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# Induction of Endotoxin Tolerance with Nonpyrogenic O-Antigenic Oligosaccharide-Protein Conjugates

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We prepared a dodecasaccharide, specific for the O-antigenic polysaccharide chain of *Salmonella typhimurium* (O-antigens 4 and 12), by the partial hydrolysis of the O-polysaccharide chain, utilizing bacteriophage 28B *endo*-α-L-rhamnosidase. The dodecasaccharide was shown by chemical and spectroscopical analyses to be totally devoid of lipid A and core oligosaccharide. By coupling this dodecasaccharide to human serum albumin, a glycoconjugate (DODECA-4809-ITC-HSA) was prepared and found to be (i) nonpyrogenic, (ii) unable to gelate a *Limulus* amoebocyte lysate, and (iii) unable to induce early-phase pyrogenic tolerance to endotoxin. Rabbits immunized either intravenously (with the glycoconjugate suspended in saline) or intrapopliteally (with the glycoconjugate suspended in Freund complete adjuvant) developed a significant although modest pyrogenic tolerance against challenge with the O-antigenic homologous *S. typhimurium* lipopolysaccharide ($P < 0.025$ and $P < 0.01$ for immunized and control rabbits, respectively). The evoked tolerance was O-antigen specific since no pyrogenic tolerance against challenge with lipopolysaccharide from *S. thompson* (possessing identical lipid A and core oligosaccharide structures but differing in the O-antigen polysaccharide chain) could be seen ($P > 0.1$). These results demonstrate that a nonpyrogenic O-antigenic polysaccharide hapten, when coupled to an immunogenic carrier protein, evokes immune responses which mediate significant, although modest, late-phase tolerance and is capable of partly reducing the pyrogenic activity of the O-antigenic homologous lipopolysaccharide.

It has long been recognized that when bacterial endotoxins are injected into healthy humans or animals, tolerance to their toxic and pyrogenic activities can be acquired. The mechanisms underlying the acquisition of such tolerance have been intensively studied by numerous investigators (for a review, see reference 4 and C. A. Johnston and S. E. Greisman *in* L. B. Hinshaw and R. A. Proctor, ed., *Endotoxin: Pathophysiology of Endotoxin*, in press). Virtually all studies have been conducted with endotoxin preparations containing the toxic lipid A component of endotoxin, either in its native state or after various chemical detoxification procedures. The chemical degradation procedures have left investigators either with an only partially detoxified product or a product which, if completely detoxified, has in most instances, been rendered non-immunogenic. In studies claiming the immunogenicity of lipid-deficient preparations, either the analytical data in support of the purity, i.e., the absence of ester- and amide-linked fatty acids, have been lacking (1), or only part of the material could be considered polysaccharide (18). It has therefore been impossible to establish with certainty whether endotoxin tolerance with specificity for the O-antigenic polysaccharide can be acquired in the absence of the lipid A component.

The present studies of endotoxin tolerance are unique in that they employ a component of endotoxin entirely devoid of its lipid A moiety, i.e., a nonpyrogenic O-specific polysaccharide isolated from the endotoxin of *Salmonella typhimurium* by means of a bacteriophage glycanase and which was rendered immunogenic by covalent coupling to human serum albumin (HSA). The findings demonstrate that this nonpyrogenic polysaccharide-HSA conjugate is capable of inducing a significant, although modest, O-antigen-specific pyrogenic tolerance to the native parent endotoxin.

## MATERIALS AND METHODS

**Bacterial strains.** *S. typhimurium* SH4809 (O-antigens 4, 5, and 12) and *S.* sp. serotype *thompson* IS40

(O-antigens 6 and 7) were available from previous investigations (22, 23). *S. typhimurium* TV119, a rough mutant devoid of the O-antigenic side chain, was also available from previous investigations. *Escherichia coli* J5 was from the strain collection of S. Greisman.

**Preparation of LPSs, oligosaccharides, and oligosaccharide-protein conjugates.** Lipopolysaccharides (LPSs) were extracted by the hot phenol-water method from batch-grown cultures of formaldehyde-killed bacteria, and the LPSs were further purified as described previously (13).

