Purification of Bacterial Endotoxins by Zonal Centrifugation

L. R. MENDIOLA,¹ B. KOUZNETZOVA, J.-C. CHERMANN, F. SINOUSSE, M. DIGEON,
AND M. RAYNAUD

Service d'Immunochimie Garches, Institut Pasteur, 92-Garches, France

Received for publication 6 March 1972

In this paper, we describe a cultivation procedure in large-capacity fermentors (200 liters) and the purification of the *Salmonella* crude extracts by means of zonal centrifugation in a sucrose gradient. Briefly, the endotoxin has been extracted from salmonellae with hypertonic solutions. The crude endotoxin was shown to contain several antigens, mainly r_1 , r_2 , r_3 , or heterologous antigen. The heavy endotoxin was isolated and purified previously by enzymatic digestion, followed by a series of gel filtrations on Sephadex or Sepharose columns. We report the isolation and purification of heavy endotoxin in sucrose gradients, with the use of a B XIV titanium zonal rotor. From 150 to 300 mg of the crude material resuspended in a solution containing 1 M sodium chloride, 0.1 M sodium citrate, and 2.5% (w/w) sucrose, has been submitted to zonal centrifugation in gradients consisting of 5 to 25% (w/w) sucrose, also containing 1 M sodium chloride and 0.1 M sodium citrate. An overlay of 200 ml of 1 M sodium chloride-0.1 M sodium citrate was introduced after the sample. The separations were obtained after centrifugation for 3.5 hr at 35,000 rev/min ($\int w^2 dt = 1.66 \times 10^{11}$) at 4°C; the heavy endotoxin sedimented as heterogeneous material, from the middle toward the distal portions of the gradient. The heterologous antigens (r_1 , r_2 , r_3), as well as bacterial proteins, remained in the sample zone. The heavy endotoxin recovered from the gradients was quite pure, as revealed by immunodiffusion tests against several antibacterial sera.

The bacterial endotoxins are firmly bound to the cell wall, and it is therefore necessary to extract them. All of the extraction procedures have in common a starting material consisting of a suspension of the bacterial pellet in the fluid of extraction. However, the resulting products are crude extracts and have to be purified before use. At least two kinds of different products can be obtained according to the method of extraction: a whole endotoxin [Boivin's (4) or Raynaud's (14) method] containing protein, lipid, and carbohydrate as chemical components, or a lipopolysaccharide [Westphal's procedure (15)] mainly composed of lipid and carbohydrate.

Various techniques have been described for the purification of the endotoxins or lipopolysaccharides; ammonium sulfate (9), alcohol (11), magnesium acetate (12), and polyethylene-glycol (5, 6) have been used as precipitating agents. However, these were in fact more concentration techniques than purification procedures, and generally the purification was achieved by one or

several ultracentrifugations (10) or by one or several gel filtrations (5, 8).

In fact, all of these techniques are tedious and time-consuming. In the present paper, we describe the isolation and purification of endotoxin from crude extracts in a single fractionation procedure by use of zonal ultracentrifugation in a sucrose gradient and analysis of the different contaminating materials present in the crude extract.

MATERIALS AND METHODS

Strains. *Salmonella typhi* 0901 (smooth form), *S. typhi* R₂ (chemotype Rb), and *S. minnesota* R 595 (chemotype Re) were used.

Culture conditions. The bacteria were grown in a basic medium containing casein hydrolysate (35 g/liter) supplemented with yeast extract (3 g/liter). The growth of the cultures was carried out in Roux bottles or in large tanks containing 200 liters of medium (fermentor Geringe, Sweden); a van Doorn glass fermentor (50 liters) was used for the strain *S. minnesota* R 595.

Stock bacteria stored in gelose were used to start a liquid culture in a 100-ml tube with a lateral inlet.

¹ Present address: Department of Biochemistry and Microbiology, Rutgers University, New Brunswick, N.J. 08903.

This culture was used as inoculum for two flasks, each containing 1,500 ml of medium and shaking in the van Doorn apparatus rotatory shaker. These 3 liters of medium containing bacteria were injected into the fermentor. Each hour, a sample was taken from the fermentor and growth was controlled by absorbance at 540 nm in a Klett apparatus or a Beckman spectrophotometer (model DB). When growth started, sterile glucose solution was added to the culture medium at a flow of 1 liter per hr with peristaltic pump. The pH of the culture was controlled with a pH electrode (Ingold) and was maintained at 7.2 by addition of 12 N sodium hydroxide. The liquid medium was stirred at about 150 rev/min, and a continuous air flow was allowed to pass through the medium.

