adrenalectomy. Data representing results of a series of titrations of each of the bacterial extracts in groups of intact and adrenalectomized mice are presented in Table I and clearly illustrate the influence of adrenal deficiency on resistance to each of these noxious agents. Challenge of the experimental animals with adequate amounts of either of the extracts induced symptoms of intoxication, such as piloerection, lethargy, diarrhea, etc., and terminated in a convulsive death within 3 to 18 hours after administration of the injurious material. Although the pharmacologic effects of these agents appeared to be quite similar, it is apparent from the data that the *E. coli* endotoxin was more potent than the *S. aureus* extract in both groups. This difference in potency was especially marked in adrenalectomized animals in which the relative potency of the *S. aureus* material was only 2.1% of the *E. coli* endotoxin *vs* a relative potency of 88% as found in the intact animals. The adrenalectomized mouse was thus a far more sensitive indicator of the quantitative differences in the lethal activity of these bacterial extracts than the intact animal.

Tumor necrosis. The necrotizing effects of endotoxins on experimental tumors has been well described by Shwartzman(9), Shear(10) and many other investigators and was readily demonstrated with each of the bacterial extracts employed in the present study. Although gross and microscopic examination of the affected tumors failed to reveal qualitative differences in the effects of these agents, the results of titration studies (Table I) indicated that the *S. aureus* material was far less effective than the *E. coli* extract, and its relative potency was equivalent to that obtained for its lethal activity in adrenalectomized mice. This similarity in the relative potencies of the *S. aureus* extract in both systems further emphasized the quantitative difference in activity of these agents. It is of interest that the ED_{50} of the *S. aureus* extract is very similar to the activity reported by Neter(4) for an extract derived from another strain of *S. aureus* cells.

Heat-killed cells of the Fritchie strain of *S. aureus* induce a fatal intoxication in adrenalectomized mice(2) which closely resembles the lethal effects of the *S. aureus* and *E. coli* extracts used in the present study. Inasmuch as it has been suggested that the toxicity of this organism is largely due to an endotoxic material(2,11), it was of interest to determine whether this organism might also have tumor-necrotizing activity. The 959 strain of *S. aureus,* which appears to have little of this endotoxic factor(11), was included in the present study for comparative purposes. Groups of tumor-bearing mice were given a single intravenous injection of 0.25 ml saline containing one of a series of graded amounts of a carefully washed and heat-killed preparation of the respective strain of bacteria. Although injections of the whole cell material did not give evidence of

TABLE II. Influence of Cortisol on Lethal Hemorrhagic Effects of Bacterial Extracts in 10-day-old Embryonated Eggs.

	Total mortality	Hemorrhagic deaths	% deaths	
			Hemorrhagic	Nonhemorrhagic
1. Control				
a. *E. coli*	15/19	15/19	100.	—
b. *S. aureus*	13/19	2/19	15.4	84.6
2. Cortisol (25 μg/embryo)				
a. *E. coli*	0/19	0/19	—	—
b. *S. aureus*	7/19	0/19	0	100.

a dose-response effect in this limited study, the tumor necrotizing activity of these preparations could be demonstrated in animals receiving 10^6 to 10^9 cells/dose. Moreover, histopathologic examination of affected tumors indicated that the observed changes were similar to those obtained with the *S. aureus* and *E. coli* extracts. Accumulated results of titrations in the above dosage range indicated that the Fritchie preparation initiated necrosis in approximately 50% (53/97) of the challenged animals *vs* an approximate 10% (8/70) effect noted in animals inoculated with the 959 strain. Neither preparation, however, appeared to be active if injected by the intraperitoneal route. Demonstration of the presence of active material in staphylococci was thus dependent on route of administration as well as on the strain of organism selected for study.

Lethality for chick embryo. The lethal potencies of the 2 extracts for the 10-day-old chick embryo were very similar, with the *S. aureus* material being only slightly less active than the *E. coli* endotoxin in this system (Table I). Qualitatively, however, the pharmalogic effects of these two agents were quite distinct. Like other endotoxins derived from gram negative bacteria(12), the *E. coli* extract induced hemorrhagic necrosis, and this change was a consistent feature in embryos injured by this material. The *S. aureus* extract, on the other hand, induced this hemorrhagic effect only on occasion, and embryos killed by this noxious material usually displayed a pale and macerated appearance.

