INFLUENCE OF THE PHYSICAL STATE OF ENDOTOXIC PREPARATIONS ON DERMAL TOXICITY

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ABSTRACT

ROBERSON, BOB S. (University of North Carolina, Chapel Hill) AND WILLIAM J. CROMARTIE. Influence of the physical state of endotoxic preparations on their dermal toxicity. J. Bacteriol. **84**:882-887. 1962.—Studies were performed with various preparations of endotoxin from *Escherichia coli* to test for a possible relation between the physical state or degree of aggregation of the toxin and dermal toxicity. Endotoxic fractions were prepared by enzymatic and physical dissolution of cell walls, phenol extraction, and ether extraction. A comparison of the toxic effects of the different preparations after a single intradermal injection into rabbits demonstrated that the degree and duration of the inflammatory response was influenced significantly by the physical state of the preparation. In addition, the least active material, the aqueous ether extract, was treated so as to yield three preparations differing only in the degree of aggregation. Dermal titration of these preparations suggests that a departure from an optimal range of particle size results in a diminished capacity of endotoxin to induce the acute and protracted manifestations of dermal response.

The general hypothesis that particles in the aggregate often behave quite differently biologically from unit particles is supported by a variety of evidence. The work of Neter et al. (1956), and of Westphal (1957), shows, respectively, that the capacity of endotoxin to modify erythrocytes and to produce a pyrogenic response is affected by aggregate size or state of dispersion. Roberson, Schwab, and Cromartie (1960) demonstrated a relationship between the local dermal toxicity of a component of streptococcal cell walls and an optimal particle size. Evidence presented by Dale and King (1953) clearly showed an analogous correlation between toxicity and the dimensions of toxic polymers of silicic acid. Certain toxic activities of complexes of antigen and antibody (Ishizaka, Ishizaka, and Campbell, 1959), and of globulin complexes alone (Christian, 1960), implicate requirements as to particle size in that these activities are not exhibited by larger, "insoluble" structures or by the smaller unaggregated units.

The present study was undertaken to determine whether a correlation exists between the local dermal effects in rabbits and the particle size of endotoxic complexes. The gram-negative endotoxin seemed an especially suitable test material, since toxicity, as determined by different techniques, varies considerably with the method of purification, even when composition appears comparable (Ribi et al., 1961). Secondly, wide variation in the state of aggregation may be inadvertently or purposely obtained by minor variations in the extraction procedure (Westphal, 1957). Finally, a single injection of endotoxin produces a dermal lesion in rabbits which can be used as a quantitative index of activity (Larson et al., 1960).

MATERIALS AND METHODS

Collection of cells and preparation of fractions. The organism used as the source of endotoxin was the Crooke's strain of *Escherichia coli*. Cells were grown for 10 to 12 hr in 4 liters of Trypticase Soy Broth (BBL), harvested by centrifugation, washed two times with the original volume of cold saline, and resuspended in water. Cell walls were collected according to a procedure previously described (Roberson and Schwab, 1960), which involved rupture of the organisms with small glass beads in a 9-kc Raytheon oscillator, differential sedimentation, and sucrose zone centrifugation. The cell walls were found by electron

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microscopy to be concentrated in the uppermost of three distinct bands in the sucrose gradient. They were removed, washed by centrifugation, and resuspended in 10 ml of phosphate buffer (pH 7, $\Gamma/2 = 0.01$). This suspension was incubated for 4 hr with 3 mg of papain and washed three times with phosphate buffer (pH 7, $\Gamma/2 = 0.1$), to yield a purified cell-wall preparation.

Cell-wall fragments of different size or density or both were obtained by sonic vibration of whole cells for 30 min without glass beads. The sonically vibrated suspension was centrifuged at 20,000 $\times$ *g* for 30 min (Spinco no. 40 rotor). The sediment was washed by resuspension and recentrifugation in phosphate buffer (pH 7, $\Gamma/2 = 0.1$), treated with papain as described for purified cell walls, and resedimented at 110,000 $\times$ *g* for 1 hr. The ratio of nitrogen to reducing sugar in this preparation was comparable with that of purified cell walls. Segregation of the cell-wall fragments was accomplished by centrifugation through discontinuous sucrose gradients. Sucrose solutions of densities 1.15 (1.25 ml), 1.10 (1.25 ml), and 1.05 (1.5 ml) were layered in cellulose centrifuge tubes, overlaid with 1 ml of the vibrated material, and centrifuged at 100,000 $\times$ *g* for 45 min in a Spinco SW-39 rotor. Fractions were collected by puncturing the tubes and aspirating the clearly visible bands into a syringe. Each fraction was dialyzed against four changes of distilled water.

