A Quantitative Estimation of Sodium Deoxycholate Remaining in Reassociated, Hybrid Endotoxins[1] (36010)

JON A. RUDBACH AND JAMES R. WEBER

Department of Microbiology, University of Montana, Missoula, Montana 59801

Endotoxins of gram-negative bacteria are thought to be quaternary complexes held together by noncovalent bonds; it is established that the addition of appropriate concentrations of a surfactant such as sodium deoxycholate reversibly dissociates these complexes into subunits (1, 2). Removal of the surfactant allows the subunits to reassociate, and in a system containing two endotoxins this reassociation results in the formation of hybrid molecules containing subunits from each of the parent endotoxins (3, 4).

From several laboratories, a considerable amount of speculation has arisen regarding the amount of surfactant retained with the reassociated parent and hybrid endotoxin molecules (5–8). Experiments described in the present report were designed to quantitate, directly, the amount of sodium deoxycholate remaining with a reassociated, hybrid endotoxin molecule. These data can be related to our previous work (1–4), on reassociated, hybrid endotoxins, because the same standard procedures were employed for treatment of endotoxins and the removal of sodium deoxycholate.

Materials and Methods. Endotoxins. Endotoxins were extracted from cell walls of *Escherichia coli* 0111:B4 and *E. coli* 0113 by the aqueous-phenol method as described previously (9, 10).

Sodium deoxycholate. ^{14}C-Sodium deoxycholate was prepared from deoxycholic acid (carboxyl-^{14}C), purchased from Nuclear Science and Engineering Corporation, Pittsburgh, PA, and it had a specific activity of 91.8 μCi/mg. One milligram of the radioactive deoxycholic acid was placed in 1 ml of 0.01 *M* NaOH and dissolved at 37°. This was added to a solution of the appropriate concentration of unlabeled sodium deoxycholate (Fischer Scientific Company, Fair Lawn, NJ) in 0.2 *M* Tris–Cl buffer (pH 8.0).

Dissociation of endotoxin and formation of hybrids. This procedure has been described in detail elsewhere (3). Briefly, 0.5 ml each of *E. coli* 0111 and *E. coli* 0113 endotoxin (2 mg/ml) were mixed with 1 ml of 4 or 2% sodium deoxycholate (NaD) and allowed to stand at room temperature (22°) for 15 min. The endotoxin was then precipitated by the addition of 6 vol of absolute ethanol; the precipitate was sedimented by centrifugation, washed once with 6 vol of ethanol, redissolved in water, and dialyzed overnight. For the control mixtures, the endotoxins were treated separately with ^{14}C-NaD and the surfactant was removed by ethanol extraction before the endotoxins were mixed and dialyzed. Another control included a mixture of endotoxins in Tris buffer, without NaD, which was precipitated with ethanol and dialyzed. Hybrid formation was detected by immunodiffusion against rabbit anti-*E. coli* 0111 and anti-*E. coli* 0113 sera (3). Radioactivity was quantitated by placing samples of the solutions into 10 ml of scintillation fluid (10 mg of POPOP; 0.4 mg of PPO; 40 ml of ethyl alcohol; 60 ml of toluene) and counting the samples at 51.4% efficiency in a Nuclear-Chicago Unilux III scintillation counter.

Results. The results of these experiments are summarized in Fig. 1 and Table I. The junction of continuity in the immunodiffusion arc generated when the hybrid endotoxin was allowed to react with the two antisera (Fig. 1) and the crossing of the precipitation arcs in the control mixtures of endotoxin indicated that the antigenic groups which previously were unique to each type of endotox-

[1] Supported by U.S. Public Health Service Grant AI09876.

