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Artificial *Salmonella* Vaccines: *Salmonella typhimurium* O-Antigen-Specific Oligosaccharide-Protein Conjugates Elicit Protective Antibodies in Rabbits and Mice

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Several saccharides representative of the O-antigenic polysaccharide chain of *Salmonella typhimurium* (O antigens 4 and 12) were used as haptenic groups covalently linked to bovine serum albumin. The disaccharide abequose1 → 3 D-mannose was synthesized, and the

abequose
$\alpha \downarrow 1$
3
(3-D-galactose $1 \underset{\alpha}{\rightarrow} 2$ D-mannose $1 \underset{\alpha}{\rightarrow} 4$ L-rhamnose $1 \underset{\alpha}{\rightarrow})_{n = 1, 2, \text{ or } 3}$

tetra-, octa- and dodecasaccharides were isolated after cleavage of isolated *S. typhimurium* O-polysaccharide chains by using bacteriophage *endo*-glycosidases. Rabbits immunized with the saccharide-protein conjugates suspended in Freund complete adjuvant readily responded with O4 antibody titers as high, or almost as high, as those elicited by heat-killed bacteria. The octa- and dodecasaccharide conjugates also gave rise to O12 antibody titers. The antibody response in mice differed in two ways from that seen in rabbits: mice did not respond with measurable antibody production against the disaccharide haptens, and the highest *S. typhimurium* lipopolysaccharide antibody response elicited by the saccharide haptens was still approximately 50-fold lower than that elicited by heat-killed bacteria. The latter difference may be a consequence of the fact that the mouse preferentially produces antibodies against the galactose residue which is terminal in the hapten but not in the native O-antigenic polysaccharide chain. Antibodies elicited in rabbits against all saccharide-protein conjugates protected passively transferred mice against intraperitoneal challenge with 100 times the 50% lethal dose of *S. typhimurium* SH 2201 (O4, 12) but not against challenge with *S. enteritidis* SH 2204 (O9, 12). The antibodies elicited by the saccharide-protein conjugates protected as well as antibodies elicited by heat-killed bacteria.

It is generally accepted that two types of immune response are involved in defense against salmonellosis, cellular and humoral. Since in attempts to control salmonellosis by vaccination living vaccines have been superior to nonviable ones, cellular immunity has been considered to be of greater importance for resistance (4). It has been firmly established in mice, however, that killed bacteria or fractions thereof can give substantial levels of protection (1, 9, 12, 17). The specificity of these vaccines has been attributed to the O antigen of the vaccine strain (6). Such observations suggest an important role for humoral immunity in resistance. Support for this hypothesis was recently obtained when immune-defect mice were used in an experimental system (16).

The O-antigen determinants of *Salmonella* are located in the polysaccharide chain of the lipopolysaccharide (LPS) of the cell envelope. The noxious endotoxic activity resides in the lipid A part of the LPS molecule. A vaccine with O-antigenic specificity but without toxic effects should therefore contain the O polysaccharide in an immunogenic form but be devoid of lipid A. We have recently prepared such nontoxic vaccines by coupling O-antigen-specific oligosaccharides to carrier protein (21-24).

In this communication we investigated the influence of the size of the hapten on the humoral antibody response in rabbits and mice. Furthermore, the influence on the immune response of the degree of substitution, i.e., the number of hapten molecules per carrier molecule, was investigated. The protection afforded by antisera elicited in rabbits was assayed by

passive transfer into NMRI mice which were subsequently challenged with a virulent *Salmonella typhimurium* strain.

MATERIALS AND METHODS

Bacterial strains. *S. typhimurium* strains SH 4809 and SH 2201 (O4, 5, 12) and *S. enteritidis* strains SH 1262 and SH 2204 (O9, 12) have been described earlier (24). The *S. thompson* IS 40 (O6, 7) and the *S. essen* (O4, 12) strains were from the laboratory strain collection.

Preparation of LPS. LPS was prepared and checked for purity as described previously (13, 14, 19).

