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Modulation of the Immune Response to Pneumococcal Type 14 Capsular Polysaccharide-Protein Conjugates by the Adjuvant Quil A Depends on the Properties of the Conjugates

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Streptococcus pneumoniae **type 14 capsular polysaccharide-bovine serum albumin (S14PS-BSA) conjugates were prepared by water-soluble-carbodiimide-mediated condensation with or without the use of *N*-hydroxysulfosuccinimide. The immunogenicities of the capsular polysaccharide (S14PS) and of the conjugates were studied in (CBA/N × BALB/c)F_1 mice and in female BALB/c mice. The response in these mice indicates that S14PS could be classified as a thymus-independent type 2 antigen. Coupling of S14PS to BSA improved the immunogenicity of this polysaccharide, and an immunoglobulin G memory response was evoked. Conjugation with *N*-hydroxysulfosuccinimide resulted in a product with a higher polysaccharide/protein ratio. This conjugate induced a greater immune response than did the classical conjugate. Quil A enhanced the immune response to S14PS and to most S14PS-BSA conjugates. The enhancement of the immune response to the conjugates seemed to depend on the coupling procedure. Our results indicate that, for the construction of immunostimulating complexes based on polysaccharide or oligosaccharide-protein conjugates, attention should be paid to the degree of cross-linking of the antigens involved.**

Pneumococcal infections are a significant cause of mortality and morbidity throughout the world (2, 30). Some of the 85 known serotypes are more prevalent and virulent than others. *Streptococcus pneumoniae* type 14 was the most commonly isolated serotype in the United States during the last decade. In young children, the immune response to this bacterium is delayed until they are 4 to 6 years old (22). The current vaccine against these microorganisms (Pneumovax R_{23}) contains capsular polysaccharides of the 23 most prevalent serotypes (3, 22). The use of bacterial polysaccharides as a vaccine, however, has some severe limitations. The immune response to these antigens is age dependent, and capsular polysaccharides are thymus-independent type 2 antigens (10, 14, 15, 17, 29, 31).

Covalent coupling of polysaccharides to proteins changes the nature of these antigens to thymus dependent. Thymus-dependent antigens induce immunological memory and therefore provide long-lasting protection. Moreover, these antigens are immunogenic in young children (12, 18).

Various methods are available for the coupling of polysaccharides to proteins (1). Water-soluble-carbodiimide-mediated condensation of polysaccharide and protein has been used for the preparation of *Haemophilis influenzae* type b and *S. pneumoniae* type S6A capsular polysaccharide-protein conjugates (23, 24, 28). The overall yield by using this method can be severely limited as the result of hydrolysis of the activated intermediate (25). In 1986, Staros et al. reported the use of *N*-hydroxysulfosuccinimide (sulfo-NHS) for enhancement of water-soluble-carbodiimide-mediated coupling reactions (25).

In this paper, we describe the use of sulfo-NHS in the preparation of pneumococcal S14 capsular polysaccharide-bovine serum albumin (S14PS-BSA) conjugates (Fig. 1). After coupling of the spacer molecule adipic acid dihydrazide to the cyanogen bromide-activated S14 polysaccharide, water-soluble-carbodiimide-mediated condensation was carried out both with and without the use of sulfo-NHS. We studied the immunogenicities of these conjugates in (CBA/N × BALB/c)F_1 mice and in female BALB/c mice. Male (CBA/N × BALB/c)F_1 mice carry an X chromosome-linked immunodeficiency and therefore, like young children, are unable to respond to thymus-independent type 2 antigens (29).

The adjuvant Quil A, a semipurified saponin, is used for constructing immunostimulating complexes (ISCOMs) (20, 21). The adjuvant activity of Quil A is considered to be based on, among others, a hydrophobic interaction between adjuvant and antigen (20, 21). Sulfo-NHS enabled us to prepare different kinds of conjugates, and we investigated whether there was a relationship between the adjuvant effect of Quil A and the type of conjugate used.

