

Self Heat-Shock Protein (hsp60) Peptide Serves in a Conjugate Vaccine against a Lethal Pneumococcal Infection

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Healthy persons manifest a high frequency of T cells reactive to epitopes of the self 60-kDa heat-shock protein (hsp60) molecule. It was reasoned that a self hsp60 peptide, p458m, might provide T cell help for a response to the T independent capsular polysaccharide of *Streptococcus pneumoniae* type 4 (PS4). The conjugate vaccine (PS4-p458m) induced resistance to challenge with >300,000 times the minimal lethal dose of pneumococci. PS4 conjugated to other immunogenic carriers (tetanus toxoid, a pneumolysin peptide, and others) or a commercial pneumococcal vaccine were far less effective. The effectiveness of the PS4-p458m conjugate was associated with an increased IgG1 antibody response to PS4, with long-term memory, and with T cell responses to the p458m peptide. Thus, T cell reactivity to a self epitope in a conjugate vaccine can be mobilized to induce help for resistance to a lethal infection.

The emergence of drug-resistant bacteria and the renewed threat of infectious diseases command the development of improved vaccines. *Streptococcus pneumoniae* and many virulent bacteria encapsulate themselves within polysaccharide coats to thwart host immune defenses [1, 2]. Such polysaccharide molecules are T independent antigens that are not recognized by T cells. Hence, immunization with these polysaccharide antigens lacks the T cell help needed by the responding B cells to switch from production of IgM to IgG antibody, and there is no affinity maturation of the antibodies and no immunologic memory [3]. To enhance T cell dependent antipolysaccharide responses, capsular polysaccharide molecules have been conjugated to foreign proteins immunogenic for T cells such as diphtheria or tetanus toxoid (TT) [4–7]. Diphtheria and TT conjugate vaccines have received attention because humans are routinely immunized against diphtheria toxoid and TT from

early childhood. However, large protein carriers have several disadvantages. First, the amount of protein in the conjugate has to be tightly controlled to enable the induction of T cell help and avoid production of anticarrier antibodies that can inhibit the antipolysaccharide immune response [8]. Second, a large protein carrier also might contain suppressor epitopes [9, 10].

Healthy persons are populated with T cells responsive to heat-shock protein (hsp) 60 epitopes of self as well as foreign origin [11, 12], apparently from birth [13]. Moreover, T cells reactive to hsp60 accumulate at sites of inflammation in large numbers [14, 15], probably because the stress of inflammation and the activation of immune cells up-regulate expression of self hsp60 components on the surfaces of antigen-presenting cells [16, 17]. Thus, hyperexpression of hsp60 can faithfully mark a site of infection for anti-hsp60 T cells. We identified a strongly immunogenic T cell epitope peptide derived from the mouse hsp60 molecule, by immunizing BALB/c mice with overlapping peptides encompassing the entire sequence of mouse hsp60. The peptide position in the hsp60 sequence was between residues 458 and 472, and therefore was named p458m. P458m (previously designated CP1m) was shown to be an effective T cell carrier epitope when conjugated to the capsular polysaccharide (Vi) of *Salmonella typhi* [18].

In the present experiments, we tested whether p458m could serve as a T cell carrier when conjugated to the type 4 pneumococcal capsular polysaccharide (PS4) and protect mice against a lethal pneumococcal infection. *S. pneumoniae* type 4 was chosen as the challenge organism because an intraperitoneal inoculation of mice with only 2 or 3 of these bacteria is lethal within 24–48 h [19]. Therefore, *S. pneumoniae* type 4 can be used conveniently in studies aimed to measure the potential of PS4 conjugates to induce active immunity to a severe infection [20].

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Materials and Methods

Mice. Female BALB/c mice (Harlan Olac, Bicester, UK) were used at 6–8 weeks of age.

S. pneumoniae capsular polysaccharide type 4 antigen. PS4 was obtained from the American Type Culture Collection (Rockville, MD). Pneumovax is a commercially available vaccine (Merck Sharp & Dohme, Rahway, NJ).

Peptides and proteins. Peptides were prepared by using an automated multiple peptide synthesizer (model AMS 422; Abimed, Langenfeld, Germany) using the company protocols for *N*- α -fluorenylmethoxycarbonyl (Fmoc) synthesis or were prepared manually by a standard solid phase method using either *N*- α -Fmoc or *N*- α -t-butyloxycarbonyl (t-Boc) strategies [21]. The peptides were purified by reversed phase high-performance liquid chromatography (HPLC) on a semipreparative C_8 -column (Lichrosorb RP-8, 7 μ m, 250 \times 10 mm; Merck, Darmstadt, Germany). The elution of peptides was achieved by linear gradients established between 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in 75% acetonitrile in water (vol/vol). The purity of the peptides was ascertained by analytical reversed-phase HPLC and amino acid analysis. The peptides we used are listed in table 1. The active peptide derived from mouse hsp60 was named p458m to indicate its starting position in the amino acid sequence and its mouse origin. This peptide was termed CP1m in a previous publication [18]. For similar reasons, the *Escherichia coli* homologue was named p437ec (it had been previously named CP1ec). TT was kindly donated by R. Arnon (Weizmann Institute of Science).

PS4 conjugation. PS4 (5 mg) was dissolved in 1 mL of double-distilled water (DDW), and the PS4 was activated with cyanogen bromide (final concentration = 2 mg/mL) in the presence of 30 mM triethylamine (Aldrich, Milwaukee) at pH 7. Two minutes later, 10 mg of the spacer 6-aminohexanoic acid (BDH Chemicals, Poole, UK) dissolved in 100 μ L of DDW were added, and the solution was incubated for 2 h at 4°C with the activated PS4. The resulting product was dialyzed against DDW and stored at 4°C. The PS4 spacer-conjugate was coupled to carrier peptides or to TT by using water-soluble diimide: 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (CDI; Aldrich). For this conjugation, 7 mg of peptide or protein was added to the PS4 spacer-conjugate in the presence of 12 mg of water-soluble CDI. The pH was adjusted to 6, and the mixture was stirred for 4 h at room temperature. The same amount of CDI was then added, and the mixture was incubated overnight and then dialyzed at 4°C against DDW. The amount of peptide or protein attached to the PS4 was determined by amino acid analysis. The carbohydrate concentration in the conjugate mixture was determined by the method of Dubois et al. ([22], see below).

