INFECTION AND IMMUNITY, Mar. 1995, p. 961–968 Vol. 63, No. 3
0019-9567/95/$04.00+0

Synthetic Peptides Representing T-Cell Epitopes Act as Carriers in Pneumococcal Polysaccharide Conjugate Vaccines

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Received 1 April 1994/Returned for modification 16 May 1994/Accepted 17 December 1994

Improvement of antibody responses to polysaccharides through their linkage to proteins is thought to be mediated by protein-specific T helper (Th) cells. To investigate whether the carrier protein of a conjugate could be substituted by a Th epitope, *Streptococcus pneumoniae* type 17F polysaccharide (PS) was bromoacetylated and coupled to different peptides via their carboxy-terminal cysteines. Two peptides, one from the mycobacterial 65-kDa heat shock protein (hsp65) and the other from influenza virus hemagglutinin, are well-known Th epitopes. Two other peptides were selected from the pneumolysin sequence by Th epitope prediction methods; one of them was synthesized with cysteine either at the carboxy or the amino terminus. Three conjugates consistently elicited in mice anti-PS immunoglobulin M (IgM) and IgG responses that were not observed upon immunization with derivatized PS without peptide. The same conjugates induced no anti-PS antibody responses in athymic (*nu*/*nu*) mice, whereas clear responses were elicited in euthymic (*nu*/+) controls, demonstrating the thymus-dependent character of these conjugates. Only the three conjugates inducing anti-PS responses were capable of eliciting antipeptide antibodies. One of the immunogenic conjugates was studied in more detail. It induced significant protection and an anti-PS IgG response comprising all subclasses. On the basis of these results and proliferation studies with peptide and conjugate-primed cells, it is concluded that linkage of Th epitopes to PS in the right orientation enhances its immunogenicity in a thymus-dependent manner. Future possibilities for using peptides as carriers for inducing antibody responses to poorly immunogenic saccharide antigens are discussed.

Coupling of bacterial polysaccharides to carrier proteins improves the immunogenicity of the polysaccharide and results in antigens with novel characteristics. Polysaccharide-protein conjugates are thymus-dependent (TD) antigens, in contrast to polysaccharides, which are thymus independent. Therefore, they are immunogenic in infants, induce immunological memory, and elicit antibody responses in which the switch of B cells towards immunoglobulin G (IgG) production is favored (reviewed in reference 42). These features of conjugate vaccines are thought to be mediated by T cells, since nude mice do not mount a response with these characteristics but rather mount a typical thymus-independent response (10) or no response at all (4).

We tested the hypothesis that substitution of the carrier protein by a T helper (Th) epitope would result in a TD antigen. We used pneumococcal polysaccharide to which different peptides were coupled as a model to investigate the cellular mechanisms behind the immune response against conjugates. Polysaccharide-peptide conjugates have some practical advantages above polysaccharide-protein conjugates (34, 36) that may be especially attractive for pneumococcal vaccines. Because of the large number of pneumococcal serotypes to be included in a vaccine, the amount of carrier used should be limited to a minimum, sufficient to induce T-cell help yet small enough to avoid excessive production of anticarrier antibodies. These antibodies have been shown to suppress subsequent responses to the conjugates in mice (38) as well as in humans (6, 7). Suppression of immune responses by preexisting antibodies to the carrier is likely to become a problem in the future if the number of conjugate vaccines containing the same carrier protein (e.g., diphtheria or tetanus toxoids) keeps growing. The use of peptides as carriers also offers the possibility for selection of suitable Th epitopes, avoiding B-cell or T suppressor cell epitopes (16).

Coupling of a Th epitope to *Haemophilus influenzae* type b polysaccharide had previously been reported to induce higher antibody levels than unconjugated polysaccharide (11). We aimed to extend experiments to Th epitopes derived from other proteins and to analyze more extensively the characteristics of the conjugates and the induced immune responses.

Peptides to be coupled to polysaccharide were selected on the basis of their (potential) Th-cell-stimulating properties (Table 1). Peptides PNL, PNc, and cPN (from the pneumococcal toxin pneumolysin) (49) were selected by using methods for predicting Th-cell antigenicity (43, 46) and on the predicted cathepsin D cleavage sites in pneumolysin (48). Peptides HAG and HSP, from influenza virus hemagglutinin and from the 65-kDa heat shock protein (hsp65) of *Mycobacterium bovis*, respectively, are proven Th epitopes (12, 21, 23, 24). HSP is a dominant epitope of hsp65 in mice. This protein, strongly conserved among different species, is an important target of T-cell reactivity in humans, probably as a result of frequent exposure during infections (25), and is a candidate carrier for future vaccines (8, 13).

We have studied pneumococcal polysaccharide type 17F (PS), which is one of the serotypes included in the 23-valent pneumococcal vaccine. PS was bromoacetylated and coupled to peptides via an additional cysteine residue. This coupling

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TABLE 1. Characteristics of the peptides used in this study

Peptide	Protein	Amino acid no.	Amino acid sequence	Features in BALB/c ($H\text{-}2^d$) mice	Reference(s)
HSP	hsp65	H_2N-153-171-Cys-COOH	DQSIGDLIAEAMDKVGNEG-C	T epitope[a]	12
HAG	Hemagglutinin	H_2N-111-120-Cys-COOH	FERFEIFPKE-C	T epitope[b]	21–24
PNL	Pneumolysin	H_2N-123-140-Cys-COOH	VRGAVNDLLAKWHQDYGQ-C	Unknown	49
PNc	Pneumolysin	H_2N-263-281-Cys-COOH	FEALIKGVKVAPQTEWKQI-C	Unknown	49
cPN	Pneumolysin	H_2N-Cys-263-281-COOH	C-FEALIKGVKVAPQTEWKQI	Unknown	49

[a] Induces in vitro proliferation of peptide or hsp65-primed cells.
[b] Induces in vitro proliferation of cells that have been primed with influenza virus, influenza hemagglutinin, or HAG coupled to a protein or to other peptides but not of cells that have been primed with peptide alone.

method results in conjugates with only slight structural and antigenic differences from PS. The IgM and IgG (subclass) responses to the conjugates, as well as the capacity of the conjugates and the free peptides to prime for peptide-specific T-cell proliferation, were examined in normal and nude mice. Furthermore, the protective capacity of one of the conjugates was assessed by challenge experiments.

MATERIALS AND METHODS

Peptides. Peptides (Table 1) were synthesized (with a terminal cysteine to enable coupling) by E. Freund (Hubrecht Laboratory, Utrecht, The Netherlands) on a Biosearch SAM-2 synthesizer by the solid-phase method of Barany and Merrifield (5). For reduction to monomers, each peptide (20 mg) was dissolved in 1 to 2 ml of 0.5 M sodium phosphate buffer (pH 8.0) together with a 100-fold molar excess of dithiothreitol (Sigma Chemical Co., St. Louis, Mo.). The solution was stirred under N_2 for 2 h at room temperature, which was found to be sufficient for total reduction of disulfide bridges (assessed with HSP, using a Sephadex G-25 column). Peptides were separated from dithiothreitol with a Sephadex G-15 column (1.6 by 60 cm) equilibrated in 0.2 M acetic acid (method adapted from that of Lee et al. [33]). Fractions eluting in the void volume were pooled and lyophilized.