MATERIALS AND METHODS

Mice and immunization. Inbred BALB/c mice and (CBA/N × BALB/c)F_1 mice were reared and maintained at the Laboratory of Microbiology, Utrecht University (breeding pairs of CBA/N mice were a generous gift from W. T. Watson, Food and Drug Administration, Bethesda, Md.). Mice were used at 10 to 11 weeks of age and were immunized intracutaneously (i.c.) with S14PS-BSA conjugates or with the *S. pneumoniae* type 14 capsular polysaccharide (S14PS). Quil A (Superflos, Vedbaek, Denmark) was used as an adjuvant in most experiments. The i.c. route was selected because it minimizes the toxic side effects of Quil A.

Polysaccharide. The capsular polysaccharide was prepared from strain ATCC 6314. S14PS was isolated and purified by ethanol-calcium chloride fractionation (5). Concomitantly, polymannan was removed by passing the crude polysaccharide through a Superflow Tm 500 radial chromatography column (Sepragen Corp.) filled with concanavalin A-Sepharose (Pharmacia). The purified polysaccharide was analyzed for protein (4) and for C-capsular polysaccharide and resid-

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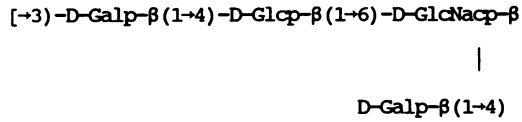

FIG. 1. Structure of the repeating unit of S14PS.

ual polymannan content (11). The final product contained less than 3% C-polysaccharide, 0.4% protein, and no polymannan.

Hydrazide derivative of S14PS. An 88-mg amount of S14PS was dissolved in bidistilled water (9 mg/ml) and activated at pH 10.5 at 4°C for 6 min with 1 mg of cyanogen bromide (Sigma Chemical Co., St. Louis, Mo.) per mg of S14PS (24). The pH was adjusted to 8.5 with 0.2 M HCl, and adipic acid dihydrazide in 0.5 M $NaHCO_3$ was added to a final concentration of 0.3 M. The reaction was allowed to proceed overnight with stirring at 8°C and was subsequently dialyzed against 0.2 M NaCl for 24 h at 8°C. In order to separate the S14PS hydrazide derivative (S14PS-AH) from the nonreacted adipic acid hydrazide and to desalt the reaction mixture, the solution was passed through a Bio-Gel P2 column (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with bidistilled water. The eluate was monitored for its polysaccharide and hydrazide content (7, 13). Fractions positive for polysaccharide and hydrazide were pooled and evaporated to dryness.

Coupling of S14PS-AH to BSA. BSA (ICN Pharmaceuticals) was covalently bound to the polysaccharide derivative by carbodiimide-mediated condensation by using 1-ethyl-3-dimethylaminopropyl carbodiimide (EDC) (Sigma) and sulfo-NHS (Pierce Chemical Co., Rockford, Ill.) (25) or EDC alone (24). The S14PS-AH was dissolved in aqua bidest (10 mg/ml). To one-half of the solution, BSA (10 mg/ml) and sulfo-NHS (7.5 mM) were added, and to the other one-half only BSA (10 mg/ml) was added. The pHs of both mixtures were adjusted to 4.9 with 0.2 M HCl, and EDC was added to a final concentration of 0.1 M. The reaction mixtures were stirred at pH 4.9 at 4°C for 3 h, dialyzed overnight against 0.2 M NaCl, and centrifuged. In the presence of sulfo-NHS, a sediment was formed; this sediment was dialyzed against aqua bidest for 72 h at 8°C and lyophilized. The supernatants were passed through a CL-6B Sepharose column (3 by 100 cm) equilibrated in 0.2 M NaCl. The eluate was monitored for polysaccharide and protein content. The first peak (void volume), containing both polysaccharide and protein, was collected. The obtained peaks of both coupling reactions were dialyzed against bidistilled water for 72 h and lyophilized. In this study, the conjugate made without the addition of sulfo-NHS is referred to as S14PS-BSA(1), the conjugate made in the presence of sulfo-NHS is referred to as S14PS-BSA(2a), and the sediment obtained is referred to as S14PS-BSA(2b). Before use, the conjugates were dissolved in sterile saline in such a way that they contained the amount of protein and polysaccharide given in Table 1.

