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Immunogenicity of *Streptococcus pneumoniae* Type 14 Capsular Polysaccharide: Influence of Carriers and Adjuvants on Isotype Distribution

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This project investigated the effects of novel carriers and adjuvants on the isotype of murine immunoglobulin G (IgG) antibody to pneumococcal capsular polysaccharide type 14 (S14PS). S14PS conjugated to bovine serum albumin induced a weak antibody response which was 100% IgG1 following injection without adjuvant. The same polysaccharide conjugated to flagella of *Salmonella typhi* induced an antibody response which was 88% IgG3. S14PS-bovine serum albumin was injected with block copolymer L121 or Quil A in squalane-in-water emulsions. The copolymer L121 was at least as effective as Quil A or complete Freund adjuvant in inducing IgG antibodies. IgG1 was the dominant subclass for all. Addition of monophosphoryl lipid A, but not the threonyl derivative of muramyl dipeptide or nontoxic *Rhodopseudomonas sphaeroides* lipopolysaccharide, to copolymer L121 increased production of the IgG2a, IgG2b, and IgG3 subclasses. S14PS-flagella with copolymer L121 induced higher titers with a markedly altered isotype distribution: 13% IgG1, 52% IgG2a, 6% IgG2b, and 29% IgG3. Monophosphoryl lipid A added to L121 reduced IgG1 antibody to 5%, but increased IgG2a antibody to 14%, IgG2b antibody to 3%, and IgG3 antibody to 78%. These studies demonstrate that both the carrier and the adjuvant can influence the titer and isotype distribution of antipolysaccharide antibody responses.

Polysaccharides of *Streptococcus pneumoniae* are thymus-independent type 2 (TI-2) antigens (27). They stimulate mainly immunoglobulin M (IgM) antibody with weak memory and readily induce tolerance (26). Thymus-dependent immune responses, in contrast, are characterized by high-titer IgG antibody synthesis and memory. Such responses to TI-2 antigens can be produced by conjugating the antigens to protein carriers (2). The immunogenicity of pneumococcal immunogens made by conjugation of whole polysaccharides or oligosaccharides to protein carriers has been extensively studied (29, 37, 39). However, the effect of various carriers and adjuvants on the antibody isotype has received little attention.

This is potentially important because the isotypes of IgG antibody differ in protective efficacy for many infections (11, 30, 34). The IgG2a and IgG3 isotypes have been reported to be particularly effective in conferring protection against *S. pneumoniae* (7–9). We have demonstrated recently that the isotype of antibody produced by hapten or peptide conjugates is influenced by the carrier. Carrier proteins commonly used for immunological studies (bovine serum albumin [BSA], tetanus toxoid, diphtheria toxoid, and keyhole limpet hemocyanin) tend to induce antibody predominantly of the IgG1 isotype in mice (1, 29). Salmonella flagellum was found to be a particularly effective carrier for inducing antibody of the IgG2a and IgG2b isotypes of hapten and peptide conjugates (17, 20).

Adjuvants also influence the antibody isotype. We studied two types of adjuvants, block copolymers and nontoxic forms of lipopolysaccharides (LPS) of gram-negative bacteria, which act via different mechanisms (15, 16, 18, 19, 33). Block copolymers are nonionic surface-active agents composed of hydrophilic polyoxyethylene and hydrophobic polyoxypropylene. Their adjuvant activity correlates with an ability to concentrate antigen and host proteins on hydrophobic surfaces, where they are effectively presented to cells of the immune system (15, 16, 19). LPS serves as an immunomodulator. It stimulates diverse cells and mediator systems. It is an effective adjuvant, but is toxic (23, 36). Recently, several nontoxic forms of LPS have been described. These include the LPS of *Rhodopseudomonas sphaeroides*, which is almost completely nontoxic (28), and monophosphoryl lipid A (MPL) (3, 4). We demonstrated recently that block copolymer adjuvants in combination with nontoxic forms of LPS can increase the production of antibody of the IgG2 isotypes while decreasing IgG1 following immunization with conjugates of a hapten or peptide on either BSA or flagella as the carrier (17, 20).

The goal of this project was to investigate the effects of BSA and flagellum carriers together with selected adjuvants and immunomodulators on the intensity, isotype, and avidity of the immune response to the pneumococcal capsular polysaccharide, type 14 (S14PS). Conjugates were injected with complete Freund adjuvant (CFA) or emulsions containing Quil A or copolymer L121. Quil A has been reported to be an effective adjuvant for protein-polysaccharide conjugates (37). CFA is the "gold standard" of adjuvant potency. Copolymer L121 in oil-in-water emulsions is the most extensively studied block copolymer adjuvant formulation (15, 16, 19). L121 was used by itself and in combination with *R. sphaeroides* LPS, MPL, and the threonyl derivative of muramyl dipeptide (tMDP). The results demonstrate that the titer, isotype, and avidity of antibody responses to polysaccharide antigens can be modulated by carriers and adjuvants which are potentially usable in humans.

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MATERIALS AND METHODS

Preparation of polysaccharide conjugates. S14PS was purified from *S. pneumoniae* as described previously by Verheul et al. (37). It was activated with cyanogen bromide (Sigma Chemical Co., St. Louis, Mo.), and amino groups were added with adipic acid dihydrazide (Sigma) as described previously (29). The chemically modified S14PS (S14PS-AH) was dialyzed against 0.2 M NaCl and fractionated on a Bio-Gel P2 column (30 by 3.6 cm; Bio-Rad Laboratories, Richmond, Calif.). The polysaccharide and amino contents of each fraction were measured by using the Dubois and trinitrobenzenesulfonic acid (TNBS) assays (10, 12). Fractions containing both components were pooled and lyophilized.

The S14PS-BSA conjugate was then prepared by the carbodiimide method (2): 20 mg of S14PS-AH and 20 mg of BSA in 2 ml of distilled water were reacted with 0.1 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. The reaction mixture was stirred for 3 h at 4°C and pH 4.9 and dialyzed overnight against 0.2 M NaCl (pH 7.0). The S14PS-BSA was purified over a Sepharose CL-6B column (30 by 3.6 cm) in 0.2 M NaCl. Characterization of the S14PS-BSA conjugate was described previously (37). Briefly, the carbohydrate content was measured by the method of Dubois et al. (12), the monosaccharide composition was measured by the method of Kamerling et al. (21), and the protein content was determined by the Lowry assay. The S14PS/protein ratio was 0.85 (wt/wt), with 17.6 mg of polysaccharide and 20.8 mg of protein per 100 ml. The monosaccharide content of the conjugate was similar to that of the original polysaccharide (Gal-Glc-GlcNAc = 2.0:1.2:0.8 in the original and 2.0:1.0:1.0 in the conjugate).