PS-peptide conjugates. PS was purified and amino groups were incorporated in it as described previously (3). The amount of amino groups introduced (29) was 88 nmol/mg of PS, which corresponds with 110 mmol of NH_2 per mol of PS-repeating units. Derivatized PS was bromoacetylated and purified by using a method originally described for proteins (9), but with a higher concentration (100-fold molar excess with regard to available amino groups) of the bifunctional crosslinker *N*-succinimidyl bromoacetate (Pierce Chemical Co., Rockford, Ill.). The efficiency of bromoacetylation, assessed by measuring the available amino groups (29), was 81%. Each lyophilized monomeric peptide was dissolved in a solution of bromoacetylated PS (3.5 to 4.0 ml; 2 mg of PS per ml in 0.1 M phosphate buffer [pH 6.1] containing 5 mM EDTA and 0.01% NaN_3) to reach a final molar ratio of 5:1 (peptide:amino groups originally present in PS). The solution was stirred for 48 h at room temperature under N_2. To block the remaining bromoacetyl groups present on PS, a 100-fold molar excess of β-mercaptoethanol was added, and the solution was stirred overnight at room temperature. As a control conjugate, we used bromoacetylated PS which was directly blocked with β-mercaptoethanol as described above but not coupled to any peptide (referred to as PS-βME). Conjugates were purified over a Sepharose CL-6B column (2.4 by 70 cm) equilibrated in 0.2 M NaCl. Fractions containing both PS and peptide, which eluted in the void volume, were pooled, extensively dialyzed against water, and lyophilized. Excess of free peptide and β-mercaptoethanol eluted from the column in a distinct peak after the conjugate. Stock solutions (1 mg/ml in saline) were kept at −20°C until use for immunizations. Conjugates were analyzed for PS content (15), and the amino acid composition was determined after 6 N HCl hydrolysis (24 h at 110°C) by cation-exchange chromatography in a fully automated system (LKB 4151 Alpha Plus amino acid analyzer [32]). *S*-Carboxymethylcysteine could not be detected, probably because of phosphate groups present in PS. Therefore, the coupling ratio was calculated on the basis of the amino acid content of the different conjugates (calculated by using norleucine as an internal standard) after subtraction of the amino acid content in PS-βME (Table 2).

Immunization with conjugates and challenge. Inbred 10- to 12-week-old female BALB/c ($H\text{-}2^d$), athymic BALB/c *nu*/*nu*, and euthymic heterozygote control (BALB/c *nu*/+) mice were supplied by Iffa-Credo (Someren, The Netherlands); they were maintained at the Central Laboratory of Experimental Animals (Utrecht University). Groups of five mice were immunized (day 0) subcutaneously in the neck with the conjugates (2.5 μg of PS per mouse) in combination with the adjuvant Quil A (see below). In previous experiments, the 2.5-μg PS dose was found to induce an optimal secondary anti-PS antibody response. At day 21, each mouse was given a booster of the same preparation used for primary immunization. Mice were bled at days 14, 28, and 35.

In another immunization experiment, one of the conjugates was studied in more detail. Female BALB/c mice (10 to 12 weeks of age) obtained from Charles River Laboratories (Wilmington, Mass.) and maintained at the National Institutes of Health (Bethesda, Md.) were immunized either with PS-cPN (2.5 μg of PS per mouse) in combination with various adjuvants (see below) or with plain PS (2.5 μg). Groups of five mice were immunized subcutaneously at two sites in the abdomen and received a booster at weeks 8 and 15. Sera were obtained at week 17, and 3 days later, mice were challenged intraperitoneally with 10^5 CFU (approximately 10 × 50% lethal doses) of *Streptococcus pneumoniae* 17F; survival was recorded for 15 days. The Wilcoxon two-tailed rank test was used to test the differences in survival time between groups immunized with PS-cPN and the control groups.

Adjuvant formulations. The adjuvants Quil A (Superfos Biosector, Vedbaek, Denmark) and Q-VAC (NOR-VET, Hvidovre, Denmark) are different saponin extracts from *Quillaia saponaria* bark. For each mouse, 20 μg of Quil A or 50 μg of Q-VAC, mixed with the antigen in saline, was used for immunization. RIBI adjuvant, containing monophosphoryl lipid A from *Salmonella minnesota* R595 (50 μg of monophosphoryl lipid A per mouse), was used as suggested by the manufacturer (Ribi Immunochem. Research, Inc., Hamilton, Mont.). Montanide Isa 740 (Seppic, Paris, France), a water-in-oil emulsion (water/oil ratio, 3/7), was mixed with a solution of PS-cPN (0.24 mg of PS per ml in saline) in a ratio of 85/35. Nonionic block copolymer surfactant L 180.5 (27) was a kind gift of R. L. Hunter (Emory University, Atlanta, Georgia). A mixture of L 180.5 and PS-cPN in saline was incorporated into a squalane-in-water emulsion, resulting in a water-in-oil-in-water emulsion with PS-cPN in the aqueous phase (referred to as W/O/W). Freund's complete adjuvant (FCA) was supplied by Difco (Detroit, Mich.). Mice immunized with PS-cPN in FCA were given boosters of PS-cPN in Freund's incomplete adjuvant (FIA). Mice immunized with PS-cPN in RIBI adjuvant or in FCA received an injection of 0.4 ml. The other groups were immunized with 0.2 ml. All groups received the same amount of PS-cPN (2.5 μg of PS per mouse).

Determination of anti-PS and antipeptide antibodies and inhibition ELISA. PS-specific antibodies were measured by an enzyme-linked immunosorbent assay (ELISA) using avidin-captured biotinylated PS (3). The horseradish peroxidase conjugates used were goat anti-mouse IgM, goat anti-mouse IgG (TAGO Inc., Burlingame, Calif.), and goat anti-mouse specific for the different mouse IgG subclasses (Southern Biotechnology Associates, Birmingham, Ala.). The specificity of the ELISA was checked by inhibition ELISA (47) using pooled mouse anti-PS-HSP antiserum (obtained at day 35) and as inhibitors PS (serotype 17F), C-polysaccharide (Statens Seruminstitut, Copenhagen, Denmark), and pneumococcal serotype 23F polysaccharide (2). PS serotype 17F, in contrast to the other inhibitors, displayed a concentration-dependent inhibition of IgG. C-polysaccharide and type 23F polysaccharide did not exceed 20% of inhibition at a concen-

TABLE 2. Properties of the PS-peptide conjugates

Conjugate	Peptide/PS (mg/g)	Peptide/PS-RU (mmol/mol)[a]	Efficiency of coupling (%)[b]	IC_{50} (nM PS-RU) (avg ± SD)[c]
PS-HSP	65.4	33.9	38	ND[d]
PS-HAG	15.5	11.9	13	16.1 ± 3.2
PS-PNL	119.0	59.5	67	50.4 ± 2.2
PS-PNc	82.6	39.4	44	27.9 ± 0.6
PS-cPN	86.2	41.0	46	38.7 ± 19.7
PS-βME				26.7 ± 5.8
PS				28.4 ± 1.9

[a] The molecular weight of the PS-repeating unit (PS-RU) is 1,250.
[b] Calculated on the basis of the available bromoacetyl groups on PS before coupling (the molar ratio of bromoacetyl to PS-repeating unit is 89.1).
[c] The 50% inhibitory concentration (IC_{50}) was measured by inhibition ELISA.
[d] ND, not determined.

tration of 100 μg/ml. Antipeptide antibodies in pooled mouse sera obtained at day 28 were measured by ELISA. A similar protocol was used as described for the determination of anti-PS antibodies (3). The only difference is that plates were coated overnight at 37°C with peptide solutions (50 μg of peptide per ml) in carbonate buffer (pH 9.6).

