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# Immunogenic Properties of Octasaccharide-Protein Conjugates Derived from *Klebsiella* Serotype 11 Capsular Polysaccharide

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**The tetrasaccharide repeating unit of the capsular polysaccharide of *Klebsiella* serotype 11, K11PS, comprises the following sequence: [→3)-β-D-Glc*p*A-(1→3)-α-D-Gal*p*-(1→3)-β-D-Glc*p*-(1→] with a 4,6-*O*-(1-carboxyethylidene)-α-D-galactopyranosyl residue linked to O-4 of the glucuronic acid residue. Octasaccharide (OS) derived from K11PS by bacteriophage ϕ11-associated glycanase, was coupled to bovine serum albumin and to keyhole limpet hemocyanin. The immunogenicity of various antigens after intraperitoneal immunization was studied by measuring the levels of circulating antibodies. Injection of BALB/c mice with K11PS resulted in induction of 2-mercaptoethanol-sensitive immunoglobulin M antibodies. The responses observed in BALB/c *nu/nu* mice and in male (CBA/N × C3H/HeN)$F_1$ mice indicate that K11PS is a thymus-independent type 2 antigen. Immunization of BALB/c mice with either OS-bovine serum albumin or OS-keyhole limpet hemocyanin resulted in the induction of circulating 2-mercaptoethanol-resistant immunoglobulin G antibodies. Results in BALB/c *nu/nu* mice indicate that the OS-protein conjugates are thymus-dependent antigens. Since the OS-keyhole limpet hemocyanin conjugate induced antibodies in both (CBA/N × C3H/HeN)$F_1$ females and males, we propose to refer to this kind of antigen as a thymus-dependent type 1 antigen, whereas OS-bovine serum albumin, which evoked immunoglobulins in (CBA/N × C3H/HeN)$F_1$ females only, can be referred to as a thymus-dependent type 2 antigen.**

Bacterial polysaccharides are generally considered to be thymus-independent (TI) antigens (4, 6) and are characterized by the induction of mainly immunoglobulin M (IgM) and IgG3 antibodies after primary immunization (17, 29), without the induction of immunological memory (35). Moreover, the immune response toward TI antigens is developed only at later stages of the ontogeny, which has important consequences for the vaccination of neonates (27, 35).

For vaccinations aiming at long-lasting protection against diseases, it would be desirable to immunize with thymus-dependent (TD) antigens (35). TD antigens are supposed to induce immunological memory and thus to provoke a state of prolonged resistance to infections (36). Furthermore, the development of TD forms of a particular TI antigen raises the possibility of immunizing young animals with more success than with the TI antigen itself.

In earlier experiments with *Streptococcus pneumoniae* type 3 capsular polysaccharide (S3PS) and hexasaccharide-protein conjugates, we showed that the S3PS behaved as a TI antigen, whereas the hexasaccharide-protein conjugates behaved as TD antigens (35). To extend the results obtained with S3PS and hexasaccharide-protein conjugates of *S. pneumoniae*, we used the capsular polysaccharide of *Klebsiella* serotype 11, K11PS, as a novel model antigen. This polysaccharide was chosen because of the availability of bacteriophage ϕ11-associated glycanase, which could be used to depolymerize K11PS. Enzymatic digestion of K11PS results in the cleavage of the β(1→3) glycosidic linkage between glucose and glucuronic acid (40), thereby generating monomers or oligomers with a repeating structure (40, 41) with glucose at the reducing end (Fig. 1).

In this study octasaccharide (OS), isolated after depolymerization of K11PS, was coupled to bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) by the method of Svenson and Lindberg (38). The resulting coupling products and K11PS were tested in euthymic BALB/c and athymic BALB/c *nu/nu* mice to study their thymus-dependence. Male (CBA/N × C3H/HeN)$F_1$ mice carry an X chromosome-linked immunodeficiency and lack Lyb $5^+$ B cells (32). As they possess an immature set of B cells (25, 32), they resemble young animals and are unresponsive to some polysaccharide antigens (1, 32). It was our aim to investigate whether oligosaccharide-protein complexes are immunogenic in these animals.

## MATERIALS AND METHODS

**Mice and immunization.** Inbred BALB/c mice were raised and maintained in the Laboratory of Microbiology, State University of Utrecht. (CBA/N × C3H/HeN)$F_1$ male and female mice raised at the Animal Production Section, National Institute of Health, were a gift from B. Merchant, Food and Drug Administration, Bethesda, Md. BALB/c nude mice (*nu/nu*) and their heterozygous littermates (*nu/+*) were obtained from Bomholtgård, Ry, Denmark. All mice were used at an age of about 10 weeks and were immunized intraperitoneally (i.p.). In some experiments mice were immunized with OS-BSA suspended in Freund complete adjuvant (FCA). OS-KLH was absorbed on bentonite.