In a cross-linking reaction between a protein and polysaccharide, it is possible that either one will preferentially cross-link to itself. The reaction conditions were developed to minimize this possibility and ensure uniform cross-linking. Polymers of BSA smaller than 12 to 15 monomers were removed by the Sepharose column. Larger monomers are extremely unlikely under the conditions used. Coupling of polysaccharide to polysaccharide during the derivatization procedure was minimized by use of excess amounts of adipic acid dihydrazide (ADH). Finally, free amino groups are incorporated at multiple sites on the S14PS molecule. Even after coupling of S14PS to S14PS, free amino groups on this complex will be available for coupling to proteins. Therefore, free S14PS (e.g., to which no BSA is coupled), will be present in the conjugates only in very low amounts, if at all. The coupling procedure leads to latticelike conjugate; e.g., more S14PS molecules and BSA molecules are present in one conjugate molecule, and they are coupled on multiple sites.

Preparation and conjugation of *Salmonella typhi* flagella. Flagella from *S. typhi* (strain TY2, type 29; American Type Culture Collection) were prepared as described previously (20). To prepare S14PS-conjugated flagella, 20 mg of lyophilized S14PS-AH was added to 2 ml (4 mg) of the flagellum preparation, which was reacted with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide as described for BSA. A precipitate formed during the reaction. Following dialysis against 0.2 M NaCl (pH 7.0) and centrifugation, the sediment was dialyzed against distilled water for 48 h and lyophilized. The supernatant was centrifuged at 160,000 × *g* (L8-70M ultracentrifuge; Beckman Instruments, Palo Alto, Calif.) for 30 min in an SW-50.1 rotor. The pellet was resuspended in phosphate-buffered saline (PBS) and lyophilized. The pellet and sediment had similar S14PS/protein ratios (1.1 and 1.4, respectively), whereas the supernatant had a much lower ratio (0.2). In preliminary experiments, the supernatant fraction was poorly immunogenic. The sediment fraction gave somewhat higher titers of total IgG than the pellet fraction. Thus, the sediment fraction was used for all of our experiments. The monosaccharide composition of the sediment was Gal-Glc-GlcNAc = 2.0:1.34:1.27, indicating a higher degree of cross-linking than in the S14PS-BSA conjugate.

Emulsion preparation and immunization. Outbred female 6-week-old ICR mice were obtained from Charles River Laboratories, Raleigh, N.C., and used at 7 to 10 weeks of age. Groups of mice (five or six per group) were injected subcutaneously with 100 μg of conjugate in 100 ml of adjuvant emulsion divided equally between the hind footpads.

The first experiment compared CFA (60% oil; GIBCO Laboratories, Chagrin Falls, Ohio), Quil A (20 μg in 2% squalane-in-saline emulsion; Superfos, Vedbaek, Denmark), and nonionic block copolymer L121 (poloxamer 401; 100 μg in 2% squalane-in-saline emulsion (CytRx Corp., Norcross, Ga.). Subsequent experiments compared the efficacy of L121 in 2% squalane-in-saline emulsions with the following immunomodulators: tMDP (gift of Anthony Allison of Syntex Corp., San Francisco, Calif.); MPL (Ribi Immunochemical, Hamilton, Mont.); *R. sphaeroides* LPS (provided by Kuni Takayama, Veteran's Administration Hospital, Madison, Wis.). MPL, tMDP, or *R. sphaeroides* LPS was used in a dose of 100 μg. In experiments with L121 or Quil A, the adjuvant was emulsified in the oil phase before addition of saline. The volume of each injection was adjusted to 100 ml with PBS containing 2% Tween 80 (Sigma). Secondary immunizations were given with the homologous antigen (100 μg) in the same type of adjuvant emulsion as the primary immunization except when the first injection contained L121 with another adjuvant. In that case, secondary immunizations used antigen in emulsions containing only L121 adjuvant. Mice were bled through the retro-orbital plexus into heparinized tubes (Natelson Capillary Tubes; Scientific Products, McGaw Park, Ill.). Plasma was stored at −70°C.

ELISAs for antibodies to S14PS (IgM, total IgG, and IgG isotypes). Antibodies to S14PS were measured by an avidin-biotin enzyme-linked immunosorbent assay (ELISA) by the method of Sutton et al. with modifications (31, 38). S14PS was biotinylated by dissolving 10 mg of S14PS-AH per ml in PBS. A 30-fold molar excess of *N*-hydroxysuccinimide-biotin (Sigma) was added in 1 ml of dimethyl formamide (pH 7.0), and the mixture was stirred overnight at 4°C. The biotinylated S14PS was purified by dialysis against 0.2 M NaCl and passage through a Bio-Gel P2 column. Fractions were assayed for polysaccharide by the Dubois method and for free and bound *N*-hydroxy-succinimide-biotin by thin-layer chromatography (25). Fractions containing polysaccharide-biotin and no *N*-hydroxysuccinimide-biotin were pooled and lyophilized. The ratio of biotin to polysaccharide, as calculated on the basis of amino group content of the S14PS-AH, was 110 nmol of biotin per mg of S14PS.