The amount of specific antibodies in serum is expressed in $\log_{10}$ titer, where the titer is calculated as the reciprocal dilution giving an absorbance of 0.5. Student's *t* test was used to assess the significance of differences between means: $P \leq 0.05$ was considered to be statistically significant. For the statistical analysis, the detection level ($\log_{10}$ titer = 1.60) was assigned to those sera with undetectable levels of one or more IgG subclasses.

The antigenicity of the PS-peptide conjugates compared with that of unconjugated PS was assessed by inhibition ELISA using a rabbit hyperimmune antiserum elicited with formalin-killed type 17F pneumococci (3).

Peptide immunizations and proliferative responses. BALB/c mice that had been immunized with conjugates as described above received an additional booster at day 42. Two to three weeks after the last immunization, spleens were removed, and cell suspensions were prepared and stored at −80°C until cell proliferation was measured. To determine whether immunization with free peptides would prime for peptide-specific T-cell responses, mice were immunized in the hind footpads with a total of 100 μg of peptide in combination with either 0.5 mg of dimethyl-dioctadecylammonium bromide or 50 μl of FIA. Eight to ten days later, draining popliteal lymph nodes were removed, and cell suspensions from each group of mice were prepared and pooled. Proliferative responses were measured by 18-h [^{3}H]thymidine incorporation in cells (2×10^5 per well) cultured for 4 days in Iscove's modified minimal essential medium supplemented with 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 μM β-mercaptoethanol, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. The mean counts per minute were measured for triplicate test cultures and divided by the mean of triplicate control cultures without antigen (stimulation index [SI]). Results are expressed as mean SI of three to five mice (or SI of triplicate cultures in the case of pooled cell suspensions) ± standard deviation (SD). As a control, the polyclonal activator concanavalin A was tested at 2 μg/ml.

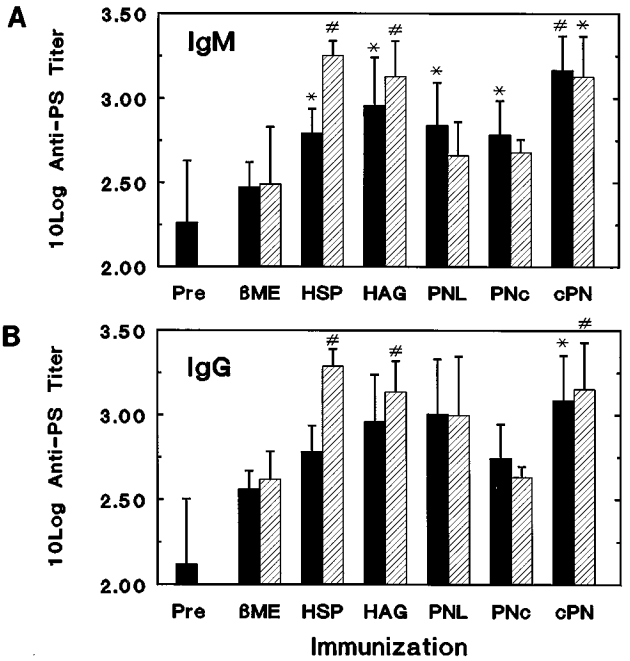

FIG. 1. Anti-PS antibody responses to the conjugates. Groups of five BALB/c mice were immunized with PS-βME (βME) or the various PS-peptide conjugates (the abbreviation of the peptide is shown) and given boosters 21 days later. Sera were obtained at day 0 (pre, preimmune), day 14 (black bars), and day 28 (hatched bars), and the antibody titers were determined by ELISA. The results, expressed as means + SD, are from a representative experiment. Titers induced by conjugates were compared with the titer induced by PS-βME: *, $P < 0.05$; #, $P < 0.01$ (Student's *t* test).

RESULTS

Characterization of PS-peptide conjugates. Bromoacetylated PS was linked to different cysteinyl peptides. The ratio of peptide to PS-repeating units (millimoles per mole) in the different conjugates varied from 12 to 59, corresponding with coupling efficiencies of 13 to 67%, respectively (Table 2). The antigenicity of the PS component of the conjugates was tested by their ability to bind to rabbit anti-PS IgG in inhibition ELISA. The reactivity of PS-βME and all PS-peptide conjugates tested was similar to that of nonderivatized PS (Table 2).

Immunogenicity of PS-peptide conjugates. Groups of five BALB/c mice were immunized with the different conjugates and given boosters 21 days later. All PS-peptide conjugates, but not PS-βME, induced statistically significant IgM responses compared with the preimmune titer, both after one immunization and after two immunizations (Fig. 1A). PS-HSP induced a clear booster response ($P < 0.01$, day 28 versus day 14), resulting in the highest titer observed at day 28. The IgM responses induced by PS-HAG and PS-cPN were relatively high, but no booster effect was observed. The conjugates PS-PNL and PS-PNc induced higher IgM levels than PS-βME only after the first dose.

The IgG response patterns induced (Fig. 1B) were similar to the IgM responses: compared with the preimmune titer, PS-βME induced a significant IgG response only at day 28 ($P < 0.05$). In contrast, all PS-peptide conjugates induced significant IgG responses above preimmune levels after one or two immunizations. PS-HSP elicited a strong secondary IgG response ($P < 0.01$ compared with day 14). Compared with PS-βME, PS-HAG and PS-cPN elicited significant responses at day 28, but again no booster effect was observed. PS-PNL and PS-PNc did not induce significant IgG responses, although the mean titer observed after immunization with PS-PNL was relatively high. The IgM and IgG patterns observed 2 weeks after booster (at day 35; data not shown) were similar to the patterns observed at day 28. It is noteworthy that PS-PNc, which differs from PS-cPN only in the position of the cysteine residue and thus in the orientation of the peptide, elicited hardly any response, whereas PS-cPN was clearly immunogenic.

PS-HSP, PS-HAG, and PS-cPN were the only conjugates capable of inducing moderate to high antipeptide antibody levels. At day 28, the increases in titer over the preimmune level ranged from 4- to 230-fold (data not shown).

