Proteins and Their Derived Peptides as Carriers in a Conjugate Vaccine for *Streptococcus pneumoniae*: Self-Heat Shock Protein 60 and Tetanus Toxoid[1]

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We induced T cell help for vaccination against *Streptococcus pneumoniae* (Pn) using self and foreign peptides and their source proteins conjugated to the capsular polysaccharide (CPS) of type 4 Pn; the carriers were self-heat shock protein 60 (HSP60) and tetanus toxoid (TT). We measured the production of IgG Abs to the CPS and the carriers, and tested resistance to challenge with highly lethal amounts of Pn injected i.p. ($LD_{50} \times 10^3$–10^6). We now report that vaccination protects old and young mice from bacterial challenge; however, there were significant differences in vaccine efficacy based on the carrier. Self-HSP60 peptide p458m was more effective than the whole HSP60 molecule and was equally effective compared with TT. Both p458m and TT were more protective than the TT-derived peptide p30 after a single vaccination. However, peptide p30 was effective in more MHC genotypes than was p458m. Unlike other vaccines, protection conferred by p458m was not related to the amount of anti-CPS Ab: mice that produced very little Ab were still protected from highly lethal doses of bacteria ($LD_{50} \times 10^5$–10^6). Furthermore, unlike the other carriers, there was no Ab response to the p458m carrier. Thus, peptides, self as well as foreign, can provide T cell help that differs functionally from that provided by the whole parent protein. ***The Journal of Immunology*****, 2003, 170: 6165–6171.**

We previously described a conjugate vaccine designed to provide T cell help for vaccination against pathogenic bacteria. This vaccine differs from standard conjugate vaccines in that its T cell carrier moiety is a peptide derived from a self-molecule: the 60-kDa heat shock protein (HSP60).[3] The HSP60 peptide, then termed CP1, was able to induce the production of anti-*Salmonella* IgG Ab when conjugated to the *Salmonella typhi* capsular polysaccharide (CPS) Vi (1). The same peptide, now termed p458m for its position in the HSP60 sequence, induced resistance to lethal bacterial challenge when used in a subunit vaccine conjugated to the CPS of *Streptococcus pneumoniae* (Pn) type 4 (PS4) (2).

In this report, we compared pairs of proteins and their derived peptides as T cell epitopes, the p458m peptide and the whole HSP60 molecule, and the foreign immunogenic protein tetanus toxoid (TT) and a T cell epitope peptide of TT. TT is being used clinically as a carrier in conjugate vaccines (3) and in several vaccines undergoing clinical trials. TT conjugated to Pn CPS was shown to induce protective levels of anti-Pn CPS Ab and a reduction in the incidence of Pn infection in both infants (4–6) and adults (7). The peptide of TT is p30, aa 947–967 of TT, previously described by Panina-Bordignon et al. (8). Peptide p30 has been used as a helper peptide fused to *Plasmodium* B cell epitopes (9, 10), and in antiviral (11) and antimelanoma vaccines (12). In addition, we used as carriers other self-T cell epitopes from HSP60 (p20) and from the self-protein lysozyme, pLys (13). We studied the various carriers conjugated to PS4 as vaccines in BALB/c mice and assayed the effect on resistance to lethal challenge of subject age, number of injections, and anti-PS4 Ab. We also investigated Abs induced to the carrier and vaccine efficacy in various strains of mice.

Materials and Methods

Mice

Female BALB/c, BALB/k, and BALB/b mice were obtained from Harlan Olac (Bicester, U.K.) and were used at the age of 8 wk unless indicated differently. Female C57BL/6, CD1, and nonobese diabetic (NOD) mice were obtained from the Weizmann Institute of Science (Rehovot, Israel). Animals were used according to the guidelines and under the supervision of the Animal Welfare Committee.

Proteins and peptides

TT was generously supplied by Pasteur Merieux Connaught (Marcy, France), through the kind mediation of Prof. R. Dagan (Ben Gurion University of the Negev, Be'er Sheva, Israel). Murine HSP60 was prepared as described (14). Peptides were synthesized using an automated multiple peptide synthesizer (Abimed model AMS 422; Langenfeld, Germany), according to the company's instructions for *N*-α-fluorenylmethoxycarbonyl synthesis. The purity of the peptides was tested by analytical reversed-phase HPLC and amino acid analysis. The peptides we used are presented in Table I.

Pn type 4

Lyophilized Pn bacteria type 4 and PS4 Ag were obtained from American Type Culture Collection (Manassas, VA).