Carbohydrate determination. This protocol is based on the observation that carbohydrates (mono-, oligo-, and polysaccharides)

and their derivatives give an orange-yellow color when treated with phenol and concentrated sulfuric acid [22]. Two milliliters of the carbohydrate solution (standard or sample, each in duplicate) was pipetted into a glass tube, and 50 μ L of 80% phenol (Sigma, Rehovot, Israel) was added. Then, 5 mL of concentrated sulfuric acid was added rapidly, and the stream of acid was directed against the liquid surface rather than against the side of the test tube in order to obtain good mixing. The tubes were allowed to stand for 10 min; they were then shaken and placed for 10 to 20 min in a water bath at 25–30°C. The absorbance of the characteristic yellow-orange color was measured at 490 nm. The amount of carbohydrate was determined by reference to a standard curve constructed in the same assay.

Immunization. Mice were injected three times subcutaneously at 14-day intervals with 2 μ g/0.1 mL of PS4 or conjugated PS4 in incomplete Freund's adjuvant (IFA), unless stated otherwise. Serum samples were collected 12 days after each injection and later by taking blood samples from the retroorbital space or the tail vein of the mice. Blood was allowed to clot at room temperature, and the serum was removed and stored at –20°C.

ELISA. For the determination of PS4-specific antibodies in serum samples, Maxisorb (Nunc) 96-well plates were coated with PS4 (1.25 μ g/well) for 16 h at 4°C. After 1 h of blocking with 1% bovine serum albumin in PBS, the plates were washed three times with 0.02% Tween 20 (Sigma) in PBS. Serum samples were diluted 1:100 in 0.5% bovine serum albumin in PBS and incubated for 2 h at 37°C. Goat anti-mouse IgG Fc-alkaline phosphatase conjugate (Jackson, West Grove, Pennsylvania) diluted 1:1000 was incubated for 1 h at 37°C to detect IgG antibodies. Different IgG subclasses and IgM antibodies were detected using rabbit anti-mouse subclass-specific antibodies attached to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). Bound antibodies were visualized by the addition of a substrate solution containing 0.6 mg/mL *p*-nitrophenylphosphate (Sigma) in diethanolamine-H₂O, pH 9.8. The optical density was read at 405 nm after 30 min of incubation.

T cell proliferative responses. Proliferation assays of spleen cells were performed 12 days after the last immunization. Splenocytes (2×10^5 /well) were cultured in the presence of the various peptides and conjugates at the indicated antigen concentrations. Cultures were grown in 200 μ L of stimulation medium composed of RPMI 1640 supplemented with 2 mM glutamine, nonessential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μ g/mL streptomycin, 0.25 μ g/mL fungizone (BioLab, Jerusalem), 5×10^{-5} M β -mercaptoethanol (Fluka, Buchs, Switzerland), and 10 mM HEPES buffer (Sigma) containing 1% syngeneic normal mouse serum, in round-bottom microtiter plates (Falcon, Cowley, UK). After 3 days of incubation, [³H]thymidine (0.5 μ Ci of 5 Ci/mmol; Nuclear Research Center, Negev, Israel) was added. At 16 h later,

Table 1. Peptides used in studies of conjugate vaccine against a lethal pneumococcal infection.

Origin	Position	Peptide name	Sequence
Mouse hsp60	458–474	p458m	NEDQKIGIEIHKRALKI
<i>Escherichia coli</i> hsp60 (GroEL)	437–453	p437ec	NEDQNVGKVALRAMEA
Pneumococcal pneumolysin	263–281	pPL	FEALIKGVKVPQTEWKQI
Murine invariant chain	85–101	CLIP	KPVSQMRMATPLLMRPN
λ repressor	12–26	λ rep	LEDARRLKAIEYKKK

the cells were harvested, and the radioactivity was counted. The results are expressed as the counts per minute of [3 H]thymidine uptake.

Preparation of bacteria. Lyophilized *S. pneumoniae* type 4 and type 3 (Danish nomenclature) were obtained from the American Type Culture Collection. The lyophilized bacterial preparations were reconstituted and subcultured on sheep blood agar, and colonies were resuspended in brain-heart infusion (BHI) broth. Aliquots of a 6-h bacterial culture were stored at -70°C in medium plus 25% glycerol. Pneumococci for infection were grown from a frozen stock for 6 h in BHI broth, and bacterial growth was estimated by the optical density reading of the culture broth at 545 nm. The bacteria were kept at 4°C from the time of dilution until injection (usually 30 min). Before dilution, encapsulation was confirmed by the agglutination of the bacteria with specific antiserum. The actual numbers of living bacteria injected in each challenge were determined immediately before injection by plating dilutions of the culture on sheep blood agar and counting the colonies on the plates 24 h later.

Determination of the minimum lethal dose (MLD). To determine the pathogenicity of *S. pneumoniae* type 4 or type 3 bacteria for our mice, we injected groups of naive BALB/c mice intraperitoneally with 0.2 mL of serial dilutions of a 6-h bacterial culture and scored the animals for survival for 14 days. The inocula were plated to determine the numbers of colony-forming units (cfu) in each experiment. All naive mice challenged with ~ 3 cfu or more of *S. pneumoniae* type 4 died within 2 days of challenge. An MLD of ~ 3 cfu has been also reported by others [23].

Protection assay. Groups of BALB/c mice were immunized as described above. Three to four weeks after the last immunization, or at the times indicated, the mice were challenged intraperitoneally with 0.2 mL of BHI broth containing the indicated cfu of pneumococci and were scored for survival for 2 weeks. All mice alive at the end of this period were considered to have survived the challenge.

Passive protection by serum. Pooled immune serum was obtained from 20 BALB/c mice immunized with the PS4-p458m conjugate or with PS4 alone. The presence of PS4-specific antibodies in the serum was verified by ELISA. Normal mouse serum was pooled from 20 age-matched naive BALB/c mice. For passive protection experiments, groups of naive BALB/c mice were injected intravenously with various amounts of serum 1 day before challenge with *S. pneumoniae* type 4. Survival was scored for 14 days.