As noted by Smith and Thomas(12) corticosteroids readily inhibit the lethal hemorrhagic effect of endotoxins in the chick embryo. Although similar hormonal effects could be readily observed in chick embryos treated with cortisol and challenged with either the *E. coli* or *S. aureus* extracts, this hormone did not inhibit the non-hemorrhagic lethal effect of the *S. aureus* material to the same degree. This differential effect is well illustrated by the results of a representative experiment shown in Table II. Groups of embryos were inoculated on the chorioallantoic membrane with 0.05 ml saline containing either: 1) 100 μg of the *E. coli* or *S. aureus* extracts singly to serve as controls, or 2) 100 μg of one of the respective extracts in mixture with 250 μg of cortisol. Observations made 24 hours later indicated that whereas 100% of the embryos killed by the *E. coli* endotoxin were hemorrhagic, only 15% of the embryos poisoned by the *S. aureus* extract showed this effect. In associated experiments it was observed that this hemorrhagic type of death could be inhibited by as little as 2.5 μg of cortisol/embryo. Yet the non-hemorrhagic type of death was highly resistant to this protective effect of cortisol, even when much larger doses of this steroid were employed (Table II). The nature of the lethal activity of the *S. aureus* extract in the chick embryo was thus quite different from that of the *E. coli* endotoxin.

Discussion. These results have indicated that a commercially available Boivin-type extract of *S. aureus* cells possesses pharmacologic properties usually attributed to endotoxins derived from gram negative bacteria, such as a marked differential toxicity in intact *vs* adrenalectomized mice, tumor necrosis, and, to a limited extent, lethality for

chick embryos. Although the presence of such a toxic factor is not a well recognized characteristic of this genus of bacteria, early work by Hartmann and Scott(13) and later studies by Higginbotham and Dougherty(2) with adrenalectomized rats and mice, respectively, have indicated that this or a similar type of noxious agent is present in cells of certain strains of *S. aureus*. The possibility that this endotoxin-like material is selectively distributed among only particular strains of *S. aureus* is suggested by the fact that whereas Shwartzman(9) was unable to detect a tumor-necrotizing activity in a strain of *S. aureus* used in his studies with the S-180 sarcoma, this activity was readily demonstrated with extracts of other strains of *S. aureus* by Mihich and Neter(4) and in the present study. The idea that this toxic factor is unequally distributed among various strains of *S. aureus* is further supported by the differences noted in tumor-necrotizing activity of heat-killed preparations of Fritchie and 959 strains of *S. aureus*.

Despite the apparent similarity in activity of the extracts of the *S. aureus* and *E. coli* with regard to lethality and tumor necrosis, it is quite apparent that the pharmacologic activity of the staphylococcal material was readily differentiated from that of the gram negative endotoxin on the basis of results in the chick embryo experiments. The reason for this difference is not clear since other more generally recognized properties of gram negative bacteria, such as pyrogenicity, may also be shared by staphylococci(14). In this regard, Marcus (personal communication) has found that *enterotoxic* extracts of the Fritchie strain of *S. aureus* possess measurable pyrogenic activity when tested in either rabbit or cats and, conversely, that pyrogenic extracts of *E. coli* are enterotoxic in the latter animal. Freedman(15), however, has observed that although the staphylococcal extract (Difco) produced the typical biphasic febrile response in rabbits which is characteristic of gram negative endotoxins, the associated leukopenic response was markedly delayed and occurred at a time when maximum fever had already developed. Thus the pharmacologic properties of the staphylococcal extract would appear to be similar but not identical with those of the classical gram negative endotoxins.

There appears to have been relatively little work done on the possible significance of this active factor in the pathogenesis of strains of *S. aureus* possessing this material. In previous work it was found that although intravenously injected cells of the Fritchie strain were rapidly cleared from the blood of adrenalectomized mice and destroyed by their reticulo-endothelial tissues, these animals died in a state of acute intoxication(2). To explain this, it was suggested that active material was released from the phagocytic cells in amounts sufficient to kill these highly sensitive animals. The deleterious influence of this material on host resistance to infection is suggested by the work of Arndt and Ritts (16) who reported that infection with an "endotoxic strain" of *S. aureus* markedly depressed resistance of mice to a concurrent infection with Pseudomonas. This point is further emphasized by the work of Neter(5), who noted that rabbits could be prepared for the epinephrine-enhanced Shwartzman response by intravenous injection of either viable cells of *S. aureus* or staphylococcal extract, and by results of the present study in which intravenously injected killed cells induced tumor necrosis as did the staphylococcal extract. It is of additional interest that the staphylococcal extract may synergize the lethal action of staphylococcal alpha toxin in mice(17). One could thus speculate that the release of such material during an infectious process could serve to decrease host resistance in such a manner as to exalt the apparent effectiveness of other virulence factors possessed by these organisms. On the other hand, Sultzer and Freedman(18) have noted that pretreatment of mice with the staphylococcal material can enhance their resistance to an otherwise lethal challenge with *E. coli* and prolong their survival time after infection with *S. typhimurium*. These apparently different effects of staphylococcal material on host resistance may indicate that the active substance can induce a biphasic effect comparable to the well recognized effects of gram negative endotoxins(6).