Preparation of *S. typhimurium* O-antigen-specific saccharide-protein conjugates. Tetra-, octa-, and dodecasaccharides (for structures, see Fig. 1) from the O-polysaccharide chain of *S. typhimurium* SH 4809 were prepared by subjecting the partially delipidated LPS (0.15 M sodium hydroxide, 100°C, 2 h) to phage 36 *endo*-rhamnosidase treatment (8, 23). Oligosaccharides obtained by preparative gel chromatography on Bio-Gel P2 and P4 columns were analyzed by thin-layer and high-performance liquid chromatography, and their structures were confirmed by proton nuclear magnetic resonance spectrometry. The spectra were recorded for solutions in D_2O at 80°C with tetramethylsilane as an external standard, using a JEOL Fx-100 instrument operated in the Pulse-Fourier transform mode. Signals at expected ratios were obtained, inter alia, at δ4.9 to 5.4 (α-anomeric protons), δ2.0 to 2.2 (*H*-3 of abequosyl groups), and δ1.2 to 1.5 (*H*-6 of abequosyl groups and L-rhamnosyl residues).

The covalent attachment of the tetra-, octa-, and dodecasaccharides to bovine serum albumin (BSA; Pentex, Miles Laboratories, Inc. Kankakee, Ill.) and *Limulus polyphemus* hemocyanine (PHC; Sigma Chemical Co., St. Louis, Mo.) was done as previously described (22). The degree of substitution was determined for each of the conjugates by measurement of sugar and protein contents by the phenol-sulfuric acid (5) and Lowry (15) methods, respectively. The synthesized disaccharide 3-*O*-(α-abequopyranosyl)-α-D-mannopyranoside conjugated to BSA (AM-BSA) (degree of substitution, ~21) was available from previous works (7, 10).

Administration of antigen to rabbits. The various saccharide-protein conjugates were suspended in Freund complete adjuvant (FCA), 1:1 (vol/vol), and injected (5 to 10 μg/dose) into the popliteal lymph nodes of New Zealand white rabbits. Antiserum against *S. essen* was obtained by intravenous inoculations with heat-killed bacteria (10^{10} cells/dose) twice a week for a total of 8 weeks followed by a booster injection 2 weeks later. The rabbits were all bled 1 week after the last injection, and the sera were stored at −20°C until assayed.

Administration of antigen to mice. Mice were given the saccharide-protein conjugate (10 μg/dose) suspended 1:1 (vol/vol) in FCA intraperitoneally. Bleedings were done by puncture of the retroorbital venous complex. The mice used were either female, specific-pathogen-free outbred NMRI mice (Anticimex, Stockholm, Sweden) weighing 18 to 20 g (6 to 8 weeks old) or female inbred BALB/c mice of the same age obtained from Bomholtgard, Ltd., Bomholtgaard, Denmark.

Determination of LD_{50}. The 50% lethal dose (LD_{50}) values were calculated as described elsewhere (18, 22a).

Passive protection experiments in mice. NMRI mice were intravenously given 0.2 ml of heat-inactivated rabbit hyperimmune serum 1 to 2 h before intraperitoneal challenge. The challenge bacteria (*S. typhimurium* SH 2201 or *S. enteritidis* SH 2204) were taken from logarithmically growing cultures and injected at doses indicated in each experiment. The mice were observed for at least 30 days.

Immunochemical methods. The enzyme-linked immunosorbent assay (ELISA) was performed as described earlier (2, 21, 24). The coating dose for all antigens was 1.0 μg/ml. The ELISA titers are, unless otherwise indicated, expressed as endpoint titers, i.e., the reciprocal of the serum dilution yielding a change of 0.1 in optical density at 400 nm in 100 min.