Test serum was depleted of PS4 antibodies by preincubation with heat-killed (60°C , 10 min) *S. pneumoniae* type 4 ($10^8/\text{mL}$) for 2 h at 4°C . The mixture was centrifuged in an Eppendorf centrifuge, and the supernatant was collected. The loss of anti-PS4 antibodies in the treated serum and the untreated serum was measured by ELISA. The sera were diluted in PBS, and a total volume of 0.4 mL was injected intravenously into naive mice. The mice were challenged 24 h later.

Anti-PS4 antibodies were purified by affinity chromatography. PS4, activated by cyanogen bromide, was coupled to 1.6 diamino-hexan-sepharose (provided by Prof. M. Wilchek, Weizmann Institute of Science). The test serum was loaded onto the column, and the effluent was collected. The column was washed with 10 vol of PBS, and the anti-PS4 antibodies were eluted with 0.1 M acetic acid, pH 3, and neutralized immediately with 1 M Tris, pH 10.

The presence of anti-PS4 antibodies in the eluate and their absence from the effluent was confirmed by ELISA (not shown). The protein concentrations of the different fractions were determined using the Bio-Rad (Richmond, CA) protein assay (based on the Bradford method [24]).

Statistical analysis. Results were analyzed for significance using Fisher's exact test, computed using software package InState 2.01 for the Macintosh computer.

Results

PS4-p458m protects against pneumococcal challenge. The PS4-p458m conjugate vaccine was compared with PS4 alone; with PS4 conjugated to a peptide from the pneumolysin molecule of *S. pneumoniae*, which has been reported to act as a carrier in vaccination to the type 17 capsular polysaccharide (PS4-pPL, [25]); with PS4 conjugated to TT, a well known carrier protein (PS4-TT [4, 26]); with PS4 conjugated to the *E. coli* homologue of the mouse p458m peptide (PS4-p437ec); with PS4 conjugated to the murine class II-associated invariant chain (CLIP) peptide (PS4-CLIP, [27]); with PS4 conjugated to the λ -repressor peptide, which has been demonstrated to bind to IA^d and IE^d (PS4- λ rep, [28]); or with unconjugated p458m peptide. All of these carriers were found to be T cell immunogens in BALB/c mice (data not shown). Table 2 shows the cumulative results of 12 different experiments. PS4-p458m was significantly more protective than the other conjugates in each separate experiment. The 97% protection provided by PS4-p458m was significantly greater ($P < .001$) than the degree of protection induced by any of the other conjugates (30%–50% survival). None of the other carrier conjugates caused increased survival above that obtained with unconjugated PS4. Peptide p458m unconjugated to PS4 did not induce any protection. When unconjugated PS4 and p458m were mixed, the protection was similar to that obtained using PS4 alone (data not shown), suggesting that the p458m peptide had to be conjugated to PS4

Table 2. PS4-p458m protects against pneumococcal challenge.

Vaccination with	No. of experiments	No. of survivors/total	% survival
PS4-p458m	12	62/64	97 ^a
PS4	9	23/51	45
PS4-pPL	4	8/26	31
PS4-TT	8	17/45	38
PS4-p437ec	2	3/10	30
PS4-CLIP	2	3/10	30
PS4- λ rep	2	4/10	40
p458m	2	0/10	
None	11	0/60	

NOTE. BALB/c mice were immunized with PS4 polysaccharide or with PS4 conjugated to indicated carrier molecules or with p458m peptide alone. Three weeks after last immunization, mice were challenged intraperitoneally with amounts of *S. pneumoniae* type 4 250–13000 times MLD and scored 2 weeks for survival. Results are presented as cumulative survival obtained in indicated no. of different experiments. PS4 = type 4 pneumococcal capsular polysaccharide; p458m = self hsp60 peptide (p437ec = *Escherichia coli* homologue); TT = tetanus toxoid; pPL = peptide of pneumolysin molecule of *S. pneumoniae*.

^a $P < .001$ compared with each of other groups of mice.

Table 3. PS4-p458m vaccinations without adjuvant.

Vaccination with	No. of survivors/total	% survival
PS4-p458m	6/6	100 ^a
PS4	1/6	17
PS4-pPL	1/6	17
PS4-TT	1/6	17
None	0/6	0

NOTE. BALB/c mice were immunized with indicated antigens in PBS. Three weeks after last immunization, mice were challenged intraperitoneally with 4×10^3 CFU of *S. pneumoniae* type 4 (1300 MLD). PS4 = type 4 pneumococcal capsular polysaccharide; p458m = self hsp60 peptide; TT = tetanus toxoid; pPL = peptide of pneumolysin molecule of *S. pneumoniae*.

^a $P < .02$ compared with each of other groups of mice.

to induce optimal protection. Full protection was obtained even when PS4-p458m was delivered in PBS alone without adding IFA to the mixture. As shown in table 3, all the mice immunized with PS4-p458m in PBS survived challenge with 4×10^3 cfu. In the other groups, PS4, PS4-TT, and PS4-pPL, only 1 of 6 mice survived. These results indicate that PS4-p458m does not need an adjuvant to induce protection.

PS4-p458m confers resistance to high doses of S. pneumoniae. To measure the degree of resistance induced by vaccination, we challenged groups of 4–9 mice with increasing numbers of bacteria and measured murine survival. Figure 1 gives some indication of the extent of the protection afforded by the PS4-p458m conjugate vaccine. Almost all (90%) PS4-p458m-vaccinated mice withstood challenge with 10^6 cfu (3.3×10^5

MLD) of *S. pneumoniae* type 4. In contrast to vaccination with PS4-p458m, vaccination with unconjugated PS4 or with a commercial vaccine (Pneumovax) induced only partial (50%) or no protection to challenge with $\gamma 1000$ cfu (333 MLD) of bacteria. Combining all the results in each group also demonstrates the advantage of PS4-p458m over the other vaccines. In total, 30 (86%) of 35 mice survived in the PS4-p458m group and only 8 (22%) of 36 in the PS4 group, 2 (12%) of 16 in the commercial vaccine group, and 0 of 36 in the naive group. This protection was immunologically specific: PS4-p458m-vaccinated mice succumbed to challenge with type 3 pneumococci (not shown), which are antigenically distinct from type 4 [29].

