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Modification of Selected Host-reactive Properties of Endotoxin by Treatment with Sodium Deoxycholate

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Endotoxin dissociated into subunits by sodium deoxycholate treatment exhibited diminished capacity to kill chick embryos, protect mice against the lethal effects of infection with *Salmonella typhi*, evoke hemorrhagic necrosis in skin inoculated with epinephrine, prepare for and provoke the dermal Shwartzman reaction, and induce pyrogenic tolerance. Surfactant-treated material which had been allowed to reaggregate displayed activity equivalent to that of untreated material. These findings were consistent with the working hypothesis that a macromolecular complex of critical size is required in order for endotoxin to elicit its characteristic effects in the host.

Although endotoxins extractable from gram-negative bacteria may differ widely in chemical composition (1), they possess, in common, a group of attributes in biological systems referred to as their host-reactive properties. The difficulties inherent in biological methods of assay, as well as the physical and chemical complexity of endotoxins, have given rise to discordant, often contradictory views on the nature of the chemical groupings responsible for the many biological activities attributed to endotoxin. Several authors (5, 11, 13, 14, 15) have reported an apparent separation of biological effects by chemical modification of the endotoxin molecule, implying that specific groupings are responsible for different host-reactive properties. Watson and Kim (8, 24, 25) have suggested the interdependence of a primary toxicity attributable to the lipid portion of the endotoxin molecule and a secondary toxicity associated with a state of hypersensitivity in the host. Westphal (26) has also emphasized the importance of the lipid portions of the molecule; however, Ribi et al. were unable to correlate lipid content with biological activity (4) and prepared a highly active endotoxin, the lipid content of which was low (17).

However, the working hypothesis tested by the experiments reported in this paper is concerned with the colloidal properties of endotoxin without referring to chemical composition. The hypothesis states simply that a critical minimal particle size is necessary in order for endotoxin to exhibit its host-reactive properties. First advanced to account for observations on the dynamics of acid hydrolysis of endotoxin (18), the hypothesis was subjected to further testing when we showed that the surfactant sodium deoxycholate (NaD) dissociated the endotoxin polymer into subunits (molecular weight, about 20,000) which could be reaggregated easily into larger particles [molecular weight, 500,000 to 1,000,000 (16)]. As predicted, endotoxin injected into rabbits in the presence of NaD exhibited diminished pyrogenicity, antigenicity, and adjuvant activity, whereas material allowed to reaggregate displayed activities equivalent to those of untreated endotoxin (16, 20). In the present study, endotoxin dissociated with NaD was used to test the validity of the hypothesis in systems which demonstrate other endotoxin activities.

MATERIALS AND METHODS

Endotoxin and its treatment with NaD. We used the conventional aqueous ether extract from fresh whole cells of *Salmonella enteritidis* strain S-795 that was described in an earlier publication (20). Stock solutions, prepared as before (20) and stored at 5 C, contained 2 mg of *S. enteritidis* endotoxin (Se) per ml in pyrogen-free 0.15 M NaCl (PFS) and 0.2% Formalin. Dilution of stock solutions in PFS provided fully potent endotoxin for assay.

Preparation of surfactant-treated endotoxin required combining stock material with a solution of NaD in PFS to give a mixture which contained 1,000 μg of Se per ml in the presence of a concentration of NaD appropriate for the assay being carried out (stock solution of Se in NaD, SSNaD-Se). Further dilution in NaD yielded the concentration of Se in NaD (NaD-Se) desired for the inoculation of animals. Alternatively, SSNaD-Se was diluted further in PFS, rather than in NaD, before injection (SSNaD-Se, diluted in saline) so that the concentration of NaD in

the inoculum was less than 0.01%, which is insufficient to maintain the endotoxin in a dissociated state (16). Se and NaD were also injected separately. One vein of an animal was inoculated with Se in saline; shortly thereafter, another vein was inoculated with NaD (NaD and Se, injected separately). NaD was injected immediately after Se when possible, but never more than 15 min afterward.

Thus, we were able to evaluate the activity of the parent endotoxin (Se), the disaggregated product (NaD-Se), and the disaggregated product which had been allowed to reaggregate (SSNaD-Se, diluted in saline). Furthermore, the influence of NaD on the host's responses to the parent endotoxin could be observed (NaD and Se, injected separately).