RESULTS

Humoral antibody response in rabbits. Sera from rabbits immunized with the various *S. typhimurium* oligosaccharide (Os)-BSA conjugates were assayed, and the titer against the carrier protein (BSA) in each instance was assigned a value of 1.0 (Table 1). The tetrasaccharide-isothiocyanatophenyl-BSA (tetra-ITC-

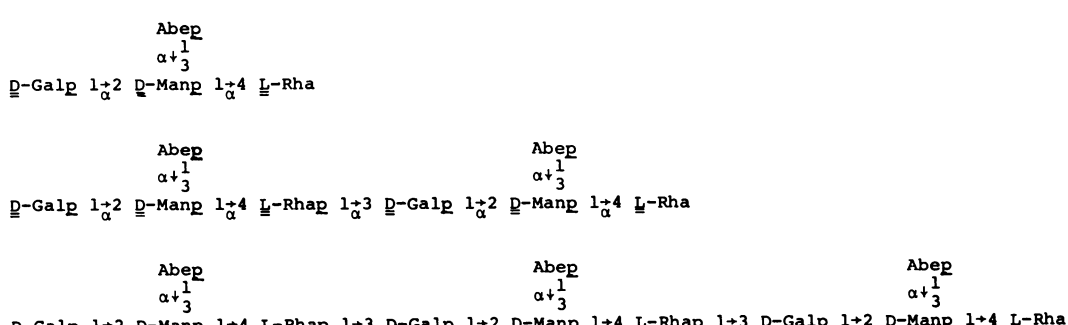

FIG. 1. *Structures of S. typhimurium O-antigen-specific oligosaccharides. Abe, 3,6-dideoxy-D-xylo-hexose; D-Manp, D-mannopyranoside; L-Rhap, L-rhamnopyranoside; D-Galp, D-galactopyranoside.*

TABLE 1. *S. typhimurium oligosaccharide hapten size and antibody response in rabbits*[a]

Immunogen	Relative ELISA titer against[b]:				
	S. typhimurium tetra-ITC-PHC	*S. typhimurium* octa-ITC-PHC	*S. typhimurium* dodeca-ITC-PHC	*S. typhimurium* LPS	BSA
Tetra-ITC-BSA	3.0	2.0	ND[c]	0.03	1.0
Octa-ITC-BSA	1.4	5.3	4.5	0.7	1.0
Dodeca-ITC-BSA	4.4	18.6	43.8	6.9	1.0

[a] New Zealand white rabbits (two per immunogen) were repeatedly injected with the indicated oligosaccharide-protein conjugates (5 to 10 μg/dose) suspended 1:1 in FCA.

[b] Antibody titers were estimated by ELISA. Figures (mean values) shown are relative titers; i.e., the anti-BSA titer for each of the immunogens was arbitrarily assigned a value of 1.0. In each instance, the coating antigen concentration was 1.0 μg/ml, and the dilution of sheep anti-rabbit immunoglobulin alkaline phosphatase conjugate was 10^{-3}.

[c] Not done.

BSA) immunogen elicited an antibody response which was highest against the tetra-ITC-PHC antigen and lowest against the *S. typhimurium* SH 4809 LPS antigen. The octa-ITC-BSA conjugate was about 20-fold and the dodeca-ITC-BSA conjugate was about 200-fold more efficient than the tetra-ITC-BSA conjugate in eliciting an antibody response detectable with the *S. typhimurium* SH 4809 LPS antigen.

The immunogenicity of the Os-BSA conjugates and the specificity of the elicited antibody response were compared with those obtained against the synthesized AM-ITC-BSA and whole heat-killed *S. essen* (O4 and O12) bacteria (Table 2). Heat-killed *S. essen* (O4, 12) appeared to be a slightly better immunogen than the saccharide-protein conjugates. However, Os-BSA conjugates also occasionally give rise to titers against the *S. typhimurium* SH 4809 LPS antigen as high as those produced by heat-killed bacteria (S. B. Svenson and A. A. Lindberg, unpublished data). The highest titers were, as expected, seen against the *S. typhimurium* SH 4809 LPS antigen. Titer determinations against the *S. enteritidis* SH 1262 LPS antigen, which has the O12 determinant in common with *S. typhimurium*, showed that the anti-O12 titer increased as a consequence of increasing size of the oligosaccharide hapten. It is interesting to note the low O12 titer elicited by immunization with heat-killed *S. essen* (O4, 12). Titers against the *S. thompson* IS 40 LPS antigen were always low and did not increase during the immunization procedure.