Polyvinyl chloride 96-well microtiter plates (Linbro Chemical Company, New Haven, Conn.) were coated overnight at room temperature with 4 μg of affinity-purified egg white avidin (Sigma) per ml in 0.1 M carbonate buffer (pH 9.6) containing 0.1% sodium azide. Plates were washed three times with 150 ml of PBS (pH 7.3 to 7.4) containing 0.05% pluronic F68 (BASF Wyandotte Corp., Wyandotte, Mich.). All subsequent washes were in this medium. A 1.0-μg

TABLE 1. Effect of L121, Quil A, and CFA on immunogenicity of S14PS-BSA conjugates in groups of five or six mice

Adjuvant	Mean antibody titer ± SE			
	IgM		IgG	
	Primary[a]	Secondary[b]	Primary	Secondary
None	344 ± 87	1,097 ± 269	404 ± 79	1,560 ± 295
Quil A	2,796 ± 125	6,245 ± 2,852	4,082 ± 1,704	15,014 ± 4,712
L121	1,905 ± 315	5,910 ± 677	7,782 ± 2,598	25,257 ± 6,597
CFA	1,982 ± 1,125	4,966 ± 2,416	2,994 ± 1,120	20,981 ± 7,954

[a] Primary sera were collected at 4 weeks.
[b] Secondary sera were collected at 6 weeks, 2 weeks after boost.

amount of S14PS-biotin in 100 μl of saline containing 0.05% F68 was added, and the plates were incubated for 1 h at 37°C and washed three times. Serum dilutions (in PBS with 0.05% F68 and 1% BSA, pH 7.3) were added, and the plates were incubated for 2 h on a shaker table (Fisher Scientific) at 200 rpm and room temperature. The first dilution was 1:40 for sera from primary immunizations or 1:80 for sera from secondary immunizations, followed by serial threefold dilutions. Sera from individual animals were tested separately, and data are expressed as means ± standard errors, unless otherwise stated. A reference serum containing IgM and IgG antibodies to S14PS was included in each run. This was a pooled hyperimmune antiserum to S14PS-BSA obtained from BALB/c mice injected with S14PS-BSA with or without Quil A.

After the washing, affinity-purified goat anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3, and IgM horseradish peroxidase-labeled antibodies (Fisher Scientific Southern Biotech, Orangeburg, N.Y.) were added and plates were incubated for 90 min at 37°C (or at room temperature for anti-IgM). On the basis of preliminary experiments, antisera to IgG, IgG1, IgG2a, IgG2b, and IgM were diluted 1:2,000 and antisera to IgG3 were diluted 1:1,000 in the same diluent as serum. After four washings, plates were incubated with *o*-phenylenediamine dihydrochloride (Sigma) in citrate buffer (pH 5.0) with 0.009% (vol/vol) of 30% hydrogen peroxide (Sigma) for 15 min. The enzyme reaction was terminated by adding 2.5 M H_2SO_4. Plates were read at 490 nm, using an automated microplate reader (model 3550; Bio-Rad). Titers were calculated by regression analysis, using the dilution resulting in an absorbance value of 1 (Microplate Manager application; Bio-Rad).

Calibration of goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 conjugates. IgG isotype values are frequently expressed simply as end-point ELISA titers. To improve the accuracy and reproducibility of this assay, we used purified myeloma proteins to determine the capacity of each peroxidase-labeled isotype-specific antiserum to bind the appropriate antigen. We also evaluated the specificity of each antiserum with IgG1, IgG2a, IgG2b, and IgG3 myeloma proteins. Isotypes were quantitated by converting the end-point ELISA titers to nanograms per milliliter by multiplying the titer of each subclass by the appropriate conversion factor, using the method of Kenney et al. (22). The conversion factors were as follows: IgG1, 7.68; IgG2a, 17.65; IgG2b, 4.82; IgG3, 10.82.

RESULTS

Effect of CFA, Quil A, and L121 on immunogenicity of S14PS-BSA. Mice were immunized with 100 μg of S14PS-BSA by itself or in emulsions containing Quil A, CFA, or L121. Without adjuvant, S14PS-BSA induced low titers of both IgM and IgG antibodies, which were increased three- to fourfold after the booster injection (Table 1). The adjuvants, CFA, Quil A, and L121, produced comparable results with S14PS-BSA. They stimulated 5.5- to 8-fold increases in primary IgM antibody and 7.4- to 19-fold increases in IgG antibody. Secondary IgG antibody titers were 9.6- to 16-fold higher with the adjuvants. L121 produced somewhat stronger IgG responses than did CFA or Quil A.

The isotype distribution of the IgG antibodies to S14PS was measured by using a calibrated ELISA. Within the limits of detection, S14PS-BSA alone produced 100% IgG1 after primary immunization (Fig. 1A). With adjuvants, the predominant isotype remained IgG1, but each of the other isotypes was also represented. L121 induced the highest proportions of IgG2a and IgG2b antibodies to S14PS (32%), followed by Quil A (25%) and CFA (<10%). The patterns were similar after secondary immunization (Fig. 1B).

Effects of selected immunomodulating agents in combination with L121. Mice were immunized with S14PS-BSA prepared in emulsions with copolymer L121 alone or in combination with MPL, *R. sphaeroides* LPS, or tMDP and boosted with antigen in L121. Addition of the immunomodulating agents to L121 had little effect on the IgM response. Primary titers ranged from 0.94- to 2.4-fold that of antigen with L121 alone, and secondary titers ranged from 1.0- to 1.5-fold that of L121 (Table 2). The effects on IgG antibody were more pronounced. During primary immunization, addition of either *R. sphaeroides* LPS or tMDP decreased primary titers compared with those obtained using antigen with L121 alone, while MPL increased titers modestly, 1.5-fold. Immunomodulators caused an even greater reduction in antibody titers following secondary injection. All groups which had initially been immunized with immunomodulators, L121, and antigen had lower IgG antibody titers after secondary immunization with antigen in an L121 emulsion than those which had received antigen and L121 alone.

The effects of immunomodulators on the isotype of IgG antibody produced in response to S14PS-BSA are shown in Fig. 2. Reduction in total IgG antibody response brought about by the immunomodulators was due largely to a reduction in the IgG1 isotype. The concentration of IgG1 antibodies was greatest in mice immunized with antigen in L121. The addition of MPL, *R. sphaeroides* LPS, or especially tMDP reduced the concentration of IgG1 antibody. All three immunomodulating agents increased proportions or, in the case of MPL, absolute amounts of IgG3 antibody. The production of IgG2a and IgG2b antibody was reduced by tMDP and *R. sphaeroides* LPS, but was not significantly changed by MPL.