IgG subclasses and protection induced by PS-cPN and influence of adjuvants. To determine which IgG subclasses were induced by PS-peptide conjugates and to investigate the protection achieved, groups of five mice were immunized with one of the most immunogenic conjugates (PS-cPN) in combination with a series of adjuvants or with nonderivatized PS. After three immunizations, mice were bled and anti-PS IgG and subclasses were measured (Table 3). Antisera raised with plain PS had a low anti-PS IgG titer, and of the subclasses, only IgG1 was detectable. In contrast, PS-cPN with saline or with adjuvants (except Q-VAC) elicited significantly higher anti-PS IgG titers than plain PS. Immunization with PS-cPN in combination with some of the adjuvants resulted also in significant rises of various IgG subclasses compared with plain PS. The W/O/W emulsion and FCA showed the strongest adjuvant effects. W/O/W increased mainly IgG1 and IgG3, whereas FCA increased predominantly the IgG2a and IgG2b titers. Only these two adjuvant preparations induced a significant increase in one or two subclasses compared with PS-cPN with saline (Table 3).

Partial protection was observed in all groups of mice immunized with PS-cPN, while nonderivatized PS did not confer any protection (Table 3). No evident improvement of survival by any adjuvant could be found above the protection induced by PS-cPN alone. When the results from all groups receiving PS-cPN were pooled, a significant increase in survival time was

TABLE 3. Anti-PS IgG subclasses and protection induced by PS-cPN conjugate in combination with adjuvants

Immunization[a]		Log_{10} titer (avg ± SD)					No. of surviving mice/5 tested[b]	Median survival time (days)
Antigen	Adjuvant	IgG	IgG1	IgG2a	IgG2b	IgG3		
PS-cPN	Saline	2.38 ± 0.40[c]	2.43 ± 0.76	1.75 ± 0.30	1.90 ± 0.34	1.75 ± 0.30	3	>15
PS-cPN	Q-VAC	1.84 ± 0.76	2.20 ± 0.86	2.13 ± 0.86	1.75 ± 0.17	1.75 ± 0.30	2	1
PS-cPN	RIBI	2.62 ± 0.40[c]	3.03 ± 0.56[d]	1.90 ± 0.51	1.90 ± 0.42	1.78 ± 0.27	3	>15
PS-cPN	Isa 740	2.51 ± 0.89[e]	2.80 ± 0.96[e]	1.78 ± 0.27	2.20 ± 0.72	2.02 ± 0.58	3	>15
PS-cPN	W/O/W	3.16 ± 0.45[c,f]	3.53 ± 0.27[c,f]	2.26 ± 0.71	2.26 ± 0.34[d]	2.32 ± 0.63[e]	4	>15
PS-cPN	FCA	3.04 ± 0.58[c]	2.56 ± 0.53[e]	2.51 ± 0.42[d,f]	2.45 ± 0.54[d]	2.05 ± 0.80	3	>15
PS	Saline	1.36 ± 0.13	1.75 ± 0.30	<1.60	<1.60	≤1.60	0	1

[a] Mice were immunized at weeks 0, 8, and 15 with PS alone or PS-cPN in combination with different adjuvants. Q-VAC is a saponin, RIBI contains monophosphoryl lipid A, Isa 740 is a water-in-oil emulsion, and W/O/W is a water-in-oil-in-water emulsion containing the nonionic block polymer L 180.5. Sera were obtained at week 17, and anti-PS IgG titers were determined by ELISA.

[b] Seventeen days after the last immunization, mice were challenged intraperitoneally with 10^5 CFU (approximately 10 × 50% lethal doses) of *S. pneumoniae* 17F, and survival was recorded for 15 days. Survival of nonimmunized mice ($n = 5$) was the same as that of PS-immunized mice.

[c] $P < 0.01$ versus PS/saline (Student's t test).

[d] $P < 0.01$ versus PS/saline (Student's t test).

[e] $P < 0.05$ versus PS/saline (Student's t test).

[f] $P < 0.02$ versus the conjugate with saline (Student's t test).

found compared with either control group (PS or nonimmunized mice) ($P < 0.02$ by the Wilcoxon two-tailed rank test).

Immunogenicity of conjugates in nude mice. To investigate the requirement of T cells for the response to PS-peptide conjugates, groups of five athymic BALB/c *nu*/*nu* and control (*nu*/+) mice were immunized with the conjugates in combination with Quil A as described above. In control euthymic mice, PS-HSP, PS-cPN, and PS-HAG induced significant anti-PS secondary responses compared with preimmune levels (Fig. 2). Compared with PS-βME, which was not immunogenic, the IgG response elicited by PS-HSP and both IgM and IgG responses induced by PS-HAG were statistically significant. In nude mice, however, no antibody responses were induced by any conjugate tested. PS-PNL did not induce any secondary response in either mouse strain (Fig. 2), consistent with findings for normal BALB/c mice (Fig. 1).

Peptide-specific proliferation by conjugate-primed spleen cells. Some of the peptides conjugated to PS were shown to enhance the anti-PS antibody responses, and these responses were not observed in nude mice. To investigate whether T cells were recruited in response to the conjugates, peptide-specific proliferation was measured in spleen cells from mice that had been primed with the different conjugates. As control groups, mice received PS-βME or saline. The results (Table 4) show that only PS-cPN-immune cells reached a significant level of proliferation to their homologous peptide (cPN), while they did not respond to a control peptide (amino acids 180 to 196 of hsp65 [12]). PS-HAG-primed cells had a slight, nonsignificant proliferative response to HAG but also to a control peptide. None of the peptides was able to induce a strong proliferative response in vitro. Responses at peptide concentrations other than 30 μg/ml were similar or lower. None of the spleens responded to PS in vitro (up to 90 μg/ml; data not shown). Spleen cells from mice that had received saline or PS-βME did not proliferate to any peptide (data not shown).

Since mice received a small conjugate dose (2.5 μg of PS), the total amount of peptide inoculated (Table 2) might have been too low to induce a detectable T-cell response. Therefore, mice were immunized as described for the former experiment with PS-HSP doses corresponding to either 2.5 or 50 μg of PS per mouse (containing 0.2 or 3.3 μg, respectively, of HSP) in combination with Quil A, and spleen cell proliferation was determined in vitro. We chose this particular conjugate because the T-cell-stimulating capacity of HSP has been demon-

