Insolubilized *Salmonella* Lipopolysaccharides as Efficient Immunoabsorbents for the Preparation of Specific *Salmonella* Antisera

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Received for publication 16 April 1975

Lipopolysaccharides (LPS) derived from a single *Salmonella* serotype or a combination of serotypes were submitted to insolubilization by means of glutaraldehyde. Cross-linked LPS proved to be efficient immunoabsorbents. They were employed for the removal of cross-reacting anti-O antibodies from various *Salmonella* antisera. LPS immunoabsorbents offer a number of advantages over bacterial cells concerning the preparation of specific bacterial antisera: they regain the original antibody binding capacity after tryptic digestion of the anti-LPS antibodies adsorbed and can be repeatedly used (15 times at least), adsorption of the sera is complete within 30 min, and the procedure is simplified. Prolonged storage (over 3 years) has no deleterious effect on the adsorption properties of the immunoabsorbents. Various applications of LPS immunoabsorbents are described.

The use of whole bacterial cells for the absorption of cross-reacting agglutinins from bacterial antisera has been the method of choice for a long time (1). This procedure, however, suffers from a number of disadvantages which cannot be disregarded: it involves numerous time- and labor-consuming steps, the bacterial mass can be used only three to four times, and the standardization of the process is not always satisfactory. That is why the elaboration of more refined methods of preparation of specific bacterial antisera seems not only attractive but desirable. The application of immunoabsorbents holds promise in this respect. Several methods of immunoabsorption have been proposed, so far, which are based on the use of: bacterial cells polymerized by means of bisdiazotized benzidine (10), bacteria incorporated in agar (9), Sephadex G-25 beads (2, 6), or polyacrylamide gel (7). Eskenazy (4) reported a procedure for the preparation of glutaraldehyde cross-linked lipopolysaccharides (LPS). The insolubilized LPS exhibited the ability of specifically binding anti-LPS antibodies.

This paper provides evidence that LPS immunoabsorbents (mono- and multicomponent) offer a reasonable alternative to bacterial cells for the preparation of specific *Salmonella* antisera.

MATERIALS AND METHODS

Antigens. Fresh bacterial cultures as well as bacterial cells which had been already utilized for absorption of *Salmonella* antisera provided the source

of LPS. LPS was extracted by the hot phenol-aqueous procedure of Westphal et al. (11). Acetone-dried bacteria (20 g) were suspended in 350 ml of distilled water maintained at 65 to 68 C. An equal volume of 90% phenol preheated to the same temperature was added, and the suspension was stirred for 20 min at 68 C. After cooling to 10 C the mixture was centrifuged at 3,000 rpm for 30 min. Centrifugation resulted in the formation of three layers: water, phenol, and insoluble residue. The water phase was taken off by suction. The phenol layer and the insoluble residue were re-extracted with 350 ml of hot phenol as described above. The water extracts were combined, poured into a dialysis bag, and dialyzed against distilled water for 3 days in the cold, changing the outer fluid twice daily. The solution was clarified by centrifugation at 3,000 rpm for 30 min, concentrated down to 100 ml under reduced pressure, and lyophilized. The LPS was utilized without further purification.

Bacterial antisera. *Salmonella* O antisera were elicited in rabbits by injection of bacterial suspensions containing 2×10^9 organisms/ml, which were killed by boiling at 100 C for 2 h. H-specific antisera were prepared by immunization of rabbits with formalinized bacteria. The rabbits received six intravenous injections of 0.5, 0.8, 1.2, 1.8, and 2 ml of the suspensions, spaced 3 to 4 days apart. Seven days after the last injection, the animals were bled by cardiac puncture. Sheep were immunized intravenously with 2, 5, 8, and 10 ml of the broth cultures, treated as described above, and allowed to rest for 3 weeks; then they received 5, 10, 15, and 20 ml of the suspensions. The intervals between the injections were the same as in the immunization scheme for rabbits.

Quantitative precipitation test. The content of

anti-LPS antibodies was assayed as described by Robbins and Uchida (8).

O agglutinin titer. The O agglutinins were quantitated by agglutination. Briefly, bacterial suspensions boiled at 100 C for 2 h and containing 1×10^9 microbial bodies/ml were added to serial twofold dilutions of 0.5 ml of the *Salmonella* antisera in test tubes. The suspensions were cautiously mixed and incubated for 18 h at 50 C. Readings were taken on the following day. The reciprocal of the final serum dilution, producing distinct agglutination pattern, was taken as the O agglutinin titer.

Sugar analysis. Total sugars were assayed by the method of Dubois et al. (3). The quantity of LPS insolubilized was determined from the difference between the quantity of LPS before and after polymerization. Glucose served as a standard.

Preparation of LPS immunoadsorbents. Insolubilization of LPS was carried out according to the following procedure: glutaraldehyde was added to 1% solution of LPS in 0.1 M phosphate buffer, pH 7.0 to obtain a 2% final concentration. An equal volume of saturated neutralized ammonium sulphate was then added, and the mixture was stirred for 1 h at room temperature. The precipitate was collected by centrifugation at 4,500 rpm for 20 min, homogenized in a Potter-Elvehjem homogenizer, repeatedly washed with distilled water until free of sulphate ions, and finally suspended in phosphate-buffered saline, pH 7.2, containing merthiolate (1:1,000). When not in use the immunoadsorbent was stored at 4 C.