Partially delipidated LPS was prepared by hydrolyzing phosphate bonds and fatty acid ester linkages in the lipid A moiety of the LPS by treatment with 0.15 M sodium hydroxide at 100°C for 2 h. After centrifugation, the pH was adjusted to 3.5, and the free fatty acids were removed by repeated chloroform extractions. After the pH was adjusted to 7.0, the partially delipidated LPS was extensively dialyzed against water and finally lyophilized. O-polysaccharide from *S. typhimurium* SH4809 was prepared by weak (1%) acetic acid hydrolysis at 100°C for 1 h of the corresponding partially delipidated LPS. This treatment selectively hydrolyzes the 2-keto-3-deoxy-D-*manno*-octulosonic acid glycoside bonds within the core oligosaccharide portion and thus yields the O-polysaccharide side chain with the adjacent core oligosaccharide. After being cooled to room temperature, the reaction mixture was extracted with chloroform, and the collected water phase was extensively dialyzed against distilled water and lyophilized. The final O-polysaccharide preparation contained less than 0.04 μg of lipid A and less than 0.01 μg of protein per mg of O-polysaccharide as estimated by analysis of the β-hydroxymyristic acid content (9) and by the method of Lowry et al. (14), respectively.

Dodecasaccharide from *S. typhimurium* SH4809 partially delipidated LPS was prepared as described previously (24). Briefly, the partially delipidated *S. typhimurium* SH4809 LPS was subjected to partial degradation by bacteriophage 28B-associated *endo*-α-L-rhamnosidase. The resulting mixture of oligosaccharides from the O-polysaccharide chain were separated and purified by gel chromatography and high-pressure liquid chromatography.

The purity and structure of each LPS, polysaccharide, and oligosaccharide preparation were checked by sugar analysis, methylation analysis, $^{1}H$ and $^{13}C$ nuclear magnetic resonance spectrometry.

Sugar analyses were performed by gas-liquid chromatography (GLC) on alditol acetates as described earlier (12). Xylose was used as an internal standard. For GLC analysis, we used a Perkin-Elmer model 990 instrument equipped with a glass column (200 by 0.3 cm) containing 3% OV-225 on Gas-Chrom Q (100/120 mesh). The peaks were measured with a Hewlett-Packard 3370-B electronic integrator.

The methylation analyses were done as previously described (12). After the saccharides were methylated, they were hydrolyzed, reduced, and acetylated, and the different permethylated sugars were identified as their alditol acetates by GLC and by GLC-direct mass spectrometry. The separation of the partially methylated alditol acetates by GLC was done with a Perkin-Elmer model 990 instrument with a glass column (200 by 0.3 cm) containing 3% OV-225 on Gas-Chrom Q (100/120 mesh). For GLC-direct mass spectrometry, a Perkin-Elmer 270 gas chromatograph mass spectrometer, fitted with an OV-225 column was used.

The 99.55-mHz proton and the 22.05-mHz $^{13}C$ nuclear magnetic resonance spectra (for solutions in $D_2O$) were recorded in the Fourier transform mode, using a JNM Fx-100 instrument. Chemical shifts were estimated by using external tetramethylsilane as the standard. The spectra were, in all instances, in accordance with the postulated structures.

The covalent attachment of the *S. typhimurium* SH4809 dodecasaccharide (Fig. 1) to HSA and *Polyphemues* hemocyanin was done as previously described (23, 26). The HSA was a kind gift from Kabi, Stockholm, Sweden, and *p*-aminophenethylamine and sodium-cyanoborohydride were purchased from EGA Chemicals, Steinheim/Albuch, West Germany. The glycoconjugates are referred to as DODECA-4809-ITC-HSA and DODECA-4809-ITC-PHC. Their degrees of substitution were 20 and 22 mol, respectively, of dodecasaccharide per mol of the carrier protein.

