Toxicity of Lipopolysaccharide and of Soluble Extracts of Salmonella typhimurium in Mice Immunized with a Live Attenuated araA Salmonella Vaccine

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Mice immunized intravenously 10 days earlier (but not those immunized 2 months earlier) with an attenuated Salmonella typhimurium SL3261 araA live vaccine and tested for delayed-type hypersensitivity by injection of crude Salmonella extracts in the footpad can die within 24 to 48 h of an unexplained allergic reaction. The lethal reaction could be prevented by prior administration of anti-tumor necrosis factor alpha serum. Injection of lipopolysaccharide (LPS) (either purified phenol-water-extracted [Westphal] LPS or protein-rich trichloroacetic acid-extracted [Boivin] LPS) was also lethal for mice immunized 10 days before. An LPS-rich crude Salmonella extract was more toxic than one which contained less LPS, suggesting that LPS may have been involved in the lethal reactions to crude antigens. Mild alkaline hydrolysis removes O-linked acyl groups from lipid A and eliminates many toxic effects of LPS; however, both Boivin LPS and Westphal LPS remained toxic for immunized mice after alkaline hydrolysis. In contrast, alkaline hydrolysis of crude whole Salmonella extracts (which caused marked protein degradation) reduced the lethal toxicity of the extracts, especially for an LPS-rich preparation. Mice immunized orally with the live vaccine did not show hypersensitivity to either LPS or crude extracts. The results suggest that the lethal reaction to crude Salmonella antigens in mice immunized 10 days earlier is complex, that tumor necrosis factor alpha is involved, and that allergic reactions to crude antigens (but not to LPS alone) can be reduced by mild alkaline hydrolysis.

We have recently shown that both antibody and T cells are important in recall of immunity to oral challenge in mice immunized with an araA Salmonella vaccine (15, 16). We are investigating the immunological correlates of protection conferred by vaccination, and footpad testing has been widely used to reveal T-cell-mediated responses to Salmonella antigens in mice immunized with salmonellae (1, 5, 6, 9, 10, 20).

However, the correlation between delayed-type hypersensitivity (DTH) and immunity to salmonellae in mice does not always hold (5, 6, 9, 10). The reason for this lack of correlation is not clear, but there may be a need for an improved test. Two problems with the footpad DTH test itself when crude Salmonella extracts are used are possible confusion of DTH with Arthus reactivity (1) and an unexplained hypersensitivity reaction seen when testing mice immunized parenterally a short time (1 to 2 weeks) earlier, which can cause mice to die within 1 to 2 days after injection of a test antigen into the footpad (5). It is known that Salmonella-infected mice become transiently hypersusceptible to lipopolysaccharide (LPS) and to tumor necrosis factor alpha (TNF-α) (17), suggesting that the reactions caused by crude antigens could be due to their LPS content.

We have shown that footpad testing for DTH of mice immunized with an araA Salmonella vaccine can yield misleading results due to long-lasting Arthus reactions to contaminating LPS which can grossly mimic DTH (14). Nonspecific reactivity to crude extracts and Arthus reactivity to purified LPS in mice immunized 8 weeks earlier disappeared after the antigens were degraded by mild alkaline hydrolysis (20). The latter procedure removes O-linked acyl groups from lipid A and disaggregates the molecule (19) without loss of O specificity (11), causing loss of complement-fixing ability and some nonspecific reactions like B-cell mitogenicity (14, 23). The footpad responses to alkali-treated crude Salmonella extracts were T cell dependent; alkali-treated LPS did not elicit a footpad response (14).

During these studies, we noticed that alkali-treated crude Salmonella extracts did not elicit lethal reactions in mice immunized 10 days earlier (our unpublished observations). The present study was undertaken to investigate the role of LPS in these lethal reactions and to see whether they are mediated by TNF-α. We also wished to compare the toxicity and effect of alkaline hydrolysis on purified phenol-water-extracted (Westphal) LPS (WLPS) and on protein-rich trichloroacetic acid-extracted (Boivin) LPS (BLPS). BLPS is more reactive than WLPS and contains endotoxin protein (EP; 18, 21), a bacterial component which can mimic some of the effects of LPS.

MATERIALS AND METHODS

Animals. Female BALB/c mice were purchased from Harlan OLAC Ltd., Blackthorn, Bicester, United Kingdom. Age-matched groups of 6- to 8-week-old mice were used.

Bacteria. Live, attenuated, aromatic-dependent Salmonella typhimurium SL3261 araA (4) was used for immunization. Virulent wild-type S. typhimurium C5 (5) was used for preparation of extracts.