PS4-p458m enhances anti-PS4 antibody production. To what immune mechanism might the resistance be attributed? Figure 2 shows that the PS4-p458m conjugate vaccine induced higher levels of IgG antibodies than did the PS4-TT or PS4-pPL conjugates or the unconjugated PS4. Note that there was a range of responders; among the mice immunized with the PS4-p458m conjugate vaccines, there were individual mice that made very little IgG antibody ($A_{405nm} < 0.2$). Nevertheless, all of the PS4-p458m-vaccinated mice survived challenge, while 50%–80% of the mice in the other groups died (marked by the asterisk).

PS4-p458m induces mainly IgG1 antibodies. Figure 3 shows that mice immunized with unconjugated PS4 produced mainly IgM anti-PS4, and to a lesser extent IgG3, as has been generally

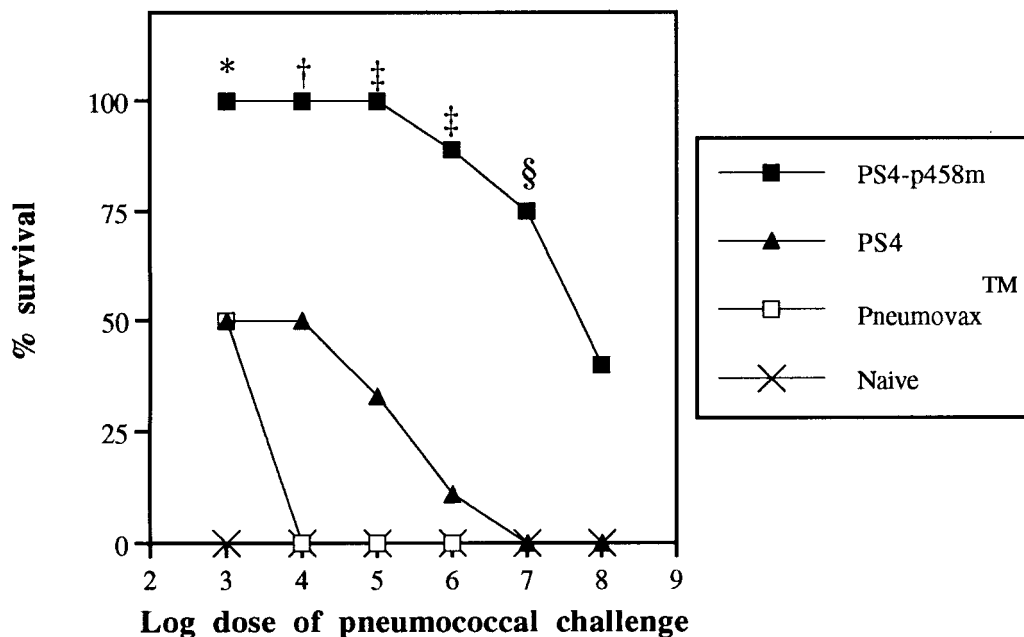


Figure 1. PS4-p458m confers resistance to high doses of *S. pneumoniae* type 4. Groups of 4 to 9 BALB/c mice were immunized with PS4-p458m, PS4, or commercial vaccine (Pneumovax). Three weeks later, mice were challenged intraperitoneally with indicated doses of *S. pneumoniae* type 4 and scored 14 days for survival. Cumulative results of 2 experiments are presented. * $P < .03$ vs. naive group. † $P < .03$ compared with naive and commercial vaccine-treated groups. ‡ $P < .005$ compared with naive, commercial vaccine-, and PS4-treated groups. § $P < .05$ compared with naive and PS4-treated groups. PS4 = type 4 pneumococcal capsular polysaccharide; p458m = self hsp60 peptide.