PS4 conjugation

All conjugates were made using a single batch of PS4. PS4 was dissolved in double-distilled water (DDW) to a final concentration of 5 mg/ml. The PS4 was activated (stirred continuously) with 0.1 ml of cyanogen bromide (20 mg/ml in acetone) in the presence of 30 mM triethylamine (Aldrich, Milwaukee, WI) in acetone at pH 7. The spacer 6-aminohexanoic acid (10 mg/ml in DDW; BDH Chemicals, Poole, U.K.) was added to the activated

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[3] Abbreviations used in this paper: HSP60, heat shock protein 60; PS, polysaccharide; CPS, capsular PS; Pn, *S. pneumoniae*; PS4, CPS of Pn type 4; TT, tetanus toxoid; NOD, nonobese diabetic; DDW, double-distilled water; BHI, brain-heart infusion.

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Table I. *Peptide carriers used in this study*

Peptide	Source Protein	Position	Sequence
p458m	Murine HSP60	458–474	NEDQKIGIEIIKRALKI
p20	Murine HSP60	286–305	LVLNRLKVGLQVVAVKAPGF
p1	Murine HSP60	3–22	RLPTVLRQMRPVSRALAPHL
p30	TT	947–967	FNNFTVSFWLRVPKVSASHLE
pLys	Murine lysozyme	105–119	IRAWVAWRAHCQNRD
pCon	None		CKKRTDKKFGEEEAAS

PS4 2 min later and then incubated for 2 h at 4°C. We then added 12 mg of water-soluble diimide–1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (CDI; Aldrich)–and 7 mg of the peptide or protein to the solution. The pH was adjusted to 6 at room temperature. Four hours later, 12 mg of CDI was again added, the mixture was incubated overnight and then dialyzed at 4°C against DDW. For the conjugate vaccines, the PS/peptide (w/w) ratios of the different batches were: PS4-p458m batches, 1:0.9, 1:0.5, and 1:0.7; PS4-p30 batches, 1:0.75, and 1:0.6; PS4-p20 batch, 1:0.3; PS4-pLys batch, 1:0.4; PS4-p1 batch, 1:0.5; PS4-pCon batch, 1:0.5. The PS/protein of the PS4-HSP60 batch was 1:1.

PS determination

The carbohydrate concentration in the conjugate solution was determined using the method of Dubois et al. (15). Briefly, the conjugate mixture or a PS standard was added to a glass tube in a final volume of 0.8 ml. We added 0.5 ml of 5% phenol (Sigma-Aldrich, Rehovot, Israel), followed by 2 ml of concentrated sulfuric acid. The tubes were allowed to stand for 10 min. The absorbance of the characteristic yellow-orange color was measured at OD_{490}. A standard reference curve was constructed using known amounts of PS4.

Protein and peptide determination

Protein concentration in solution was determined by the Bradford assay (16). Amino acid analysis (Chemical Services, Weizmann Institute) was used to determine the amount of the peptide coupled to the PS4.

Immunization

Mice were injected one to three times s.c. on the back at 2-wk intervals with 2 μg of PS4 per mouse, either conjugated or native, emulsified in IFA. The mice were bled 1 mo after the last boost or as indicated. The blood samples were centrifuged at 10,000 $\times$ *g* for 10 min, and the sera were collected and stored at -20°C.

Serology

Maxisorb 96-well plates (Nunc, Roskilde, Denmark) were coated with PS4 or with the various carriers (10 μg/ml in PBS) overnight at 4°C. The plates were washed three times with 0.02% Tween 20 in PBS between the different stages. Each plate was blocked with 2% skim milk (Difco, Detroit, MI) in PBS. Serum samples were diluted 1/50 or serial-diluted in 0.2% skim milk in PBS and incubated for 2 h at 37°C. The detection of IgG Ab was done using goat anti-mouse IgG coupled to alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1/1000 and incubated for 45 min at 37°C. Substrate solution containing 0.6 mg/ml *p*-nitrophenylphosphate (Sigma-Aldrich) in diethanolamine-H_2O pH 9.8 was then added. The OD_{405} was read when a strong color was detectable, usually after 15 min to 1 h of incubation. The titer was determined as the dilution at which the OD was double the OD of sera from mice vaccinated with IFA. To simplify presentation, most of the OD results are shown at the 1/50 dilution. The 1/50 dilution reflected the serial dilution titer (see Fig. 5).