Immunization. Bacteria were grown as stationary overnight 37°C cultures in tryptic soy broth (Oxoid). Aliquots (0.5 ml) were snap frozen and stored in liquid nitrogen. For vaccination, a vial was rapidly thawed and diluted in phosphate-buffered saline (PBS). Mice were immunized with a single intravenous injection of ca. 10⁶ S. typhimurium SL3261 organ-
isms. The inoculum size was checked by pour plating in tryptic soy agar (Oxoid).

For oral immunization, bacteria were grown in stationary overnight 37°C Luria-Bertani broth cultures, harvested by centrifugation, and resuspended in PBS to a concentration of approximately 5 × 10⁴/ml. Mice were given 0.2 ml intragastrically by gavage tube under light ether anesthesia. The inoculum size was checked by pour plating.

LPS. LPS from smooth *S. typhimurium* were obtained from Sigma (Poole, Dorset, United Kingdom). WLPS (catalog no. L6251), chromatographically purified WLPS (P-WLPS; <1% protein; catalog no. L2262), and BLPS (catalog no. L7261) were dissolved in PBS and stored at −20°C. For use, appropriate dilutions were made in PBS.

**Salmonella extracts.** Crude extracts from virulent *S. typhimurium* C5, C5 soluble extract (CSSE), and footpad antigen (FPAg) were similar to those we had previously used for DTH testing of mice and were prepared as previously described (23). Briefly, whole-cell CSSE was prepared from overnight stationary 37°C Tryptic soy broth cultures washed with EDTA to reduce the amount of LPS (12) and disrupted by sonication. The supernatant was clarified by centrifugation and filtered through a 0.22-µm-pore-size filter. For spent-medium FPAg, the cell-free supernatant of a 2-day culture in Cohn’s medium was precipitated with 4 volumes of ethanol, redissolved in PBS, and clarified by centrifugation. The protein concentration was estimated by using the BCA kit (Pierce Biochemicals, Rockford, Ill.) by following the manufacturer’s instructions. The amount of antigen used is expressed as its protein content.

The relative amount of LPS present as a functional endotoxin in the protein-rich antigens was estimated by a semiquantitative *Limulus* assay (Sigma) by following the instructions of the manufacturer. FPAg was found to contain approximately 10 times more functional LPS than was CSSE. Positive gelling reactions were given by a solution of phenol-extracted *S. typhimurium* LPS at 0.1 mg/ml, spent-medium FPAg at 1 ng/ml, and whole-cell CSSE at 10 ng/ml. We have recently reported (14) that the O-antigen content of these same preparations (as measured by inhibition of hemagglutination of LPS-coated erythrocytes) was 10 times higher for spent-medium FPAg than for whole-cell CSSE.

**Alkali treatment of salmonella antigens and LPS.** The preparations were incubated with NaOH at a final concentration of 0.25 M for 3 h at 37°C and neutralized with HCl with phenol red as a pH indicator. Antigen without alkali was similarly incubated as a control. We have reported that alkali-treated LPS still produced a typical LPS ladder pattern on silver-stained sodium dodecyl sulfate-polycrylamide gel electrophoresis gels while alkali treatment of the proteinaceous antigens resulted in extensive protein degradation in Coomassie blue-stained sodium dodecyl sulfate-polycrylamide gel electrophoresis gels (23). We have reported (14) that, as expected, the alkali-treated LPS preparations described here retained their O specificity as measured by inhibition of hemagglutination of LPS-coated erythrocytes but showed a marked modification in the ability to fix complement, which showed a very pronounced prozone effect.

**Anti-TNF-α antibodies.** A rabbit anti-TNF-α serum was raised in our laboratories as previously described (13), by using recombinant TNF-α kindly provided by G. R. Adolf, Boehringer Ingelheim, Vienna, Austria. Whole globulins were used after 40% ammonium sulfate precipitation and dialysis against PBS. A 2-µg sample of anti-TNF-α globulins neutralized the biological activity of ca. 2 × 10⁴ U of recombinant TNF-α in the L929 cytotoxicity assay, as previously described (13). Controls were given normal rabbit globulins (Sigma).

**Toxicity test.** Mice were immunized as described above. Ten days later, they were injected in the footpad with serial twofold dilutions of the test antigens in 25 µl of PBS. Controls received a similar volume of sterile PBS. Mortality was recorded over 48 h.

### RESULTS

**In vivo toxicity of footpad antigens and LPS for mice immunized intravenously with attenuated salmonellae.** CSSE is a whole-cell soluble extract of mouse-virulent *S. typhimurium* C5. FPAg is a concentrated spent-medium culture supernatant of C5 which contains over 10 times more LPS than does CSSE. CSSENaOH and FPAgNaOH are alkali-treated preparations of CSSE and FPAg. WLPS was used untreated or after alkaline hydrolysis (WLPSNaOH). Groups of four mice immunized intravenously with 10⁶ CFU of *S. typhimurium* SL3261 10 days earlier were injected in the footpad with graded doses of these preparations in 25 µl, and deaths were recorded over 48 h (Table 1).