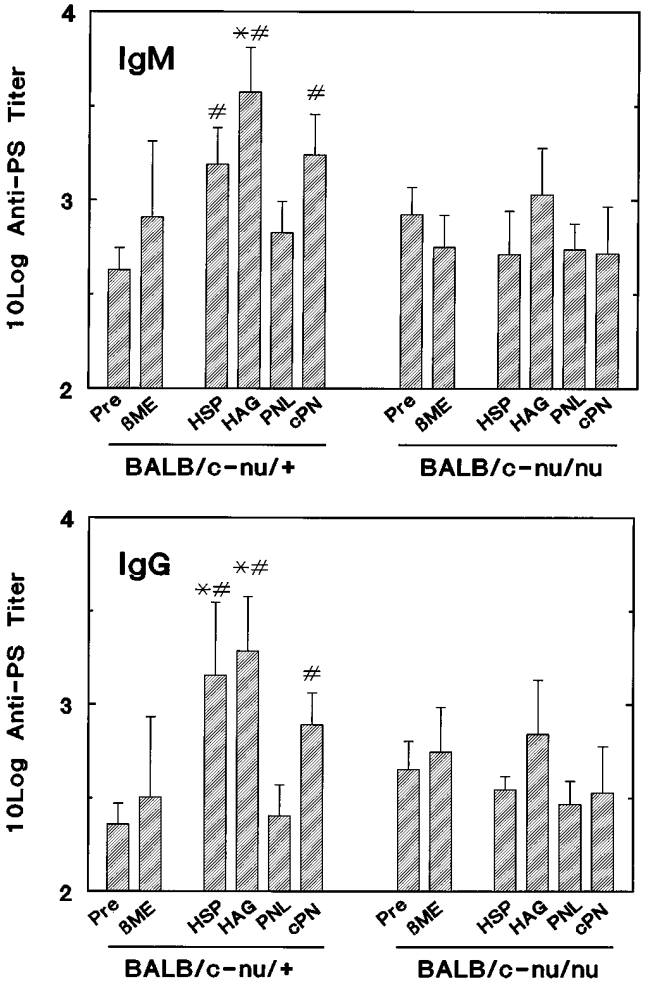

FIG. 2. Anti-PS antibody responses to PS-βME (βME) and PS-peptide conjugates (the abbreviation of the peptide is shown) in nude mice. Groups of five athymic BALB/c *nu*/*nu* mice and their controls (BALB/c *nu*/+ mice) were immunized as described in the legend to Fig. 1. Sera were obtained at day 35, and the antibody titers were determined by ELISA (data expressed as means + SD). Pre, preimmune titer. Mean titers induced by conjugates were compared with the preimmune titer or the titer induced by PS-βME: *, $P < 0.05$ versus PS-βME; #, $P < 0.01$ versus preimmune serum (Student's t test).

TABLE 4. Proliferative responses[a] of conjugate-primed spleen cells

Immunogen	SI (avg ± SD) with test antigen (30 μg/ml):		Avg cpm, no peptide
	Homologous peptide	Control peptide	
PS-HSP	2.4 ± 1.4	0.7 ± 0.2	1,513
PS-HAG	1.6 ± 0.6	1.8 ± 1.0	1,020
PS-PNL	1.2 ± 0.1	1.4 ± 0.6	2,750
PS-PNc	1.5 ± 0.5	1.3 ± 0.5	1,684
PS-cPN	2.1 ± 0.6[b]	0.9 ± 0.1	3,400

[a] Mice were immunized three times subcutaneously with conjugates (2.5 μg of PS per mouse) in combination with Quil A. Proliferation was measured in individual cell suspensions (three to five for each group). The mean response (SI ± SD) to concanavalin A was 73.5 ± 71.6.
[b] Significantly different from control peptide ($P < 0.01$).

strated (12). When HSP was added to the culture medium in a concentration of 90 μg/ml, cells primed with 2.5 μg of conjugate showed a mean SI ± SD of 1.6 ± 0.1, whereas cells primed with 50 μg of conjugate showed a significantly higher proliferative response: 2.9 ± 0.1 ($P < 0.001$). In this experiment, both immunization doses resulted in significant peptide-specific proliferation ($P < 0.01$ compared with the cultures without antigen).

Peptide-specific proliferation by peptide-primed lymph node cells. Since the proliferative response upon in vivo priming with PS-HSP was limited by the low dose of conjugate used for immunization, we investigated the ability of larger doses of peptides to prime for in vitro proliferation. Mice were immunized with unconjugated peptide (100 μg per mouse) because the amount of conjugate needed to reach the usual peptide doses for this kind of experiment would be too large. Mice were immunized once in the footpads, with peptides in FIA or in dimethyl-dioctadecylammonium bromide, and proliferation was measured in freshly isolated lymph node cells (Table 5). The only peptides inducing proliferation above background levels were PNc and cPN, which differ from each other only in the position of the terminal cysteine. As expected, both peptides were able to prime for proliferation to each other but not to a nonrelated peptide (data not shown). The other peptides, including HSP, did not induce any proliferative response. Lymph node cells from nonimmunized mice did not proliferate to any peptide (data not shown).

DISCUSSION

In this study, the feasibility of substituting a carrier protein by a peptide representing a Th epitope was investigated by testing whether polysaccharide-peptide conjugate vaccines behave as TD antigens. Furthermore, the IgG subclasses and the protection against *S. pneumoniae* induced by one of the conjugates were assessed.

TABLE 5. Proliferative responses[a] of peptide-primed lymph node cells

Immunization	SI (mean ± SD)	
	FIA	DDA
HSP	1.1 ± 0.1	0.9 ± 0.1[b]
HAG	0.9 ± 0.1	0.8 ± 0.1[b]
PNL	1.1 ± 0.1	0.8 ± 0.0
PNc	1.9 ± 0.1[c]	4.9 ± 1.7[c]
cPN	2.0 ± 0.2[c]	3.0 ± 0.2[c]

[a] Mice were immunized intracutaneously with 100 μg of peptide (in FIA or with dimethyl-dioctadecylammonium bromide [DDA]). Proliferation was measured at 10 μg of peptide per ml. Mean responses to concanavalin A were 33.2 ± 4.9 and 11.5 ± 6.9 for the FIA and DDA groups, respectively. Counts per minute with medium alone ranged from 550 to 2,360.
[b] Proliferation was assayed at 20 μg of peptide per ml.
[c] Significantly different from mean SI without peptide ($P < 0.02$).

Peptides used in our study were selected on basis of their demonstrated or predicted Th-cell-stimulating properties (Table 1). HSP is a dominant Th epitope from mycobacterial hsp65 (12). HAG, from influenza virus hemagglutinin, has been shown to prime for T-cell proliferation when coupled to a carrier protein (24). The same peptide, colinearly synthesized to a B-cell epitope, was able to provide Th activity for the production of antibodies (21). It was therefore intriguing to determine whether it would also act as a Th epitope when linked to PS. Pneumolysin (or its toxoids), from which sequence peptides PNL and PNc/cPN were synthesized, is a promising carrier candidate for pneumococcal conjugate vaccines. Antibodies against pneumolysin have been shown to partially protect mice against pneumococcal disease (37). So far, no T-cell epitopes from this protein have been described.

The degrees of peptide incorporation in the conjugates differed (Table 2), possibly because of differing solubilities of the peptides, either during the treatment with dithiothreitol (resulting in only partial reduction to monomers) or at the coupling stage. However, these differences did not seem to influence the immunogenicity of the conjugates (see below). The abilities of nonderivatized PS, PS-βME, and the different PS-peptide conjugates to inhibit binding of rabbit anti-PS IgG in ELISA were similar, demonstrating that the antigenicity of the PS component of the conjugates was not substantially changed by this coupling method.