**Miscellaneous methods.** Protein contents were determined by the method of Lowry et al. (14), and the total amount of hexoses was determined by the phenol-sulphuric acid method (3). The β-hydroxymyristic acid contents were measured by GLC as described previously (9).

**ELISA.** The enzyme-linked immunosorbent assay (ELISA) was performed as described earlier (2) and adapted to microtiter plates (Microelisa System, plate M129B; Dynatech Laboratories, Inc., Alexandria, Va.). Alkaline phosphatase (calf intestinal mucosa type VII; Sigma Chemical Co., St. Louis, Mo.) conjugated to sheep anti-rabbit immunoglobulin (estimates immunoglobulin A [IgA], IgG, and IgM). NBL preparation A51+52 was utilized, and the absorbance was measured at 405 nm in a Titertek Multiscan Plate Reader (made for Flow Laboratories by Eflab. Oy., Helsinki, Finland).

**Immunizations with glycoconjugates.** The animals were healthy male New Zealand albino rabbits weighing 2.2 ± 0.3 kg at the onset of immunization. The sera for antibody titrations were obtained by bleeding from the central ear artery immediately before starting immunization and 24 h before the tolerance testing.

Group I rabbits were immunized with the DODECA-4809-ITC-HSA conjugate dissolved in 1 ml of pyrogen-free sterile 0.9% saline and given intravenously according to the following schedule: 50 μg on day 0 and 25 μg on days 6, 9, 15, 18, 20, 22, 25, 27; half of group I received an additional 50 μg on days 34, 44, 53, 55, 57, and 60. The control animals were given intravenous injections of 1 ml of pyrogen-free sterile 0.9% saline. Testings for pyrogenic tolerance were performed 4 to 9 days after the last injection. Groups II and III rabbits were immunized with the DODECA-4809-ITC-HSA conjugate that had been dissolved in pyrogen-free sterile saline and then emulsified in Freund complete adjuvant (FCA; GIBCO Laboratories, Grand Island, N.Y.) in a 1:1 ratio. A 0.1-ml amount of this emulsion containing 20 μg of the DODECA-ITC-HSA conjugate was injected into the shaven popliteal space on days 0, 7, 24, 34, and 46. On day 46, an additional 20 μg of DODECA-ITC-HSA in 0.4 ml of pyrogen-free sterile saline was administered intraperitoneally. The control animals received comparable intraperitoneal injections of pyrogen-free sterile saline emulsified in FCA, as well as 0.4 ml of the

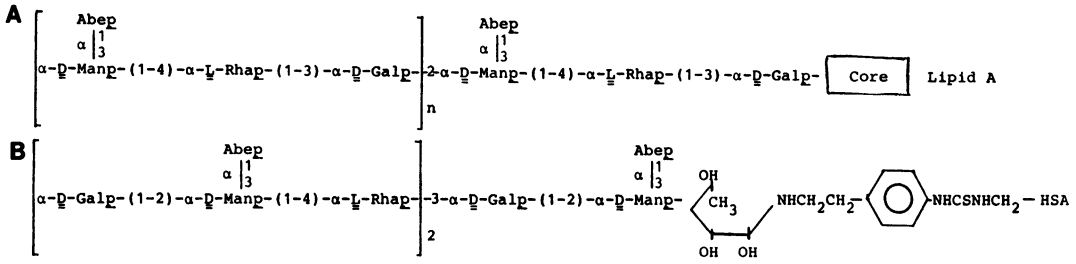


FIG. 1. Structure of *S. typhimurium* LPS (A) and *S. typhimurium* DODECA-4809-ITC-HSA conjugate (B) used in study of late-phase tolerance.

saline on day 46. Testings for pyrogenic tolerance were performed 8 to 10 days after completion of the immunizations.