All of the mice survived inoculation with 200 µg of CSSE; one mouse died after this dose of CSSENaOH. Conversely, four of four mice died after injection with 50 or 25 µg of spent-medium FPAg. However, only one of four mice died when injected with 50 µg of FPAgNaOH, and no deaths occurred after injection with 25 µg of FPAgNaOH.

Conversely, WLPS was highly toxic for immunized mice and this toxicity did not disappear after alkaline hydrolysis. Injection with 25 µg of WLPS or WLPSNaOH killed four of four mice, while 10 µg killed three of four and two of four mice, respectively, and 5 µg had no effect. No deaths occurred in equal numbers of naive mice injected with the highest concentrations of each antigen or in immunized mice injected with PBS.

Thus, the crude extract with the higher LPS content proved more toxic in *Salmonella*-infected mice. Whereas alkaline hydrolysis markedly reduced the lethal effect of LPS-rich FPAg, it had little effect on the lethal effect of WLPS.

### TABLE 1. Comparison of the lethal reactivity of *Salmonella*-immune mice of crude *Salmonella* extracts and of WLPS before and after alkaline hydrolysis of the antigens

<table>
<thead>
<tr>
<th>Dose (µg)</th>
<th>CSSE</th>
<th>CSSENaOH</th>
<th>FPAg</th>
<th>FPAgNaOH</th>
<th>WLPS</th>
<th>WLPSNaOH</th>
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<tbody>
<tr>
<td>200</td>
<td>0/4</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
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</tr>
</tbody>
</table>

* Mice were immunized intravenously with ca. 10⁶ of *S. typhimurium* SL3261 and used 10 days later.

* For crude extracts, the dose is expressed as micrograms of protein content.

* ND, not done.
TABLE 2. Anti-TNF-α serum reverses the lethal effect of crude extracts and WLPS in Salmonella-immune mice

<table>
<thead>
<tr>
<th>Dose (μg)</th>
<th>FPAg</th>
<th>FPAgNaOH</th>
<th>FPAg anti-TNF-α</th>
<th>WLPS</th>
<th>WLPSNaOH</th>
<th>WLPS anti-TNF-α</th>
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<td>1/4</td>
<td>0/4</td>
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</tr>
</tbody>
</table>

* Mice were immunized with ca. 10⁹ CFU of S. typhimurium SL3261 and used 10 days later.
* Number of mice dead/number inoculated after 48 h.
* For crude extracts, the dose is expressed as micrograms of protein content.
* Mice were given 2 × 10⁴ neutralizing units of rabbit anti-TNF-α serum 2 h before antigen injection.
* ND, not done.

occurred in parallel groups of naive mice injected with the highest concentrations of each antigen or in immunized mice injected with PBS as a control. TNF-α is therefore a mediator of the toxic effects displayed by WLPS and FPAg in Salmonella-infected mice.

In vivo toxicity of BLPS and P-WLPS in mice immunized intravenously. The toxicity of protein-rich BLPS was compared with that of P-WLPS. BALB/c mice were immunized as described above and challenged in the footpad 10 days later with decreasing amounts of BLPS and P-WLPS as the native preparations or after alkaline hydrolysis (BLPSNaOH and P-WLPSNaOH).

There was little difference in activity between BLPS and WLPS. BLPS and BLPSNaOH both killed four of four mice at doses of 25, 12, and 6 μg. P-WLPS and P-WLPSNaOH killed four of four mice at doses of 25 and 12 μg. No deaths occurred in groups of naive mice injected with the highest concentrations of LPS or in immunized mice injected with PBS.

Thus, BLPS and WLPS were both toxic for Salmonella-infected mice. Alkali treatment had no effect on the toxicity of either preparation.

Lack of toxicity of LPS and crude extracts for mice immunized orally. Groups of eight mice were immunized orally or intravenously with SL3261 and tested 10 days later with 50 μg of crude FPAg, including eight unimmunized controls. Deaths were recorded over 48 h. All of the intravenously immunized mice died, whereas all of the orally immunized mice and all of the naive controls survived.

Groups of four mice were immunized orally or intravenously and tested 10 days later with 50 μg of WLPS, along with four unimmunized controls. Deaths were recorded over 48 h. Again, all of the intravenously immunized mice died, whereas all of the orally immunized mice and all of the naive controls survived.