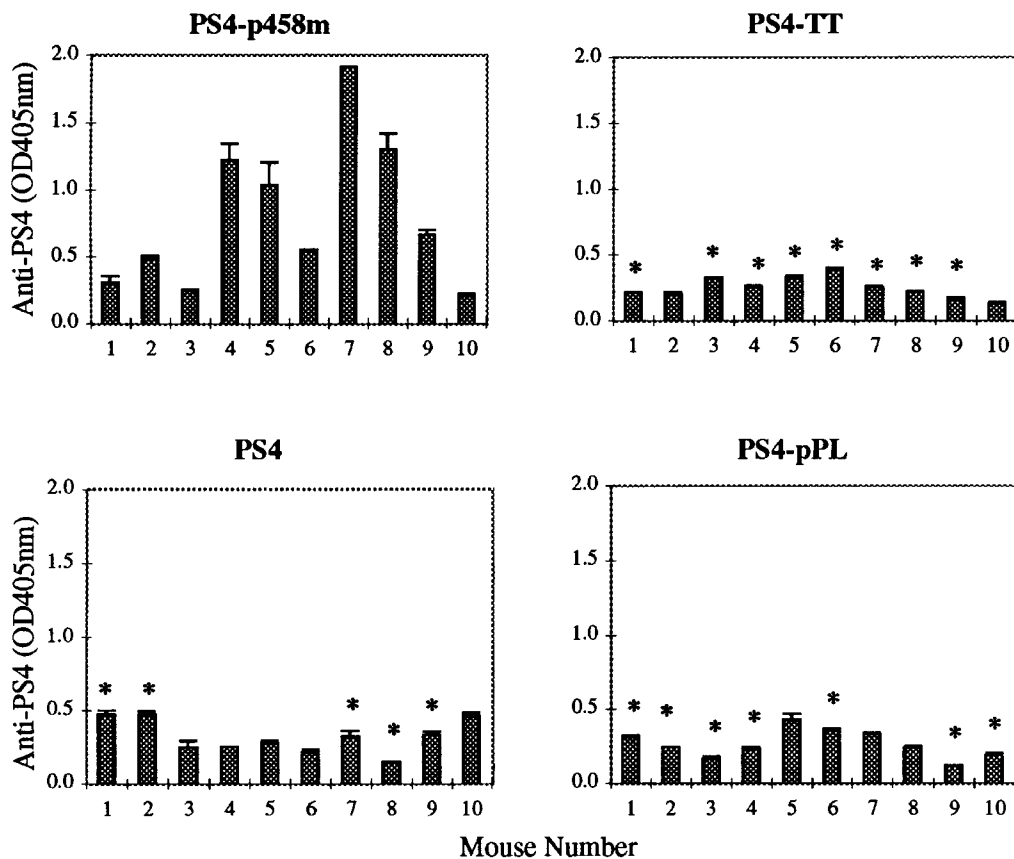


Figure 2. Effects of PS4-p458m conjugate vaccine on anti-PS4 antibodies and resistance to pneumococcal challenge. Groups of 10 BALB/c mice were immunized with PS4-p458m, PS4-TT, PS4-pPL, or PS4 immunogens. Sera were collected 12 days after the last immunization and assayed by ELISA for IgG anti-PS4 antibodies. On following day, mice were challenged intraperitoneally with 6000 cfu (2000 MLD) of pneumococci type 4 and followed for 14 days for survival. *Mice that died following challenge (experiment was done 4 times with similar results). PS4 = type 4 pneumococcal capsular polysaccharide; p458m = self hsp60 peptide; TT = tetanus toxoid; pPL = peptide of pneumolysin molecule of *S. pneumoniae*.

reported for T independent antigens [3]. In contrast, the PS4-p458m conjugate elicited high anti-PS4 IgG levels, with a high proportion of PS4-specific IgG1 antibodies (figure 3).

Passive protection transferred by PS4-p458m serum. To learn whether anti-PS4 antibodies can mediate passive protection to challenge, various amounts of sera obtained from mice immunized with unconjugated PS4 or with the PS4-p458m conjugate were transferred intravenously to naive mice 1 day before challenge with a dose of *S. pneumococcus* equivalent to ~1000 times the MLD (~3000 cfu). Control mice were not injected or were injected intravenously with normal mouse serum (we did not test sera from mice injected with p458m alone because such mice were not resistant; table 2). As shown in table 4, all the control mice and the mice that had received 0.2 or 0.5 mL of serum from PS4-immunized mice died 1 day after challenge. In contrast, all the mice receiving 0.05–0.5 mL of the PS4-p458m immune serum were completely protected. There was no protection afforded by 0.02 mL of the PS4-p458m serum.

To confirm that anti-PS4 antibodies mediated the protection,

we depleted the PS4-specific antibodies using whole heat-killed *S. pneumoniae* type 4 or a PS4 affinity column (table 4). The protective effect of the PS4-p458m serum was lost by either adsorption procedure. The protective effect was recovered with the eluate of the PS4 column (table 4), which contained the affinity purified anti-PS4 antibodies (demonstrated by ELISA; data not shown). This suggests that the presence of anti-PS4 antibodies in the serum was crucial for the protection induced against *S. pneumoniae* infection.

T cell proliferative responses. To analyze whether T cells were activated by immunization with the PS4 conjugates, T cell proliferation was measured in splenocyte populations of mice immunized with different PS4 conjugates: PS4-p458m, PS4-TT, or PS4-pPL. The different groups (10 mice/group) were divided into 2: 6 mice were then challenged with 4×10^3 cfu of pneumococci to document their resistance; the other 4 mice were sacrificed, and the proliferative responses of their spleen T cells were assayed. With regard to resistance, all of the mice vaccinated with PS4-p458m survived challenge, and all the mice

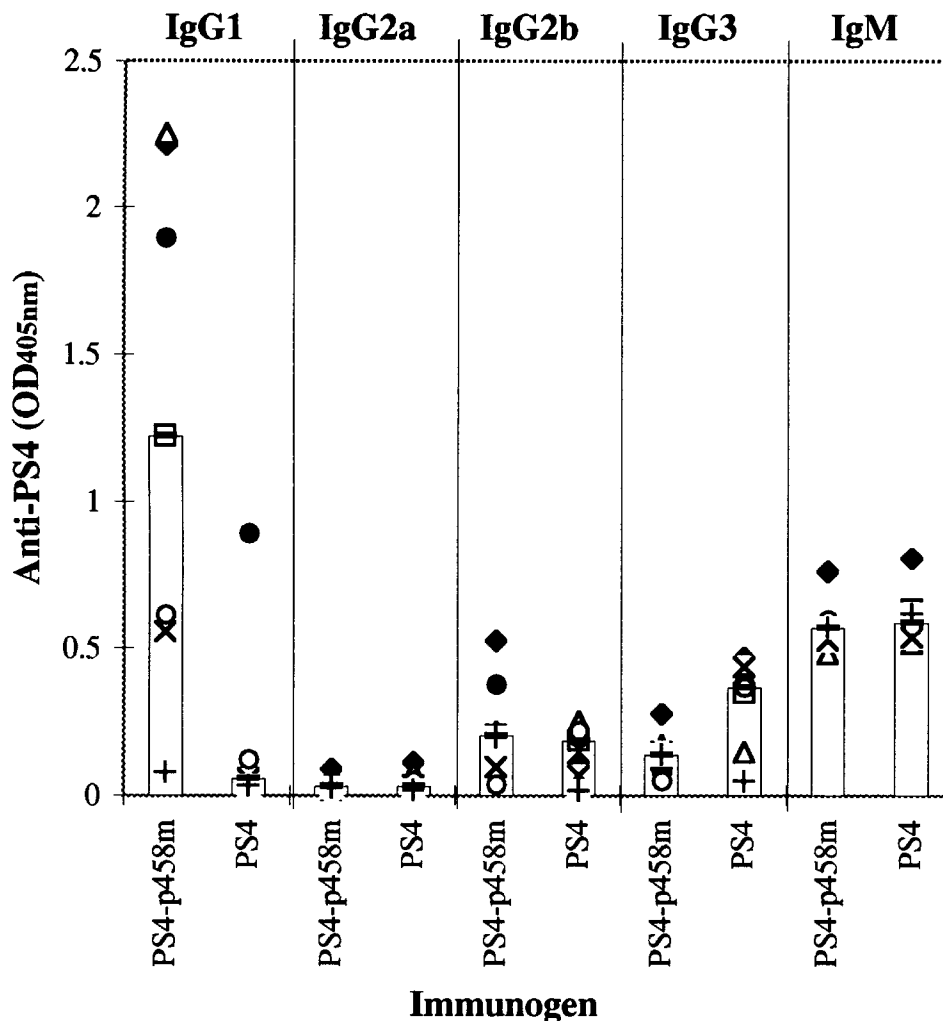


Figure 3. PS4-p458m induces IgG1 anti-PS4 antibodies. Seven BALB/c mice per group were immunized with PS4-p458m or with PS4; sera were tested individually by ELISA for presence of PS4-specific IgG1, IgG2a, IgG2b, IgG3, and IgM antibodies as indicated. Bars indicate median values. PS4 = type 4 pneumococcal capsular polysaccharide; p458m = self hsp60 peptide.