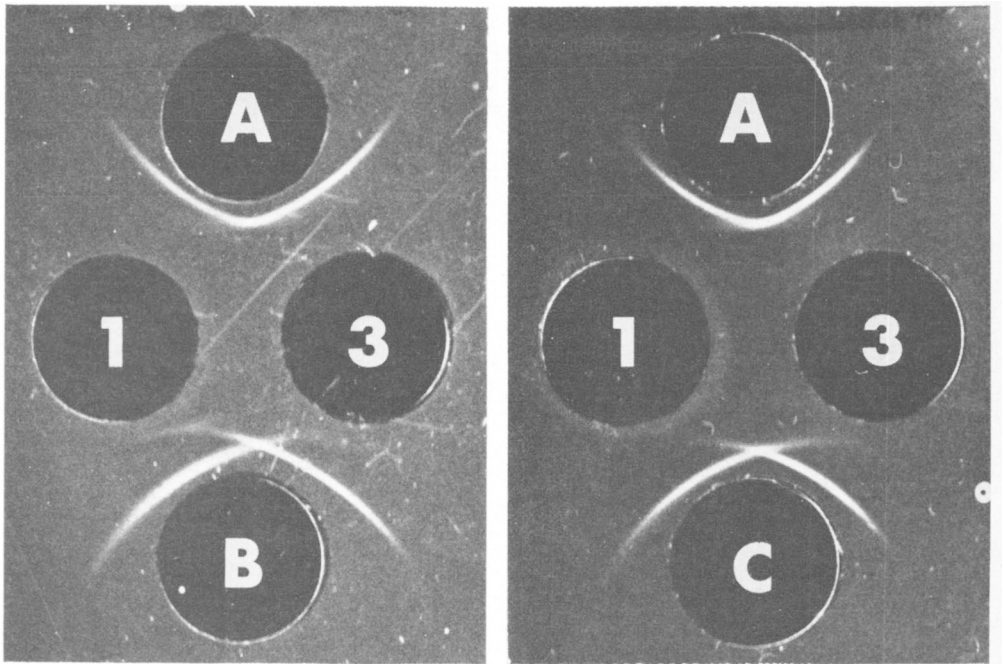

FIG. 1. Hybrid formation between *E. coli* 0111 and *E. coli* 0113 endotoxins: (1) antiserum to *E. coli* 0111; (3) antiserum to *E. coli* 0113; (A) hybrid formed between *E. coli* 0111 and *E. coli* 0113 endotoxins; (B) endotoxins from *E. coli* 0111 and *E. coli* 0113, which were treated separately with NaD, precipitated with ethanol, and mixed; (C) a mixture of *E. coli* 0111 and *E. coli* 0113 endotoxins, which was only precipitated with ethanol. The total endotoxin concentration was 1 mg/ml, *i.e.*, 500 μg of each endotoxin/ml.

in had combined to form a single molecular species and that this hybridization was mediated through NaD. The validity of this interpretation was established previously (3). Also, the demonstration of hybridization indicated that the endotoxin molecules had been dissociated by NaD and then had reassociated after removal of the NaD by ethanol extraction (3, 4). These reassociated, hybrid endotoxins did contain some NaD which was not removed by the ethanol extractions (Table I). When the hybrid was formed by reaction of the endotoxins with 2% NaD less than 1% of total NaD added remained in the samples. However, this weight of NaD was 4 to 7% of the weight of endotoxin in the sample. In Expt. II all of the added NaD was accounted for, and lower values for residual amounts of surfactant in the samples were obtained. These latter values, approximating 4% NaD per unit weight of endotoxin are probably more accurate than the former, higher values. In Expt. III, hybrid formation was induced by 1% NaD and the percentage NaD remaining in the samples relative to the amount of NaD added was similar to that obtained in Expt. II. However, the percentage of NaD left in the sample per unit weight of endotoxin was about half that obtained in the previous experiment in which 2% NaD was employed. Thus, it appears that two extractions of the endotoxin–surfactant mixture with ethanol leave behind a constant percentage of NaD, the absolute weight of which varies with the amount added initially.

Discussion. In some of the initial work on treatment of endotoxins with NaD the surfactant was removed from the sample by dialysis (3). However, after more than a week of dialysis, occasional preparations still contained sufficient NaD to cause foaming when they were shaken. Therefore, ethanol extraction was attempted to effect a more complete and consistent removal of NaD from endotoxins. This procedure has been used in our laboratory for several years and was

TABLE I. Amount of Sodium Deoxycholate (NaD) Remaining in Samples of Hybrid Endotoxins and Control Mixtures.