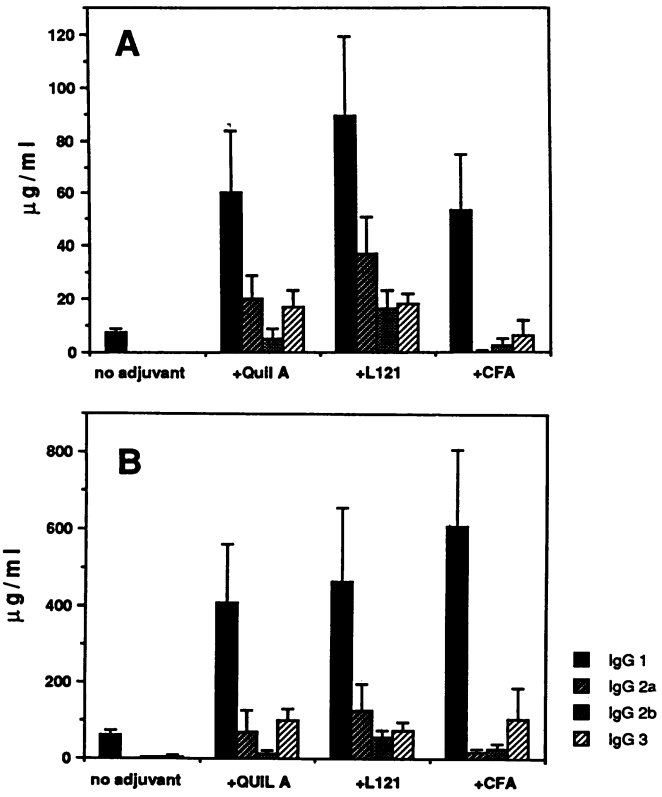

FIG. 1. Effect of L121, Quil A, and CFA on IgG isotype distribution of antibodies to S14PS induced by S14PS-BSA conjugates. Animals were immunized with S14PS-BSA alone or in adjuvant emulsions. Primary sera were collected at 4 weeks, and the animals were boosted with S14PS-BSA in the homologous adjuvant. Secondary sera were collected at 6 weeks. Concentrations of anti-S14PS of each isotype were measured in individual sera, using the calibrated avidin-biotin S14PS ELISA. Results are expressed as means ± standard errors (n = 5 or 6 per group) (A) Results of primary immunization; (B) results of secondary immunization. Symbols: ■, IgG1; ▨, IgG2a; ▩, IgG2b; ▨, IgG3.

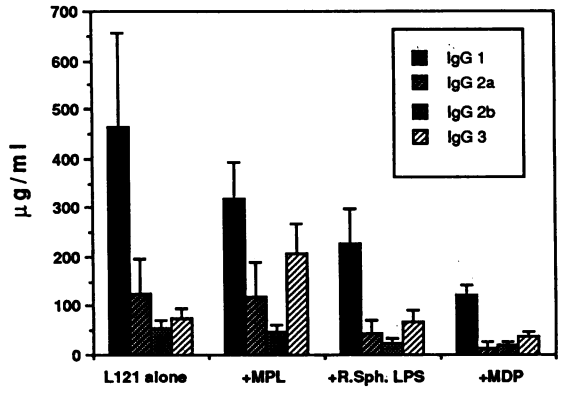

FIG. 2. IgG isotype responses to S14PS-BSA conjugates injected in adjuvant emulsions of copolymer L121 with or without the immunomodulators MPL, *R. sphaeroides* LPS, and tMDP. Groups of five or six mice were immunized with S14PS-BSA in L121 adjuvant emulsions with or without MPL, *R. sphaeroides* LPS, or tMDP. The animals were boosted with S14PS-BSA in L121 adjuvant emulsions after 4 weeks. Data shown are for secondary sera collected at 6 weeks. Concentrations of anti-S14PS antibodies of each isotype were measured in individual sera, using the calibrated avidin-biotin S14PS ELISA. Results are expressed as means ± standard errors.

The persistence of the antibody responses of each isotype was also examined (Fig. 3). Antigen with L121 produced the highest prolonged IgG1 antibody response, with significant levels remaining for as long as 14 weeks. The IgG1 titers in the other groups also persisted at easily measurable levels, although the titers were lower. The patterns of IgG2a and IgG2b antibody responses were similar. Groups immunized with antigen in L121 with MPL produced the highest concentrations of IgG2a and IgG2b antibodies both before and after boost. These soon tapered but persisted for at least 14 weeks. The highest relative and absolute concentrations of IgG3 antibodies were produced by animals immunized with L121 and MPL.

Immunogenicity of S14PS-flagellum conjugates with L121 and immunomodulators. Mice were immunized subcutaneously with 100 μg of S14PS-flagellum conjugate with and without adjuvants and bled for antibody titers at intervals (Fig. 4). S14PS-flagella by itself produced low but persistent antibody titers. L121 adjuvant increased the titers approximately 10-fold, while L121 with MPL produced only a 4-fold increase in titer. Secondary immunization produced little increase in total IgG antibody, but the titers were nearly stable for as long as 7 weeks.

The isotype distribution of IgG antibody after primary and secondary immunization with S14PS-flagellum conjugates was very different from that seen with S14PS-BSA conjugates (Fig. 5). In the primary response, only a small proportion of the antibody to S14PS was IgG1. S14PS-flagella by itself induced predominantly IgG3 antibody. L121 shifted the response to predominantly IgG2a (52% of total antibody), followed by IgG3 (28%), IgG1 (8%), and IgG2b (6%). Although addition of MPL to L121 decreased total IgG anti-

TABLE 2. Effect of copolymer L121 with or without the immunomodulator MPL, *R. sphaeroides* LPS, or tMDP on antibody response to S14PS-BSA conjugates in groups of five or six mice

Adjuvant	Mean antibody titer ± SE			
	IgM		IgG	
	Primary[a]	Secondary[b]	Primary	Secondary
L121	1,905 ± 315	5,910 ± 1,677	7,782 ± 2,598	25,257 ± 6,597
L121 + MPL	4,677 ± 1,222	9,063 ± 2,905	12,113 ± 4,428	19,875 ± 5,319
L121 + LPS	3,988 ± 1,336	7,972 ± 2,214	5,480 ± 1,362	8,179 ± 2,531
L121 + tMDP	1,800 ± 595	6,063 ± 2,875	3,740 ± 1,166	6,379 ± 1,565

[a] Primary sera were collected at 4 weeks.
[b] Secondary sera were collected at 6 weeks, 2 weeks after boost.