vaccinated, in this experiment, with PS4-TT or PS4-pPL, died within 2 days. Figure 4 shows that immunization with PS4-p458m gave rise to spleen cells that responded significantly ($P < .05$) to the p458m peptide (figure 4A). The PS4-TT-primed spleen cells also showed a significant ($P < .05$) proliferative response to TT and to PS4-TT (figure 4B). Thus, the TT conjugate vaccine was immunogenic for proliferative T cells. In contrast, vaccination with PS4-pPL elicited very weak proliferative responses to the pPL peptide or to the PS4-pPL conjugate (figure 4C) that were not stronger than the background proliferation of naive spleen cells in response to these antigens (figure 4D). None of the spleens showed a proliferative response to unconjugated PS4. These findings indicate that both the PS4-p458m and the PS4-TT conjugates elicited proliferative T cells. Nevertheless, the PS4-p458m conjugate was more effective in

inducing anti-PS4 antibodies and protection to challenge (see table 2 and figure 2).

PS4-p458m elicits long-lasting memory. To examine the long-term effects of PS4-p458m immunization, we followed up the anti-PS4 immune response over various periods of time after a second booster injection. The PS4-specific antibody titers of mice immunized with the PS4-p458m conjugate did not fall 6 months after the last boost but actually increased (figure 5A). Indeed, the titers continued to rise even after 1 year (not shown). This was not the case for sera obtained after immunization with unconjugated PS4. Although 1 mouse (no. 8) showed an enhanced primary immune response, this antibody response had disappeared by 6 months later (figure 5A). In protection assays performed 6–17 months following immunization, almost 90% of the PS4-p458m immunized mice survived, whereas only

Table 4. PS4-p458m immune serum induces passive protection.

	Volume (mL)	No. of survivors/total
Serum		
None	—	0/4
Naive	0.5	0/4
PS4	0.5	0/3
	0.2	0/5
PS4-p458m	0.5	4/4 ^a
	0.2	5/5 ^b
	0.1	3/3 ^a
	0.05	3/3 ^a
	0.02	0/3
PS4-p458m serum depletion by incubation with inactivated type 4 pneumococci		
PS4-p458m	0.15	4/5 ^c
PS4-p458m (depleted)	0.15	0/5
PBS	—	0/4
Affinity purification of PS4-p458m serum on PS4 column		
Anti-PS4 antibodies (eluate)	25 μ g	5/5 ^b
PS4-p458m (effluent)	2500 μ g	0/5
PBS	—	0/4

NOTE. Naive BALB/c mice were injected intravenously (iv) with indicated amounts of naive mouse serum or with serum obtained from mice that had been immunized with PS4-p458m or with PS4. One day later, mice were challenged with 3200 cfu (1000 MLD) of *S. pneumoniae* type 4 and scored for survival. For PS4-p458m serum depletion of anti-PS4 antibodies by adsorption with heat-killed pneumococcus, mice were challenged with 2600 cfu (850 MLD). For anti-PS4 antibodies affinity-purified using column of PS4-coupled to Sepharose, mice were injected iv with either eluate or effluent and challenged with 5800 cfu (2000 MLD). PS4 = type 4 pneumococcal capsular polysaccharide; p458m = self hsp60 peptide.

^a $P = .029$ compared with "PBS" or "None" controls.

^b $P = .008$ compared with "PBS" or "None" controls.

^c $P = .048$ compared with "PBS" or "None" controls.

30%–50% of the mice in the other groups survived (figure 5B). Thus, immunization with PS4-p458m induced long-lasting anti-PS4 antibodies and protection.

Discussion

We previously reported that peptide p458m could be used in a conjugate vaccine to obtain T cell help for high titers of antibody production against the capsular polysaccharide (Vi) of *Salmonella typhi* [18]. The present investigation was done to extend the initial antibody studies and to learn whether the p458m conjugate could induce active immunity to a lethal bacterial infection. We chose to use *S. pneumoniae* as an indicator organism because of its high virulence for mice.

The results presented here show that peptide p458m can indeed provide powerful help for an immune response to PS4 and can induce resistance to lethal pneumococcal challenge. Protection was associated with enhanced PS4-specific antibody titers, a T cell response to the p458m carrier, and long-term memory. Moreover, PS4-p458m appeared to be effective even when administered in an aqueous solution without an adjuvant. The protection induced by PS4-p458m vaccination was several orders of magnitude greater than that induced by a commercially available vaccine or by PS4 conjugated to TT or to other carrier T cell epitopes.

It was shown previously that TT linked to PS4 by the use of a spacer, 6-aminohexanoic acid, resulted in a T cell-dependent antibody response to the carbohydrate moiety of the conjugate [26]. We applied the same coupling procedure for the

formation of our constructs, since a spacer molecule might enhance the incorporation of the carrier into the conjugate and avoid steric hindrance, allowing a more efficient interaction between the carrier and helper T cells [30]. However, PS4-TT in our studies failed to induce protective titers of anti-PS4 antibodies greater than those evidenced by unconjugated PS4, even though T cell proliferation to TT was demonstrated. Similarly, the amounts of specific antibodies to Vi induced by the Vi-p458m conjugate, in our previous report, were ~4- to 5-fold higher than the amounts of anti-Vi antibody induced by a Vi-TT conjugate [18]. An IgG anti-PS4 response to a PS4-TT conjugate was lower in BALB/c mice than in NIH or SJL mice [5]. At present we do not know why the PS4-TT conjugate we used was not effective. As we did not control the amount of TT in the conjugate, perhaps we injected doses of TT that might have induced carrier T cell suppression rather than T cell help [8]. Other peptides, such as pPL, p437ec, CLIP, and λ rep, which have been found by us and others to be T cell epitopes in BALB/c mice, were also relatively inefficient T cell carriers for PS4.