Toxicity precluded intravenous (iv) administration of more than 10 mg of NaD to rabbits, 0.125 mg to chick embryos, and 0.625 mg to mice. The concentrations of NaD chosen for each assay were, except in the skin tests, the greatest that could be administered without producing toxic manifestations. It had been shown previously (16) that at least a 0.5% concentration of NaD is required to bring about maximal dissociation of Se and reduction in pyrogenicity, but that measurable effects are obtainable with 0.125% or less. Since no buffers were employed and the endotoxin itself was neutral in solution, the *p*H of the mixtures in PFS varied with the concentration of NaD. At 0.125% NaD, the *p*H was 6.9; at 0.5%, the *p*H was 7.7; and at 4%, the *p*H was 8.1.

Lethality for chick embryos. Preparations administered iv (0.1-ml volumes) killed 11-day-old chick embryos under conditions described previously (12). The concentration of NaD in NaD-Se and SSNaD-Se was 0.125%. Mortality in all tests included in the tabulations bracketed the 50% end point. In a limited number of embryos in which two veins were exposed, Se and NaD were injected separately.

Protection of mice infected with S. typhi. We determined the ability of materials (0.25-ml volumes, injected iv) to enhance the resistance of mice to the lethal effect of infection with *S. typhi* strain Ty 2, as described previously (4). The effect measured is termed "nonspecific resistance," since endotoxins antigenically unrelated to *S. typhi* were as protective as homologous preparations. The concentration of NaD in NaD-Se was 0.25% for this assay.

Dermal Shwartzman reaction. First, we tested the ability of Se and NaD-Se to prepare skin for a Shwartzman reaction provoked by 30 μg of Se administered iv 24 hr later. The shaved lateral surfaces of New Zealand white rabbits (1.5 to 2 kg) from the colony at the Rocky Mountain Laboratory received intracutaneous (ic) inoculations (0.2 ml) of graded doses of Se and NaD-Se. Two series of dilutions, one of Se and one of NaD-Se, were placed in rows on each side. Products were arranged so that one series of dilutions from each preparation had its most dilute material cephalad, whereas the other series from the same preparation had its most dilute material caudad.

Se which was inoculated ic (10 μg in 0.2 ml) at two sites on a shaved lateral surface of 10 rabbits tested the capacity of graded doses of Se and NaD-Se (0.5% NaD) administered iv 24 hr later (1-ml volumes) to provoke the Shwartzman reaction.

Examination of reactions in the gross, 24 hr after the provoking dose, allowed us to distinguish positive sites readily. Blue-black hemorrhagic lesions at least 4 mm in diameter were considered positive. Though NaD alone neither prepared skin nor provoked a reaction in skin prepared with endotoxin, sites in the skin inoculated with NaD or NaD-Se (0.5% NaD) displayed depressed white lesions, 2 to 4 mm in diameter, even in the absence of the provoking material. The size and color of the lesions distinguished them unmistakably from typical Shwartzman reactions.

All of the Shwartzman tests were carried out from October to January.

Hemorrhagic necrosis produced by epinephrine in the skin of rabbits. We followed the procedure described by Thomas (21). Inoculation (ic, 0.2-ml volume) of 100 μg of epinephrine (adrenalin chloride; Parke, Davis & Co.) into the midline of the shaved abdomen of New Zealand white rabbits (0.8 to 1.5 kg) immediately preceded iv administration (1-ml volumes) of the materials shown in Table 5. After 24 hr, the length and width of the elliptical hemorrhagic reactions were measured and their areas were calculated. The concentration of NaD in the NaD-Se and SSNaD-Se was 0.5% for this assay.

Tolerance to the pyrogenic effect of endotoxin. The method employed in our laboratory for measuring the febrile response of rabbits to pyrogen has been well standardized (4). The fever index (FI) indicated the height and duration of the response occurring over a period of 6 hr (4, 7).

We attempted to induce tolerance to the pyrogenic effect of 0.2 μg of Se (inoculated iv) by administering iv injections of Se (1 μg) or NaD-Se (1 μg of Se in 4% NaD) 24 hr earlier. The unusually heavy concentration of NaD was employed to assure maximal dissociation of Se, because it has been found that as little as 0.001 μg of endotoxin in saline induces definite tolerance under these conditions (Milner and Rudbach, *unpublished data*). Each curve in Fig. 1 was constructed from responses of six rabbits.