Characterization of the conjugates. The conjugates were analyzed for protein and polysaccharide content (4, 7) and molecular monosaccharide composition (11). The results and the molecular monosaccharide composition of the original capsular polysaccharide are listed in Table 1.

Preparation of the biotinylated S14PS. S14PS was biotinylated by the method of Sutton et al. (26), with a few modifications. In brief, S14PS-AH was dissolved in phosphate-buffered saline (pH 7.4), and NHS-biotin (Sigma) in dimethylformamide was added. The solution was stirred overnight at 8°C. In order to remove the free NHS-biotin and to desalt the reaction mixture, the material was passed through a Bio-Gel P2 column (1.5 by 30 cm). The polysaccharide-positive fractions were pooled and freeze-dried. A thin-layer chromatography method was used for the qualitative determination of free and bound NHS-biotin. The method was a modification of the method described by McCormick and Roth (19). Thin-layer chromatography was performed on cellulose F254 plates (E. Merck AG, Darmstadt, Federal Republic of Germany) with *n*-butanol–acetic acid–water (2:1:1, vol/vol/vol) as the ascending solvent. The spray reagent was comprised of a 1:1 mixture of a 2% solution of H_2SO_4 in ethanol and a 0.2% solution of *p*-dimethylaminocinnaldehyde (Sigma) in ethanol. The extent of coupling with biotin was estimated on the basis of the decrease in hydrazide groups (26).

TABLE 1. Properties of the antigens used in this study

Antigen	Content (μg/100 μl) of[a]:		S14PS/protein ratio (μg/μg)	Monosaccharide content[b]		
	Protein	Polysaccharide		Gal	Glc	GlcNac
S14PS-BSA(1) (−NHS)	20.8	17.6	0.9	2.0	1.0	1.0
S14PS-BSA(2a) (+NHS)	20.8	34.2	1.6	2.0	1.2	1.2
S14PS-BSA(2b) (+NHS, sediment)	20.0	10.5	0.5	2.0	1.6	1.4
S14PS				2.0	1.2	0.9

[a] Protein content was determined by the method of Bradford (4) with BSA as the standard. Polysaccharide content was analyzed by the method of Dubois et al. (7) with a galactose–glucose–*N*-acetylglucosamine mixture (molecular ratio, 2:1:1) as the standard. The results were confirmed by sugar analysis (11).

[b] Monosaccharide composition was determined by gas chromatographic sugar analysis. Molecular ratios of monosaccharides were calculated with galactose (Gal) taken as 2.0. Glc, Glucose; GlcNAc, *N*-acetylglucosamine.

Antibody determination in serum. To determine antibody concentrations in serum, blood was withdrawn from the retroorbital venous plexus and serum was prepared and stored at −20°C. Specific antibodies were detected by an avidin-biotinylated S14PS enzyme-linked immunosorbent assay (ELISA). Polyvinylchloride 96-well microdilution plates (Titertek, 173-05 activated; Flow Laboratories, Zwanenburg, The Netherlands) were coated with 4 μg of affinity-purified egg white avidin (Sigma) per ml in 0.1 M carbonate buffer (pH 9.6) overnight at room temperature. The plates were washed with 0.074% EDTA–0.85% NaCl–0.03% Tween 20 in demineralized water as washing fluid. Next, the plates were incubated with 1 μg of S14PS-biotin per ml (60 nmol of bound biotin per mg of S14PS) in saline containing 0.05% Tween 20 for 1 h at 37°C. After being washed, the plates were incubated with a test serum dilution in saline containing 0.05% Tween 20 and 1% normal rabbit serum for 2 h at 37°C. The serum dilutions in general started with a 1/50 dilution, followed by $10^{-1/2}$ dilutions. Following the washing procedure, the plates were incubated for 2 h at 37°C with a goat anti-mouse immunoglobulin G (IgG) horseradish peroxidase-labeled antibody (Nordic Immunological Laboratories, Tilburg, The Netherlands) or a goat anti-mouse IgM horseradish peroxidase-labeled antibody (TAGO Immunologicals, Burlingame, Calif.) dissolved in the same diluent as the serum. After being washed, the plates were incubated for 20 min (for IgG) or for 10 min (for IgM) with a tetramethylbenzidine (Sigma)-H_2O_2 substrate solution. The enzyme reaction was terminated by the addition of 1 M H_2SO_4, and the A_{450} was measured with a spectrophotometer. The amount of a certain isotype present in a test serum was compared with the amount present in a reference serum. The effective