**Proteins.** BSA was purchased from Miles Laboratories, Elkhart, Ind., and KLH was purchased from Calbiochem, La Jolla, Calif.

**Oligosaccharides.** *Klebsiella* serotype 11 (strain 390) was a gift from S. Stirm, Center for Clinical Biochemistry, Giessen, Federal Republic of Germany. For the isolation of their capsular polysaccharides, the strain was grown on 1.5% Worfel Ferguson agar (Difco Laboratories, Detroit, Mich.) and extracted by the phenol-water-cetyltrimethylammonium

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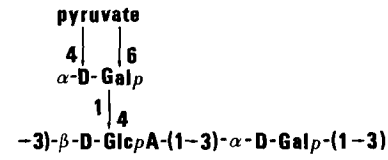


FIG. 1. Tetrasaccharide repeating structure of oligosaccharides generated after bacteriophage ϕ11-associated glycanase treatment of K11PS.

bromide procedure (19, 40). The lipopolysaccharide content of K11PS was determined by a Limulustest (Coatest endotoxin; Kabi Vitrum, Stockholm, Sweden). The K11PS contained 0.34% lipopolysaccharide. Bacteriophage ϕ11 was isolated from sewage (37). High-titer phage stocks ($3 \times 10^{11}$ to $2 \times 10^{12}$) were prepared by Diaflo ultrafiltration (HIP100; nominal molecular weight cutoff, 100,000; Amicon Corp., Lexington, Mass.) and purified by subsequent isopycnic centrifugation through a continuous CsCl gradient (41, 43).

Tetrasaccharide (TS) and OS were split from the capsular polysaccharide by bacteriophage ϕ11-associated glycanase (31, 41). The oligosaccharides were isolated via Sephadex G-25 superfine (Pharmacia Fine Chemicals, Uppsala, Sweden) gel filtration. The purity of eluted fractions was checked by thin-layer chromatography on silica gel plates (DC-Alufolien Kieselgel 60; E. Merck AG, Darmstadt, Federal Republic of Germany) with *n*-butanol–pyridine–water (6:5:5, vol/vol/vol) as a solvent. Detection was realized by spraying the plates with methanol-sulfuric acid (4:1, vol/vol) and heating to 120°C for 10 min. Only fractions containing one visual oligosaccharide spot or minor contaminations were pooled for further purification on Bio-Gel P4 (Bio-Rad Laboratories, Richmond, Calif.) gel filtration columns. Again, purity was checked by thin-layer chromatography. The fractions containing only one oligosaccharide were pooled and characterized by gaschromatographic sugar analysis and 500-MHz $^1H$ nuclear magnetic resonance spectroscopy.

Gas chromatographic sugar analysis was performed by standard procedures (22, 42). Incomplete (only 30%) hydrolysis of the pyruvate acetal group in the nonreducing D-galactose end groups (40) gave rise to two unknown peaks in the gas chromatogram ($R_{mannitol}$ = 0.84 and 0.88; 3:1), which were identified by gas chromatography-mass spectrometry as methyl 4,6-*O*-(1-carboxyethylidene methyl ester)-2,3-di-*O*-trimethylsilyl α- and β-D-galactopyranoside.

The sugar analysis of TS after standard procedures with $NaBH_4$ treatment was as follows: glucose, 0; galactose, 1.4; glucuronic acid, 1.0; 4,6-O-(1-carboxyethylidene)-galactose, 0.7; and glucitol, 1.2. The sugar analysis of OS after the same treatment was as follows: glucose, 1.0; galactose, 2.6; glucuronic acid, 2.0; 4,6-O-(1-carboxyethylidene)-galactose, 1.3; glucitol, 1.0.

The 500-MHz $^1H$ nuclear magnetic resonance spectra of underivatized TS and OS were recorded with a Brucker WM 500 spectrometer in the pulsed Fourier transform mode after proton exchange in $D_2O$. The data obtained (Table 1) confirmed the anomeric configurations of the constituting monosaccharides of TS and OS (Fig. 1).

The isolated TS and OS were analyzed immunochemically by testing the inhibition of the antigen-antibody complex formation in the K11PS–anti-K11PS antibody system. Quantitative precipitations were carried out as described by Heidelberger et al. (16). A 60-μg amount of OS inhibited the precipitin reaction by 50%. The TS was less inhibitory; approximately 120 μg caused 50% inhibition. This suggests that OS resembles the antigenic determinant of K11PS more closely than TS does (14).