Pn type 4 bacteria

Lyophilized bacteria were reconstituted and subcultured on sheep's blood agar (Hy Laboratories, Rehovot, Israel) and the colonies were resuspended in brain-heart infusion (BHI) broth (Hy Laboratories). After 6 h, the bacterial cultures were aliquoted and stored at -70°C in medium with 25% glycerol. Pneumococci for challenge were grown from frozen stock for 7 h in BHI broth at 37°C, and then maintained at 4°C until injection. Bacterial growth was estimated by turbidity at OD_{545}. The actual dose of viable bacteria injected in each challenge was determined by plating dilutions of the culture on sheep's blood agar for 24 h to determine the number of CFU. Pneumococcal virulence was maintained by periodic passage in mice: mice were injected with $LD_{50} \times 100$, and 20 h later the spleens were harvested, passed through a wire mesh, and seeded on sheep's blood agar. The bacteria were then prepared as indicated above.

Determination of minimal lethal dose

Naive BALB/c mice were injected i.p. with 0.2 ml of serially diluted bacterial cultures in BHI broth. Survival was determined daily for 2 wk. All naive mice challenged with two or more CFU died within 2 days of challenge. The resulting LD_{50} was determined to be one bacterium per mouse. The LD_{50} determination was done with large numbers of mice. In addition, in each challenge experiment, two naive mice were challenged with $LD_{50} \times 100$ to verify bacterial virulence.

Challenge assay

Vaccinated mice were injected i.p. with Pn 1 mo after the last boost or as indicated in *Results*. The number of bacteria injected is indicated as a multiple of the LD_{50}. Survival was estimated after 2 wk.

Statistics

Results were analyzed using Fisher's exact test for Figs. 1–3 and Table II; the Student *t* test or the alternate Welch *t* test were used for Figs. 4–7.

Results

Whole HSP60, p458m, TT, and p30 carriers compared

We previously compared the carrier function of HSP60 peptide p458m to that of whole TT in conjugate vaccines to PS4 (2). In this study, we extended the investigation to include whole HSP60, in addition to its p458m peptide, and the p30 peptide of TT, in addition to the whole TT. The carriers conjugated to PS4 are termed PS4-p458m, PS4-HSP60, PS4-TT, and PS4-p30. We used unconjugated PS4 as a control vaccine. The protein-peptide pairs were injected three times and the mice were tested for their resistance to lethal Pn challenge ($LD_{50} \times 10^5$–10^6) (Fig. 1). The PS4-p458m vaccine performed similarly to the PS4-TT and PS4-p30 vaccines in providing nearly complete protection to challenge. However, the PS4-HSP60 vaccine failed to protect 50% of the mice, a significant decrease ($p < 0.015$) compared with the other conjugate vaccines. Thus a peptide of 17 amino acids derived from HSP60 can be a more effective carrier than the whole parent molecule with its 540 amino acids. Nevertheless, the PS4-HSP60 vaccine was able to protect better than PS4 alone ($p < 0.006$). The PS4 vaccine was least effective (5% survival; $p < 0.0001$). Based on these results, we omitted HSP60 from the remainder of the studies.

Effect of number of injections

The above protocol used three injections of vaccine. Could the different conjugate vaccines be discriminated by their ability to protect mice after only one or two injections? We found that two

Table II. *Protection in different murine strains*[a]

Strains	Vaccines			
	PS4-p458m	PS4-p20	PS4-p30	PS4
BALB/k	4/4[b]	5/5[b]	4/4[b]	0/5
BALB/b	0/4	2/5	4/4[c]	2/5
C57BL/6	1/5	2/5	NT	3/5
CD1	2/4	1/5	4/4[d]	1/5
NOD	0/5	0/5	4/5[e]	0/5

[a] BALB/k, BALB/b, C57BL/6, CD1, and NOD mice were immunized three times with PS4-p458m, PS4-p20, PS4-p30, or with PS4 alone in IFA. One month after last boost, the mice were challenged with $LD_{50} \times 10^5$ Pn and were scored for survival. NT, not tested.

[b] $p < 0.0079$ compared to PS4.

[c] $p < 0.06$ compared to PS4-p458m, PS4-p20, or PS4.

[d] $p < 0.0762$ compared to PS4-p458m, PS4-p20, or PS4.

[e] $p < 0.0476$ compared to PS4-p458m, PS4-p20, or PS4.

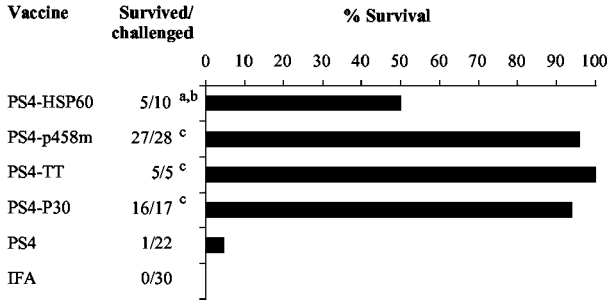

FIGURE 1. Vaccination with conjugate vaccines: challenge after three injections. BALB/c mice were immunized three times with PS4-HSP60, PS4-p458m, PS4-TT, PS4-p30, PS4, or IFA. One month later, the mice were challenged with $LD_{50} \times 10^5$–10^6 Pn and scored for survival. The data were collected from one to five individual experiments. [a]Value of $p < 0.0152$ compared with the other tested vaccines; [b]$p < 0.006$ compared with PS4 and IFA; [c]$p < 0.0001$ compared with PS4 and IFA.