All conjugates elicited anti-PS antibody responses above the preimmune level and frequently higher than those induced by PS-βME (Fig. 1). The PS-peptide conjugates PS-HSP, PS-HAG, and PS-cPN elicited consistent anti-PS antibody responses of both isotypes above the levels induced by PS-βME. PS-PNL did not induce substantial IgG responses in BALB/c mice, although the mean titer observed was relatively high. In BALB/c *nu*/+ mice, it induced no response at all. Of the conjugates tested, PS-PNc had the lowest immunogenicity. The different anti-PS antibody responses induced by the conjugates might be related to differences in timing of the primary and/or secondary responses to the various conjugates or to administration of suboptimal doses of some of the conjugates. However, the most likely explanation for the observed differences is the amino acid sequence of the peptide used as a carrier. The inability of PS-HSP, PS-HAG, and PS-cPN to elicit antibody responses in nude mice (Fig. 2) demonstrates that these three conjugates are TD antigens. Furthermore, the induction of all IgG subclasses by PS-cPN (Table 3) confirms its TD character. In addition to their consistent anti-PS antibody responses, PS-HSP, PS-HAG, and PS-cPN were the only conjugates inducing antipeptide antibodies. This finding supports the presumed ability of these peptides to stimulate Th cells, since no IgG will be normally produced against a peptide in the absence of Th activity. Of the three most immunogenic conjugates, only PS-HSP showed significant booster IgM and IgG responses. The lack of booster response upon immunization with PS-HAG and PS-cPN might be related to the anti-PS antibody levels present at the time of secondary immunization (day 21), since PS-HSP induced a lower primary response than PS-HAG and PS-cPN.

In vitro peptide-specific proliferation was measured upon immunization with either PS-peptide conjugates or unconjugated peptides (Tables 4 and 5). PS-cPN and PS-HSP were the only PS-peptide conjugates able to prime for in vitro proliferation. When the priming ability of unconjugated peptides was

assessed, only PNc and cPN induced in vitro peptide-specific proliferation, independently of the adjuvant used for immunization. In vivo, however, not only cPN but also HSP and HAG were able to provide help for anti-PS antibody production. These data suggest that the T-cell-priming ability of a peptide, as measured by in vitro T-cell proliferation, is not predictive for the induction of high anti-PS antibody responses (45). Moreover, T-cell-priming ability (as displayed by PNc) is not necessarily correlated with efficient help for antibody production (see below). Mapping of Th epitopes on the basis of proliferation experiments may fail to detect Th epitopes with strong in vivo activity or may detect epitopes without functional T-B helper activity. From the literature, we know that in some cases a dichotomy in T-cell responses can be observed: a proliferative response (thought to be mainly of the Th1 subset) that is able to induce delayed-type hypersensitivity, or a T-B helper response (ascribed to the Th2 subset) that predominantly results in antibody formation, does not prime for in vitro proliferation (or does so to a lesser extent), and also may give rise to immediate-type hypersensitivity (26, 28, 45). Whether one of these two responses, or both of them, is induced seems to depend on the immunogen structure and its dose, the adjuvant used, and the major histocompatibility complex (MHC) of the animals used (30, 31, 35, 44). Although a satisfactory explanation for this diversity in responses is not yet provided, distinct antigen-presenting cells have been shown to lead the response towards a proliferative or a T-B helper response (26). The role of antigen-presenting cells, which differ in cytokine secretion pattern, has been suggested to be based on the distinct cytokine requirements of Th1 and Th2 cells for autocrine growth (20, 22). Another possible explanation is the density of the peptide-MHC complexes formed on the surface of the antigen-presenting cells, resulting in different levels of signaling through the T-cell receptor/CD3 complex (45), which is in accordance with the different second messenger pathways that seem to be utilized by Th1 and Th2 cells (19).

Under some circumstances, activation of Th1 and Th2 cells appears to act antagonistically. Gamma interferon, produced by Th1 cells, inhibits Th2 cell proliferation (18) and B-cell activation (40, 41). Similarly, Th2 cells produce a factor which inhibits cytokine production by Th1 cells (17). Furthermore, human Th1 cell clones have been shown to directly inhibit B-cell responses, probably by their cytolytic activity on antigen-specific B cells (14). This antagonistic action of the Th1 and Th2 subsets may explain why, upon immunization with certain antigens, only one type of response, and not a combination of both proliferative and helper responses, is observed.

The results obtained with HAG and its PS-conjugate may be explained by such a dichotomy. This peptide, either alone or conjugated to PS, did not prime for HAG-specific proliferation, whereas PS-HAG induced strong anti-PS antibody responses. For this particular conjugate, other, not mutually exclusive explanations are also possible. (i) The Th epitope present in HAG does not induce strong proliferation (21), and the HAG/PS ratio of this conjugate is low (Table 2). Therefore, immunization with a higher dose of PS-HAG may be required to detect HAG-specific proliferation. (ii) HAG-specific T-cell proliferation has been observed after priming with HAG, but exclusively when this peptide was coupled to a protein (24) or to another peptide (21). It is possible that this peptide in its unconjugated form is more susceptible to degradation in vivo than when it is linked to other structures. (iii) The lack of proliferative response to HAG may also be due to more efficient processing and/or presentation of the peptide when flanked by other structures (39), since intact hemagglutinin was shown to be $\sim 10^6$ times more efficient on a molar basis than an HAG variant (amino acids 111 to 119) in stimulating the proliferation of a T-cell line (23).

The strong difference in immunogenicity between PS-PNc and PS-cPN, observed for both the anti-PS and the antipeptide antibody responses, confirms earlier results demonstrating the importance of the orientation of a peptide for its immunogenicity (reference 21 and references therein). Since PNc and cPN have the same sequence except for the terminal cysteine and the conjugates have similar PS/peptide ratios (Tables 1 and 2), PS-PNc seems to be the ideal built-in control to assess T-cell help induction by PS-cPN. When this peptide was linked to PS via its amino terminus (PS-cPN), but not via its carboxy terminus (PS-PNc), it was able to prime for T-cell proliferation. In contrast, both PNc and cPN in the unconjugated form efficiently primed for in vitro proliferation to the homologous peptide and to each other, which means that both peptides are recognized by T cells. From the proliferation data, it can be concluded that the difference in immunogenicity between PS-PNc and PS-cPN is most probably due to the inability of antigen-presenting cells to cleave the proper T-cell-stimulating peptide from PS and thus the inability to present PNc in the context of MHC class II molecules to Th cells. Regardless of the orientation, the consistent priming ability of either peptide demonstrates that amino acids 263 to 281 from pneumolysin contain a Th epitope.

Studies of polysaccharide-peptide conjugates have been limited. Recently, Paradiso and colleagues (36) coupled a Th epitope from the diphtheria toxin mutant CRM_{197} to oligosaccharides derived from the capsular polysaccharide of *H. influenzae* type b and *S. pneumoniae* serotype 14. Both conjugates were immunogenic. Lett et al. (34) constructed conjugates consisting of mannan and *Streptococcus mutans* polysaccharide. These polysaccharides were linked to two peptides, one containing a B-cell epitope and the other containing both a B- and a T-cell epitope. Only the latter was able to induce anti-polysaccharide responses.