**Coupling of OS to proteins.** The OS was covalently linked to the proteins BSA and KLH by the method of Svenson and Lindberg (38) as described before (35) and resulted in two conjugates, OS-BSA and OS-KLH. A 32-mg amount of OS yielded 30 mg of OS-2-(4-aminophenyl)-ethylamine derivative (38). This was treated with thiophosgene; after the addition of 50 mg of KLH and dialysis, 40 mg of conjugate was isolated. In the same manner 40 mg of OS-BSA conjugate was prepared from 32 mg of OS and 50 mg of BSA. The purity of the conjugates was checked on a Sephacryl S300 (Pharmacia) gel filtration column. Protein eluted as a single peak, followed by residual, nonbound oligosaccharides (±40%). The degree of conjugation (molar ratio of OS to protein) was calculated after quantitative protein and sugar determination by the methods of Lowry et al. (24) and Dubois et al. (11), respectively. The sugar content of the conjugates was corrected for the amount of sugar measured with nonconjugated carrier protein.

The following OS-protein conjugates were prepared: $OS_5$-BSA (100 μg of conjugate contained 16.3 μg of OS, of which 9.8 μg was bound to BSA) and $OS_{18}$-KLH (100 μg of conjugate contained 18.5 μg of OS, of which 11.1 μg was bound to KLH), wherein the subscript refers to the molar ratio of conjugated OS to protein. For immunizations, 100 μg (dry weight; i.e., sugar and protein) of these conjugates was injected i.p. This dose had earlier proved to be optimal for OS-protein conjugates (data not shown).

**Antibody detection in serum.** Blood was withdrawn from the retroorbital venous plexus, and serum was prepared and stored at −20°C. The presence of antibodies was determined by a hemagglutination (HA) assay, with indicator sheep erythrocytes optimally derivatized with K11PS by the method of Baker et al. (5). Twofold dilutions of heat-inactivated (20 min, 56°C) serum were made in microtiter plates (Greiner, Nürtingen, Federal Republic of Germany) with saline supplemented with 1% normal rabbit serum (heat inactivated and absorbed with sheep erythrocytes) as the diluent. A 50-μl amount of a suspension of indicator erythrocytes was mixed with 50 μl of the serum dilution. To detect 2-mercaptoethanol (2-ME)-resistant antibodies (IgG), serum was preincubated for 30 min in the presence of 0.05 M

TABLE 1. $^1H$ nuclear magnetic resonance spectral data of the anomeric protons of K11PS-derived TS and OS

| Monosaccharide constituent | TS | | OS | |
|---|---|---|---|---|
| | δ[a] (ppm) | $J_{1,2}$[b] (Hz) | δ (ppm) | $J_{1,2}$ (Hz) |
| →3)-α-Glucose | 5.220 | 3.8 | 5.239 | 3.8 |
| →3)-β-Glucose | 4.645 | 7.9 | 4.662 | 8.0 |
| →3)-β-Glucose-(1→ | | | 4.954 | 8.0 |
| →3)-αGalactose-(1→ | 5.370[c] | 3.2 | 5.392[d] | 3.5 |
| | 5.377 | 3.8 | | |
| | | | 5.387 | 4.6 |
| →4)-β-Glucuronic acid-(1→ | 4.650 | 7.8 | 4.688[d] | 7.9 |
| | | | 4.669 | 7.8 |
| 4,6-O-(1-carboxyethylidene)-α-galactose-(1→ | 5.453 | 3.0 | 5.500[d] | 3.2 |
| | | | 5.477 | 2.1 |

[a] Chemical shift in parts per million.
[b] Coupling constant.
[c] Two signals observable due to anomerization effect of the reducing glucose.
[d] Two signals of internal and terminal monosaccharide.

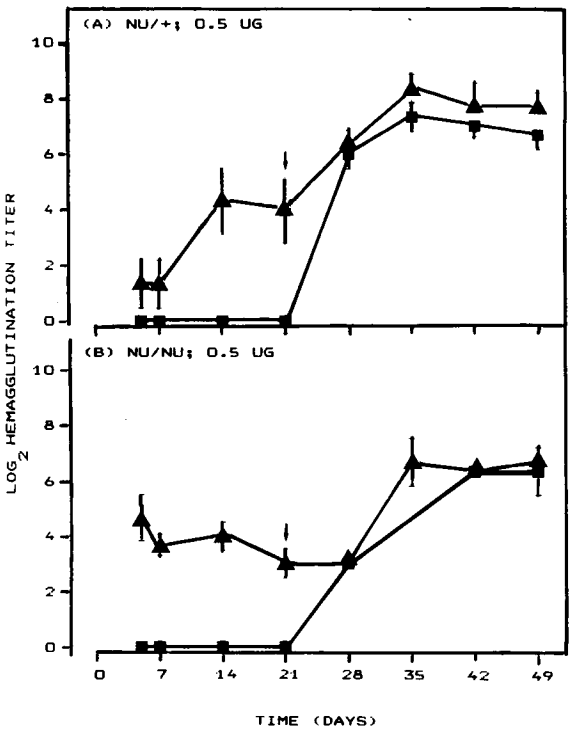


FIG. 2. Serum antibody response of nude mice to immunization with K11PS. Groups ($n$ = 5) of BALB/c *nu*/+ and *nu*/*nu* female mice were immunized i.p. with 0.5 μg of K11PS in saline and boosted 3 weeks later (arrow) i.p. with 1.0 μg of K11PS in saline. The mice were bled weekly, and the serum anti-K11PS antibody titers were determined by HA, both in the absence (▲) and in the presence (■) of 2-ME. A, *nu*/+ mice immunized primarily with 0.5 μg of K11PS, boosted with 1.0 μg of K11PS; B, *nu*/*nu* mice immunized as in A. Vertical bars indicate standard errors of the mean.