**Pyrogen assay.** Before testing for endotoxin tolerance in the rabbits immunized with DODECA-4809-ITC-HSA, we established endotoxin dose-response relationships in nonimmune rabbits by titrating the pyrogenic activity of *S. typhimurium* SH4809 LPS and *S.* sp. serotype *thompson* LPS. Healthy 2.5- to 3.0-kg New Zealand albino rabbits of mixed sex, never previously injected with any materials, were loosely restrained by chain collars in individual stalls in an air-conditioned room at 70°F (ca. 21°C) and acclimatized for 5 to 7 h/day for several days. On the evening before the study, thermistor probes were inserted 6 in (ca. 15 cm) into the rectum and connected to a recording telethermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio). The next morning, temperatures were monitored every 30 min for 3 h before the injection of LPS. Animals with temperatures greater than 104°F (ca. 40°C) or varying more than 0.3°F (ca. 0.1°C) during any 30-min control period were excluded. Solutions of the *S. typhimurium* and *S.* sp. serotype *thompson* LPS in pyrogen-free sterile 0.9% saline were warmed to 37°C and injected intravenously in 1.5-ml volumes. The quantities of LPS injected were 0.005, 0.05, 0.5, and 1.0 μg/kg, with groups of four rabbits for each dose. After LPS injection, temperatures were monitored every 30 min for 6 h. Fever indexes were calculated from the area under the 6-h fever curve; a 1°F (ca. 0.4°C) increment in rectal temperature sustained for 1 h = 4 $cm^2$. The results are given in Fig. 2. For the assessment of endotoxin tolerance after immunization with the DODECA-4809-ITC-HSA, we conducted pyrogen assays in the same manner as described above, utilizing test quantities of LPS that evoked fever near the upper portion of the sensitive dose-response range, i.e., 0.5 μg of *S. typhimurium* 4809 LPS per kg and 1.0 μg of *S.* sp. serotype *thompson* IS40 LPS per kg. The total test doses of endotoxin given to immunized and control animals in each of the experimental groups were comparable since weights at the time of tolerance testing were comparable.

**Limulus amoebocyte lysate assay.** In addition to the pyrogen assay, the endotoxin content of the reagents employed was assessed by the *Limulus* amoebocyte lysate gelation test. Lysate was purchased from M. A. Bioproducts, Walkersville, Md.

## RESULTS

**Purity of artificial glycoconjugate.** It was deemed essential to demonstrate that both the dodecasaccharide, obtained by phage *endo*-α-L-rhamnosidase hydrolysis of the O-polysaccharide chain of partially delipidated LPS from *S. typhimurium* SH4809, and the HSA preparations, as well as the resulting DODECA-4809-ITC-HSA glycoconjugate, were free of contaminating protein and endotoxin. The following analyses of the dodecasaccharide were done. (i) The partially delipidated LPS was prepared by treatment with 0.15 M sodium hydroxide at 100°C for 2 h, and the O-polysaccharide was prepared by boiling at 100°C for 1 h in 1% acetic acid. The O-polysaccharide preparations contained <0.04 μg of lipid A and <0.01 μg of protein per mg of O-polysaccharide as estimated by analysis of the content of β-hydroxymyristic acid and by the method of Lowry (14), respectively. (ii) The dodecasaccharide was prepared from the O-polysaccharide by enzymatic hydrolysis. The dialysis procedure employed in this hydrolysis excluded molecules with a molecular weight of >8,000. In the gel and high-pressure liquid chromatographic purification steps, only the dodecasaccharide with a molecular weight of 1,800 could be detected. Furthermore, in the $^{1}H$ and $^{13}C$ nuclear magnetic resonance spectra (data not shown), all signals obtained were assignable to the expected dodecasaccharide.

The HSA preparation used as the carrier protein was negative in the *Limulus* amoebocyte lysate gelation test at a concentration of 1,600 μg/ml. The DODECA-4809-ITC-HSA conjugate, purified by gel chromatography, failed to gelate the *Limulus* lysate at the highest concen-

tration tested (200 μg/ml). In contrast, the native *S. typhimurium* 4809 LPS produced a strong gelation at a concentration of 0.001 μg/ml and weak but definite gelations at 0.0001 μg/ml. Moreover, when injected intravenously in a concentration of 25 μg/kg in acclimatized healthy rabbits, no febrile responses were elicited by the DODECA-4809-ITC-HSA conjugate (Fig. 2). In contrast, unequivocal febrile responses were evoked by the injection of 5,000-fold-smaller concentrations (0.005 μg/kg) of the native *S. typhimurium* SH4809 LPS (Fig. 2).