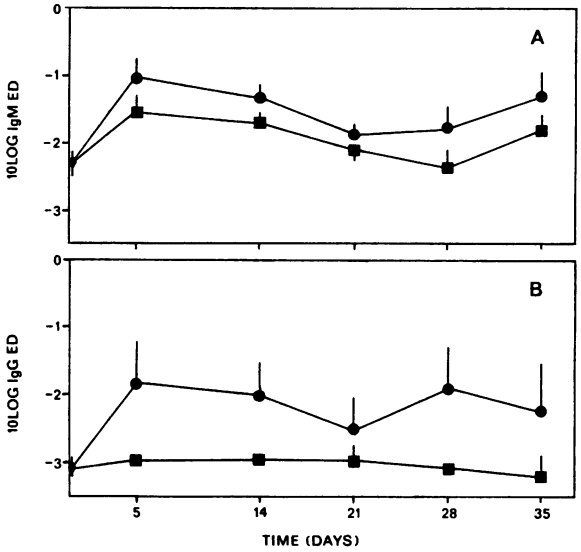

FIG. 2. Adjuvant effect of Quil A on the immune response to S14PS in BALB/c mice. Female BALB/c mice (n = 5) were injected i.c. either with 10 μg of S14PS or with 10 μg of S14PS mixed with 20 μg of Quil A at days 0 and 21. Serum was obtained, and IgM (A) and IgG (B) ED values were determined by ELISA. ED values on day 0 indicate preimmune levels of IgM and IgG. Results are expressed as means ± standard deviation. Symbols: ●, S14PS + Quil A; ■, S14PS.

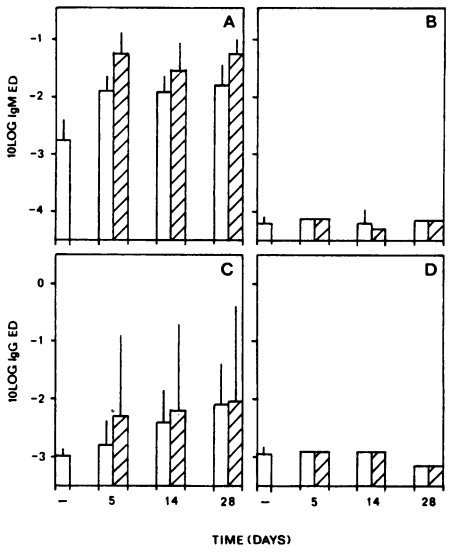

FIG. 3. Influence of Quil A on the immune response of S14PS in (CBA/N × BALB/c)F_1 mice. Both male (B and D) and female (A and C) (CBA/N × BALB/c)F_1 mice (n = 5) were immunized i.c. either with 10 μg of S14PS or with 10 μg of S14PS in combination with 20 μg of Quil A. A booster injection was given with antigen and adjuvant at day 21. Mice were bled on days 5, 14, and 28, and IgM (A and B) and IgG (C and D) ED values were determined by ELISA. ED values on day 0 indicate preimmune levels of IgM and IgG. Results are expressed as means ± standard deviation. Symbols: ⊠, S14PS + Quil A; □, S14PS.

dose (ED) (16) was calculated as follows: ED = $\log_{10}$ (dilution of reference serum giving an A_{450} of 0.500) − $\log_{10}$ (dilution of test serum giving an A_{450} of 0.500). As reference sera, an anti-S14PS-BSA hyperimmune serum (IgM and IgG antibodies) and a serum obtained after immunization of mice with Formalin-killed bacteria (mostly IgM antibodies) were used.