Statistical analyses. Calculations by the method of Litchfield and Wilcoxon (10) established values and 95% confidence limits for the median lethal doses (LD_{50}) of various preparations in chick embryos and the median effective doses (ED_{50}) for materials inducing resistance in mice infected with *S. typhi*. The method of Larson et al. (9) gave an estimate of the mean threshold dose (MTD) of materials necessary to provoke, or prepare skin for, the Shwartzman reaction. This parameter [designated SPD_{50} by Larson et al. (9)] is essentially an ED_{50} (6) calculated from reactions at individual skin sites. Standard errors, however, were calculated according to the number of animals employed rather than according to the number of skin sites inoculated. There were half as many animals as skin sites at each dose level.

RESULTS

In all instances, the biological assays revealed that NaD significantly altered endotoxic potency.

It diminished the lethality of Se for chick embryos (Table 1, A versus B), even though toxicity of the NaD itself forced us to use a concentration which only incompletely dissociated the endotoxin (16). However, when a mixture of endotoxin and surfactant was diluted in saline rather than in NaD and the concentration of NaD was thereby reduced below the level required to dissociate the Se (16), the original lethality of the endotoxin was reestablished (Table 1, C). NaD injected separately from Se did not alter endotoxin activity (Table 1, D).

NaD influenced other properties of endotoxin similarly. It impaired the capacity of Se to protect mice challenged with virulent *S. typhi* (Table 2, A versus B), but only when the endotoxin and surfactant were injected as a mixture (Table 2, B versus C). Preparation of the skin of rabbits for the Shwartzman reaction required larger amounts of Se in NaD than Se in saline (Table 3); in skin prepared with 10 μg of endotoxin, Se in 0.5% NaD was far less active in provoking the reaction than Se in saline (Table 4). Surfactant-treated endotoxin also produced less dermal hemorrhagic necrosis than untreated material in rabbits whose skin was inoculated with epinephrine (Table 5, A versus B). Again, Se and NaD injected separately, but simultaneously, produced reactions comparable to those produced by Se alone (Table 5, A and C); and Se in NaD, diluted in saline rather than NaD, though producing lesions somewhat smaller than the untreated material, elicited reactions in as great a percentage of animals as Se (Table 5, A and D). Finally, whereas NaD-Se, though slightly pyrogenic (FI = 6.4), did not induce tolerance to the pyrogenic effect of endotoxin administered 24 hr later (Fig. 1, B), untreated Se did (Fig. 1, A). The pyrogenic activity of 0.2 μg of Se (Fig. 1, D) was essentially unaffected by prior inoculation of NaD alone (Fig. 1, C).

DISCUSSION

We determined the influence of NaD on the host-reactive properties of endotoxin and attempted to correlate this influence with the capacity of NaD to reversibly dissociate the endotoxin polymer (16). Our present and earlier findings (16, 20) have indicated that the subunits (molecular weight, about 20,000) formed by the reaction of endotoxin with NaD are far less active than the parent material (molecular weight, 1,000,000 or greater) in assays for pyrogenicity, antigenicity, adjuvant activity, and lethality for chick embryos, in ability to protect mice infected with *S. typhi*, in capacity to prepare for and provoke the dermal Shwartzman reaction, in ability to evoke hemorrhagic necrosis in skin inoculated with epinephrine, and in capacity to induce pyrogenic tolerance.

Earlier studies on the kinetics of acid hydrolysis demonstrated that the host-reactive properties of endotoxin were abolished at a rate which paralleled the disruption of the endotoxic complex into particles approximately 1/100 the size of the original material (18). This finding led to the

TABLE 1. *Influence of sodium deoxycholate on lethality of endotoxin for chick embryos*

Prepn[a]	Per cent deaths at various doses (μg)								LD_{50} (μg)	95% confidence limits (μg)	No. of embryos inoculated at each dose level
	0.0008	0.004	0.04	0.02	0.2	0.1	1.0	5.0			
A. Se	0.83	28.3		96.7		100			0.0058	0.0047–0.0071	120
B. NaD-Se[b]			0.0		13.1		95.7	100	0.39	0.29–0.53	46
C. SSNaD-Se, diluted in saline[c]	0.0	6.7		87.8		100			0.0095	0.0076–0.011	90
D. Se and 0.1 ml of 0.125% NaD, injected separately	0.0	25		100		100			0.0064		8

[a] A. Untreated *S. enteritidis* endotoxin (Se). B. Se injected in the presence of NaD; that is, the disaggregated product (NaD-Se). C. The disaggregated product allowed to reaggregate as the concentration of NaD was reduced by diluting in saline (SSNaD-Se, diluted in saline). D. Separate injections of Se and a quantity of NaD equal to that given in NaD-Se (Se and 0.1 ml of 0.125% NaD, injected separately). All preparations were injected in a volume of 0.1 ml.