The above findings made us conclude that the DODECA-4809-ITC-HSA glycoconjugate was devoid of any contaminating substances with pyrogenic and endotoxic activities. Further support for this conclusion was obtained by demonstrating that rabbits which failed to develop fever upon injection with the DODECA-4809-ITC-HSA glycoconjugate were capable of normal fever responses when given parent LPS intravenously 4 h later (data not shown). These latter findings also demonstrate the inability of the DODECA-4809-ITC-HSA conjugate to evoke early-phase pyrogenic tolerance.

**Endotoxin tolerance in rabbits.** The rabbits immunized intravenously with the DODECA-4809-ITC-HSA conjugate responded with quite low antibody titers against the *S. typhimurium* SH4809 LPS and the dodecasaccharide hapten. However, all rabbits exhibited high titers against the HSA carrier protein (Table 1). When the DODECA 4809-ITC-HSA conjugate was suspended in FCA and administered to the area of the popliteal lymph nodes, the titers against the *S. typhimurium* SH4809 LPS, as well as the HSA, were 50- to 100-fold higher (Table 1). The sera were also assayed for antibodies against the core structure of both *S. typhimurium* (strain TV119) and *E. coli* (strain J5) LPS. The titers were uniformly low, and in no instance did they differ in sera from the same rabbit whether collected before or after immunization (allowing for ±20% variation in interassay testing) with the glycoconjugates.

When tested for tolerance to the parent *S. typhimurium* SH4809 LPS, the intravenously immunized rabbits (group I) showed a moderate but definite tolerance (Fig. 3). The mean 6-h fever index (square centimeters) plus or minus 1 standard error was 49.0 ± 6.0 as compared with 78.6 ± 7.4 in the control group ($P < 0.025$). When the rabbits had been immunized with the DODECA-4809-ITC-HSA glycoconjugate suspended in FCA (group II), the evoked pyrogenic tolerance was even more striking. The mean 6-h fever index was 39.2 ± 5.2 versus 72.9 ± 8.3 in the control group ($P < 0.01$). For both groups I and II, no individual correlation was seen between humoral antibody titers as estimated by

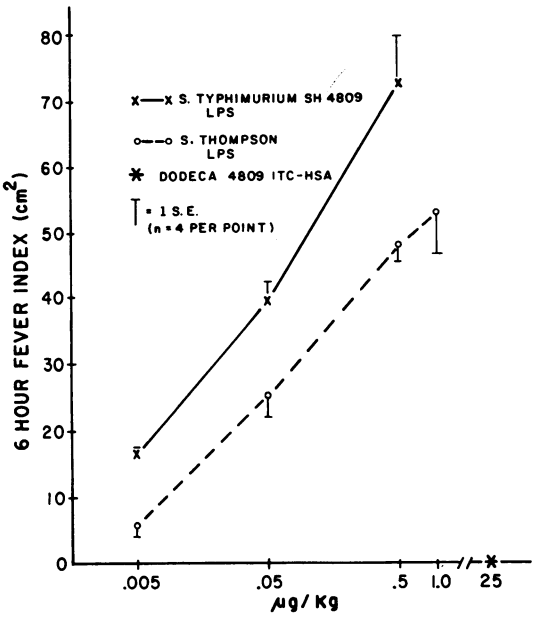


FIG. 2. Six-hour fever indexes in nonimmunized rabbits. For experimental details, see the text.

ELISA and the level of evoked pyrogenic tolerance (data not shown).