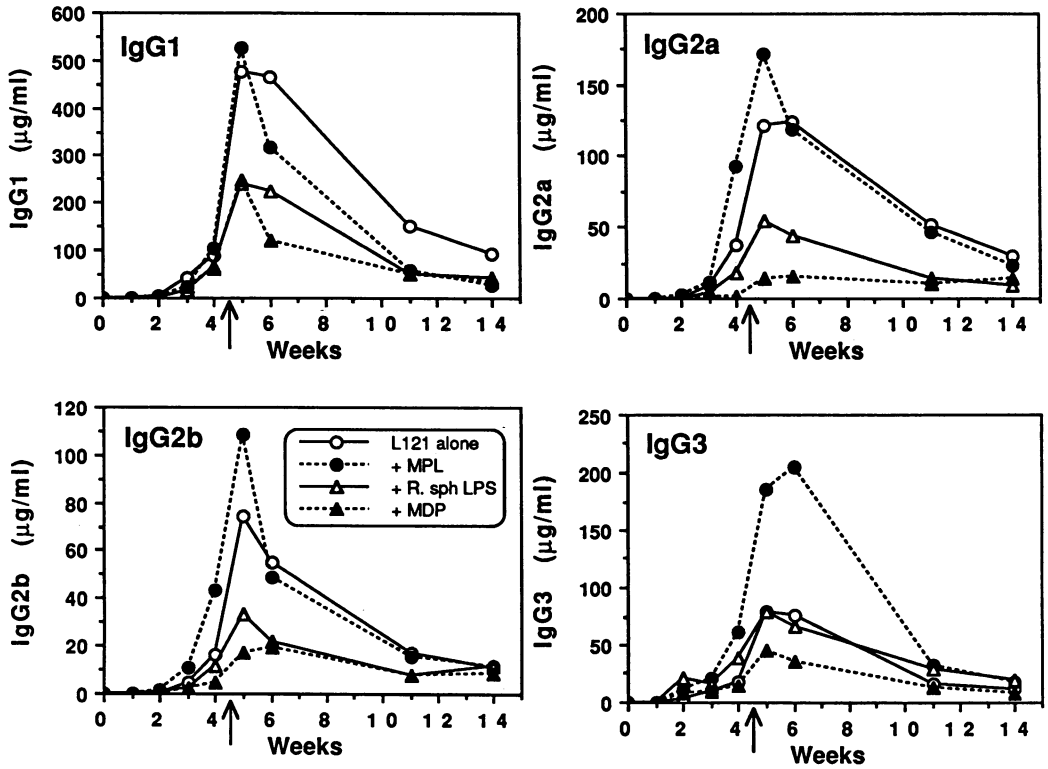

FIG. 3. Time course of IgG isotype response to S14PS-BSA conjugates injected in adjuvant emulsions of copolymer L121 with or without the immunomodulators. Groups of five to six mice were immunized with S14PS-BSA in L121 adjuvant emulsions with or without MPL, *R. sphaeroides* LPS, or tMDP and were boosted with S14PS-BSA in L121 adjuvant emulsions (arrow). Concentrations of anti-S14PS antibodies of each isotype were measured in individual serum samples collected at several time points. Results are expressed as means.

body production, it increased the concentration of IgG3 to over 70% of the total antibody to S14PS.

After secondary immunization with S14PS-flagella (alone for the no adjuvant group or with L121 for either adjuvant group), the isotype patterns shifted further (Fig. 5B). The group immunized with L121 showed an increase in IgG1 and IgG3 antibody but no change in IgG2a or IgG2b. The secondary response of animals which received MPL showed no change for IgG3, but an increased IgG2a and continued suppression of IgG1 antibody against S14PS.

Interestingly, the range of individual variability in antibody response to S14PS-flagella differed depending on the IgG isotype. Outbred mice were used to facilitate evaluation of the effects of the immunogen formulations on variability of responses. Figure 6 shows results at 4 weeks for individual mice immunized with L121. Note the broad range of IgG2a and IgG2b antibody concentrations in contrast to the clustered IgG1 and especially IgG3 antibody concentrations.

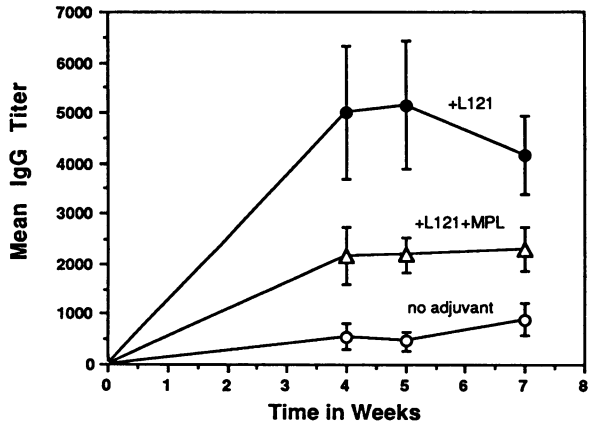

FIG. 4. Time course of IgG response to S14PS-flagellum conjugates injected alone or in adjuvant emulsions of copolymer L121 with or without MPL. Groups of mice were immunized with S14PS-flagella alone or in L121 adjuvant emulsions with or without MPL. Sera were collected, and the animals were boosted at 4 weeks with S14PS-flagella in L121 adjuvant emulsions. Concentrations of anti-S14PS IgG were measured in individual animals. Results are expressed as mean titers ± standard errors.

DISCUSSION

The present experiments demonstrate that modifications of both carrier and adjuvant profoundly influence the isotype of antibody to S14PS. The most important factor in determining antibody isotype was the carrier. Conjugation of S14PS to either BSA or flagella changed the S14PS-specific response from a mainly IgM response to a combined IgG and IgM response. This confirms the observation of others that protein-polysaccharide conjugates induce the diversified responses characteristic of proteins rather than the restricted responses of polysaccharides (1, 5, 24).