2-ME at 37°C. The HA was read after 16 h. The reciprocal value of the last dilution still giving agglutination was taken as the titer.

**Statistical analysis.** Results are expressed as the arithmetic mean of $n$-independent observations ± standard error of the mean. Significance of differences was analyzed with Student's $t$-test.

## RESULTS

**Immunogenicity of K11PS in various mouse strains.** Groups of BALB/c mice immunized with different doses of K11PS gave rise to various levels of circulating antibodies, as determined by a HA assay with K11PS-coated sheep erythrocytes. Immunization with 0.5 to 1.0 μg of K11PS resulted in optimal antibody titers of about 5. Since the HA reaction was 2-ME sensitive, the antibodies involved are considered to be of the IgM isotype.

To determine more precisely the immunogenic properties of the K11PS antigen, BALB/c *nu*/*nu* and *nu*/+ mice were immunized with 0.5 or 1.0 μg of K11PS, and blood was collected at different intervals. Both doses gave similar patterns of antibody production (for 0.5 μg, see Fig. 2). BALB/c *nu*/+ and *nu*/*nu* mice mounted a comparable antibody response, with optimal antibody titers ranging from 3.4 to 5.0 and no significant difference between the two mouse strains (Fig. 2). Three weeks after primary immunization all mice were boosted with 1 μg of K11PS. Both groups of mice responded with further increases of the levels of circulating antibodies, which were mainly of the 2-ME-resistant IgG isotype (Fig. 2). The responses were again comparable in both groups of mice. Since K11PS induced an antibody response in congenitally athymic mice, it can be characterized as a TI antigen.

Immunization with 1 μg of K11PS resulted in a primary IgM response in (CBA/N × C3H/HeN)$F_1$ female mice without the appearance of a secondary immune response after boosting 3 weeks later (Fig. 3). This is in contrast to the $F_1$ males, who did not respond at all (data not shown). The results suggest that K11PS is a TI type 2 (TI-2) antigen.

**Immunogenicity of OS-protein conjugates in BALB/c mice.** A single injection of BALB/c mice with 100 μg of OS-BSA in FCA resulted initially in the induction of circulating 2-ME-sensitive IgM antibodies as determined by the HA assay (Fig. 4A); on day 13 there was a difference between the HA-titer in the absence and the presence of 2-ME. Afterward, IgG predominated with high IgG levels persisting for up to 9 weeks, when the experiment was discontinued. Augmentation of these IgG antibody levels could be accomplished by means of a booster injection with 100 μg of OS-BSA 5 weeks after the primary immunization.

The pattern of antibody formation after immunization with 100 μg of OS-KLH on bentonite was comparable to that obtained with OS-BSA, with slight differences (Fig. 4B). The difference between the HA titers in the absence and the presence of 2-ME was larger during the first 6 weeks. Boosting after 7 weeks with 100 μg of OS-KLH on bentonite raised the contribution of 2-ME-resistant antibodies to the level observed after immunization with OS-BSA.

The injection of BALB/c mice with various amounts (1 to 20 μg) of nonconjugated OS did never lead to measurable HA titers. Furthermore the addition of nonconjugated BSA or KLH did not overcome the inability of nonconjugated OS to stimulate circulating antibody levels (data not shown).

**Immunogenicity of OS-protein conjugates in nude mice.** To obtain information on the thymus dependence of the OS-protein conjugates, the conjugates were injected in *nu*/*nu* and *nu*/+ mice. After primary immunization the antibody levels

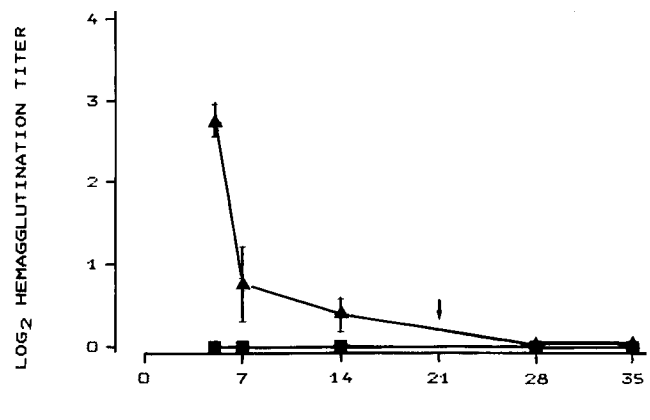


FIG. 3. Immunogenicity of K11PS in (CBA/N × C3H/HeN)$F_1$ female mice. One group ($n$ = 5) of (CBA/N × C3H/HeN)$F_1$ female mice was immunized i.p. with 1.0 μg of K11PS in saline and boosted i.p. 3 weeks later (arrow) with the same dose of K11PS. The procedure for the determination of the HA titer was as described in the legend to Fig. 2. One group of male $F_1$ mice ($n$ = 5) immunized in the same way did not demonstrate detectable antibody titers. Symbols: (▲) HA titer in the absence of 2-ME; (■) HA titer in the presence of 2-ME.