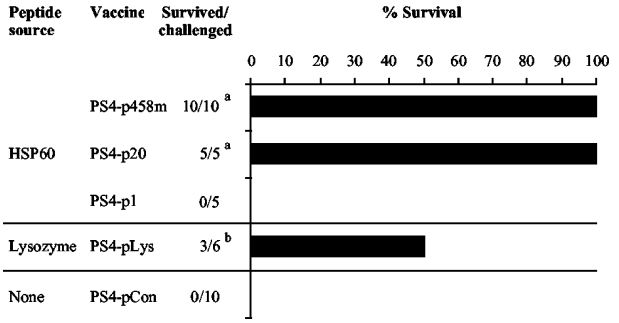

FIGURE 3. Survival of mice vaccinated with self-peptide carries. BALB/c mice were immunized three times with PS4-p458m, PS4-p20, or PS4-p1, all derived from HSP60, with PS4-pLys, derived from lysozyme, or with PS4-pCon, a random control peptide. One month later, the mice were inoculated with $LD_{50} \times 10^5$ of Pn. The mice were scored for survival. [a]Value of $p < 0.0079$ compared with PS4-p1 or PS4-pCon; [b]$p < 0.0487$ compared with PS4-p1 or PS4-pCon.

injections were effective for each of the three vaccines: PS4-p458m, PS4-TT, and PS4-p30 (not shown). However, one immunization revealed differences in efficacy between the vaccines (Fig. 2). Under these conditions, the PS4-p30 conjugate vaccine protected only 40% of the mice, whereas both PS4-p458m and PS4-TT protected ~80% of the mice. Hence, suboptimal vaccination can reveal differences in the effectiveness of vaccines that seem equally effective after adequate boosting.

Carrier p458m compared with other self-peptide carriers

We compared p458m to two other HSP60peptides: p20, a T cell epitope, and p1, which is not a T cell epitope and does not induce T cell proliferation (data not shown). In addition, we used a self-T cell epitope derived from a mouse lysozyme, pLys, and a control peptide, pCon, composed of a random sequence of amino acids. These peptides were conjugated to PS4: PS4-p458m, PS4-p20, PS4-p1, PS4-pLys, and PS4-pCon. Groups of mice were vaccinated three times and challenged 1 mo after the last boost with $LD_{50} \times 10^5$ of Pn. Fig. 3 shows that the PS4-pLys vaccine was able to protect 50% of the mice ($p < 0.02$ compared with vaccination with the unconjugated PS4). However, the PS4-p20 conjugate protected 100% of the mice, as did the p458m conjugate. Both PS4-p1 and PS4-pCon vaccines elicited no protection. Thus, the two immunogenic HSP60 peptides were more effective than an immunogenic peptide derived from a different self-molecule, pLys. The nonimmunogenic peptides were not effective carriers.

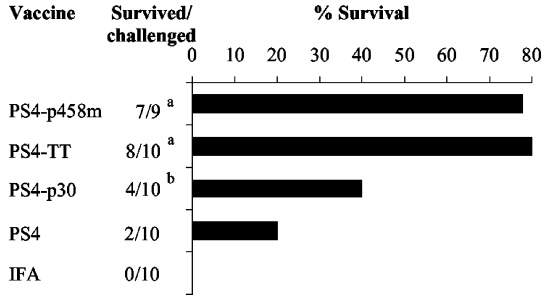

FIGURE 2. Vaccination with conjugate vaccines: challenge after one injection. BALB/c mice were immunized once with PS4-p458m, PS4-TT PS4-p30, PS4, or IFA. One month later, the mice were challenged with $LD_{50} \times 10^4$ Pn and scored for survival. The data were collected from two experiments. [a]Value of $p < 0.023$ compared with PS4 and IFA; [b]not significantly different from PS4 and IFA.