Inasmuch as administered endotoxins can markedly influence host resistance(6,7) and the properties of the *S. aureus* extract are similar in certain respects to those of gram negative endotoxins, it may be speculated that this toxic material may be an important virulence factor in those strains of *S. aureus* which possess this substance. Unfortunately, the frequency of distribution of this active material among various pathogenic strains of *S. aureus* does not appear to have been studied. However, in a limited series of tests conducted in this laboratory using tumor necrosis, differential toxicity in intact *vs* adrenalectomized mice or virulence by the intraperitoneal route as criteria for the presence of this factor, activity has been noted in the following mouse-virulent strains: J651 (received from Dr. W. F. Arndt), A and 304 (received from Dr. P. B. Carter), as well as in Fritchie and Smith strains. In addition, this factor also appeared to be present in 3 out of 15 mouse-virulent strains of *S. aureus* freshly isolated from lesions in human patients. However, since resistance of experimental animals to the noxious effects of the *S. aureus* extracts or viable cells can be decreased by adrenalectomy and enhanced by adrenal steroids(2,3,11), it may very well be that the net influence of this endotoxic substance on the pathogenesis of these organisms may be inversely related to the adequacy of adrenal function in the host.

Summary. Comparative dose-response studies on the pharmacologic effectiveness of an *S. aureus* extract and *E. coli* endotoxin have been performed with regard to 1) lethal effect in intact and adrenalectomized mice, 2) tumor-necrotizing activity, and 3) lethal and hemorrhagic effects in chick embryo. Results indicate that properties of *S. aureus* extract are distinctive but similar to those of gram negative endotoxin. It is suggested that this material may be an important virulence factor in certain strains of *S. aureus* and that its effectiveness in this regard is inversely related to adrenal function of the host.

The authors wish to acknowledge the invaluable technical assistance of Miss Miriam Mallari.

1. Elek, S. D., *Staphylococcus Pyogenes and Its Relation to Disease,* E. & S. Livingstone, Ltd., pub. London, 1959.

2. Higginbotham, R. D., Dougherty, T. F., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 253.

3. Higginbotham, R. D., *J. Allergy,* 1962, v33, 35.

4. Mihich, E., Neter, E., PROC. SOC. EXP. BIOL. AND MED., 1961, v106, 97.

5. Neter, E., Anzai, H., Gorzynski, E. A., *J. Infect. Dis.,* 1960, v107, 332.

6. Landy, M., *Ann. N. Y. Acad. Sci.,* 1956, v66, 292.

7. Dubos, R. J., Schaedler, R. W., *J. Exp. Med.,* 1960, v111, 497.

8. Miller, L. C., Tainter, M. L., PROC. SOC. EXP. BIOL. AND MED., 1944, v57, 261.

9. Shwartzman, G., *Phenomenon of Local Tissue Reactivity and Its Immunological Pathological and Clinical Significance*, Paul E. Hoeber, Inc., New York, 1937.

10. Shear, M. J., Turner, F. C., *J. Nat. Cancer Inst.,* 1943, v4, 81.

11. Higginbotham, R. D., Bass, J. A., *Texas Rep. Biol. and Med.,* 1961, v19, 283.

12. Smith, R. T., Thomas, L., *J. Exp. Med.,* 1956, v104, 217.

13. Hartman, F. H., Scott, W. J. M., *ibid.,* 1932, v55, 63.

14. Probey, T. F., Pittman, M., *J. Bact.,* 1945, v50, 397.

15. Freedman, H. H., *J. Exp. Med.,* 1960, v112, 619.

16. Arndt, W. F., Ritts, R. E., PROC. SOC. EXP. BIOL. AND MED., 1962, v108, 166.

17. Bass, J. A., Higginbotham, R. D., *Bact. Proc.,* 1961, p109.

18. Sultzer, B. M., Freedman. H. H., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 60.

Received October 14, 1963. P.S.E.B.M., 1964, v116.