The S14PS-BSA conjugate by itself induced an almost pure IgG1 antibody response. Addition of adjuvants to S14PS-BSA resulted in much higher titers as well as induction of all IgG isotypes and IgM. Whether or not adjuvant was used, however, IgG1 antibodies predominated. The

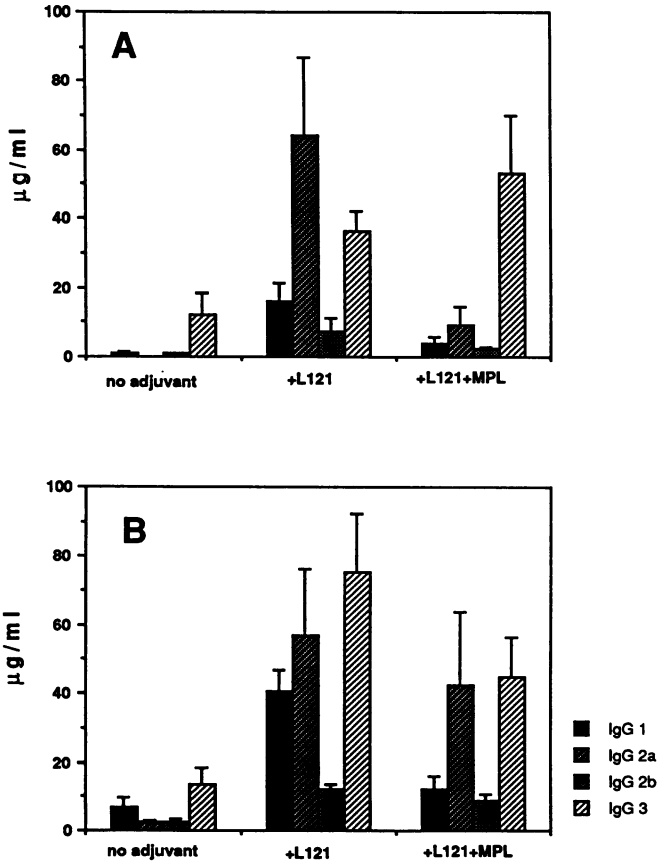

FIG. 5. IgG isotype response to S14PS-flagellum conjugates injected alone or in adjuvant emulsions of copolymer L121 with or without MPL. Groups of mice were immunized with S14PS-flagella alone or in L121 adjuvant emulsions with or without MPL. Primary sera were collected at 4 weeks (A), and the animals were boosted with S14PS-flagella in L121 adjuvant emulsions. Secondary sera were collected at 5 weeks (B). Concentrations of anti-S14PS antibodies of each isotype were measured in individual sera, using the calibrated avidin-biotin S14PS ELISA. Results are expressed as means ± standard errors. Symbols: ■, IgG1; ▨, IgG2a; ▩, IgG2b; ▨, IgG3.

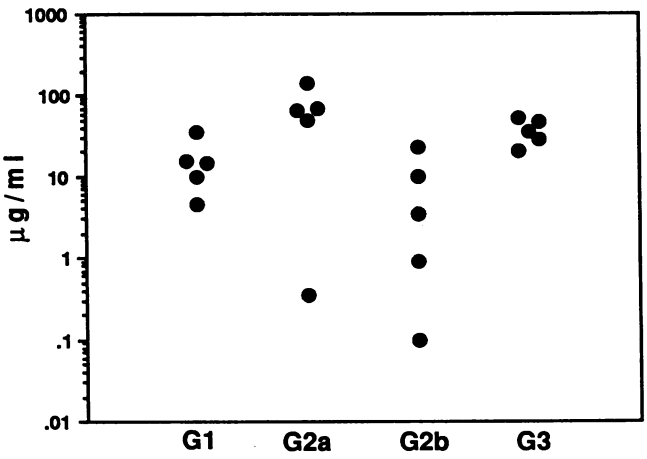

FIG. 6. Individual variability of IgG isotype response to S14PS-flagellum conjugates injected in adjuvant emulsions of copolymer L121. Groups of ICR mice were immunized with S14PS-flagella in L121 adjuvant emulsions. Concentrations of anti-S14PS antibodies of each isotype were measured at 4 weeks. Each point represents the value from a single animal.

copolymer L121 was marginally superior to Quil A and CFA. These results with BSA are typical of those reported with other thymus-dependent protein antigens such as egg albumin, keyhole limpet hemocyanin, and tetanus toxoid as carriers in that soluble thymus-dependent protein immunogens tend to induce a large proportion of IgG1 antibody (18, 33, 37).

S14PS-flagellum conjugates induced an isotype pattern different from that of S14PS-BSA. Without adjuvants, IgG3 antibodies predominated. Addition of L121 and MPL increased the titers of IgG3 and induced each of the other subclasses, whereas addition of L121 without MPL favored production of IgG2a antibodies. Similar effects of flagellum carriers on antibody of the IgG2a isotype were seen in studies with a malaria peptide as the antigen (20). However, a large increase in IgG3 antibody was not observed in previous studies. IgG3 is normally present only in very low concentrations and tends to be produced following immunization with polysaccharides, which are thymus-independent antigens (7–9).

Flagellum is an unusual protein carrier since it is able to function as both a thymus-independent and a thymus-dependent immunogen. It can stimulate B cells without the participation of T cells in a fashion typical of TI-2 immunogens, but also has T-cell epitopes and induces strong thymus-dependent responses (27). It is localized avidly in germinal centers, which are a site of B-cell differentiation (13, 27). It seems likely that these factors contribute to the unusual distribution of isotypes induced by flagella.

The observation that groups of mice immunized with S14PS-flagella did not contain any IgG3 nonresponders is interesting. The variability of responsiveness within groups was routinely examined by scattergrams in all experiments. Adjuvant formulations which increased titers almost always increased variability. The relative uniformity of the IgG3 responses to the flagellum conjugates was an exception. It might be of practical importance because this isotype has been reported to be highly protective against pneumococcal infections (7). Since IgG3 is induced by polysaccharides which are TI-2 immunogens, it seems likely that the thymus-independent properties of flagella also contributed to enhancement of IgG3 antibodies. The same factors might explain the uniformity of responsiveness of the IgG3 responses. Genetic variability of immune responses is frequently mediated by T cells (6).

The total IgG and IgM antibody titers induced by flagellum conjugates were lower than those induced by BSA conjugates. Our previous studies with the hapten trinitrophenol (TNP) found flagella to be potent carriers for inducing high and long-lasting titers (17). The lower titers observed in this system could be due to the size of the antigen (the polysaccharide S14PS is much larger than hapten), differences in the degree of cross-linking between antigen and carrier, the presence of small amounts of free polysaccharide, or other features of the conjugation procedure. In future studies, it will be necessary to evaluate thoroughly the properties of conjugates which contribute to modulation of isotype responses.