Sample		(cpm)[a]	NaD in sample calculated as: % of total NaD added	NaD in sample calculated as: % of NaD/unit wt of ET[b]
Expt. I (using 2% NaD)				
A	Hybrid ET	1.13×10^4	0.35	7.0
B	Mixture of ET	1.36×10^4	0.43	8.5
C	2% NaD	3.20×10^6	100	—
D	1st ethanol sup. of A	2.94×10^6	82	—
E	2nd ethanol sup. of A	1.20×10^3	0.038	—
Expt. II (using 2% NaD)				
F	Hybrid ET	1.60×10^4	0.19	3.8
G	Mixture of ET	1.99×10^4	0.23	4.7
H	2% NaD	8.52×10^6	100	—
I	1st ethanol sup. of F	8.45×10^6	99	—
J	2nd ethanol sup. of F	1.25×10^5	1.5	—
Expt. III (using 1% NaD)				
K	Hybrid ET	1.12×10^4	0.14	1.4
L	Mixture of ET	1.78×10^4	0.23	2.2
M	1% NaD	7.90×10^6	100	—
N	1st ethanol sup. of K	7.45×10^6	96	—
O	2nd ethanol sup. of K	1.07×10^5	1.4	—

[a] The counts per minute (cpm) are all corrected to correspond to a sample volume of 2 ml which contained 2 mg of ET.

[b] Abbreviations: ET = endotoxin; sup. = supernatant fluid.

thought to have extracted almost all of the surfactant. Data presented above show that after dissociation of endotoxins with NaD and then removal of the NaD by two ethanol extractions the endotoxins reassociated; this was demonstrated by hybrid formation. However, these reassociated endotoxins still contained 4 to 7% NaD.

The role of the residual NaD in maintaining the quaternary structure of the reformed endotoxins (or the hybrids) is unknown. When phenol–water extracted endotoxins were dissociated with surfactant and then allowed to reassociate, they were more monodisperse than the parent materials but they possessed quantitatively similar biological and immunological properties. Also, regarding the role of residual NaD in hybrid endotoxins: it should be noted that in all three experiments shown on Table I the control mixtures, in which the endotoxins were dissociated and reassociated before they were mixed, contained more NaD than did the hybrid preparations. Therefore, if the NaD is playing a role in holding the hybrid endotoxin together it must be an ancillary function. However, the possibility did exist that the sites on the lipopolysaccharide to which the NaD was binding were the same sites that supply cohesion in the micellar bundle of subunits. This facet of the problem needs further investigation.

Summary. Endotoxins which had been dissociated by sodium deoxycholate (NaD) and reassociated into hybrids still contained 1.5 to 7% surfactant. Control mixtures which were treated separately with NaD and did not form hybrids contained 2.2 to 8.5% NaD after two extractions with ethanol. When the amount of NaD remaining in the sample was calculated as percentage of that originally added, the ethanol extractions removed all but 0.2 to 0.5%.

1. Ribi, E., Anacker, R. L., Brown, R., Haskins, W. T., Malmgren B., Milner, K. C., and Rudbach, J. A., J. Bacteriol. 92, 1493 (1966).

2. Rudbach, J. A., Anacker, R. L., Haskins, W. T., Johnson, A. G., Milner, K. C., and Ribi, E., Ann. N.Y. Acad. Sci. 133, 626 (1966).

3. Rudbach, J. A., Milner, K. C., and Ribi, E., J. Exp. Med. 126, 63 (1967).

4. Rudbach, J. A., J. Infec. Dis. 122, 139 (1970).

5. Olins, A. L., and Warner, R. C., J. Biol. Chem. 242, 4994 (1967).

6. McIntire, F. C., Sievert, H. W., Barlow, G. H., Finley, R. A., and Lee, A. Y., Biochemistry 6, 2363 (1967).

7. McIntire, F. C., Barlow, G. H., Sievert, H. W., Finley, R. A., and Yoo, A. L., Biochemistry 8, 4063 (1969).

8. Beer, H., Staehelin, T., Douglas, H., and Braude, A. I., J. Clin. Invest. 44, 592 (1965).

9. Fukushi, K., Anacker, R. L., Haskins, W. T., Landy, M., Milner, K. C., and Ribi, E., J. Bacteriol. 87, 391 (1964).

10. Ribi, E., Haskins, W. T., Landy, M., and Milner, K. C., J. Exp. Med. 114, 647 (1961).

Received July 2, 1971. P.S.E.B.M., 1971, Vol. 138.