Protection of mice with passively transferred rabbit antisera. The protective capacity of the various antisera was evaluated by intravenous administration to mice, before intraperitoneal challenge, of virulent *S. typhimurium* SH 2201 (O4, 5, 12) and *S. enteritidis* SH 2204 (O9, 12). All mice given 0.2 ml of undiluted hyperimmune serum against the AM-ITC-BSA, octa-ITC-BSA, and *S. essen* heat-killed bacteria were fully protected over the 30-day observation period against both 10 and 100 times the LD_{50} of *S. typhimurium* SH 2201 (O4, 5, 12) (Table 3). No protection was seen in mice given hyperimmune serum against BSA only or phosphate-buffered saline. Rabbit normal serum, collected before immunization, was likewise nonprotective, although the mean survival time increased from 3 to 5.5 days as compared with mice given phosphate-buffered saline only.

Mice challenged with *S. enteritidis* SH 2204 (O9, 12) were not protected when given rabbit anti-AM-ITC-BSA, normal rabbit serum, or anti-BSA serum. Half of the mice which had been given anti-octa-ITC-BSA serum and subsequently challenged with 10 times the LD_{50} were protected, and a slight increase in the mean survival time of the succumbing mice was seen. Whether this was a consequence of the relatively high anti-O12 titer in the octa-ITC-BSA serum remains unclear. Mice given the *S. essen* (O4, 12) antiserum were fully protected when challenged with 10 times the LD_{50}, and the mean survival time was increased, from 4.5 (BSA control) to 12 days, when the challenge dose was 100 times the LD_{50}. The anti-O12 antibody titer in the anti-*S. essen* antiserum was lower than in the anti-octa-ITC-BSA antiserum (Table 2), which suggested that protective antibodies directed against surface structures other than the O antigen had been elicited by heat-killed *S. essen*.

A comparison of the protective capacity of the antisera was obtained by giving mice serial dilutions of the various rabbit antisera before challenge with 25 times the LD_{50} of *S. typhimurium* SH 2201 (O4, 5, 12). When the percentage of surviving mice was plotted against the *S. typhimurium* SH 4809 LPS titer, all curves clustered: 50% protection was seen at titers ranging from 3.0×10^5 to 1.0×10^6 (Fig. 2). Thus, there appeared to be a close parallel between the protective efficacy of the given anti-O-antigen-

TABLE 2. *S. typhimurium oligosaccharide hapten size and specificities of antibody response in rabbits*[a]

Immunogen	ELISA endpoint titer ($\times 10^3$) against[b]:			
	S. typhimurium SH 4809 (O4, 5, 12) LPS	*S. enteritidis* (O9, 12) LPS	*S. thompson* (O6, 7) LPS	O9, 12/O4, 12 ratio
3-*O*-(α-abequopyranosyl)-α-D-mannopyranoside-ITC-BSA	8,250 (5,700–10,800)	10 (8–12)	20 (18–22)	<0.005
Tetra-ITC-BSA	900 (795–1,000)	65 (30–100)	3 (3–3)	0.07
Octa-ITC-BSA	3,320 (2,660–3,980)	735 (250–1,220)	30 (15–45)	0.22
Dodeca-ITC-BSA	1,430 (1,410–1,450)	675 (230–1,120)	20 (10–30)	0.47
Heat-killed *S. essen* (O4, 12)	10,000 (7,300–12,700)	80 (70–90)	40 (15–65)	0.008

[a] New Zealand white rabbits (two per immunogen) were repeatedly injected with the indicated saccharide-protein conjugates (10 μg/dose) or heat-killed *S. essen* (10^8 cells/dose). The immunogens were in all instances given suspended 1:1 in FCA.

[b] The antibody titers were determined by ELISA. Figures are mean values; the range is indicated within parentheses. The coating antigen concentration was 1.0 μg/ml, and the dilution of sheep anti-rabbit immunoglobulin alkaline phosphatase conjugate was 10^{-3}.