An additional endotoxic preparation was extracted by the phenol-water method of Westphal, Lüderitz, and Bister (1952). Partially purified cell walls were heated at 65 C for 10 min in a mixture of phenol and water (1:1, w/v). On cooling, the aqueous layer containing the endotoxin was separated from the phenol, and residual phenol was removed with ether. Traces of ether were removed under vacuum. Reduction of the aggregate size of the phenol-extracted material was accomplished by the method described by Neter et al. (1956), which consisted of treatment with 0.25 N NaOH for 6 min at 56 C. By this method, they found the particle size to be reduced from a molecular weight of 10 to 20 million to 200,000 without any loss of toxicity as determined by mouse lethality and pyrogenicity. After such treatment, the solution retained only a moderate amount of the original turbidity.

The final preparation was an aqueous ether extract of viable cells prepared according to the method described by Ribi, Milner, and Perrine (1959). Freshly harvested cells were washed with cold saline and resuspended in saline, and placed in a separatory funnel with excess ether. The mixture was vigorously shaken for 1 min and allowed to stand at room temperature overnight. The ether was then removed under vacuum. After centrifugation, the resulting supernatant fluid was dialyzed and filtered through an O2 Selas filter to yield a clear and colorless liquid.

Chemical determinations. Total reducing sugar was determined by a modification of the Somogyi technique (Nelson, 1944). Nitrogen was estimated by nesslerization according to the modification of Koch and McMeekin (1924).

Histological methods. Blocks of tissue for histological examination were fixed in 10% formalin solution. Paraffin sections were stained with hematoxylin and eosin.

RESULTS

The first estimate of dermal toxicity was based on a comparison of the lesions produced by the injection of comparable amounts of the different preparations. For this comparison, each preparation was diluted to contain, after hydrolysis, 30 μg of reducing sugar per injection (0.2 ml). Figure 1 shows the average size (mm^2) of the lesions produced in four New Zealand white rabbits. The dermal reactions induced by the cell walls and the cell-wall fragments were comparable throughout the observation period, and, initially (first 5 days), they were significantly less than the reactions produced by the phenol-water extracts. However, the cell-wall preparations produced a more extended toxic effect than did the phenol-water extracts, as shown by a comparison of the lesions from the 5th through the 20th day. The aqueous ether extract produced the least reaction of any of the preparations, regardless of time of comparison.

A more sensitive index of dermal toxicity was provided by serial titration in rabbits of endotoxic preparations similar to those used in the previous experiment. For each titration, seven twofold dilutions containing 6.0 to 0.09 μg of polysaccharide, estimated as glucose, were prepared and 0.2 ml of each dilution was injected into each of four rabbits. The injection sites were observed daily, and the highest dilution producing a lesion greater than 5 mm^2 was recorded. Figure 2 shows for each fraction the lesion index, which is the