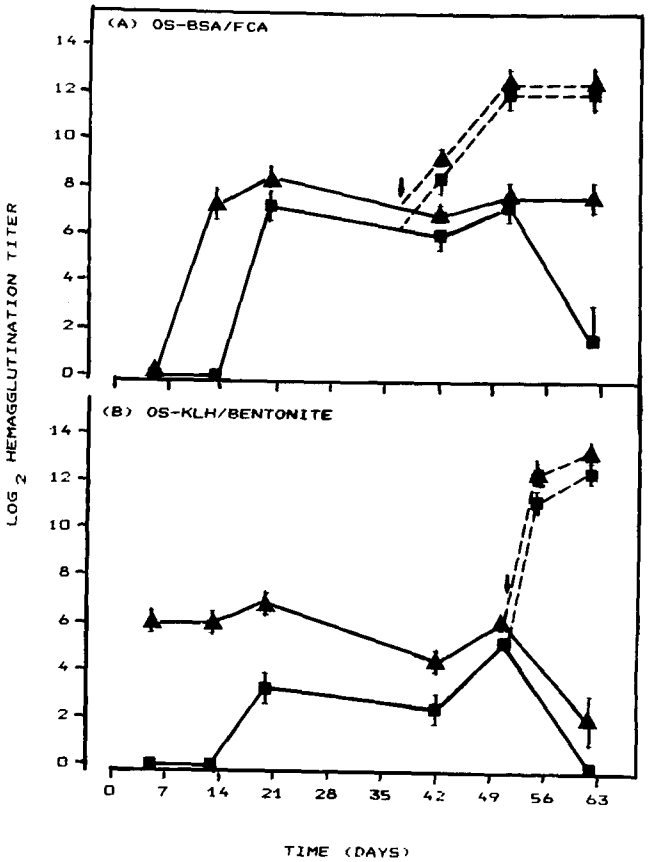


FIG. 4. Serum antibody levels in BALB/c mice in response to i.p. immunization with OS-protein conjugates. Groups of BALB/c mice ($n = 6$) were immunized i.p. with either 100 μg of OS-BSA in FCA (A) or 100 μg of OS-KLH on bentonite (B). Two groups received i.p. booster injections (arrows) of the homologous antigen on day 37 (OS-BSA) or day 51 (OS-KLH). Determination of the HA titer was performed as described in the legend to Fig. 2. A, BALB/c mice immunized with OS-BSA in FCA; B, BALB/c mice immunized with OS-KLH on bentonite. Symbols: (—) antibody titers after one immunization; (- - -) antibody titers after boosting; (▲) antibody titers in the absence of 2-ME; (■) antibody titers in the presence of 2-ME. Vertical bars indicate standard errors of the mean.

were quite low in both *nu/nu* and *nu/+* mice, except for *nu/+* mice immunized with OS-KLH on bentonite, wherein antibody titers rose immediately (Fig. 5). Antibodies formed by *nu/+* mice were partially of the IgG isotype (Fig. 5D through F). Boosting of the mice 3 weeks later with 100 μg of the homologous antigen revealed large differences between *nu/+* and *nu/nu* mice. The injection of 100 μg of OS-BSA and 100 μg of OS-KLH on bentonite in *nu/+* mice underlay further increases of the antibody levels in these mice. This phenomenon was not observed in the *nu/nu* mice, which displayed continuing low serum antibody titers. The absence of a response in nude mice is stressed by the fact that use of FCA at primary immunization, which stimulates the response in *nu/+* mice (Fig. 5D and E), is without effect in *nu/nu* mice (Fig. 5A and B). From these experiments the conclusion can be drawn that the OS-protein antigens are TD.

**Immunogenicity of OS-protein conjugates in (CBA/N × C3H/HeN)$F_1$ mice.** OS-protein conjugates were injected in (CBA/N × C3H/HeN)$F_1$ male and female mice (Fig. 6). The male mice were unable to respond to 100 μg of OS-BSA, in contrast to the females, who gave rise to low levels of circulating antibodies. The antibody levels in female mice could be elevated by primary immunization with OS-BSA suspended in FCA followed by a second injection 3 weeks later, a treatment which did not result in antibody formation in male mice. As there is a qualitative difference in the response to OS-BSA between male and female mice, there is only a quantitative difference in the response to OS-KLH on bentonite. Although OS-KLH on bentonite evoked circulating antibodies only in females initially, there appeared antibodies in males also after boosting with 100 μg of the homologous antigen. In males, however, the titers were significantly lower than in female mice after booster injections.