Statistical analysis. Results are expressed as the arithmetic mean of n independent observations ± standard deviation. Significance was tested with the Student t test. P values smaller than 0.05 were considered to be significant.

RESULTS

Immunogenicity of S14PS. Groups of female BALB/c mice (n = 5) were immunized i.c. either with 10 μg of S14PS or with 10 μg of S14PS mixed with 20 μg of Quil A. Anti-S14-specific IgM and IgG antibody titers expressed as ED values were determined by ELISA. Mice immunized with S14PS formed only IgM antibodies, while a booster injection at day 21 did not significantly increase these titers (Fig. 2). Addition of Quil A resulted in enhanced IgM antibody levels and in the induction of IgG antibodies before and after the booster.

In order to classify the S14PS antigen, male (CBA/N × BALB/c)F_1 mice (n = 5) were injected i.c. with 10 μg of S14PS at days 0 and 21. As a control group, female (CBA/N × BALB/c)F_1 mice (n = 5) were used. IgM and IgG ED values were determined at days 5, 14, and 28. Male mice immunized with S14PS did not respond, while S14-specific IgM and IgG antibodies were induced in the control group (Fig. 3).

To study the influence of Quil A on the immunogenicity of S14PS in (CBA/N × BALB/c)F_1 mice, both males and females were injected i.c. with 10 μg of S14PS mixed with 20 μg of Quil A. After 3 weeks, a booster injection with antigen and adjuvant was given.

Quil A enhanced the IgM response in the female mice to a minor extent, while no immune response was observed in the male mice (Fig. 3).

Immunogenicities of S14PS-BSA conjugates in BALB/c mice. Female BALB/c mice (n = 5) were injected i.c. with 100 μl of either S14PS-BSA(1) or S14PS-BSA(2a). After 3 weeks, mice were given booster injections of the homologous antigen. IgM and IgG ED values were determined at days 5, 14, and 28.

The patterns of antibody formation after injection of mice with S14PS-BSA(1) or S14PS-BSA(2a) were comparable (Fig. 4). Both conjugates induced IgM and IgG antibodies after the first injection, while a significant increase occurred after the booster injection ($P < 0.001$), resulting in similar levels of IgG antibodies. One minor difference between the groups was observed. Mice immunized with S14PS-BSA(2a) developed a significantly higher IgG antibody level at day 14 ($P < 0.001$).

In the next experiment, groups (n = 5) of female BALB/c mice were immunized i.c. with the conjugates mixed with 20 μg of Quil A. Quil A enhanced the IgM and IgG responses to both conjugates (Fig. 4), but some differences in the effects of the conjugates were observed between the groups of mice. At day 14, a 15-fold increase in the IgG antibody titer was noted for mice immunized with S14PS-BSA(1) and a 2.5-fold increase was noted for mice immunized with S14PS-BSA(2a). Moreover, the IgG response induced by S14PS-BSA(1) was significantly higher on that day ($P < 0.05$).

Mice given booster injections of the homologous antigen plus Quil A at day 21 displayed an enhancement of the IgG response of the same magnitude (three- to sixfold increase). The detected amounts of IgG antibodies in both groups were comparable.

Immunogenicities of S14PS-BSA conjugates in (CBA/N × BALB/c)F_1 mice. Male and female (CBA/N × BALB/c)F_1