That the elicited tolerance was specific for the O-antigens was tested for by injecting the O-antigenic heterologous *S.* sp. serotype *thompson* LPS into an additional group of rabbits immunized with the DODECA-4809-ITC-HSA conjugate (group III). The *S.* sp. serotype *thompson* LPS has a lipid A and core region identical to that of *S. typhimurium*, but the structure of the O-antigenic polysaccharide chain is different. When injected with the *S.* sp. serotype *thompson* LPS, no pyrogenic tolerance was seen: the 6-h fever index was 62.8 ± 7.0 as opposed to 49.2 ± 3.5 in the control group ($P > 0.1$).

## DISCUSSION

When endotoxins are administered to experimental animals or humans, resistance is acquired to their pyrogenic and toxic activities. Such resistance is termed tolerance. (This must be distinguished from immunological tolerance, which refers to the inability of antigen-specific B lymphocytes to respond to endotoxins by the synthesis and secretion of antibodies directed against the endotoxin molecule). It is now known that resistance to the fever-producing activity of endotoxins, i.e., pyrogenic tolerance, develops in two temporally distinct phases. An early phase appears within hours and then wanes within days if the endotoxin injections are not continued. This phase is directly proportional to the initial pyrogenic response, is not associ-

TABLE 1. ELISA titrations of rabbit antisera after immunization with *S. typhimurium* O-antigen-specific polysaccharide-HSA conjugates

| Vaccine | No. of rabbits | ELISA titer[a] of rabbit artisera for antibodies to: | | | | | |
|---|---|---|---|---|---|---|---|
| | | *S. typhimurium* SH4809 LPS | *S. thompson* IS40 LPS | *S. typhimurium* DODECA-ITC-PHC[b] | *S. typhimurium* TV119 LPS | *E. coli* J5 LPS | HSA |
| None | 18 | <1 | <1 | <1 | 11 ± 10 | 2 ± 2 | <1 |
| DODECA-4809-ITC-HSA intravenously (group I) | 6 | 29 ± 13 | <1 | 30 ± 9 | 14 ± 4 | 6 ± 6 | 348 ± 126 |
| DODECA-4809-ITC-HSA + FCA intrapopliteally (group II) | 5 | 348 ± 58 | <1 | 576 ± 88 | 10 ± 12 | <1 ± 2 | 11,840 ± 8300 |
| DODECA-4809-ITC-HSA + FCA intrapopliteally (group III) | 4 | 148 ± 16 | <1 | 245 ± 37 | 8 ± 4 | 3 ± 2 | 31,650 ± 15,360 |

[a] The titers are given as endpoint titers, i.e., the highest serum dilution giving an absorbance of 0.1 at 400 nm/60 mins. The coating antigen concentration was 10 μg/ml, and the dilution of the sheep anti-rabbit immunoglobulin-alkaline phosphatase conjugate was $5 \times 10^2$. The mean values plus or minus the standard deviation are given.

[b] PHC, *Polyphemues* hemocyanin.

ated with increments in antiendotoxin antibodies, is not transferable with serum, and possesses no interendotoxin specificity. It appears to represent an acquired refractoriness of those target cells that are triggered by endotoxin to synthesize and release endogenous pyrogens, i.e., the macrophages, particularly the hepatic Kupffer cells (4, 5, 8; Johnston and Greisman, in press). Based upon all of the known characteristics of the early phase of pyrogenic tolerance, this phase appears to be mediated by the toxophore component of endotoxin, i.e., lipid A (4; Johnston and Greisman, in press).