Adsorption capacity of the immunoadsorbents. The adsorption capacity was determined by adding different volumes of serum to a constant weight of adsorbent. The suspensions were gently stirred for 30 min at room temperature. The adsorbed sera were separated by centrifugation at 4,500 rpm for 15 min, and the supernatants were tested for the presence of unbound O agglutinins by agglutination. The quantity of immunoadsorbent (in terms of milligrams) ensuring complete removal of cross-reacting agglutinins from 1 ml of antiserum determined the adsorption capacity of the immunoadsorbent. Routine adsorptions were performed using this amount. Since various *Salmonella* antisera differed widely in the level of anti-LPS antibodies, the adsorption capacity was determined in each particular case.

Regeneration of the immunoadsorbents. After adsorption of the sera, the immunoadsorbents were washed with phosphate-buffered saline until the absorbances of the supernatants were 0 at 280 nm. The anti-LPS antibodies bound to the immunoadsorbents were digested with trypsin, which was dissolved in 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.1 (ratio of adsorbent to enzyme 20:1 wt/wt). Incubation took place for 20 h at 37 C. After copious washing with physiological saline, the immunoadsorbents were employed again.

Three experimental approaches were tried with the objective of studying diverse applications of LPS immunoadsorbents: (i) removal of anti-LPS antibodies from antisera with a high level of H agglutinins; (ii) preparation of *Salmonella* O factor sera by cross-adsorption on appropriate immunoadsorbents; and

(iii) utilization of multicomponent immunoadsorbents for the preparation of specific anti-O and anti-H sera.

RESULTS

The yield of LPS derived from various *Salmonella* strains was found to be close to that reported by Westphal et al. (11). For example, 0.58 g of LPS was extracted from 5.2 g (dry weight) of *Salmonella nottingham* bacteria (11% yield).

The analysis of the total sugars revealed that glutaraldehyde brought about more than 90% insolubilization of the LPS originally present in the solution. This was found to be true for all cases examined.

LPS immunoadsorbent was prepared from a strain of *S. nottingham*. It was designed for the removal of anti-O16 antibodies from an anti-H:gm serum produced against the same strain. The effect of various conditions, namely quantity of immunoadsorbent and duration of adsorption, was ascertained in preliminary experiments. The experimental data showed that the reduction of the agglutination data depended on the amount of immunoadsorbent added (Table 1). When the anti-H:gm serum was allowed to react with the immunoadsorbent for 15 min, the O agglutination titer dropped to values comparable to those obtained for longer reaction times. Adsorption for half an hour resulted in practically complete depletion of anti-LPS antibodies and this time period was chosen as optimal.

It was established that *S. nottingham* bacteria and the corresponding immunoadsorbent manifested similar adsorption properties when compared on a weight basis.

The results of the quantitative precipitation test are presented in Fig. 1. Calculations showed that the anti-H:gm serum employed contained 1.4 mg of anti-O16 antibodies per ml.

On application of the above immunoadsorbent under production conditions (526.5 mg of immunoadsorbent and 190 ml of antiserum), it is evident that a single adsorption of anti-H:gm

TABLE 1. Effect of the quantity of *S. nottingham* LPS immunoadsorbent on the degree of adsorption of anti-O16 antibodies

Immunoadsorbent (mg/ml of anti-H:gm serum)	Titer of O agglutinins	
	Before absorption	After adsorption
1.46	2,560	16
2.92	2,560	8
5.80	2,560	4
11.7	2,560	0

FIG. 1. Quantitative precipitation reaction of phenol-water extracted LPS from *Salmonella nottingham* bacterial cells.

serum led to a 160-fold decrease (from 2,560 before) of O agglutinin titer, the O agglutinins being completely adsorbed in another step. When the adsorbed anti-H:gm serum was examined for H specificity, it was demonstrated that the titer of H agglutinins (1:32,768) remained unchanged. This may be taken as an indication that the immunoadsorbent failed to bind antibodies other than O-specific ones.

Other experiments were performed to determine whether *Salmonella* O factor sera could be prepared by cross-adsorption on appropriate immunoadsorbents. Thus, *Salmonella give* immunoadsorbent (O antigenic factors 3 and 10) was used to bind anti-O3 and anti-O10 antibodies from an antiserum prepared by immunization of sheep with a mixed bacterial culture of *S. give* (3, 10) and *Salmonella görlitz* (3, 15). With 10.6 mg of immunoadsorbent and 1 ml of antiserum added, the O agglutinin titer decreased from 2,560 before (checked by agglutination with *S. give* and *S. görlitz*) the O agglutinin titer decreased to 160 after adsorption when checked by agglutination with *Salmonella new-haw* (O3, O15) or to complete adsorption when checked by agglutination with *S. give*. Thus, after adsorption O15-specific antiserum was obtained. The immunoadsorbent has been reused 15 times in the course of 3.5 years without losing its adsorption capacity.