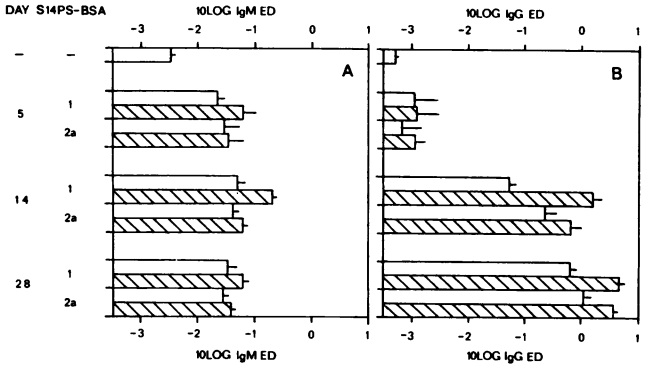

FIG. 4. Antibody response of BALB/c mice to S14PS-BSA conjugates and adjuvant effects of Quil A. Groups of female BALB/c mice (n = 5) were injected i.c. with 100 μl of S14PS-BSA(1), 100 μl of S14PS-BSA(2a), or the conjugates in combination with 20 μg of Quil A. The mice were given booster injections of the homologous antigen plus Quil A at day 21. IgM (A) and IgG (B) ED values were measured at days 5, 14, and 28 by ELISA. ED values at day 0 indicate preimmune levels of IgM and IgG. Results are expressed as means ± standard deviation. Symbols: ▨, conjugate + Quil A; □, conjugate alone.

mice (n = 5) were immunized i.c. with 100 μl of S14PS-BSA(1), S14PS-BSA(2a), or S14PS-BSA(2b) at days 0 and 21. IgM and IgG ED values were determined at days 5, 14, and 28 (Fig. 5). Immunization of female (CBA/N × BALB/c)F_1 mice with S14PS-BSA(1) or S14PS-BSA(2a) gave rise to almost identical immune responses, as was observed in the female BALB/c mice (Fig. 4 and Fig. 5A and B). The pattern of antibody formation induced by S14PS-BSA(2b), a conjugate not included in the earlier experiments, was comparable with that induced by the two other conjugates.

In striking contrast to the (CBA/N × BALB/c)F_1 females, hardly any antibodies were formed in the male mice (Fig. 5C and D). Only mice given booster injections of the S14PS-BSA(2a) conjugate produced IgG antibody titers that were above background level ($P < 0.001$).

To investigate the adjuvant effect of Quil A on the immune response to the S14PS-BSA conjugates in (CBA/N × BALB/c)F_1 mice, both male and female mice (n = 5) were injected i.c. with the conjugates mixed with 20 μg of Quil A. A booster injection containing the homologous antigen plus adjuvant was given at day 21. The adjuvant effect on the immune response to S14PS-BSA(1) and S14PS-BSA(2a) was