## DISCUSSION

The data presented, extend our results obtained with S3PS and hexasaccharide-protein conjugates of *S. pneumoniae*. Like S3PS, K11PS is a TI antigen. Upon injection, octasaccharide-protein conjugates gave rise to antibodies that bound to K11PS-coated sheep erythrocytes, indicating that the OS represents an antigenic determinant of K11PS. This was already suggested by the experiments described above, for in these experiments it was shown that OS efficiently inhibited the binding of anti-K11PS antibodies to K11PS. Furthermore, as was stated earlier by Thurow et al. (40), the immunodominant group of K11PS is the 4,6-*O*-(1-carboxyethylidene)-D-galactose. This group is located at the nonreducing end of oligosaccharides, which are generated by bacteriophage ϕ11-associated glycanase treatment of K11PS. Since the oligosaccharides are coupled via the reducing end to proteins, the immunodominant group is located at the distal end of the oligosaccharide chains linked to the carrier protein. Our choice of coupling OS instead of TS to proteins as immunizing complex is based on the results of Geyer et al. (14), who found that OS derived from *Klebsiella* serotype 2 was far more protective than TS when coupled to proteins and that two repeating subunits of the K2PS were necessary for a good expression of the serological specificity of the polysaccharide.

The thymus independence of K11PS, as manifested by equal primary (IgM) responses in *nu/nu* and *nu/+* mice, has also been demonstrated for several other bacterial polysaccharides: pneumococcal polysaccharides (12, 35), lipopolysaccharides (6), meningococcal polysaccharides (8, 10), and others (33). The elevated antibody level after secondary immunization of *nu/nu* and *nu/+* mice with K11PS (Fig. 2), which is mainly IgG, is not characteristic for the response to TI antigens (6). This TD-like response, however, might be the result of the minor lipopolysaccharide contamination (0.34%) of the K11PS, since lipopolysaccharide is a potent adjuvant for the humoral immune response; as a consequence, IgG antibodies might be formed in response to TI antigens even in *nu/nu* mice (18, 26). The absence of a secondary response and IgG antibodies in (CBA/N × C3H/HeN)$F_1$ mice after immunization with K11PS from the same batch as used for the *nu/nu* and *nu/+* mice, however, rules out this explanation. Another possibility might be a reduced neonatal induction of T-suppressor cells due to the specific pathogen-free housing conditions of the *nu/nu* and *nu/+* mice (34). The initial difference observed between the antibody response in *nu/nu* and *nu/+* mice might be due to the complete absence of T-suppressor cells in *nu/nu* mice, concordant with their athymic condition, whereas in *nu/+* mice an only partially reduced T-cell-mediated suppression limited the antibody response. Investigation of the influence of purified T-suppressor cells from various mouse strains on