The cells were harvested at the exponential phase of growth in a Sharples centrifuge and were washed two or three times with physiological saline in a PR 6 refrigerated centrifuge (International Equipment Co.) at 3,000 rev/min for 30 min.

Preparation of the crude endotoxin. The extraction of the endotoxin from the cells with a hypertonic solution of 1 M sodium chloride-0.1 M sodium citrate was accomplished by a method described previously (14). The extracts were subsequently dialyzed against distilled water and lyophilized. The resulting dry powder constituted the crude starting material for the various experiments.

Sera. Sera were prepared by hyperimmunization of horses, as previously described (5, 8). The properties and characteristics of all sera are reported in Table 1.

Zonal centrifugation. Amounts of 150 to 300 mg of the crude endotoxin preparations were resuspended in 10 ml of 2.5% (w/w) sucrose, containing 1 M sodium chloride and 0.1 M sodium citrate. The opalescent suspension was introduced into a rotor containing a linear gradient of 5 to 25% (w/w) sucrose, also containing 1 M sodium chloride-0.1 M sodium citrate. The titanium B-XIV rotor was operated in a B-60 ultracentrifuge (International Equipment Co.). The general conditions for rotor loading and unloading were as described by Anderson et al. (1) and Price and Hirvonen (13). The sample was followed by an overlay of 200 ml of 1 M sodium chloride-0.1 M sodium citrate. Centrifugations were carried out at 35,000 rev/min for approximately 3.5 hr

$[\int w^2 dt = 1.68 \times 10^{11}]$ at 4 C for *S. typhi* 0901 and R₂, and at 40,000 rev/min for 5 hr $[\int w^2 dt = 4.13 \times 10^{11}]$ for *S. minnesota* R 595. Unloading was accomplished by displacement of the gradient with 45% (w/w) sucrose, at a rate of 20 to 30 ml/min. The effluent was collected in 10-ml fractions. The absorbancy at 280 nm of each fraction was determined. Every other fraction was also used for immunodiffusion tests against several sera.

RESULTS AND DISCUSSION

Analysis of crude extracts of endotoxin reveals the presence of several nontoxic, antigenic components, along with the heavy endotoxin, r₄ (Fig. 1a). The extraneous antigens consist of the heterologous antigens r₁, r₂, and r₃ (common to all enterobacteria), antigens of the cell wall proteins, and finally the flagella. Previously, the heavy endotoxin (r₄) has been isolated from such crude extracts, thus eliminating the extraneous antigens, by a series of purification steps which are rather tedious and time-consuming, as mentioned above. In the final step of purification, i.e., gel filtration on Sephadex G-200, the heavy endotoxin (r₄) is obtained in the exclusion volume, whereas the heterologous antigens are retained by the gel. The extraneous proteins of the wall had been previously removed by digestion with Pronase.

On the other hand, by directly subjecting the crude material to zonal centrifugation in sucrose gradients, we have obtained purification of the heavy endotoxin (r₄) in a single step. Figure 2 shows the absorbancy profile from a typical zonal centrifugation run of a crude extract of *S. typhi* R₂. The distribution of the different antigens and wall proteins is also indicated. Under the conditions employed, the heterologous antigens r₁, r₂, and r₃, as well as soluble proteins, remained in the sample zone and did not migrate through the gradient, whereas the flagella migrated towards the densest portion of the gradient. The heavy endotoxin (r₄) migrated as a heterogeneous material from about the middle toward the heavier portions of the gradient, and traces were also found in the peak corresponding to the flagella. The heterogeneity of the endotoxin has previously been observed by other workers. Certainly, the heterogeneity exhibited in the zonal runs can be attributed to different sizes or states of aggregation of the particles, since the immunodiffusion test against several sera revealed the presence of only the r₄ component in these fractions. Furthermore, after the fractions (38-58) were pooled, dialyzed extensively against distilled water, and concentrated by lyophilization, the resulting material still showed, on immunodiffusion, the presence of only the heavy

TABLE 1. *Properties of the sera*

Serum no.	Antibodies against
13475	Heterologous antigens r ₁ r ₂ r ₃ , endotoxin from <i>S. typhi</i> R ₂
90193	Endotoxin from <i>S. typhimurium</i> TV 119
90319	Endotoxin from <i>S. typhi</i> R ₂
13525	Flagella, proteins of the wall, endotoxin from <i>S. typhi</i> R ₂
483	Endotoxin from <i>S. typhi</i> 0901
90307	Endotoxin from <i>S. typhi</i> 0901
90516	Endotoxin from <i>S. minnesota</i> R 595