Our study has demonstrated that coupling of Th epitope peptides to PS can result in constructs with most of the typical characteristics of TD antigens, provided that they are coupled to PS in the right orientation. The ability of a peptide to induce help to B cells for the production of anti-PS antibodies is not always correlated to their T-cell proliferative responses. It is noteworthy that protection experiments performed with PS-cPN showed a significant level of protection. This was similar to the protection induced by PS-pneumolysin and PS-hsp65 conjugates (1). Research on polysaccharide-peptide conjugate vaccines, therefore, appears to be not only an interesting experimental approach; it may even result in the development of efficacious vaccines. For successful application in humans, limitations due to MHC restriction should be circumvented, for instance by using colinearly synthesized peptides suitable for various MHC types or by using "promiscuous" peptides that can interact with multiple MHC alleles (13). The use of (poly)peptides (36) is especially attractive for polyvalent pneumococcal conjugate vaccines, in order to avoid excessive amounts of carrier protein that may induce antibodies with suppressive effects on subsequent responses (6, 7, 38). For pneumococcal vaccines, peptides from pneumolysin or other pneumococcal proteins (like pneumococcal surface protein A) should be preferred above peptides from heterologous carriers. On the other hand, since the number of conjugate vaccines against a variety of pathogens is expanding, the need for efficient and safe universal carriers will grow. Heat shock proteins are major targets of continuously challenged T-cell reactivity in humans (25). Since some regions of hsp65 have been suspected to be involved in the induction of autoimmune diseases (25),

selection of peptides unable to break self-tolerance but still having Th-cell-stimulating properties may open new approaches to the development of efficient and safe universal carriers.

ACKNOWLEDGMENTS

S. M. Anderton is supported by a Wellcome Trust postdoctoral research fellowship to Western Europe.

We thank J. M. Van Noort and E. J. H. J. Wiertz for help in the analysis of putative T-cell epitopes and for useful suggestions with regard to the choice of the peptides. H. A. T. Dekker is gratefully acknowledged for her technical assistance, and R. G. Feldman is acknowledged for his valuable advice.