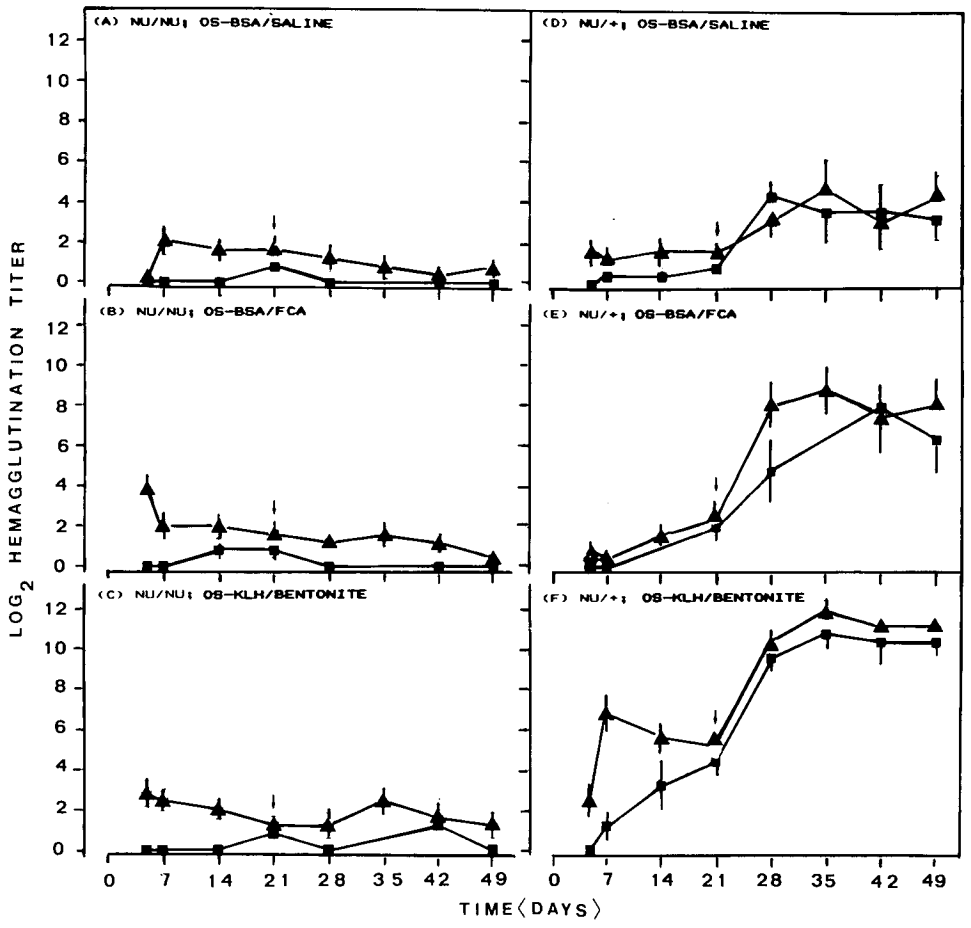


FIG. 5. Induction of circulating antibodies in congenitally nude mice by immunization with OS protein conjugates. BALB/c *nu/nu* and *nu/+* mice ($n = 5$ in each group) were immunized i.p. with 100 μg of OS-BSA in saline (A and D), 100 μg of OS-BSA in FCA (B and E), or 100 μg of OS-KLH on bentonite (C and F). Mice were boosted i.p. 3 weeks after primary immunization (arrows) with the homologous antigen (OS-BSA in saline, OS-KLH on bentonite). The HA titer was determined as described in the legend to Fig. 2. Symbols: (▲) antibody titers in the absence of 2-ME; (■) antibody titers in the presence of 2-ME. Vertical bars indicate standard errors of the mean.

the in vitro antibody production could test this hypothesis. The absence of circulating antibodies in (CBA/N × C3H/HeN)$F_1$ males after immunization with K11PS indicates that this polysaccharide is a TI-2 antigen (32). As is usual for TI antigens, reimmunization of these $F_1$ mice with K11PS led to neither increased antibody levels nor immunological memory (6, 35).

For vaccination purposes, K11PS is not quite suitable, since TI-2 antigens fail to induce an immune response in CBA/N mice (32) or neonates (27, 35). Furthermore TI antigens in general are unable to induce immunological memory (6, 35). To improve the properties of the antigen in these respects the antigenic determinant of K11PS was coupled to protein carriers. Coupling of oligosaccharides as haptens or polysaccharides to protein carriers has been a procedure performed for a long time now to increase the antigenicity of saccharide antigens. In the 1930s Avery and Goebel (3, 15) coupled both polysaccharides and disaccharides to proteins to induce hapten-specific antibodies. Later, *Haemophilus influenzae* capsular polysaccharides (2, 33) and meningococcal capsular polysaccharides (7–10, 20) were coupled to proteins to intensify the studies on modulation of the humoral response. Jennings et al. and Svenson et al. did not couple intact polysaccharides but isolated oligosaccharides that were coupled to protein (21, 39). From various experiments carried out by these different groups it became clear that saccharide-protein conjugates induced higher antibody levels than polysaccharides (2, 7, 20, 35), and that they behaved, in contradiction to the polysaccharides, as TD antigens (8, 10, 33, 35), whereas the induced antibodies retain their capacity to bind the original polysaccharide.

OS-protein conjugates behaved strikingly differently when compared with K11PS. After booster injections with OS-KLH or OS-BSA, immunological memory and high IgG levels were demonstrable. This response was limited to *nu/+* mice; *nu/nu* mice did not react. This suggests that OS-protein conjugates are TD and have the same characteristics as saccharide-protein conjugates made by others. As became clear from earlier experiments with saccharide-protein conjugates, the carrier used, the number of oligosaccharide repeating units in the hapten, and the number of haptens on a protein are of importance for the induced antibody response (39). The two conjugates used in this study were chosen because they contained approximately the same amount of oligosaccharide per milligram of protein. The presence of free OS did not interfere with the reaction on the conjugate since the free OS neither induced antibodies nor impaired the antibody response (9).