FIG. 1. Immunodiffusion patterns. (a) The center well contains crude extract of *S. typhi* R₂. The sera 90193 and 90319 are specific for the rough endotoxin R II (chemotype Rb). Serum 13475 also reveals the heterologous antigens r₁, r₂, and r₃, and 13525 shows the proteins of the cell wall and the flagella. (b) The center well contains *S. typhi* R₂ pure heavy endotoxin purified by zonal centrifugation as described in the text. The outer wells have the same disposition of sera as in 1a. (Note the immunological heterogeneity of the pure endotoxin. Two molecules with common antigenic determinants; references 9, 11, 12, 14, 15.) (c) The center well contains serum 13525 (see Table 1); anti-crude endotoxin extracted from *S. typhi* R₂. The outer wells contain proteins remaining in sample zone from: 1, *Salmonella arachevelata* (Institut Pasteur collection); 2, *E. coli* O 111 B₄ (Institut Pasteur collection); 3, *S. typhi* R₂; 4, *S. typhimurium* TV 161 Rou A 417; 5, *S. paratyphi* B (Institut Pasteur collection); 6, *S. minnesota* 1117 R₃, extreme-rough.

FIG. 2. Absorbancy profile of a separation of heavy endotoxin (r_4) from a crude extract of *S. typhi* R_2 (rough). A 300-mg amount of the crude material was resuspended in 10 ml of 2.5% (w/w) sucrose, 1 M sodium chloride and 0.1 M sodium citrate. The conditions for centrifugation are described in the text. The presence of the antigens was determined by immunodiffusion on gels of every second fraction, against several sera.

FIG. 3. Absorbancy profile of a separation of heavy endotoxin from a crude extract of *S. typhi* 0901 (smooth). A 200-mg amount of crude material was employed; otherwise the conditions of centrifugation were identical to those of Fig. 2.

endotoxin (Fig. 1b). Preliminary experiments performed with purified heavy endotoxin in an analytical ultracentrifuge also revealed that the material was heterogeneous, with a mean sedimentation coefficient of 53S.

As had been observed with the heavy endotoxin (r_4) preparations obtained before (e.g., by Pronase digestion and gel filtration), the material purified by zonal centrifugation gave a single continuous precipitation line with sera 90193 and 90319, two distinct lines were formed with sera 13525 and 13475, each being continuous with the single line given by the two former sera (Fig. 1b).

The behavior of the purified endotoxin on immunodiffusion on gels against several sera and its immunological heterogeneity have been discussed previously (8).

The endotoxin purified by zonal centrifugation was biologically active. It was toxic to 10-day-old chick embryos at a dose of 10^{-8} μ g and was pyrogenic for rabbits. When crude extracts of *S. typhi* 0901 (smooth) were subjected to zonal centrifugation, a profile similar to those given by crude extracts of the rough form was obtained. Here again, the heavy endotoxin migrated as heterogeneous particles, in similar positions in the gradient (Fig. 3). With the extracts from *S. minnesota*, longer centrifugation times were necessary to obtain separation of the endotoxin, although the latter also migrated as heterogeneous particles (Fig. 4).

Thus, the heavy endotoxin can be isolated from crude endotoxin of smooth or rough forms of *S. typhi* or *S. minnesota* by zonal centrifugation in sucrose gradients. This single fractionation procedure may be easily applied to the purification of endotoxins for enterobacteria.

During purification of endotoxins extracted from various other bacteria (such as *E. coli*, Shiga, or some other strains of *Salmonella*), we found that the proteins remaining in the sample zone were common to all *Enterobacteriaceae* (as shown in Fig. 1c), and probably similar to the

FIG. 4. Absorbancy profile of a separation of endotoxin from a crude extract of *S. minnesota* R 595 (KDO-Lipid A). A 321-mg amount of the crude material was resuspended in 10 ml of 2.5% (w/w) sucrose, 1 M sodium chloride, and 0.1 M sodium citrate. Conditions for centrifugation are the following: $\int \omega^2 dt = 4,040 \times 10^8$, corresponding to 5 hr at 40,000 rev/min. The type of gradient is described in the text.

"protein determinants" described by Barber et al. (2, 3).