Different conjugates induce different amounts of anti-PS4 IgG Ab

Fig. 4 shows the amounts of anti-PS4 Ab induced by the different vaccines. The responses of individual mice are shown. It can be seen that the PS4-p20, PS4-TT, and PS4-p30 vaccines induced relatively high amounts of Ab to PS4 ($p < 0.0005$, compared with PS4-pCon and to PS4). The mice vaccinated with PS4-pLys that produced Ab also survived; mice that did not produce detectable amounts of Ab did not survive challenge. Although equally as effective as PS4-TT and PS4-p20 in inducing resistance (see Figs. 1–3), the PS4-p458m conjugate vaccine induced relatively low amounts of anti-PS4 Abs in ~50% of the vaccinated mice. The mice vaccinated with PS4-p458m, as a group, manifested significantly less anti-PS4 Abs than did the PS4-p20, PS4-TT, and PS4-p30 groups ($p < 0.043$). However, the PS4-p30 vaccine was still not as protective as the PS4-p458m after only one immunization (see Fig. 2). None of the mice vaccinated with PS4-pCon or PS4 made significant amounts of Ab to PS4, and all of these mice (excluding two mice that received PS4 vaccine) died after challenge with $LD_{50} \times 10^5$. Thus, unlike the other carriers, we could

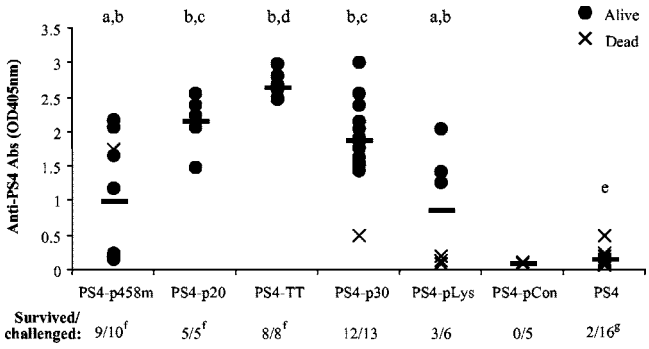

FIGURE 4. Anti-PS4 Ab in sera of vaccinated mice. Sera from mice vaccinated three times with PS4-p458m, PS4-p20, PS4-TT, PS4-p30, PS4-pLys, PS4-pCon, or PS4 were tested by ELISA (dilution of 1/50) for Ab binding to PS4. The sera were collected from one to two experiments. The mice were then challenged with $LD_{50} \times 10^5$–10^6 Pn and scored for survival. [a]Value of $p < 0.043$ compared with PS4-p20, PS4-TT, or PS4-p30; [b]$p < 0.0971$ compared with PS4-pCon or PS4; [c]$p < 0.06$ compare to PS4-TT or PS4-pLys. [d]Value of $p < 0.0036$ compared to PS4-p30 or PS4-pLys; [e]$p < 0.0302$ compared with PS4-pCon; [f]$p < 0.079$ compared with PS4-pCon or PS4; [g]$p < 0.001$ compared with PS4-pCon

not find a clear association between the degree of resistance induced by the p458m conjugate and the amount of anti-PS4 Ab it induced.

The p458m carrier does not induce Abs to itself

It is believed that a T cell response to the carrier is required to induce IgG Abs to the PS4 moiety of the conjugate (17). Nonimmunogenic carriers are not effective (see Table II and Ref. 18). However, Abs induced to the carrier might compete with immunity induced to the conjugated target Ag and negatively affect the vaccination (19). To investigate the induction of IgG Ab to the carrier, we immunized mice with the different conjugate vaccines and then tested the sera for Abs to the different carriers. The sera were also assayed for Ab binding to the source proteins of the peptide carriers. Likewise, sera of mice vaccinated with protein carriers were also tested for Abs to the peptide carriers (Fig. 5). The PS4 conjugates using whole HSP60, whole TT, peptide p30, or peptide p20 induced IgG Ab to the carrier. In contrast, PS4-p458m did not induce detectable IgG Abs to the carrier ($p < 0.0006$ compared with other tested vaccines). Furthermore, peptide p458m failed to induce anti-p458m IgG Abs even when conjugated to a carrier such as the keyhole limpet hemocyanin protein (not shown). The possibility that the p458m was not attached to the plate was ruled out by coating ELISA plates with p458m conjugated to BSA (data not shown). Interestingly, the PS4-TT vaccine induced Abs that bind to p30 ($p < 0.0037$, compared with the other vaccines) and Abs from PS4-p30-injected mice bind the whole TT protein ($p < 0.0009$). Thus, different carrier moieties in effective conjugate vaccines can induce greater or lesser amounts of IgG Ab to the carrier. The p458m carrier is notably free of the induction of Abs to itself, although it provides help for effective immunity to Pn.