The later phase of pyrogenic tolerance to endotoxin develops several days after the initial injection of the endotoxin. It is not proportional to the intensity of the initial pyrogenic response, persists for weeks to months, can be passively transferred with serum or sensitized spleen cells, is dependent upon the immunogenicity of the endotoxin, and can be related, in part at least, to antiendotoxin antibodies of both the IgM and IgG classes (4, 6, 16; Johnston and Greisman, in press). Moreover, both the titer of antiendotoxin antibodies and the effectiveness of the passive transfer of tolerance can be enhanced by repetitive injections of the endotoxin into the donor animals. Thus, in contrast to the rapidly developing early phase of tolerance, the later phase is based, in part at least, upon classic immunization with the endotoxin molecule. However, the precise antigenic component(s) of the endotoxin molecule which elicit(s) the later phase of pyrogenic tolerance has been a matter of controversy (16, 17, 19; Hinshaw and Proctor, in press). This is so because an immune response can be elicited against three distinct portions of the lipopolysaccharide molecule: the O-antigen polysaccharide chain, the core oligosaccharide, and the lipid A. Lipid A is considered by most investigators to be required for the immunogenicity of endotoxin (15), and virtually all studies of endotoxin tolerance have employed products containing lipid A either in its native state or after various chemical modification procedures (1, 4, 17, 18; Johnston and Greisman, in press). Therefore, it has been impossible to study whether immunity directed solely against the polysaccharide (either the O-polysaccharide chain or the core) can mediate tolerance.

The present studies demonstrate unequivocally that the O-polysaccharide antigens alone are sufficient for the induction of at least part of the late phase of pyrogenic tolerance. This could be shown by employing a DODECA polysaccharide isolated and purified from the O-antigenic polysaccharide of *S. typhimurium* 4809 and rendered immunogenic by covalent attachment to HSA. The important progress made in this study is the use of such a dodecasaccharide, obtained through the phage glycanase hydrolysis of the O-polysaccharide chain. The dodecasaccharide has been shown to contain the antigenic determinants of the native O-antigenic polysaccharide chain (10). Such a saccharide, free of core polysaccharide (and lipid A residues), is otherwise impossible to obtain by chemical degradation of the LPS. The saccharide as such is incapable of eliciting any measurable immune

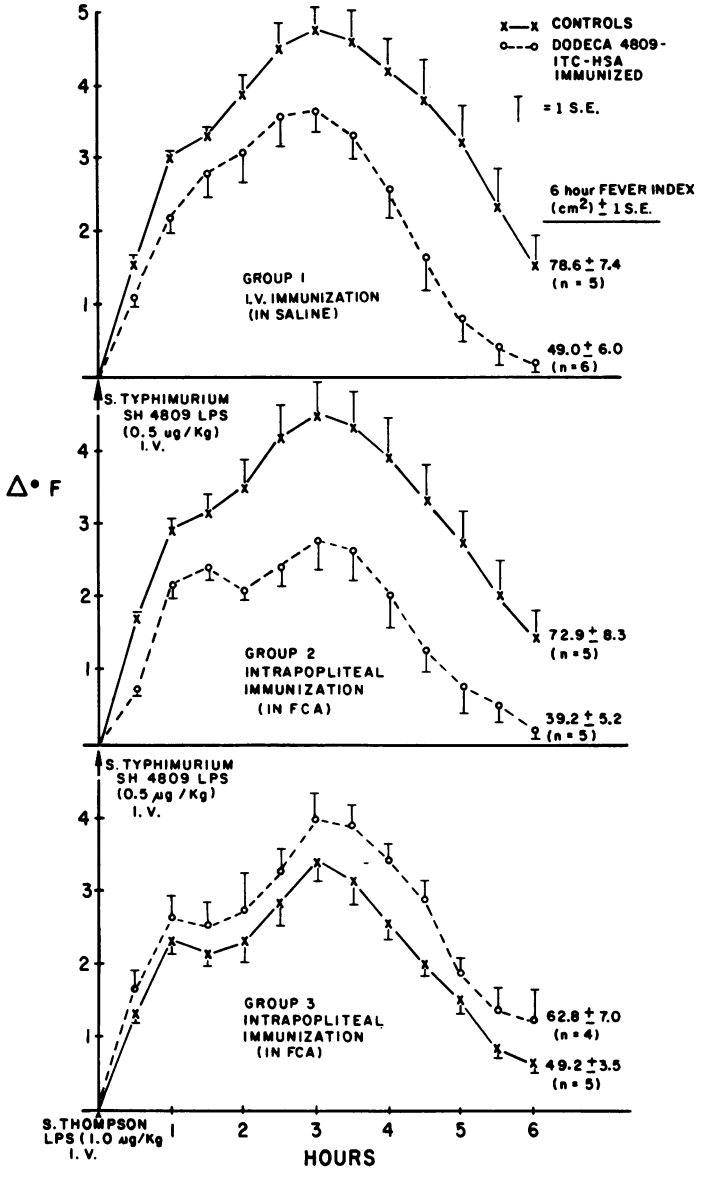


FIG. 3. Pyrogenic responses in immunized rabbits. For experimental details, see the text. I.V., Intravenous.