The preparation of an immunoadsorbent composed of several LPS components seemed to be especially promising. "Hybrid" LPS carrying the O antigenic factors of the constituent LPS was extracted by the method of Westphal et al. (11) from a mixture of acetone-dried "waste" bacterial cells (absorbing cells already lost the capacity to interact with O agglutinins). They originated from the following group B *Salmonella*: *S. abortus-equi* (O factors 4,12), *S. essen* (4,12), *S. kisangani* (1,4, /5/,12), *S. arechavaleta* (4, /5/,12), *S. abony* (1,4, /5/,12), *S. derby* (1, 4, /5/,12), and *S. heidelberg* (1,4, /5/,12). The hybrid LPS was submitted to cross-linking as mentioned above. The multicomponent immunoadsorbent was applied to the adsorption of antibodies directed to antigenic factors O1, O4, and O12 from *Salmonella schwarzengrund* (1,4,12,27). Since the hybrid LPS and the *S. schwarzengrund* LPS shared common O factors (1,4,12) it seemed likely that the polyadsorbent would be able to adsorb antibodies to the common antigenic determinants. The experimental data corroborated this assumption. With the immunoadsorbent at 3,600 mg and 200 ml of antiserum added, the O agglutination titer decreased from 6,400 before to 160 after adsorption. A single adsorption resulted in the preparation of O27-specific antiserum. The specificity of the O27 antiserum was verified by cross-

agglutination, using the following strains: *Salmonella tinda* (O factors 1,4,12,27), *S. uppsala* (4,12,27), *S. schleissheim* (4,12,27), *S. bredeney* (1,4,12,27), *S. ball* (1,4,5/12,27), and *S. tafo* (1,4,12,27). The same immunoadsorbent has been successfully applied to the preparation of specific Henx, Heh, OB, and OE antisera. To date, the polyimmunoadsorbent has been reused 17 times without qualitatively reducing its capacity to remove undesired antibodies.

In addition, another multicomponent immunoadsorbent was made from LPS extracted from the following *Salmonella* serotypes: *S. chingola* (11), *S. orientalis* (16), *S. berlin* (17), *S. blukva* (18), *S. spartel* (21), *S. jolo* (35), *S. wandsworth* (39), *S. nowawes* (40), *S. waycross* (41), and *S. uphill* (42). This immunoadsorbent proved to be especially suitable for the production of specific anti-OA-OE serum which was difficult to render specific applying the routine procedure.

DISCUSSION

The experimental results suggest that the glutaraldehyde induced cross-linking of LPS does not impair the O antigenic determinants. The resulting insolubilized LPS exhibit the ability of specifically adsorbing homologous anti-O antibodies.

It is generally recognized that animals respond to immunization with whole bacterial cells by the formation of a heterogeneous population of antibodies, manifesting specificity to various bacterial antigens. This is true in the case of immunization with gram-negative bacteria in particular. It is not surprising, therefore, that high levels of anti-LPS antibodies are frequently found in *Salmonella* antisera of marked H specificity. The elimination of O agglutinins in such cases is quite often accompanied by considerable difficulties. It is for this reason that repeated absorptions with cells from different serotypes should be carried out to obtain specific antisera. LPS immunoadsorbents seem to offer a good alternative along these lines. They can adsorb only anti-LPS antibodies since the phenol extraction procedure yields LPS devoid of protein components. This accounts for the fact that no reduction of H agglutination titer occurs when LPS immunoadsorbents are employed.

Immunoadsorbents are superior to whole bacteria in many respects. The bacterial cells can be reused four times after heating as shown by Gray et al. (5). The antibody molecules remain attached to the O antigenic determinants which results in a loss of adsorption capacity.

Contrary to bacterial cells, immunoadsorbents are easily regenerated and regain the original antibody binding capacity. Tryptic digestion of the anti-LPS antibodies bound to the adsorbent exerts no effect on the polysaccharide matrix, and antigenic determinants are set free and become accessible for interaction with homologous antibodies. This permits the reuse of immunoadsorbents (15 times at least) which cheapens the whole production process. In addition, the depletion of the antisera of undesired antibodies proceeds more rapidly (30 min compared to 2 h in the case of bacterial cells). Immunoadsorbents withstand prolonged storage (longer than 3 years) without deterioration of their adsorption properties. They possess compact structure (occupy a smaller volume than bacteria), the adsorption of large serum volumes being facilitated.

Polyimmunoadsorbents merit special attention. The data indicate that they can be used for the preparation of a wide variety of anti-H and anti-O sera which attests to their versatility. Polyimmunoadsorbents may be regarded as an efficient tool for the production of specific bacterial antisera by simplified procedures (the application of a seven-component *Salmonella* immunoadsorbent allowed the preparation of a specific O27 antiserum in a single adsorption step which is practically impossible to achieve by the classic procedure).

It should be pointed out that immunoadsorbents should be used in combination with bacterial cells to remove antibodies to any trace protein antigens.

We believe that this procedure should be applicable to the preparation of specific antisera to other gram-negative bacteria as well.

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