To examine the immunogenicity of the OS-protein conjugates in neonates, the (CBA/N × C3H/HeN)$F_1$ mice were

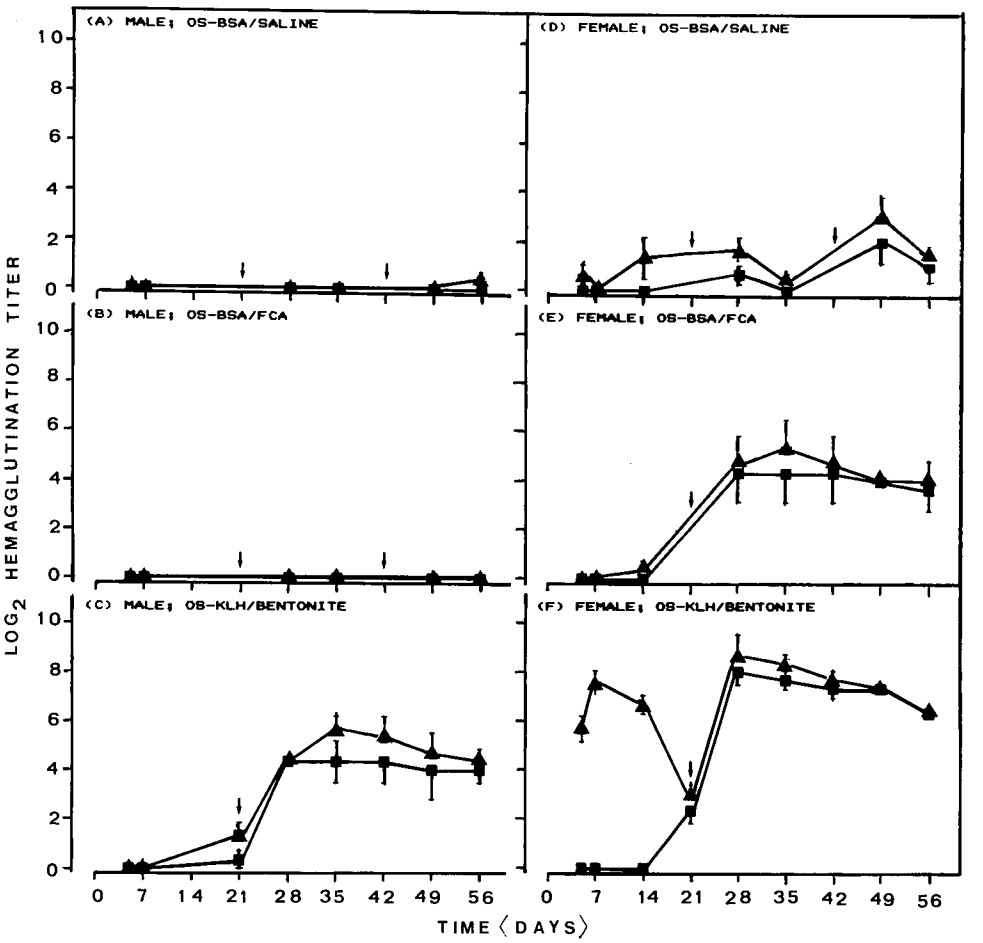


FIG. 6. Antibody response of (CBA/N × C3H/HeN)$F_1$ mice to OS-protein conjugates. Groups of $F_1$ males and females ($n$ = 5) were injected i.p. with 100 μg of OS-BSA in saline (A and D), 100 μg of OS-BSA in FCA (B and E), or 100 μg of OS-KLH on bentonite (C and F). Mice were boosted i.p. 3 weeks after primary immunization, and some groups (A, B, and D) were boosted again 6 weeks after primary immunization (arrows) with the homologous antigen (OS-BSA in saline, OS-KLH on bentonite). The HA titer was determined as described in the legend to Fig. 2. Symbols: (▲) antibody titers in the absence of 2-ME; (■) antibody titers in the presence of 2-ME. Vertical bars indicate standard errors of the mean.

again used as a model system. The $F_1$ males were able to discriminate between OS-BSA and OS-KLH and gave a humoral immune response against the latter antigen only, in contrast to female mice, which responded to both antigens. From these results and experiments with phosphocholine (23, 30) and sheep erythrocytes (13), it was already comprehensible that CBA/N mice carrying the X chromosome-linked immunodeficiency are not only unresponsive to TI-2 antigens, but are also unresponsive to some TD antigens. We propose to call these antigens TD-2 antigens. The consequence of this finding is that the formation of TD-forms of certain antigens is not a sole condition for antibody production in CBA/N mice or neonates. Certain carrier determinants present on KLH, but not on BSA, seem to be required for the induction of antibodies with OS-protein conjugates in CBA/N mice. We propose to call the TD antigens that induce an antibody response in CBA/N mice TD-1 antigens. This is in analogy with the TI-1 antigens, which induce an antibody response in CBA/N mice. We prefer to extend the classification of TD and TI antigens rather than to abandon it, as was proposed by Nossal and Pike (28). We do agree, however, that each classification has exceptional cases and might not be valid under all circumstances, as was found by these authors with in vitro stimulation of single B cells of CBA/N mice with various TI antigens (28).

The experiments presented here confirm the observation that isolation of oligosaccharides from polysaccharides and subsequent coupling to proteins yields a TD form of an initially TI antigen. The choice of the carrier protein is important since it determines whether B cells of CBA/N mice carrying the X chromosome-linked immunodeficiency, and probably also neonates, respond to the compound antigen. This might be of importance for the development of vaccines to be used in younger persons.

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