Thus, the hypersensitivity reaction was not observed when mice were immunized orally instead of intravenously.

**DISCUSSION**

In the present report, we show that crude Salmonella extracts of the type we have used for DTH testing and also LPS can induce lethal toxic reactions in mice immunized intravenously 10 days earlier with a live, attenuated, aromatic-dependent Salmonella vaccine. It must be noted that this hypersensitivity reaction did not appear in mice immunized orally. The toxic reactions seemed to correlate with the LPS contents of the extracts, could be induced by LPS alone, and could be prevented by in vivo injection of anti-TNF-α antibodies. Alkali treatment of a crude extract reduced its toxicity, whereas alkali-treated LPS remained toxic. The results indicate that this is a reaction mediated not by normal endotoxicity but by a different cytokine-mediated phenomenon.

Allergic reactions caused by vaccination during typhoid outbreaks are well known (for a review of earlier literature on provocation disease caused by vaccination during the incubation period of typhoid fever, see reference 24). Infection with gram-negative and gram-positive bacteria sensitizes mice to the lethal effects of LPS, and α-galactosamine-treated mice become sensitized to LPS and to gram-positive or gram-negative bacteria; TNF-α is an important mediator in these reactions (2). Mice infected with Salmonella become transiently susceptible to TNF-α and LPS (17). The present results are consistent with these findings in that both crude extracts and LPS were toxic for immunized mice and the effects could be neutralized with anti-TNF-α sera.

The observation that the LPS-rich extract was the more potent of the two further suggests that LPS is an important mediator in these reactions. However, the effect of alkali treatment indicates that the reaction to crude extracts is complex, as alkali treatment reduced the lethal reactions to endotoxin-rich FPAg but had little effect on the toxicity of purified LPS, which remained lethal at doses of 2.5 to 5 μg. Two conclusions stem from this observation.

(i) The results indicate that the TNF-α-mediated lethal toxicity of crude Salmonella antigens is enhanced, at least in part, by components (proteins?) other than LPS itself. It is known that Salmonella components other than LPS can trigger TNF-α production. Whole Salmonella, but not LPS, were shown to stimulate TNF-α production in LPS nonresponder mice in vivo (unpublished data) and in vitro in macrophage cultures (2). Purified and virtually LPS-free S. typhimurium porins can induce TNF-α production in human monocytes (3).

Ep (18, 21), which would be present in BLPS but not in WLPS, are known to mimic some effects of bacterial endotoxins, and EPs could be responsible for some of the effects of crude antigens seen here. However, the contribution of EPs to the toxic effect seen here could not be determined, as both WLPS and BLPS remained toxic after hydrolysis, so that a reduction in the toxicity of BLPS due to degradation of EPs could have been masked by the residual toxicity of LPS.

(ii) Our observations imply that the mitogenic activity and the unusually long-lasting Arthus reaction observed by us in DTH studies (14) and the toxic lethal reaction described here are mediated by different parts of the LPS molecule. We have shown that the Arthus reactions and the mitogenic ability of LPS disappear after alkali treatment (23), whereas the present results indicate that a lethal reaction persists. The drop in mitogenic activity and Arthus reactivity after hydrolysis is probably due to disaggregation of the molecule and loss of complement-fixing ability, whereas lethal reactivity to LPS in infected mice has been reported to lie within the LPS core region (8). It has recently been reported that the state of disaggregation of LPS by sodium deoxycholate reduces its toxicity for α-galactosamine-sensitized mice (20a).

This hypersensitivity reaction has not previously been reported in mice immunized with live Aro Salmonella vaccines. It must be stressed that the time at which the mice are tested is very important for the appearance of the allergic reaction. We have seen it only in mice immunized 1 to 2 weeks earlier by the intravenous route, whereas the number of bacteria in the mononuclear phagocyte system are at their peak (7). We have never seen this reaction a month or later following intravenous inoculation.
immunization or at any time after vaccination in mice immunized orally. Phase 1 trials with humans and live Aro salmonella vaccines given orally have shown great promise (22), and the correlates of immunity conferred by these vaccines remains to be determined. A Salmonella elicitor could be useful for studies on correlates of immunity to salmonellae in humans. We stress that we have never seen these lethal hypersensitivity reactions in mice immunized orally. The detoxified, alkali-treated whole-cell soluble extracts described elicit higher in vitro T-cell responses than do the native preparations (23) and are effective in DTH tests, eliciting true T-cell-mediated responses without the hypersensitivity reactions seen with LPS-rich extracts in intravenously immunized mice (14). It remains to be seen whether this type of preparation could be useful for future investigations into cell-mediated responses to S. typhi in humans.

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REFERENCES