[b] The concentration of NaD in the product injected was 0.125%.

[c] The concentration of NaD in the product injected was <0.01%.

TABLE 2. *Influence of sodium deoxycholate on the ability of endotoxin to protect mice challenged with S. typhi strain Ty 2*

Prepn[a]	ED_{50} (μg)	95% confidence limits (μg)
A. Se	0.32	0.21–0.48
B. NaD-Se[b]	3.5	2.2–5.6
C. Se and 0.25 ml of 0.25% NaD, injected separately	0.19	0.08–0.43

[a] *See* footnote *a* of Table 1 for explanations of abbreviations. Preparations were injected iv in 0.25-ml volumes.

[b] The concentration of NaD in the product injected was 0.25%.

TABLE 3. *Influence of sodium deoxycholate on the capacity of endotoxin to prepare skin for the Shwartzman reaction in rabbits*[a, b]

Skin prepared with Se[c]		Skin prepared with NaD-Se[d]	
Dose (μg)	Per cent positive	Dose (μg)	Per cent positive
10	95	100	100
5	62.5	50	84.4
2.5	60	25	59.4
1.25	32.5	12.5	28.1
0.625	20	6.25	3.1
0.313	7.5	3.08	0.0
0.156	5	1.54	0.0
0.078	2.5	0.77	0.0

[a] See footnote *a* of Table 1 for abbreviations.

[b] Provoked by iv inoculation of 30 μg of Se (0.5 ml) 24 hr after preparing the skin with ic inoculation of Se or NaD-Se in 0.2-ml volumes.

[c] Forty skin sites were tested at each dose level. Mean threshold dose (μg) was 2.06 (95% confidence limits, 1.69 to 2.43 μg).

[d] Thirty-two skin sites were tested at each dose level. Mean threshold dose (μg) was 21.87 (95% confidence limits, 19.79 to 23.95 μg).

formulation of a hypothesis that a macromolecular complex of critical size is required in order for endotoxin to elicit its characteristic effects in the host. The capacity of NaD to dissociate endotoxin in a reversible manner has now allowed us to demonstrate not only diminution in host-reactive potency in the dissociated product, but also restoration of activity in material allowed to reaggregate.

Thus, in the systems that we have described, it is clear that the presence of NaD in a solution of endotoxin is associated concurrently with diminished host-reactive potency and dissociation of the endotoxic polymer. The critical question, not yet definitively answered, is whether dissociation of endotoxin and its diminished biological activity are related as cause and effect. We postulate that this is the case but emphasize again that size is probably not the only, or even necessarily the most important, factor determining toxicity. Results in our laboratory (*unpublished data*) confirm

TABLE 4. *Dermal Shwartzman reactions provoked by Se and NaD-Se in the skin of rabbits prepared with 10 μg of Se*[a]

Dose (μg)	Provoking material[b]	
	Se	NaD-Se[c]
1,000	ND[d]	+[e]
200	ND	+
40	+	—
8	+	—
1.6	—	—
0.32	—	—

[a] *See* footnote *a* of Table 1 for explanation of abbreviations.

[b] Inoculated iv (1 ml) 24 hr after the skin was prepared by ic inoculation of 10 μg of Se (0.2 ml).

[c] Concentration of NaD in the product injected was 0.5%.

[d] Not done.

[e] Positive (+) or negative (—) reactions at each of two skin sites on the shaved lateral surface of one rabbit.

TABLE 5. *Hemorrhagic necrosis produced by 100 μg of epinephrine in the skin of rabbits receiving iv injections of Se and NaD-Se*[a]

Prepn given iv[b]	Hemorrhagic necrosis (no. positive / no. tested)	Mean area (cm^2)
A. Se	13/13	12.3
B. NaD-Se[c]	2/22	3.2
C. Se and NaD[d], injected separately	4/5	9.5
D. SSNaD-Se, diluted in saline[e]	4/4	7.1
E. NaD[d]	0/5	0.0
F. None	0/7	0.0

[a] *See* footnote *a* of Table 1 for abbreviations.

[b] Preparations A, B, C, and D each contained 5 μg of Se and were injected in a volume of 1 ml.

[c] Concentration of NaD in the product injected was 0.5%.

[d] A 1.0-ml amount of 0.5% NaD.

[e] Concentration of NaD in the product injected was <0.01%.