Strain-dependent protection

To test the various peptide carriers in mice of different MHC backgrounds, we immunized several strains with PS4-p458m, PS4-p20, and PS4-p30. We used PS4 as a control vaccine. Female mice were immunized three times at 2-wk intervals beginning at age 8 wk. Mice were challenged with $LD_{50} \times 10^5$ 1 mo after the last immunization. Whereas the TT peptide p30 was efficacious in all the strains tested, both HSP60 peptides were protective only in mice of the H-2^d or H-2^k genotypes (Table II). Lymph node cells from each strain were tested for T cell proliferation following footpad injection with the peptides in IFA. As expected, the ability of T cells from each strain to proliferate in response to each peptide correlated with the protective efficacy of that peptide conjugated to PS4 (data not shown).

Induction of long-term protection

The susceptibility of the elderly to Pn infection is a major health problem (20). We performed two kinds of experiments to test whether the conjugate vaccines could protect old mice: the resistance of old mice vaccinated in their youth and the efficacy of vaccination done in old mice. In the first protocol, we vaccinated 8-wk-old mice with the different conjugate vaccines: PS4-p458m, PS4-TT, or PS4-p30. We used PS4-pCon, PS4, and IFA as controls. The vaccines were injected three times at 2-wk intervals. Sera were collected from the mice at 1 and 11 mo from the last boost and the serum IgG Ab to PS4 was determined. The mice were then challenged with Pn ($LD_{50} \times 10^3$) 1 year after the last boost, at 15 mo of age. Fig. 6 shows that both the PS4-p458m and PS4-TT vaccines induced long-term protection; they protected 100% of the 15-mo-old mice. Indeed, the titer of serum Ab to PS4 remained high over time ($p < 0.0001$, compared with the controls). In contrast, the low Ab response induced in some of the mice immunized with PS4 alone did not persist over time. We found that unconjugated PS4 induced IgM and IgG3 Abs (data not shown and Ref. 2). Both the IgM and the IgG3 isotypes are short-lived and this can explain the decrease in Ab reactivity to PS4 with time following vaccination with PS4 alone (21).

Vaccination at 1 year of age is effective

In the second protocol, we vaccinated mice at the age of 1 year with the PS4-p458m or PS4-p30 vaccines. We used PS4 and IFA as controls. The mice were bled 1 mo after the second boost and the sera were tested for IgG Abs to PS4. The mice were challenged with Pn ($LD_{50} \times 10^3$). The results in Fig. 7 indicate that the mice were protected at the age of 15 mo, and the amount of anti-PS4 Ab did not differ from that detected in young mice (not shown). The PS4 vaccine failed to protect the mice. Based on the combined

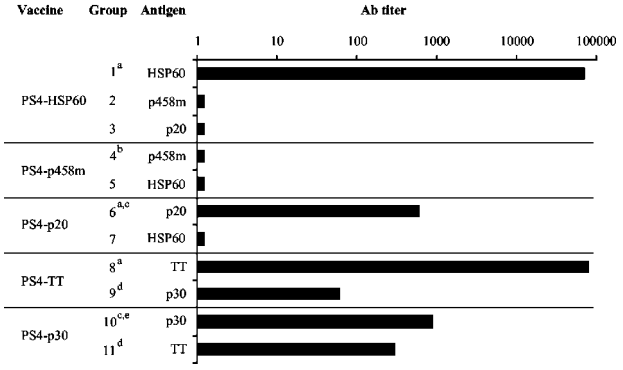

FIGURE 5. Ab to the carriers induced by conjugate vaccines. Groups of five to six mice were immunized with PS4-HSP60, PS4-p458m, PS4-p20, PS4-TT, or PS4-p30 conjugate vaccines. The sera were collected 1 mo after the last boost and pooled. The pooled sera were serially diluted and tested for IgG Abs to the different carriers by ELISA. The results are presented as the log of the serum titer. [a]Value of $p < 0.0008$ group 1 compared with groups 2 and 3, group 6 to group 7, and group 8 to group 9; [b]$p < 0.0006$ compared with groups 1, 6, 8, and 10; [c]$p < 0.0272$ compared with groups 1 and 8. [d]Value of $p < 0.0037$ compared with groups 2, 3, 5, and 7; [e]$p < 0.00669$ compared with group 11.