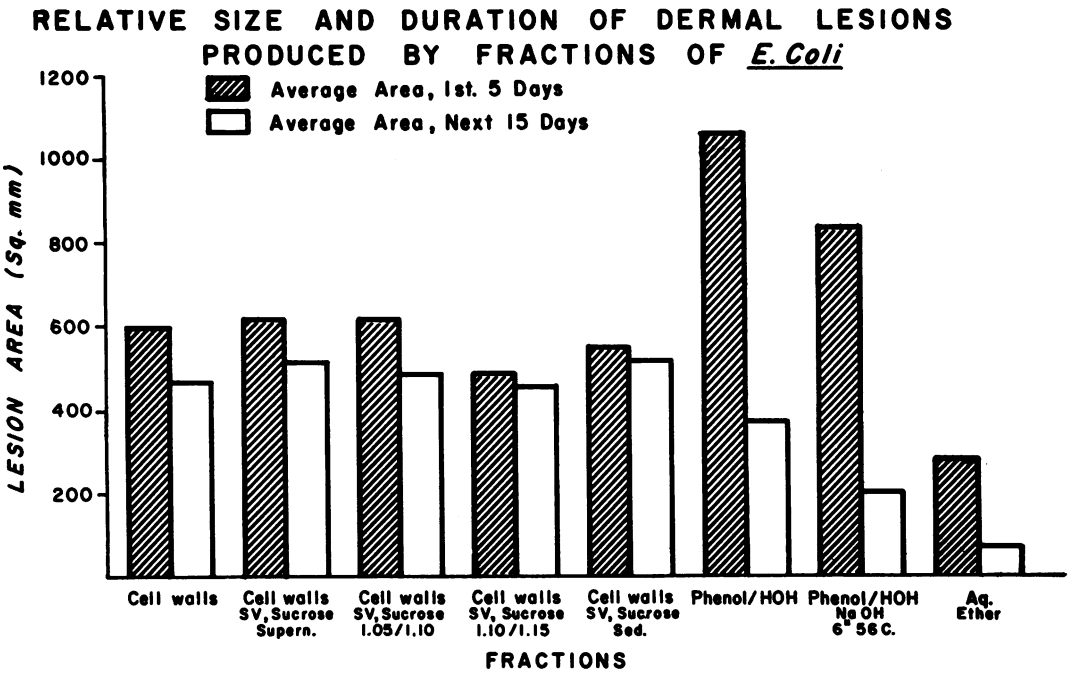


FIG. 1. *Comparison of the dermal toxicity of endotoxin preparations in varying physicochemical states. The designation of the fraction of cell walls dispersed by sonic vibration* (SV) *corresponds to the zone of collection from sucrose zone centrifugation. Each area given is the average of daily readings from four rabbits.*

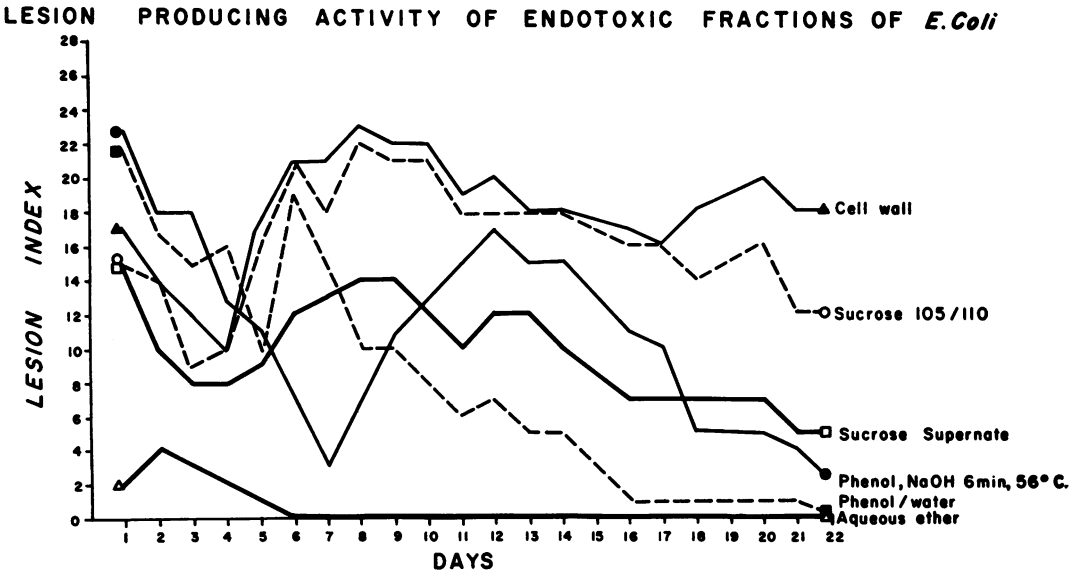
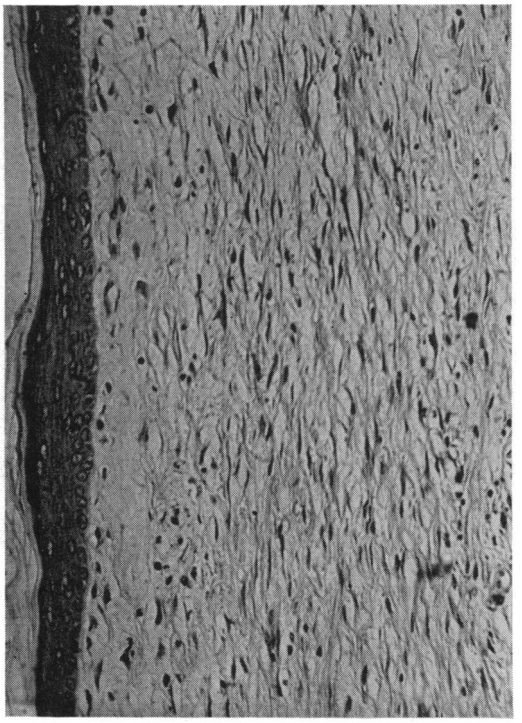


FIG. 2. *Comparative daily levels of dermal reaction to twofold serial dilutions of endotoxin. The lesion index is the sum of the highest positive dilution* (log_2) *from four dermal titrations.*

sum of the highest positive dilution (log_2) from each of the four titrations, plotted against the time in days.