REFERENCES

1. **AlonsoDeVelasco, E., A. F. M. Verheul, and H. Snippe.** Unpublished data.
2. **AlonsoDeVelasco, E., A. F. M. Verheul, A. M. P. Van Steijn, H. A. T. Dekker, R. G. Feldman, I. M. Fernández, J. P. Kamerling, J. F. G. Vliegenthart, J. Verhoef, and H. Snippe.** 1994. Epitope specificity of rabbit immunoglobulin G (IgG) elicited by pneumococcal type 23F synthetic oligosaccharide- and native polysaccharide-protein conjugate vaccines: comparison with human anti-polysaccharide 23F IgG. Infect. Immun. **62:**799–808.
3. **AlonsoDeVelasco, E., A. F. M. Verheul, G. H. Veeneman, L. J. F. Gomes, J. H. van Boom, J. Verhoef, and H. Snippe.** 1993. Protein-conjugated synthetic di- and trisaccharides of pneumococcal type 17F exhibit a different immunogenicity and antigenicity than tetrasaccharide. Vaccine **11:**1429–1436.
4. **Anderson, P., and R. A. Insel.** 1988. Prospects for overcoming maturational and genetic barriers to the human antibody response to the capsular polysaccharide of *Haemophilus influenzae* type b. Vaccine **6:**188–191.
5. **Barany, G., and R. B. Merrifield.** 1980. Solid-phase peptide synthesis, p. 1–284. *In* E. Gross and J. Meienhofer (ed.), The peptides, vol. 1. Academic Press, New York.
6. **Barington, T., A. Gyhrs, K. Kristensen, and C. Heilmann.** 1994. Opposite effects of actively and passively acquired immunity to the carrier on responses of human infants to a *Haemophilus influenzae* type b conjugate vaccine. Infect. Immun. **62:**9–14.
7. **Barington, T., M. Skettrup, L. Juul, and C. Heilmann.** 1993. Non-epitope-specific suppression of the antibody response to *Haemophilus influenzae* type b conjugate vaccines by preimmunization with vaccine components. Infect. Immun. **61:**432–438.
8. **Barrios, C., A. R. Lussow, J. Van Embden, R. Van der Zee, R. Rappuoli, P. Costantino, J. A. Louis, P.-H. Lambert, and G. Del Giudice.** 1992. Mycobacterial heat-shock proteins as carrier molecules. II. The use of the 70-kDa mycobacterial heat-shock protein as carrier for conjugated vaccines can circumvent the need for adjuvants and Bacillus Calmette Guérin priming. Eur. J. Immunol. **22:**1365–1372.
9. **Bernatowicz, M. S., and G. R. Matsueda.** 1986. Preparation of peptide-protein immunogens using N-succinimidyl bromoacetate as a heterobifunctional crosslinking reagent. Anal. Biochem. **155:**95–102.
10. **Beuvery, E. C., F. Van Rossum, and J. Nagel.** 1982. Comparison of the induction of immunoglobulin M and G antibodies in mice with purified pneumococcal type 3 and meningococcal group C polysaccharides and their protein conjugates. Infect. Immun. **37:**15–22.
11. **Bixler, G. S., Jr., R. Eby, K. M. Dermody, R. M. Woods, R. C. Seid, and S. Pillai.** 1989. Synthetic peptide representing a T-cell epitope of CRM197 substitutes as carrier molecule in a *Haemophilus influenzae* type b (HIB) conjugate vaccine. Adv. Exp. Med. Biol. **251:**175–180.
12. **Brett, S. J., J. R. Lamb, J. H. Cox, J. B. Rothbard, A. Mehlert, and J. Ivanyi.** 1989. Differential pattern of T cell recognition of the 65-kDa mycobacterial antigen following immunization with the whole protein or peptides. Eur. J. Immunol. **19:**1303–1310.
13. **Del Giudice, G.** 1992. New carriers and adjuvants in the development of vaccines. Curr. Opin. Immunol. **4:**454–459.
14. **Del Prete, G. F., M. De Carli, M. Ricci, and S. Romagnani.** 1991. Helper activity for immunoglobulin synthesis of T helper type 1 (Th1) and Th2 human T cell clones: the help of Th1 clones is limited by their cytolytic capacity. J. Exp. Med. **174:**809–813.
15. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **3:**350–356.
16. **Etlinger, H. M., D. Gillessen, H.-W. Lahm, H. Matile, H.-J. Schönfeld, and A. Trzeciak.** 1990. Use of prior vaccinations for the development of new vaccines. Science **249:**423–425.
17. **Fiorentino, D. E., M. W. Bond, and T. R. Mosmann.** 1989. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J. Exp. Med. **170:**2081–2095.
18. **Gajewski, T. F., and F. W. Fitch.** 1988. Anti-proliferative effect of IFN-γ in immune regulation. I. IFN-γ inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. J. Immunol. **140:**4245–4252.
19. **Gajewski, T. F., S. R. Schell, and F. W. Fitch.** 1990. Evidence implicating utilization of different T cell receptor-associated signaling pathways by Th1 and Th2 clones. J. Immunol. **144:**4110–4120.
20. **Germann, T., A. Partenheimer, and E. Rüde.** 1990. Requirements for the growth of TH1 lymphocyte clones. Eur. J. Immunol. **20:**2035–2040.
21. **Golvano, J., J. J. Lasarte, P. Sarobe, A. Gullón, J. Prieto, and F. Borrás-Cuesta.** 1990. Polarity of immunogens: implications for vaccine design. Eur. J. Immunol. **20:**2363–2366.
22. **Greenbaum, L. A., J. B. Horowitz, A. Woods, T. Pasqualini, E.-P. Reich, and K. Bottomly.** 1988. Autocrine growth of CD4+ T cells. Differential effects of IL-1 on helper and inflammatory T cells. J. Immunol. **140:**1555–1560.
23. **Hackett, C. J., B. Dietzschold, W. Gerhard, B. Ghrist, R. Knorr, D. Gillessen, and F. Melchers.** 1983. Influenza virus site recognized by a murine helper T cell specific for H1 strains. Localization to a nine amino acid sequence in the hemagglutinin molecule. J. Exp. Med. **158:**294–302.
24. **Hackett, C. J., J. L. Hurwitz, B. Dietzschold, and W. Gerhard.** 1985. A synthetic decapeptide of influenza virus hemagglutinin elicits helper T cells with the same fine recognition specificities as occur in response to whole virus. J. Immunol. **135:**1391–1394.
25. **Harboe, M., and A. J. Quayle.** 1991. Heat shock proteins: friend and foe? Clin. Exp. Immunol. **86:**2–5.
26. **Hayakawa, K., and R. R. Hardy.** 1988. Murine CD4+ T cell subsets defined. J. Exp. Med. **168:**1825–1838.
27. **Hunter, R. L., M. Olsen, and S. Buynizky.** 1991. Adjuvant activity of non-ionic block copolymers. IV. Effect of molecular weight and formulation on titre and isotype of antibody. Vaccine **9:**250–265.
28. **Katsura, Y., M. Takaoki, S. Nishikawa, and S. Muramatsu.** 1977. Cell-mediated and humoral immune responses in mice. IV. Difference of the functional cell population between helper activity and delayed-type hypersensitivity. Immunology **32:**237–245.
29. **Kemp, H. A., and M. R. A. Morgan.** 1986. Studies on the detrimental effects of bivalent binding in a microtiter plate ELISA and possible remedies. J. Immunol. Methods **94:**65–72.
30. **Kraaijeveld, C. A., G. la Rivière, B. J. Benaissa-Trouw, J. Jansen, T. Harmsen, and H. Snippe.** 1983. Effect of the adjuvant dimethyl dioactadecyl ammonium bromide on the humoral and cellular immune responses to encephalomyocarditis virus. Antiviral Res. **3:**137–149.
31. **Krzych, U., A. V. Fowler, A. Miller, and E. E. Sercarz.** 1982. Repertoires of T cells directed against a large protein antigen, β-galactosidase. I. Helper cells have a more restricted specificity repertoire than proliferative cells. J. Immunol. **128:**1529–1534.
32. **Lambrechts, H., H. Ravestein, and C. Kollöffel.** 1992. Temperature dependent redistribution of organic nitrogen during 'dry' storage of tulip bulbs cv. Apeldoorn. Physiol. Plant. **86:**97–103.
33. **Lee, A. C. J., J. E. Powell, G. W. Tregear, H. D. Niall, and V. C. Stevens.** 1980. A method for preparing β-hCG COOH peptide-carrier conjugates of predictable composition. Mol. Immunol. **17:**749–756.
34. **Lett, E., S. Gangloff, M. Zimmermann, D. Wachsmann, and J.-P. Klein.** 1994. Immunogenicity of polysaccharides conjugated to peptides containing T- and B-cell epitopes. Infect. Immun. **62:**785–792.
35. **Murray, J. S., J. Madri, J. Tite, S. R. Carding, and K. Bottomly.** 1989. MHC control of CD4+ T cell subset activation. J. Exp. Med. **170:**2135–2140.
36. **Paradiso, P. R., K. Dermody, and S. Pillai.** 1993. Novel approaches to the development of glycoconjugate vaccines with synthetic peptides as carriers. Vaccine Res. **2:**239–248.
37. **Paton, J. C., R. A. Lock, C.-J. Lee, J. P. Li, A. M. Berry, T. J. Mitchell, P. W. Andrew, D. Hansman, and G. Boulnois.** 1991. Purification and immunogenicity of genetically obtained pneumolysin toxoids and their conjugation to *Streptococcus pneumoniae* type 19F polysaccharide. Infect. Immun. **59:**2297–2304.
38. **Peeters, C. C. A. M., A.-M. Tenbergen-Meekes, J. T. Poolman, M. Beurret, B. J. M. Zegers, and G. T. Rijkers.** 1991. Effect of carrier-priming on immunogenicity of saccharide-protein conjugate vaccines. Infect. Immun. **59:**3504–3510.
39. **Perkins, D. L., G. Berriz, T. Kamradt, J. A. Smith, and M. L. Gefter.** 1991. Immunodominance: intramolecular competition between T cell epitopes. J. Immunol. **146:**2137–2144.
40. **Rabin, E. M., J. J. Mond, J. Ohara, and W. E. Paul.** 1986. Interferon-γ inhibits the action of B cell stimulatory factor (BSF)-1 on resting B cells. J. Immunol. **137:**1573–1576.
41. **Reynolds, D. S., W. H. Boom, and A. K. Abbas.** 1987. Inhibition of B lymphocyte activation by interferon-γ. J. Immunol. **139:**767–773.
42. **Robbins, J. B., and R. Schneerson.** 1990. Polysaccharide-protein conjugates: a new generation of vaccines. J. Infect. Dis. **161:**821–832.
43. **Rothbard, J. B., and W. R. Taylor.** 1988. A sequence pattern common to T cell epitopes. EMBO J. **7:**93–100.
44. **Snijders, A., B. J. Benaissa-Trouw, H. J. Visser-Vernooy, I. Fernández, and H. Snippe.** 1992. A delayed-type hypersensitivity-inducing T-cell epitope of Semliki Forest virus mediates effective T-helper activity for antibody production. Immunology **77:**322–329.
45. **Soloway, P., S. Fish, H. Passmore, M. Gefter, R. Coffee, and T. Manser.** 1991.

Regulation of the immune response to peptide antigens: differential induction of immediate-type hypersensitivity and T cell proliferation due to changes in either peptide structure or major histocompatibility complex haplotype. J. Exp. Med. **174:**847–858.

46. **Spouge, J. L., H. R. Guy, J. L. Cornette, H. Margalit, K. Cease, J. A. Berzofsky, and C. DeLisi.** 1987. Strong conformational propensities enhance T cell antigenicity. J. Immunol. **138:**204–212.
47. **Van Dam, G. J., A. F. M. Verheul, G. J. W. J. Zigterman, M. J. De Reuver, and H. Snippe.** 1989. Estimation of the avidity of antibodies in polyclonal antisera against *Streptococcus pneumoniae* type 3 by inhibition ELISA. Mol. Immunol. **26:**269–274.
48. **Van Noort, J. M., and A. C. M. Van der Drift.** 1989. The selectivity of cathepsin D suggests an involvement of the enzyme in the generation of T-cell epitopes. J. Biol. Chem. **264:**14159–14164.
49. **Walker, J. A., R. L. Allen, P. Falmagne, M. K. Johnson, and G. J. Boulnois.** 1987. Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae*. Infect. Immun. **55:**1184–1189.