It has been claimed that antibodies specific for a pneumococcal cell wall polysaccharide, which may contaminate preparations of capsular polysaccharides, can mask a correlation between capsule-specific antibodies and protection [31]. However, we did not find any change in the titer of anti-PS4 antibodies measured by ELISA after preincubation of individual mouse sera with pneumococcal cell wall polysaccharide, and the anti-PS4 antibodies did not cross-react with the cell wall C-polysaccharide (data not shown).

To learn the extent of the resistance induced by PS4-p458m,

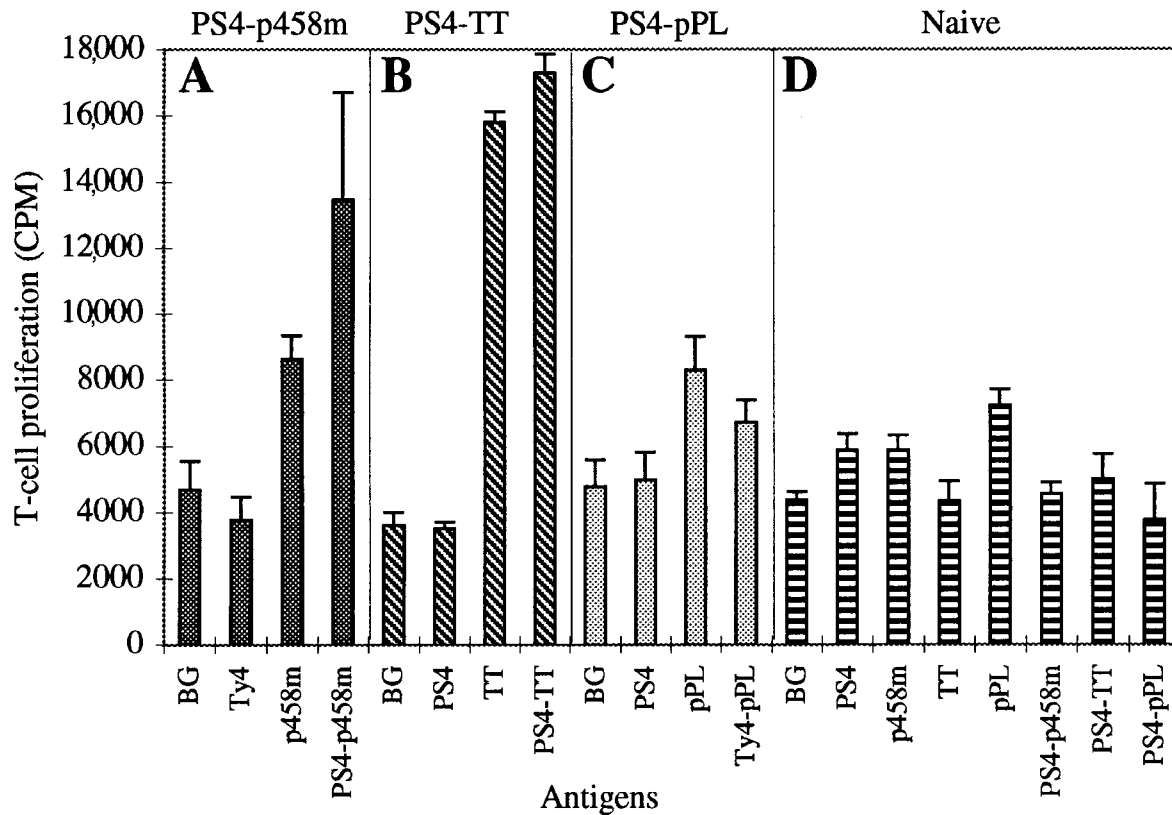


Figure 4. P458m and TT induce T cell proliferative responses. BALB/c mice (4/group) were immunized subcutaneously with PS4-p458m (A), PS4-TT (B), or PS4-pPL (C) or were untreated (D). Cell suspensions of pooled spleens were prepared two weeks after last injection and assayed for proliferation to indicated antigens. Concentrations of antigens used, either as conjugates or nonconjugates, were 2 mg/mL PS4, 3 mg/mL p458m, 3.2 mg/mL TT, and 15.4 mg/mL pPL (BG = background). Results are representative of 3 experiments. Each assay was done in triplicate (SDs are indicated). PS4 = type 4 pneumococcal capsular polysaccharide; p458m = self hsp60 peptide; TT = tetanus toxoid; pPL = peptide of pneumolysin molecule of *S. pneumoniae*.

we used large doses of bacteria for challenge. For comparison, the pneumolysin peptide (pPL) conjugated to pneumococcus type 17 capsular polysaccharide was reported to confer only partial protection upon challenge with 10 times the LD₅₀ [25]. In contrast, PS4-p458m-immunized mice were almost fully (90%) protected against challenge with ~1 million pneumococci, despite the fact that only 3–4 bacteria of *S. pneumoniae* type 4 are sufficient to kill a mouse. The commercial vaccine Pneumovax, consisting of 23 purified pneumococcal polysaccharides, induced only a low level of protection against a lower challenge dose of bacteria.

Although we used inbred BALB/c mice for immunization, there were individual variations in antibody levels to PS4. Similar variations have also been reported by others [5] using PS4-TT to vaccinate inbred BALB/c or outbred NIH mice. Of interest, the correlation between antibody titers and protection at the level of individual mice varied. Mice with high titers of anti-PS4 antibodies usually survived challenge, but mice with apparently low levels of specific antibody might or might not be protected, depending on the conjugate used for vaccination.

All mice immunized with PS4-p458m, expressing either low or high amounts of antibody, survived. Significant modification of the structure of PS4 in the different conjugates is unlikely, since the conjugates were equally well recognized by a commercial rabbit anti-PS4 serum in ELISA studies (not shown). We do not know whether the antibodies to PS4 elicited by the different conjugates vary in their affinity for PS4 or in their potential to activate phagocytic cells. It seems, therefore, that conjugation with an immunogenic carrier molecule alone is not enough to induce protection, but full protection seems to be influenced by other properties of the specific carrier.