MPL, *R. sphaeroides* LPS, and tMDP are immunomodulators used to enhance antibody production. The suppression of antibody titers observed in these studies was unexpected. The suppression in total IgG antibody, however, was

due largely to suppression of the IgG1 isotype. In the case of MPL, this occurred at the same time IgG2a titers were being increased. A similar increase of IgG2a and IgG2b isotypes was observed previously with MPL and *R. sphaeroides* LPS in studies with hapten and peptide antigens conjugated to BSA (17, 20, 33). The mechanisms of these effects are not known. It is also known that LPS influences cytokine production by multiple types of cells and that cytokines influence the function of T-helper cells and antibody isotype selection (23). More specifically, it is suggested (35) that the adjuvant effect of MPL is mediated by gamma interferon. This could explain the suppression of the IgG1 isotype as well as the stimulation of the IgG2a isotype. Recent studies on the immunogenicity of S3PS have demonstrated that MPL inactivates suppressor T-cell activity (3) probably due to a process which starts off with the binding of MPL to activated suppressor T cells (4). Whether this also holds true for S14PS is under investigation. The effect of MPL on isotypic patterns after immunization with unconjugated S14PS will also be studied. The observation that MPL decreases the IgG1 response to the flagellum conjugates might also be explained by the addition of a secondary amphipatic structure (MPL), resulting in a modified antigen presentation or exposure.

Vaccine efficacy depends on not only the titer but also the class and isotype and avidity of the antibody. It has long been known that IgM antibody confers protection against pneumococci (14). Some controversy exists about which IgG isotypes contribute to protection against these infections. Briles et al. showed that IgG3 antibody is especially protective against pneumococcal infections in mice (7). In later studies, however, they showed that the other IgG isotypes can also be protective (8, 9). Studies on the role of various isotypes of antibody in protection have been hampered by the paucity of experimental models. It is anticipated that development of means of selectively stimulating antibody of particular isotypes will eventually provide improved models for characterizing protective immune responses as well as an improved rationale for production of more effective vaccines. Even though humans frequently differ in their response to antigens in comparison to mice, it seems likely that similar factors will influence the isotypes of antibodies in most species.

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REFERENCES

1. **Anderson, P., M. Pichichero, K. Edwards, C. R. Porch, and R. Insel.** 1987. Priming and induction of *Haemophilus influenzae* type b capsular antibodies in early infancy by Dpo 20, an oligosaccharide-protein conjugate vaccine. J. Pediatr. **111**:644–650.
2. **Aplin, J. D., and J. C. Wriston.** 1981. Preparation, properties and applications of carbohydrate conjugates of proteins and lipids. Crit. Rev. Biochem. **5**:259–307.
3. **Baker, P. J., J. R. Hiernaux, M. B. Fauntleroy, B. Prescott, J. L. Cantrell, and J. A. Rudbach.** 1988. Inactivation of suppressor T-cell activity by nontoxic monophosphoryl lipid A. Infect. Immun. **56**:1076–1083.
4. **Baker, P. J., J. R. Hiernaux, M. B. Fauntleroy, P. W. Stashak, B. Prescott, J. L. Cantrell, and J. A. Rudbach.** 1988. Ability of monophosphoryl lipid A to augment the antibody response of young mice. Infect. Immun. **56**:3064–3066.
5. **Barrett, D. J., C. G. Lee, A. J. Ammann, and E. M. Ayoub.** 1984. IgG and IgM pneumococcal polysaccharide antibody responses in infants. Pediatr. Res. **18**:1067–1071.
6. **Benoist, C., and D. Mathis.** 1990. Regulation of major histocompatibility complex class-II genes: X, Y, and other letters of the alphabet. Annu. Rev. Immunol. **8**:681–715.
7. **Briles, D. E., J. L. Claflin, K. Schroer, and C. Forman.** 1981. Mouse IgG3 antibodies are highly protective against infection with *Streptococcus pneumoniae*. Nature (London) **294**:88–90.
8. **Briles, D. E., C. Forman, J. C. Horowitz, J. E. Volanakis, Jr., W. H. Benjamin, L. S. McDaniel, J. Eldridge, and J. Brooks.** 1989. Anti-pneumococcal effects of C-reactive protein and monoclonal antibodies to pneumococcal cell wall and capsular antigens. Infect. Immun. **57**:1457–1464.
9. **Briles, D. E., C. Forman, S. Hudak, and J. L. Claflin.** 1984. The effects of subclass on the ability of anti-phosphocholine antibodies to protect mice from fatal infection with *Streptococcus pneumoniae*. J. Mol. Cell Immunol. **1**:305–309.
10. **Coon, J., and R. L. Hunter.** 1975. Properties of conjugated protein immunogens which selectively stimulate delayed-type hypersensitivity. J. Immunol. **114**:1518–1522.
11. **Coutelier, J. P., J. T. M. van der Logt, F. W. A. Heessen, A. Vink, and J. van Snick.** 1988. Virally induced modulation of murine IgG antibody subclasses. J. Exp. Med. **168**:2373–2378.
12. **Dubois, M., K. H. Gillis, J. K. Hamilton, A. A. Rebers, and R. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
13. **Hanna, M. G., and R. L. Hunter.** 1971. Localization of antigen and immune complexes in lymphatic tissue, with special reference to germinal centers, p. 257–279. *In* K. Lindahl-Kiessling, G. Alm, and M. G. Hanna (ed.), Morphological and fundamental aspects of immunity. Plenum Press, New York.
14. **Hirschmann, J. V., and B. A. Lipsky.** 1981. Pneumococcal vaccine in the United States. A critical analysis. J. Am. Med. Assoc. **246**:1428–1432.
15. **Hunter, R. L., and B. Bennett.** 1984. The adjuvant activity of nonionic block polymer surfactants. II. Antibody formation and inflammation related to the structure of triblock and octablock copolymers. J. Immunol. **133**:3167–3175.
16. **Hunter, R. L., and B. Bennett.** 1986. The adjuvant activity of nonionic block polymer surfactants. III. Characterization of selected biologically active surfaces. Scand. J. Immunol. **23**: 287–300.
17. **Hunter, R. L., B. Bennett, D. Howerton, S. Buynitzky, and I. J. Check.** 1989. Nonionic block copolymer surfactants as immunological adjuvants: mechanisms of action and novel formulations, p. 133–144. *In* G. Gregoriadis, A. C. Allison, and G. Poste (ed.), Immunological adjuvants and vaccines. Plenum Press, New York.
18. **Hunter, R. L., M. Olsen, and S. Buynitzky.** 1991. Adjuvant activity of nonionic block copolymers. IV. Effect of molecular weight and formulation on titer and isotype of antibody. Vaccine **9**:250–256.
19. **Hunter, R. L., F. Strickland, and F. Kezdy.** 1981. Studies on the adjuvant activity of nonionic block polymer surfactants. I. The role of hydrophile-lipophile balance. J. Immunol. **127**:1244–1250.
20. **Kalish, M. L., I. Check, and R. L. Hunter.** Murine IgG isotype responses to the Plasmodium cynomolgi circumsporozoite protein (NAGG)5. I. Effects of carrier, copolymer adjuvants, and nontoxic LPS on isotype selection. J. Immunol., in press.
21. **Kamerling, J. P., G. J. Gerwig, and J. P. F. Vliegenthart.** 1975. Characterization by gas liquid chromatography-mass spectrometry and proton magnetic resonance spectroscopy of pertrimethylsilyl methyl glycosides obtained in the methanolysis of glycoproteins and glycopeptides. Biochem. J. **151**:491–495.
22. **Kenney, J. S., B. W. Hughes, M. P. Masada, and A. C. Allison.** 1989. Influence of adjuvants on the quantity, affinity, isotype and epitope specificity of murine antibodies. J. Immunol. Methods **121**:157–166.
23. **Le Garrec, Y.** 1986. Immunomodifiers of bacterial origin. Comp. Immunol. Microbiol. Infect. Dis. **9**:137–141.