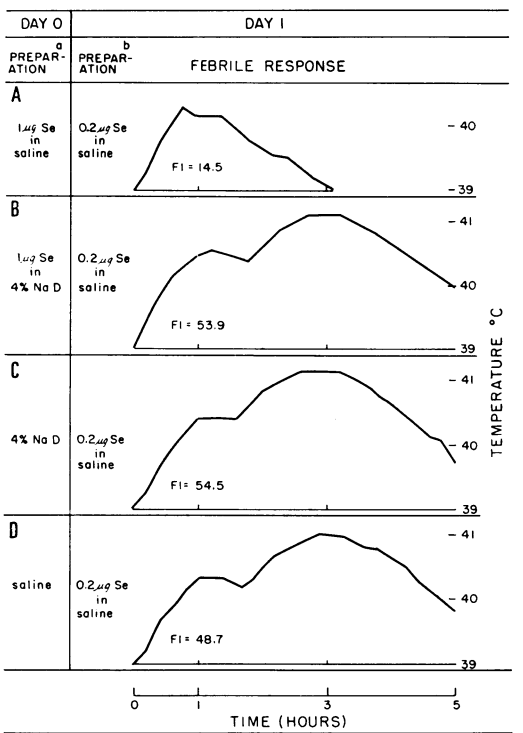

FIG. 1. *Capacity of a single injection of Salmonella enteritidis endotoxin (Se) or its sodium deoxycholate-treated product (NaD-Se) to induce tolerance to a pyrogenic dose of Se. Footnote a, 0.25-ml volumes, injected iv. Footnote b, 1-ml, volumes injected iv. FI denotes fever index of the corresponding curve.*

reports (22) that detoxification is readily accomplished by treatments which do not reduce particle size below a molecular weight of 400,000. Furthermore, by acylation with long-chain fatty acids (23), we have been able to produce aggregates of nontoxic O-specific substance (native protoplasmic polysaccharide), very similar to active endotoxin in size, without bestowing endotoxic properties upon this material (*unpublished data*).

However, fully endotoxic particles usually have a molecular weight of not much less than 400,000 and treatments which produce cleavages resulting in smaller particles generally also produce detoxification. In the present investigation, the diminished activity of these smaller particles was confirmed, and this allowed us to regard as still tenable the hypothesis that a minimal critical particle size is necessary in order for endotoxin to exhibit host-reactive properties. Factors other than size can unquestionably play a role in determining endotoxic potency, and our hypothesis may require modification as these factors are scrutinized more closely.

Mechanisms in the host which might account for the diminished activity of NaD-Se have not yet been examined. Interference with the host's capacity to respond cannot be considered as a possibility, since NaD injected separately from Se had no discernible effect on host-reactive potency of the endotoxin.

The factors responsible must explain the uniform type of alteration observed in ostensibly diverse reactions. A simple hypothesis would postulate a single type of tissue receptor, or possibly antibody, which allows the manifestation of different physiological reactions when activated by endotoxin administered by different routes, in different doses, or under disparate conditions of immunity or susceptibility in the host. NaD might decrease potency by altering the steric properties of endotoxin, thus preventing proper coupling of effector with receptor.

Another factor which would account for decreased potency is enhancement of the host's ability to excrete or metabolize NaD-Se. How this might occur was suggested by our demonstration that subunits formed under the influence of NaD apparently bind to constituents of human plasma in vitro and remain biologically inactive when so bound, even though the concentration of NaD is reduced below those levels which allow the NaD, by itself, to inactivate the endotoxin (16, 19). Such a mechanism operating in vivo might enhance binding of NaD-Se to a constituent in the plasma, in extracellular fluid, on cell surfaces, or within cells. The binding, in turn, could facilitate excretion, enhance degradation, or inhibit interaction with receptors mediating reactions in the host.

Another physiological system which may account for differences between the responses of various hosts to Se and NaD-Se is the complement system. The recent demonstration that endotoxins from three sources fixed at least one component of the complement system in vitro (2) raises the possibility that a similar reaction may be important in vivo. The necessity of studying the complement system in this context is further indicated by evidence (3) which implicates it in an important nonimmunological homeostatic mechanism which may play a role in processes, such as the dermal Shwartzman reaction, characterized by infiltration with polymorphonuclear leukocytes.

These are only a few of the mechanisms which must be investigated. The complexity of the phenomena we are studying is immense, but NaD should prove useful in future investigations

because of its ability to reversibly dissociate endotoxin, probably without breaking covalent bonds, into subunits of low molecular weight and diminished biological activity.

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