It is generally assumed that type-specific anticapsular antibodies play a decisive role in host defense against pneumococcal infection, probably by activation of the classical complement pathway and induction of phagocytosis [20, 32, 33]. Antibodies also played a role in host defense against pneumococcal infection in our experimental system. Cross-protection studies revealed that the antibodies elicited by the PS4-p458m conjugate were type-specific; they could not confer protection against challenge with *S. pneumoniae* type 3. Furthermore, passive ad-

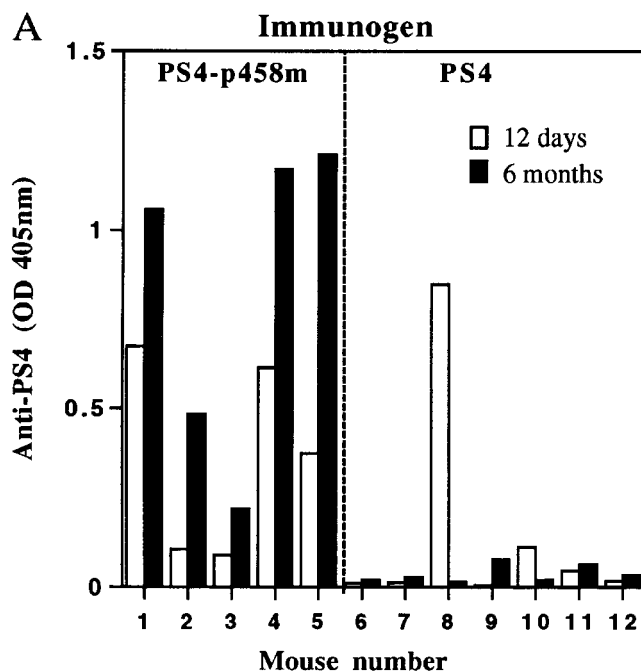


Table 5B: Long term protection

Vaccination	Survivors/total	% survival
PS4-p458m	15/17	88*
PS4-TT	6/13	46
PS4	10/30	33
Pneumovax	3/10	30

Figure 5. PS4-p458m vaccination induces long-lasting immunity. *A*, BALB/c mice were immunized with PS4-p458m or with PS4, and sera were assayed for PS4-specific IgG antibodies 12 days and 6 months after last booster injection. *B*, BALB/c mice were immunized as indicated, and 6–17 months after last immunization, mice were challenged with 4000–90,000 cfu of *S. pneumoniae* type 4. Cumulative results of several time points are presented. Mice were scored 14 days for survival after challenge. * $P < .001$ vs. other groups. PS4 = type 4 pneumococcal capsular polysaccharide; p458m = self hsp60 peptide.

ministration of small amounts of PS4-p458m immune serum (0.05 mL) to naive mice was protective, whereas an amount of PS4-induced serum that was 10 times higher (0.5 mL) failed to protect the mice.

Why did the p458m peptide appear to exert a more efficient carrier effect than did the TT and other carrier molecules we tested, and why was there no absolute correlation between the titer of antibodies and resistance to infection? The TT conjugate induced T cell proliferation that was comparable to that in-

duced by p458m. However, an hsp60 peptide may have other properties. The p458m conjugate, for example, was effective in inducing resistance even when administered in PBS without adjuvant. Thus far, we have been unable to demonstrate in vitro that p458m might activate macrophages directly (not shown), but there may be direct in vivo effects of hsp60 peptides yet to be detected.

The ubiquitous distribution of hsp60, its high sequence conservation, and its universal expression during inflammatory processes might activate a prolonged stimulation of the immune system, leading to an expansion of hsp60-reactive T cells, including T lymphocytes specific for peptide p458m, at any site of physiologic stress [34–36]. Challenging a mouse with *S. pneumoniae* is clearly a stressful situation for the bacterium as well as for the mouse and consequently will lead to an enhanced expression of bacterial and murine hsp60 at the site of the infection. Thus, the induced p458m T cells might accumulate and expand locally at the site of hsp60 expression, secrete specific cytokines, and create an environment that, in concert with anti-PS4 B cells, might enhance the ability of the vaccinated mouse to overcome the infection. The sequence of the pneumococcal hsp60 molecule has not been published, so we do not know whether the mouse p458m sequence and the pneumococcal homologue are similar or cross-reactive. In any case, the p458m peptide is an immunogenic self peptide in BALB/c mice, and thereby the vaccine response involved T cell autoimmunity by definition [18].

T lymphocytes specific for TT are induced by immunization with the PS4-TT conjugate, but in contrast to hsp60 epitopes, no local accumulation of TT carrier-reactive T cells at the site of infection with *S. pneumoniae* would be expected; TT is exclusively expressed in *Clostridium tetani* [37]. Previous studies have shown that pneumolysin and its derivatives provide some degree of protection against subsequent challenge with virulent pneumococci [38, 39]. Moreover, a pneumolysin derivative was also conjugated to pneumococcal capsular polysaccharide type 19F and was found to induce high antibody responses to 19F PS, but a protection assay was not presented [40]. Because pneumolysin is present at the site of pneumococcal challenge, we also used a carrier peptide derived from pneumolysin, described elsewhere [25]. However, the pneumolysin peptide conjugated to PS4 did not appear to be very effective in our hands. Moreover, p437ec, the *E. coli* homologue of p458m, was not able to induce protective immunity, suggesting that p458m may have an advantage over a carrier derived from a bacterial hsp60. We propose that T cells primed to hsp60 epitopes may do more than provide help for the initial induction of antibody production. These anti-hsp60 T cells might be recycled to the site of infection because up-regulation of anti-self hsp60 accompanies inflammation [17, 41]. Thus, irrespective of the inciting agent, appropriate hsp60 peptide epitopes might serve universally in conjugate vaccines.

Until recently, autoimmunity was considered to be forbidden

because it was logically incompatible with the immune system's duty to discriminate between self and foreign [42]. Now it is becoming clear that the aim of the immune system is not merely to discriminate nonself from self but also to protect the individual from "danger" [43, 44]. There are situations, such as that shown here, in which autoimmunity can contribute to fitness [45, 46]. The infallible expression of hsp60 at sites of stress might make autoimmunity to hsp60 advantageous in the natural acquisition of resistance to infection and not only in artificial vaccination. Perhaps this hsp60 self peptide conjugate vaccine is effective because hsp60 belongs to the limited set of self antigens for which there is healthy, regulated autoimmunity, the immunologic homunculus [45, 47].

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