The r_1 , r_2 , and r_3 heterologous antigens were detected by the 13475 antiserum in all tested extracts: Westphal's phenol-water (aqueous phase) and Boivin's or Raynaud's crude extract. They are probably lipoproteins and contain 39% amino acids, but no tyrosine and cysteine. These antigens are nontoxic for the chick embryo and nonpyrogenic for the rabbit. They are different from the Kunin's common antigen (7) and consequently are not a part of the endotoxin (S. Hammarstrom, 1st Int. Congr. Immunol., Washington, D.C., 1971).

ACKNOWLEDGMENTS

We thank O. Lüderitz (Max Plank Institut, Freiburg-Zähringen, Germany) for the *S. minnesota* mutants, L. Le Minor (Centre International des Salmonellae, Institut Pasteur, Paris, France) for several of the strains used, B. A. D. Stocker (Stanford University, Stanford, Calif.) for the *S. typhimurium* TV strain, L. Muller for the cultures, and A. Carlin and F. Garcia-Pons for their excellent technical assistance.

L. Mendiola, J.-C. Chermann, and M. Digeon were workers from INSERM (Institut National de la Santé et de la Recherche Médicale), INSERM contract 72 4 016.

LITERATURE CITED

1. Anderson, N. G., D. A. Waters, W. D. Fischer, G. B. Cline, C. E. Nunley, L. M. Elrod, and C. T. Rankin, Jr. 1967. Analytical techniques for cell fractions. V. Characteristics of the B-XIV and B-XV zonal centrifuge rotors. *Anal. Biochem.* 21:235-252.
2. Barber, C., E. Eylan, and J. Keydar. 1968. Sur les déterminants communs des Enterobacteriaceae. *Pathol. Microbiol.* 31: 321-327.
3. Barber, C., I. R. Vladoianu, and G. Dimache. 1967. Contributions to the study of *Salmonella*. II. Protein determinants shared by *S. typhi*, *S. enteritidis* and *S. typhimurium*. *Immunology* 12:411-416.
4. Boivin, A., I. Mesrobian, and L. Mesrobian. 1933. Extraction d'un complexe toxique et antigénique à partir du bacille d'Aertrycke. *C. R. Seances Soc. Biol. Filiales* 114: 307-310.
5. Chermann, J.-C., M. Digeon, B. Kouznetzova, and M. Raynaud. 1971. Smooth and rough heavy endotoxins and lipopolysaccharides: preparation and properties. *Symp. Series Immunobiol. Stand.* 15:123-130.
6. Chermann, J.-C., M. Digeon, and M. Raynaud. 1967. Une nouvelle méthode de purification des endotoxines: la précipitation par le polyéthylène-glycol. *C. R. Acad. Sci. Sér.* 265:1251-1252.
7. Chermann, J.-C., M. Digeon, and M. Raynaud. 1967. Non identité des antigènes hétérologues r_1 r_2 r_3 et de l'antigène commun de Kunin. *Ann. Inst. Pasteur (Paris)* 112:77-85.
8. Chermann, J.-C., M. Raynaud, and M. Digeon. 1967. Etudes des divers antigènes élaborés par *Salmonella typhi* rough (souche R₂). II. Séparation et propriétés. *Ann. Inst. Pasteur (Paris)* 113:375-398.
9. Crutchley, M. J. 1971. Free endotoxin. *Symp. Series Immunobiol. Stand.* 15:115-122.
10. Fromme, I., O. Lüderitz, A. Nowotny, and O. Westphal. 1958. Chemische Analyse des Lipopolysaccharids aus *Salmonella abortus equi*. *Pharm. Acta Helv.* 33:391-400.
11. Galanos, G., O. Lüderitz, and O. Westphal. 1969. A new method for the extraction of R lipopolysaccharides. *Eur. J. Biochem.* 9:245-249.
12. Osborn, M. J., S. M. Rosen, L. Rothfield, and B. L. Horecker. 1962. Biosynthesis of bacterial lipopolysaccharide. I. Enzymatic incorporation of galactose in a mutant strain of *Salmonella*. *Proc. Nat. Acad. Sci. U.S.A.* 48:1831-1838.
13. Price, C. A., and A. P. Hirvonen. 1967. Sedimentation rates of plastids in an analytical zonal rotor. *Biochim. Biophys. Acta* 148:531-538.
14. Raynaud, M., and M. Digeon. 1949. Sur une nouvelle toxine du bacille typhique extraite des formes rough. *C. R. Acad. Sci.* 229:564-566.
15. Westphal, O., O. Lüderitz, and F. Bister. 1952. Über die Extraktion von Bakterien mit Phenol/Wasser. *Z. Naturforsch.* 7b:148-155.