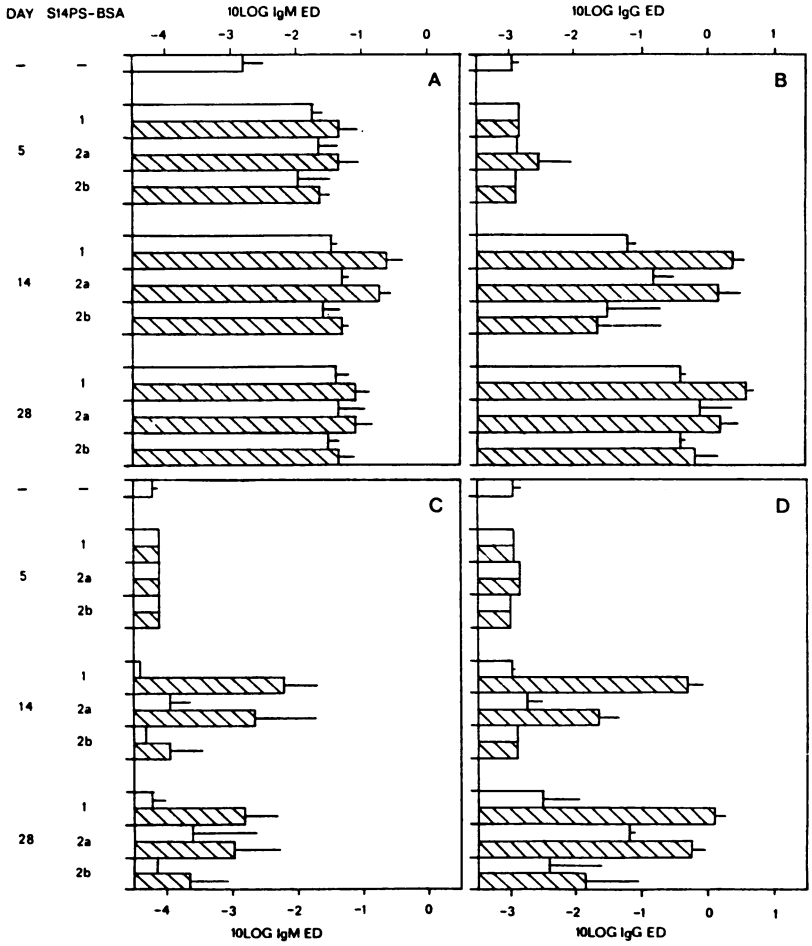

FIG. 5. Modulation of the immune response to S14PS-BSA conjugates in (CBA/N × BALB/c)F_1 mice by Quil A. Male (C and D) and female (A and B) (CBA/N × BALB/c)F_1 mice (n = 5) were immunized i.c. with 100 μl of S14PS-BSA(1), 100 μl of S14PS-BSA(2a), or 100 μl of S14PS-BSA(2b). Other groups of male and female mice were injected i.c. with the same S14PS-BSA conjugates mixed with 20 μg of Quil A. A booster injection with antigen and adjuvant was given at day 21. IgM (A and C) and IgG (B and D) ED values were determined at days 5, 14, and 28 by ELISA. ED values at day 0 indicate preimmune levels of IgM and IgG. Results are expressed as means ± standard deviation. Symbols: ▨, conjugate + Quil A; □, conjugate alone.

comparable with those obtained in the female BALB/c mice (Fig. 4 and 5A and B). In the male offspring, levels of antibody to S14PS-BSA(1) were greatly elevated by the addition of Quil A (160-fold for IgM and 340-fold for IgG on day 14) while the immune response to the S14PS-BSA(2a) conjugate was only moderately enhanced (20- and 13-fold increase for IgM and IgG, respectively, on day 14). Moreover, male (CBA/N × BALB/c)F_1 mice given booster injections of S14PS-BSA(1) plus Quil A displayed a significantly higher IgG antibody titer than did the female offspring immunized with the conjugate alone ($P < 0.001$).

Quil A, however, did not augment the immune response to all the S14PS-BSA conjugates. No enhancement of the immune response to the S14PS-BSA(2b) conjugate was observed in either male or female (CBA/N × BALB/c)F_1 mice.

DISCUSSION

S14PS displays all the characteristics of a thymus-independent type 2 antigen. Primary immunization did not result in memory induction (Fig. 2), and no immune response was observed in the male (CBA/N × BALB/c)F_1 mice (Fig. 3B and D), while IgM and IgG antibodies were detected in the female control group (Fig. 3A and C). In contrast, only an IgM response was observed in the BALB/c mice. The data presented seemed to suggest that the IgG response to S14PS is mouse strain dependent, but control experiments revealed that the observed difference could be attributed to the use of different batches of S14PS, containing probably slightly different amounts of contaminants, for the immunization of the two mouse strains (data not shown).

To use S14PS as a vaccine, the immunogenicity of this antigen should be improved (10). Coupling of polysaccharides to proteins changes the nature of this type of antigen to thymus dependent, which results after immunization in memory induction and the ability to evoke an immune response in young children (12, 17, 23, 24, 28). Various coupling procedures are now available, and an often used method is water-soluble-carbodiimide-mediated condensation (1, 23, 24, 28). The overall yield of this method, however, can be severely limited by the hydrolysis of the activated intermediate, but it is possible to enhance the efficacy of the procedure by using sulfo-NHS (25).

From the same batch of S14PS-AH we prepared S14PS-BSA conjugates with and without the use of sulfo-NHS. Three conjugates were obtained, all of which had different polysaccharide/protein ratios (Table 1). As shown by sugar analysis, the monosaccharide compositions of S14PS-BSA (1) and S14PS-BSA(2a) were similar to the composition of the native polysaccharide, while a difference was observed for S14PS-BSA(2b) (Table 1). This change in monosaccharide composition is a result of the sulfo-NHS/EDC-mediated coupling because the same batch of S14PS-AH was used for the preparation of the three conjugates.