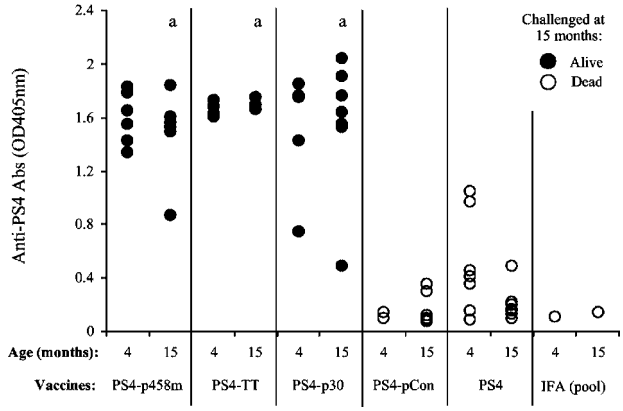

FIGURE 6. Long-lasting protection to challenge and IgG Ab to PS4. Groups of seven mice were immunized with conjugates PS4-p458m, PS4-TT, PS4-p30, or PS4-pCon. PS4 and IFA were controls. The mice were bled at the age of 4 mo (1 mo after immunization) and at the age of 15 mo (11 mo after immunization). The sera were tested by ELISA for Ab to PS4. Two days after the second bleeding, the mice were challenged with Pn ($LD_{50} \times 10^3$) and scored for survival. [a]Value of $p < 0.0001$ compared with controls at 15 mo.

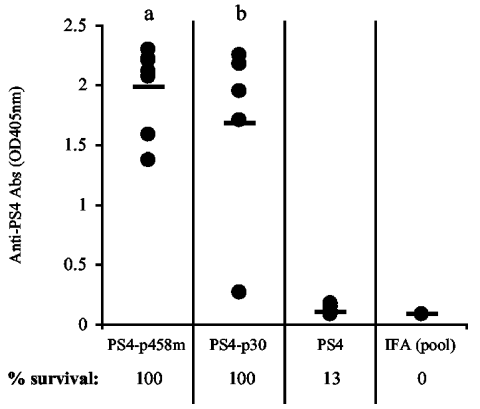

FIGURE 7. One-year-old mice respond to PS4 conjugate vaccines. Groups of five to eight BALB/c mice were immunized at the age of 12 mo with the conjugates PS4-p458m or PS4-p30. PS4 and IFA were used as controls. The mice were bled 1 mo from the last boost. The sera were tested by ELISA for IgG Ab to PS4. Two days after bleeding (age of ~15 mo), the mice were challenged with Pn ($LD_{50} \times 10^3$) and scored for survival. [a]Value of $p < 0.0001$ compared with controls; [b]$p < 0.022$ compared with controls.

results shown in Figs. 6 and 7, we can conclude that the performance of the PS4-p458m vaccine is not different from that of the PS4-TT vaccine and slightly better than the PS4-p30 vaccine in protecting old mice.

Discussion

In this work, we found that the protective capability of the p458m carrier conjugated to PS4 was either equal or superior to other tested carriers in BALB/c mice: TT, now being tested in clinical trials (6, 22), its peptide epitope p30 (23), lysozyme peptide pLys (13), and HSP60 peptides p20 and p1. The protective capability of p458m was evident in vaccination of both young and old mice. The PS4-p458m conjugate vaccine, after two to three injections, induced nearly complete (96%) resistance to challenge with very high amounts of Pn ($LD_{50} \times 10^5$–10^6). Similar results were obtained with PS4-TT, PS4-p30, and PS4-p20. However, a single immunization revealed differences between the vaccines: the PS4-p30 vaccine induced relatively low protection in comparison to that induced by PS4-p458m or PS4-TT. PS4-p458m led to resistance even in mice that made very low amounts of anti-PS4 Ab. Moreover, vaccination with PS4-p458m did not induce detectable Abs to the p458m carrier peptide. All the effective vaccines protected mice vaccinated at 1 year of age. In addition, the vaccines induced long-lasting serum Ab to PS4 and protected old mice that had been vaccinated 1 year before challenge. Finally, PS4-p30 was protective in the various strains we tested, whereas PS4-p458m and PS4-p20 were restricted to particular MHC genotypes. To the best of our knowledge, this is the first demonstration that a TT-derived peptide can be a protective carrier in a bacterial CPS conjugate vaccine. All conjugates were made with the same batch of PS4 and administered in IFA adjuvant. Therefore, differences between the various conjugates cannot be explained by differences in the PS4 moiety or in the adjuvant, but are likely to have resulted from the carriers themselves.

Proteins are highly efficient at recruiting T cell help for T-independent (nonprotein) molecules (24), and are widely used as carriers in vaccine strategies. If, as is shown in this study, peptides and proteins can serve similarly in subunit vaccines, what might be the advantage of using peptides instead of whole proteins as carriers? Immunologically, proteins can be viewed as a collection of epitopes; some epitopes might contribute to a desirable response while others might interfere or suppress the response (19, 25). Indeed, the specific interference of a protein carrier with the response to its conjugated Ag has been reported (26, 27). Peptide carriers might circumvent such problems.