TABLE 3. *Protection of NMRI mice by passively transferred rabbit antibodies*[a]

Rabbit antiserum raised against:	No. of survivors/no. of dead mice with given challenge strain			
	S. typhimurium SH2201, 10 × LD_{50}	*S. typhimurium* SH2201, 100 × LD_{50}	*S. enteritidis* SH2204, 10 × LD_{50}	*S. enteritidis* SH2204, 100 × LD_{50}
3-*O*-(α-abequopyranosyl)-α-D-mannopyranoside-ITC-BSA	6/0 (>30)[b]	6/0 (>30)	0/6 (6)	0/6 (4)
Octa-ITC-BSA	6/0 (>30)	6/0 (>30)	3/3 (8)	0/6 (3)
Heat-killed *S. essen* (O4, 12)	6/0 (>30)	6/0 (>30)	6/0 (>30)	0/6 (12)
BSA	0/6 (5.8)	0/6 (4)	0/6 (6.5)	0/6 (4.5)
Rabbit normal serum	0/6 (5.5)	0/6 (4)	1/5 (6.3)	0/6 (2.5)

[a] NMRI mice (groups of six) were given intravenous injections (0.2 ml) of indicated undiluted rabbit antisera 1 to 2 h before intraperitoneal challenge with *S. typhimurium* SH 2201 (O4, 5, 12) or *S. enteritidis* SH 2204 (O9, 12). The LD_{50} values for *S. typhimurium* SH 2201 and *S. enteritidis* SH 2204 were 4.4×10^4 and 5.1×10^5, respectively. After challenge with indicated doses, dead mice were recorded daily for 30 days.

[b] Numbers in parentheses give the mean survival time (days) of succumbing mice.

specific serum and the avidity and content of antibodies with ability to bind to the corresponding LPS.

Humoral antibody response in mice. Groups of BALB/c mice were immunized with the Os-BSA conjugates and with heat-killed *S. essen* (O4, 12), and the antibody titers were estimated (Table 4). The disaccharide AM-ITC-BSA conjugate elicited no, or only a very low, antibody response reactive with the saccharide-containing antigens. The anti-BSA response was, however, as high as that elicited by the other Os-ITC-BSA conjugates. The tetra- and octa-ITC-BSA conjugates elicited antisaccharide responses; the titers were highest against the homologous saccharide hapten (Table 4). An antibody response measurable with the *S. typhimurium* SH 4809 and *S. enteritidis* SH 1262 LPS antigens was also seen; the *S. typhimurium* SH 4809 LPS antibody titers were up to 13-fold higher than the *S. enteritidis* SH 1262 LPS titers. The highest antibody response against the *S. typhimurium* SH 4809 LPS antigen was elicited by immunization with heat-killed *S. essen* (O4, 12): it was about 55- and 2,200-fold higher than that seen after immunization with the octa- and tetrasaccharide-ITC-BSA conjugates, respectively. However, when the antibody titers were estimated against the saccharide-ITC-PHC conjugates as antigens, higher titers were seen in sera from Os-BSA-immunized mice than in sera from *S. essen* (O4, 12)-immunized mice (Table 4).

When BALB/c mice were immunized with octa-ITC-BSA conjugates in which the molar ratio of the saccharide to carrier BSA was varied from 7 to 23, the antibody response increased 22- and 15-fold, respectively, when the molar ratio of octasaccharide in the immunogen was increased from 7 to 23 (Table 5). The increase in antibody content reacting with the *S. enteritidis* SH 1262 LPS paralleled that seen against the *S. typhimurium* SH 4809 LPS antigen.

Immunization of the outbred NMRI mice with the same set of octa-ITC-BSA conjugates gave results similar, although not as pronounced, to those seen in BALB/c mice. The anti-O-specific antibody titers were in all instances about 10-

fold lower than in the BALB/c mice.

DISCUSSION

Data presented here establish that *Salmonella* O-antigen-specific saccharides covalently attached to BSA elicit a humoral antibody response in rabbits and mice. As the size of the saccharide hapten increased, the antibody titers elicited and the titers estimated with the native *S. typhimurium* SH 4809 (O4, 5, 12) LPS as antigen increased in rabbits as well as in mice (Tables 1, 2, and 4). Thus, the dodeca-ITC-BSA conjugate was about 200-fold more efficient than the tetra-ITC-BSA conjugate in rabbits (Table 1). In BALB/c mice, the corresponding difference was of the same magnitude (Table 4).