response in rabbits, mice, and calves (unpublished data). The glycoconjugate (DODECA-ITC-HSA) was shown to lack both lipid A and core oligosaccharide as determined by chemical, physical, and biological analyses. Rabbits immunized with the DODECA-ITC-HSA glycoconjugate developed significant tolerance to the parent native endotoxin ($P < 0.025$ and $P < 0.01$ after immunizations with the conjugate in saline and in CFA, respectively). This tolerance could not be attributed to endotoxin contamination. The DODECA-ITC-HSA was pyrogen free (amounts 5,000-fold greater than the pyrogenic quantities of the parent endotoxin elicited no fever), gave no gelation of a *Limulus* amoebocyte lysate in concentrations as high as 200 µg/ml (whereas gelation was produced by 0.0001 µg of the parent endotoxin per ml), and failed to elicit early-phase pyrogenic tolerance. Moreover, the tolerance was specific since the DODECA-4809-ITC-HSA was not capable of evoking pyrogenic tolerance to *S.* sp. serotype *thompson* endotoxin, which possessed the same lipid A and core oligosaccharides as the *S. typhimurium* endotoxin but differs in the structure of the O-polysaccharide chain. The low and, upon immunization with the glycoconjugate, unchanged titers against two core LPS

antigens such as those of *S. typhimurium* TV119 and *E. coli* J5 (Table 1) also speak against the involvement of immunity directed against the basal parts of the endotoxin molecule in this instance. The exquisite O-antigenic specificity suggests that anti-O-antigen immune mechanisms are involved.

The fact that cell-mediated immunity specific for the O-antigenic polysaccharide chain of *S. typhimurium* recently has been demonstrated in an experimental model (20, 21) raises the possibility that cell-mediated, as well as humoral, immune mechanisms can be involved in the development of pyrogenic tolerance to endotoxins. The possibility of humoral, as well as cell-mediated, immune reactions being important, combined with the variations in the intrinsic responsiveness of individual rabbits to endotoxin, may explain why we did not observe individual correlations between antibody titers and the degree of acquired late-phase tolerance. Differences in the levels of the subclasses of anti-O-immunoglobulins may also have been a contributing factor. In any event, the present findings indicate that a portion of the O-polysaccharide component of endotoxin, devoid of lipid A and core oligosaccharide, is capable of eliciting a specific although modest tolerance against endotoxin-induced pyrogenicity. Such findings are the best evidence to date that immune mechanisms directed solely against a nontoxic portion of the endotoxin molecule are capable of reducing the pyrogenic activity of the toxophore (i.e., lipid A) component. Moreover, this tolerance, obtained with the nonpyrogenic DODECA-4809-ITC-HSA glycoconjugate, was entirely comparable to that previously observed in our laboratory when native *S. typhimurium* LPS was used as the immunogen to elicit similar increments in LPS antibodies, and testing was delayed for similar intervals after immunization. These observations directly support the previously postulated importance of O-specific polysaccharide antigens in the late phase of tolerance to wild-type bacterial endotoxins (4; Johnston and Greisman, in press). The degree of late-phase tolerance, although statistically significant, was by no means complete. Repeated or even single injections of native LPS rapidly induce a much more pronounced fever tolerance through the mechanisms of early-phase tolerance. These mechanisms are activated by LPS components other than the O-polysaccharides. The consideration of the nature of these mechanisms is outside the scope of the present study and constitutes the subject of two recent exhaustive reviews (4; Johnston and Greisman, in press).

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