Initially, the phenol-water extracted material and that exposed to 0.25 N NaOH for 6 min at 56 C produced greatest response. The ether-extracted endotoxin at the lowest dilution produced only minimal lesions. The cell-wall prepa-



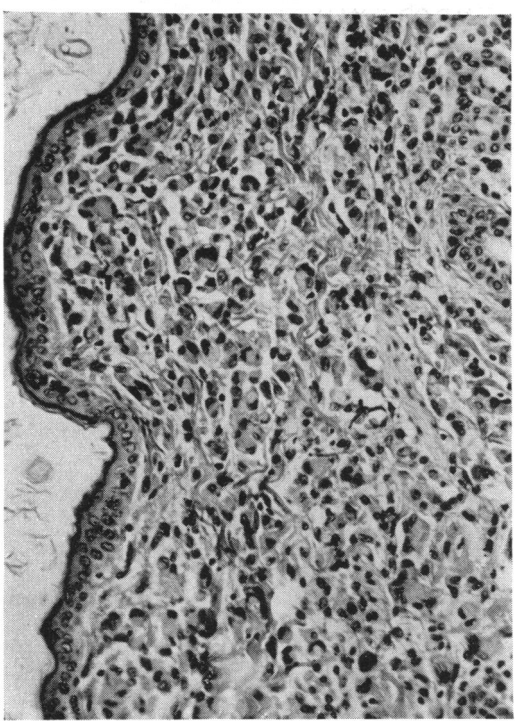


FIG. 3 (top). *Scar formation at site of injection of the phenol-water extract, 36 days after injection. Hematoxylin and eosin stain.* × *400.*

rations, on the other hand, while moderately active initially, induced higher levels of late dermal response with a marked persistence over that of the phenol-extracted material. This plot also reflects the fact that certain of these lesions tended to subside and exacerbate.

The rabbits were killed 36 days after injection, and histological sections were prepared from the various sites of injection. The observed microscopic changes reflected the findings noted in the gross and recorded in Fig. 2. At the site of injection of the phenol-water extract, there was complete healing and scar formation without chronic inflammatory changes. Sections from the sites of injection of the cell wall and the gradient fraction collected between sucrose densities of 1.05 and 1.10 showed a marked chronic inflammatory response which consisted of focal and diffuse accumulation of macrophages, and giant cells associated with fibroblast proliferation and increased number of capillaries. These changes are illustrated in Fig. 3 and 4.

To exclude the possibility that variation in toxicity among the preparations was due to differences in composition, physical changes were produced in a single preparation, the relatively less toxic aqueous ether extract. Previously, the aqueous ether material had been filtered through an O2 Selas filter to remove the sediment which formed on dialysis. In this experiment, the extract was filtered before dialysis. The flocculent precipitate which developed during dialysis was retained. The procedure of Neter et al. (1956), using 0.25 N NaOH for 6 min at 56 C, was again used to reduce the size of the aggregate. All samples were made physiological with NaCl and diluted on the basis of original volume. Figure 5 shows an electron micrograph of the three preparations. The sparsity of particulate matter in the crude extract is indicative of the small aggregate size of the endotoxic components (left frame, Fig 5). The middle frame shows aggregations of the dialyzed endotoxin which suggests a tendency of the particles to cord. The right frame shows the dispersal of the aggregate into a smaller unit on treatment with NaOH. The results of dermal titration of these preparations are shown in Fig. 6. The clear, crude aqueous ether extract and the

FIG. 4 (bottom). *Injection site of the 1.05/1.10 fraction, 36 days after injection. The presence of many macrophages and an occasional giant cell is the most striking feature of this reaction. Hematoxylin and eosin stain.* × *400.*