No detectable IgG Ab-response to the p458m carrier was observed throughout our experiments with the PS4-p458m vaccine. In contrast, large amounts of anti-carrier Ab to the TT and the HSP60 carriers, and moderate amounts of Abs to the p30 and the p20 carriers were produced. Thus, the effective p30 and p20 carriers provided both T cell and B cell epitopes. Indeed, Lett et al. (28), who conjugated Pn CPS to carrier peptides, concluded that peptides that are both T cell and B cell epitopes could serve as good carriers. Nevertheless, p458m, which does not induce a B cell response to itself, served as a very effective carrier. In fact, carriers that do not elicit Abs to themselves may be advantageous because high titer anti-carrier Abs may eventually suppress the reaction to the attached CPS (29). Suppression caused by multiple administrations of a carrier is a real concern because only a few proteins have been approved thus far for use as carriers (17). Recent data raise the issue of carrier suppression both in humans (30–32) and in animal models (19, 33, 34).

Limitations in the binding of a single T cell epitope peptide to different MHC alleles is a potential drawback to the use of peptides as carriers in conjugate vaccines. Indeed, the PS4-p458m vaccine was effective in only a few mouse strains. In contrast, the universal p30 carrier (8) conjugated to PS4 was protective in all of the murine strains we have tested so far. Protection correlated with T cell proliferative responses to the peptide (data not shown). Similarly, Alexander et al. (35) designed a synthetic peptide, PADRE, that could bind multiple HLA alleles. PADRE-PS conjugate vaccines were able to induce high titers of IgG Abs to PS in mice (36). Thus, a single peptide may be able to replace many of the carrier functions of TT or other carrier proteins in CPS conjugate vaccines.

Most vaccines are evaluated by their ability to induce high titers of Ab to the relevant B cell epitopes (20, 24, 37). Indeed, the mice that were vaccinated with PS4-p20, PS4-TT, PS4-p30, or with PS4-pLys and survived the Pn challenge produced Abs to PS4. Because the protection induced by vaccination was serotype-specific (2), we can conclude that resistance to challenge was not due to immunization to the nonprotein backbone of the cell wall or other bacterial structures common to different serotypes. The amount of Abs to PS4 induced by the PS4-p458m vaccine was variable. Nevertheless, most of the mice vaccinated with PS4-p458m survived a highly lethal dose of Pn, even if they produced little detectable anti-PS4 Ab. We are currently investigating the mechanisms that might explain this phenomenon. Perhaps the T cells themselves might add a measure of resistance. For example, the expression of self-HSP60 is up-regulated in inflammation generally (38). Hence, HSP60 peptide epitopes, such as p458m, might provide T cell help at the site of infection and not only during vaccination (39, 40). Thus, serum Ab is not necessarily the only way to evaluate and predict the ability of a given vaccine to induce protection.

Interestingly, the ability of HSP60-derived peptides to protect mice was significantly better than that of the whole HSP60 protein. This result is surprising because HSP60 is very immunogenic and induces both T and B cell responses toward itself when injected alone (not shown). Perhaps an excessive number of B cells responding to HSP60 might interfere with the anti-PS4 B cells. Indeed, healthy mice show detectable IgM Abs to self-HSP60, but

not to foreign proteins such as purified protein derivative of mycobacteria (41). It is important to note that throughout our extensive use of the p458m conjugate in young and old mice, overt autoimmune disease was not observed. Vaccination with the HSP60-based conjugate, too, did not lead to autoimmune disease.

Pn is one of the major causes of death among the elderly (20, 42, 43). Old mice, like elderly people, suffer from decreased immunity (20), suggesting that old mice can serve as a model for Pn vaccination in the elderly. It was reported that the response of older people to a commercial vaccine containing unconjugated PS from 23 Pn serotypes (23-valent) was variable and marked by inadequate or ineffective Ab responses (44). Likewise, the response of old mice to the 23-valent vaccine also declined with age (42). In our experiments too, mice older than 1 year, in contrast to young mice, were not protected from even low amounts of Pn when immunized with unconjugated PS4. However, the PS4-p458m, PS4-TT, and PS4-p30 conjugate vaccines were able to induce anti-PS4 Abs and protect old mice, and old mice that had been vaccinated when young. Finding an effective vaccine for the elderly is a critical step toward reducing mortality from Pn infection.

The rise of antibiotic resistance among invasive bacteria demands greater focus on vaccine strategies. The design of novel and varied carrier molecules represents one way to improve vaccine potency and overcome problems associated with the repetitive use of the same protein carrier, such as TT or diphtheria toxoid (17). Defined immunogenic foreign peptides such as TT-derived p30 may eventually diversify the carrier pool for bacterial PS conjugate vaccines. Stress protein-derived peptides, such as the self-HSP60 p458m, might provide a significant advantage especially if they can be proved not to induce autoimmune disease and still provide strong T cell help without eliciting B cell responses to themselves.

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