24. **Makela, O., P. Mattila, N. Rautonen, I. Seppala, J. Eskola, and Kayhty.** 1987. Isotype concentrations of human antibodies to *Haemophilus influenzae* type b polysaccharide (Hib) in young adults immunized with the polysaccharide as such or conjugated to a protein (diphtheria toxoid). J. Immunol. **139**:1999–2004.
25. **McCormick, D. B., and J. A. Roth.** 1970. Specificity, stereochemistry, and mechanism of the color reaction between P-dimethylamino-cinaldehyde and biotin analogs. Anal. Biochem. **34**:226–236.
26. **Perlmutter, R. M., D. Hansburg, D. E. Briles, R. A. Nicolotti, and J. M. Davie.** 1978. Subclass restriction of murine anti-carbohydrate antibodies. J. Immunol. **121**:566–572.
27. **Pike, B. L., and G. J. V. Nossel.** 1984. A reappraisal of "T-independent" antigens. I. Effects of lymphokines on the response of single adult hapten-specific B lymphocytes. J. Immunol. **132**:1687–1695.
28. **Salimath, P. V., J. Weckesser, W. Strittmatter, and H. Mayer.** 1983. Structural studies on the non-toxic lipid A from *Rhodopseudomonas sphaeroides* ATCC 17023. Eur. J. Biochem. **136**: 195–200.
29. **Schneerson, R., J. B. Robbins, J. C. Parke, C. Bell, J. J. Schlesselman, A. Sutton, Z. Wang, G. Schiffman, A. Karpas, and J. Shiloach.** 1986. Quantitative and qualitative analyses of serum antibodies elicited in adults by *Haemophilus influenzae* type b and pneumococcus type 6A capsular polysaccharide-tetanus toxoid conjugates. Infect. Immun. **52**:519–528.
30. **Spiegelberg, H. L.** 1974. Biologic activities of immunoglobulins of different classes and subclasses. Adv. Immunol. **19**:259–294.
31. **Sutton, A., W. F. Vann, A. B. Karpas, K. E. Stein, and R. Schneerson.** 1985. An avidin-biotin based ELISA for quantitation of antibody to bacterial polysaccharides. J. Immunol. Methods **82**:215–224.
32. **Tai, J. Y., P. P. Vella, A. A. McLean, A. F. Woodhour, W. J. McAleer, A. Sha, C. Dennis-Sykes, and M. R. Hilleman.** 1987. *Haemophilus influenzae* type b polysaccharide-protein conjugate vaccine. Proc. Soc. Exp. Biol. Med. **184**:154–161.
33. **Takayama, K., M. Olsen, P. Datta, and R. L. Hunter.** 1991. Adjuvant activity of nonionic block copolymers. V. Modulation of antibody isotype by lipopolysaccharides, Lipid A and precursors. Vaccine **9**:257–265.
34. **Takehara, H., A. A. Perini, M. H. da Silva, and I. Mota.** 1981. *Trypanosoma cruzi* role of different antibody classes in protection against infection in the mouse. Exp. Parasitol. **52**:137–146.
35. **Tomai, M. A., and A. G. Johnson.** 1989. T cell and interferon-gamma involvement in the adjuvant action of a detoxified endotoxin. J. Biol. Response Modif. **8**:625–643.
36. **Ukei, S., J. Iida, T. Shiba, S. Kusumoto, and I. Azuma.** 1986. Adjuvant and antitumor activities of synthetic lipid A analogues. Vaccine **4**:21–24.
37. **Verheul, A. F. M., A. A. Versteeg, M. J. De Reuver, M. Jansze, and H. Snippe.** 1989. 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. Infect. Immun. **57**:1078–1083.
38. **Verheul, A. F. M., et al.** 1990. Measurement of the humoral immune response against Streptococcus pneumoniae type 14-derived antigens by an ELISA and ELISPOT assay based on biotin-avidin technology. J. Immunol. Methods **126**:79–87.
39. **Zigterman, G. J. W. J., K. Schotanus, E. B. H. W. Ernste, G. J. Van Dam, M. Jansze, H. Snippe, and J. M. N. Willers.** 1989. Nonionic block polymer surfactants modulate the humoral immune response against *Streptococcus pneumoniae*-derived hexasaccharide-protein conjugates. Infect. Immun. **57**:2712–2718.