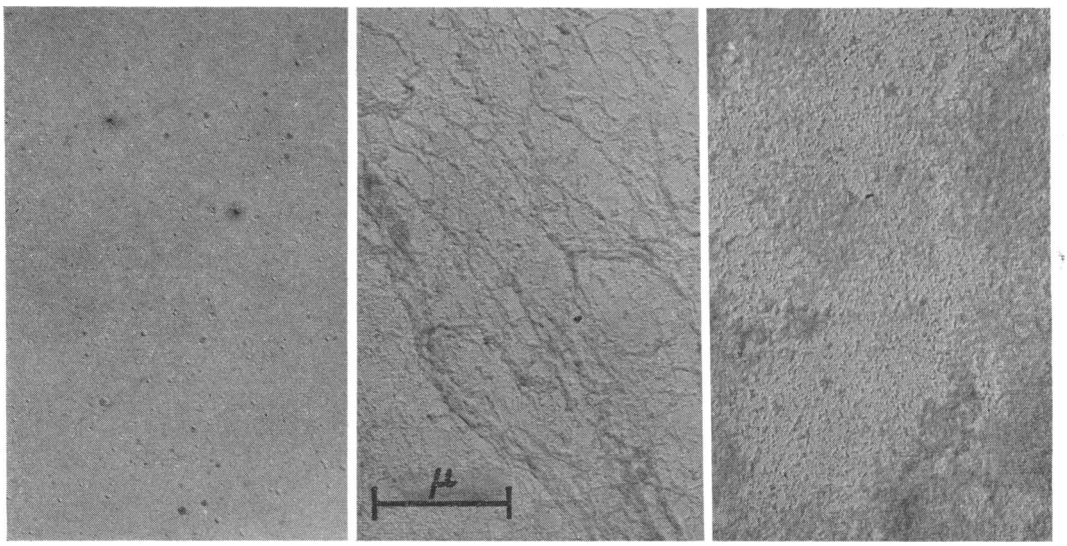


FIG. 5. *Electronmicrographs of particles in aqueous ether extracts transferred from agar by collodion pseudoreplica and shadowed with chromium. Represented are: (left) a filtered, aqueous ether extract; (center) the same extract following dialysis; and (right) the dialyzed extract following NaOH treatment.*

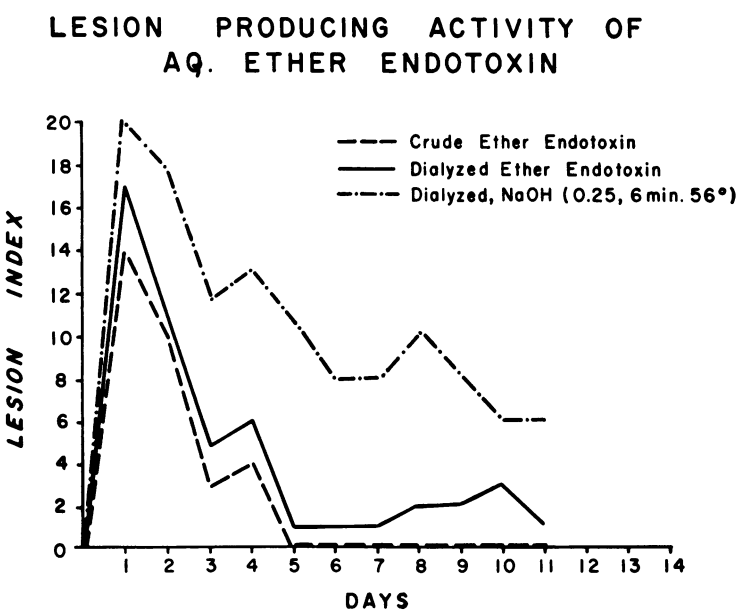


FIG. 6. *Comparison of dermal response to the aqueous ether extracts shown in Fig. 5. Each point represents the lesion index obtained from four dermal titrations.*

aggregated dialyzed material showed only minor differences in dermal toxicity. The NaOH-treated fraction, which contained aggregates of an intermediate size, exhibited the greatest degree of dermal toxicity.

DISCUSSION

Evaluation of procedures for extracting and purifying endotoxin depends on biological testing methods, such as animal lethality, production of fever, changes in leukocytes, or production of

skin lesions, for estimating potency. Such methods usually emphasize the production of a specific reaction with the least amount of material. Characterization of the extracted material is then often based on chemical methods which tend to disregard the physical properties of the endotoxin. However, evidence presented here indicates that the physical dimensions of the components can be an important parameter in the production of the localized dermal reaction. Such a dependency might account for changes in toxicity observed after attempts to purify the toxin.

An optimal range of particle size, as suggested by these data, would imply that a variation in either direction would result in a less toxic preparation. These results are consistent with those of the experiments reported previously with particles derived from streptococcal cell walls (Roberson et al., 1960), which show a correlation between maximal toxicity and particles of intermediate size.

The variation in toxicity attributable to state of aggregation may have a bearing on the initial, acute dermal lesion as well as the subsequent course of the reaction. The latter facet of toxicity may not necessarily be related in degree to the extent of the initial lesion. Although not usually considered in estimation of endotoxic potency, the protracted toxic reaction, as illustrated by the chronic lesions which developed in response to cell-wall material, may contribute considerably under natural infectious processes to the total of the toxic manifestations produced by endotoxins.

ACKNOWLEDGMENT

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