In contrast to rabbits, the BALB/c mice failed to respond with antibody production against the 3-*O*-(α-abequopyranosyl)-α-D-mannopyranoside disaccharide hapten (Table 4). This is not unique to BALB/c mice, but was also observed for outbred NMRI mice as well as inbred C57BL, A/J, C_3H and CBA mouse strains when the immunogen dose was varied from 0.025 to 250 μg per injection, the immunogen was administered with or without FCA, and the route and the schedule of immunization were varied (data

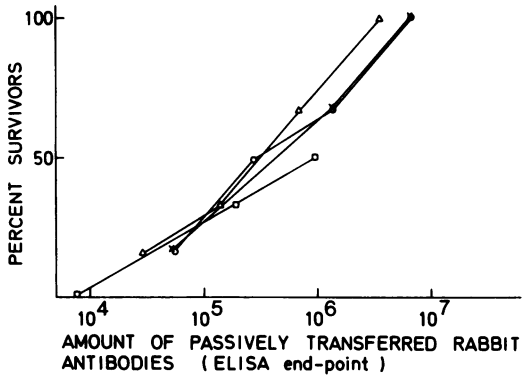

FIG. 2. *Protective efficiency of passively transferred rabbit antisera raised against different S. typhimurium O-antigen-specific immunogens. Groups of outbred NMRI mice (six per group) were given various S. typhimurium O-antigen-specific antisera before intravenous challenge with 25 times the LD_{50} dose (1.1 × 10^6 cells) of S. typhimurium SH 2201. The percentage of survivors was plotted against the ELISA titers (expressed as endpoint titers) as determined against the S. typhimurium SH 4809 LPS. Symbols: (□) anti-S. essen (O4, 12 specific); (△) anti-dodeca-ITC-BSA (O4, 12 specific); (×) anti-octa-ITC-BSA (O4, 12 specific); (○) anti-AM-ITC-BSA (O4 specific).*

TABLE 5. *Humoral antibody response to S. typhimurium O-antigen-specific octasaccharide-BSA conjugates in BALB/c mice: influence of degree of substitution*[a]

Mouse strain	Degree of substitution (mol of saccharide/mol of BSA)	ELISA endpoint titer (× 10^3) against[b]: *S. typhimurium* octa-ITC-PHC	*S. typhimurium* SH 4809 LPS	*S. enteritidis* SH 1262 LPS
BALB/c	7	710	45.0	<1
BALB/c	16	3,550	280	5.0
BALB/c	23	15,800	630	20.0
NMRI	7	70	55	<1
NMRI	16	65	30	<1
NMRI	23	1,580	70	<1

[a] Groups of mice (10 per group) were intraperitoneally given *S. typhimurium* O-antigen-specific octasaccharide-BSA conjugates of the indicated degree of substitution. The various octa-ITC-BSA conjugates were given suspended 1:1 in FCA and at doses of 10 μg on days 0, 14, and 28. Mice were bled on day 36, and the antisera from each group were pooled and titrated by ELISA.

[b] Coating antigen concentration was 1.0 μg/ml, and dilution of rabbit anti-mouse immunoglobulin alkaline phosphatase conjugate was 10^{-3}.

TABLE 4. *Humoral antibody response to S. typhimurium O-antigen-specific immunogens in mice: influence of hapten size*[a]

Immunogen	ELISA endpoint titer (× 10^3) against[b]: *S. typhimurium* tetra-ITC-PHC	*S. typhimurium* octa-ITC-PHC	*S. typhimurium* SH 4809 LPS	*S. enteritidis* SH 1262 LPS
3-*O*-(α-abequopyranosyl)-α-D-mannopyranoside-ITC-BSA	<1.0	<1.0	<1.0	<1.0
Tetra-ITC-BSA	500	79.5	1.50	1.50
Octa-ITC-BSA	82.0	1,995	65.0	5.0
Dodeca-IIC-BSA	360	1,010	280	10.0
Heat-killed *S. essen* (O4, 12)	39.8	280	3,550	710

[a] Groups of mice (10 per group) were intraperitoneally inoculated with the indicated immunogens suspended 1:1 in FCA (10 μg/dose) on days 0, 14, and 28. The mice were bled on day 36, and the antisera from each group were pooled and titrated by ELISA.