The immunogenicities of these conjugates were comparable in the female BALB/c mice and in the female (CBA/N × BALB/c)F_1 mice (Fig. 4 and Fig. 5A and B). After the mice were given a booster injection, a significant but sometimes slight increase in IgG antibody titer was induced by all the conjugates. Moreover, the conjugates induced significantly higher IgG antibody titers as compared with S14PS. In contrast, only S14PS-BSA(2a) elicited a substantial IgG antibody titer in the male (CBA/N × BALB/c)F_1 mice, while only minor amounts of IgM and IgG antibodies were induced by the two other conjugates (Fig. 5C and D). These results indicate that the use of sulfo-NHS enabled us to prepare a conjugate with a high S14PS/protein ratio [S14PS-BSA(2a)], which induced a strong immune response.

The difference between the immunological properties of the conjugates can probably be attributed to different S14PS/protein ratios, but factors such as the degree of cross-linking and the solubility might also play a role (27, 31). S14PS-BSA(2b), which was obtained as a sediment, was less soluble than the other conjugates, and the change in monosaccharide composition (Table 1) could indicate a greater degree of cross-linking.

The adjuvant Quil A is a semipurified saponin that is used for constructing ISCOMs (20, 21). It consists of two polysaccharide chains linked to titerpenoid quillaic acid. The cage-like globular structure of an ISCOM is based on a hydrophobic interaction between antigen and adjuvant, and it contains about 0.5 μg of Quil A. No adverse side effects have been reported after the use of ISCOMs in mice, rats, rabbits, and monkeys (21). Therefore, it is regarded as a candidate for use in human vaccines (21). The mode of action of Quil A in an ISCOM or merely in a mixture with an antigen is not well known.

Localization and improved presentation of the antigen seemed to play a role in the immune response enhancement by Quil A, but direct interaction between Quil A and cellular components of the immune system has also been suggested as a possible mechanism (6, 8, 9, 20, 21). Quil A stimulates the immune response to both thymus-dependent and thymus-independent antigens but, similar to other adjuvants, cannot induce an IgG memory response to thymus-independent antigens (8, 9). Furthermore, no immune response is elicited in male (CBA/N × BALB/c)F_1 mice after immunization with a thymus-independent type 2 antigen (2,4,6-trinitrophenyl-Ficoll) plus Quil A (8, 9).

Our experiments confirm these results. Quil A enhanced the immune response to the thymus-independent type 2 antigen S14PS in female BALB/c mice (Fig. 2) and in female (CBA/N × BALB/c)F_1 mice (Fig. 3A and C), while no antibodies were detected in the male (CBA/N × BALB/c)F_1 mice (Fig. 3B and D). Furthermore, no IgG memory response was demonstrated. The immune response to most of the S14PS-BSA conjugates was also augmented by Quil A, but some interesting differences were observed (Fig. 4 and 5). Levels of antibody to S14PS-BSA(1) were greatly elevated, yet those to S14PS-BSA(2a) were elevated only moderately. No enhancement occurred after immunization with S14PS-BSA(2b) plus Quil A. The differences were even more pronounced in male (CBA/N × BALB/c)F_1 mice (Fig. 5C and D). These results suggest that there is a relationship between the adjuvant effect of Quil A and the way a conjugate was prepared. We hypothesized that, when Quil A is not used in an ISCOM, the adjuvant effect is dependent on a hydrophobic interaction between antigen and adjuvant. The prepared conjugates differed in the polysaccharide/protein ratio, solubility, monosaccharide composition, and probably degree of cross-linking (Table 1). A higher degree of cross-linking might result in a conjugate with less hydrophobic regions and thus a decreased ability to interact with Quil A. Therefore, the observed differences in immunostimulation of the conjugates by Quil A could be due to a different degree of cross-linking of the conjugates.

In conclusion, the use of sulfo-NHS improves the immunogenic properties of the S14PS-BSA conjugates. Furthermore, Quil A enhances the immune response both to the thymus-independent type 2 antigen S14PS and to most S14PS-BSA conjugates. There seems to be a relationship

between the adjuvant effect of Quil A and the way a conjugate is prepared. This could have consequences for the construction of ISCOMs based on polysaccharide- or oligosaccharide-protein conjugates.

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