[b] Coating antigen concentration was 1.0 μg/ml, and dilution of rabbit anti-mouse immunoglobulin alkaline phosphatase conjugate was 10^{-3}.

not shown). All mouse strains invariably responded with antibody production against the carrier protein, and in some instances also against the ITC linkage arm used to attach the disaccharide hapten (data not shown). These findings suggest that a disaccharide is too small a hapten to elicit an antibody response in mice that is detectable with our assay systems.

The immune response is the sum of many clonal responses. Analyses of immunoglobulins with specificity for polysaccharides have, in both rabbits and mice, revealed that antibodies with specificity for terminal as well as intrachain antigenic determinants and of varying affinities are elicited (3, 20). The observation, particularly prominent when mice were immunized, that the anti-*S. typhimurium* SH 4809 LPS titers elicited with the saccharide hapten conjugates were more than 50-fold lower than those elicited with heat-killed *S. essen* (O4, 12) (Tables 2 and 4) may be explained by the structure of the antigenic determinants. The tetra-, octa-, and dodecasaccharides all have as the terminal nonreducing sugar an α-D-galactosyl residue which is not present in a terminal position in the native O-polysaccharide chain (Fig. 1). As a consequence, antibodies with specificity for the terminal α-D-galactosyl residue may be elicited by the Os-BSA conjugates, which could explain the relatively high titers seen when the OS-ITC-PHC conjugates were used as coating antigens in ELISA (Table 4). With these coating antigens also, relatively low titers were detected in the antisera from mice immunized with heat-killed *S. essen* (O4, 12) (Table 4). The observation that the 3-*O*-(α-abequopyranosyl)-α-D-mannopyranoside disaccharide hapten conjugate to BSA in rabbits elicited a higher anti-*S. typhimurium* LPS titer than the Os-BSA conjugates, possessing the terminal α-D-galactosyl residue, emphasizes the need of oligosaccharides which have a terminal nonreducing sugar identical to that in the native LPS.

The tetra-, octa-, and dodecasaccharide-BSA conjugates all elicited antibodies with O4 specificity (Tables 1, 2, and 4). The antibody response with O12 specificity was, however, more evident the larger the saccharide hapten. This is understandable since the octasaccharide hapten is the smallest hapten which contains the complete saccharide structure of the native bacterial LPS antigen (Fig. 1).

Not only the size of the oligosaccharide hapten but also the molar ratio of oligosaccharide to carrier protein was important in eliciting a high antibody response. Increases in degree of substitution from 7 to 23 in the octa-ITC-BSA conjugates increased the anti-*S. typhimurium* LPS response more than 10-fold in the BALB/c mice but seemingly less in the outbred NMRI mice.

Based on the findings presented in this and the accompanying paper (11), an artificial immunogen representative of the *S. typhimurium* O antigen should be composed of a saccharide hapten of the size of an octa- or dodecasaccharide, and the degree of substitution of the saccharide-protein conjugate should be about 20 molecules of saccharide per molecule of carrier. The terminal nonreducing end of the hapten should also preferably be identical to that of the native antigen.

Passive transfer of rabbit antisera against all saccharide-protein conjugates tested efficiently protected mice against the highest challenge dose ($100 \times LD_{50}$) of *S. typhimurium* strain SH 2201 (O4, 5, 12), supporting the hypothesis that humoral antibodies are important for protection. Since mice challenged with *S. enteritidis* strain SH 2204 (O9, 12) (identical to strain SH 2201 except for the structure of the 3,6-dideoxyhexose substituent in the O-antigenic polysaccharide chain) were not protected, we conclude that the immune response directed against the terminal O4 determinant was more important than that directed against the intrachain O12 determinant.

Although these data by no means exclude the importance of cell-mediated immunity in the defense against salmonellosis, they do support the hypothesis that humoral immunity also contributes to an effective defense against experimental mouse typhoid.

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