significant (baseline versus 5-min test, $t_{12} = 0.24$; baseline versus 24-hr test, $t_{12} = 1.9$). Input resistance was measured by injecting a 1-s 1-nA constant current hyperpolarizing pulse into the motor neuron.
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Use of Prior Vaccinations for the Development of New Vaccines

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There is currently a need for vaccine development to improve the immunogenicity of protective epitopes, which themselves are often poorly immunogenic. Although the immunogenicity of these epitopes can be enhanced by linking them to highly immunogenic carriers, such carriers derived from current vaccines have not proven to be generally effective. One reason may be related to epitope-specific suppression, in which prior vaccination with a protein can inhibit the antibody response to new epitopes linked to the protein. To circumvent such inhibition, a peptide from tetanus toxoid was identified that, when linked to a B cell epitope and injected into tetanus toxoid–primed recipients, retained sequences for carrier but not suppressor function. The antibody response to the B cell epitope was enhanced. This may be a general method for taking advantage of previous vaccinations in the development of new vaccines.

IT WOULD BE EXTREMELY USEFUL IF highly immunogenic proteins widely used in vaccines [such as tetanus toxoid (TT)] could be used as carriers to develop new vaccines for poorly immunogenic protective epitopes (such as small peptides). Unfortunately, overall effectiveness with this approach has not been generally achieved (*1, 2*). Since the antibody response to a hapten coupled with a carrier protein can be inhibited when the recipient has been previously immunized with the unmodified protein (*3*), it is possible that poor immunogenicity of the hapten + carrier is due to prior vaccinations.

The phenomenon, termed epitope-specific suppression, is related in part to the presence of carrier-specific B cells (*4*) and suppressor T cells (*4, 5*). The observation that peptides recognized by suppressor and helper T cells can be distinct (*6*) suggested that some peptides might only be functionally recognized by the latter cell type in carrier-primed animals. We therefore attempted to identify a peptide with helper but not suppressor function. The involvement of carrier-specific B cells in epitope-specific suppression provided a criterion for peptide selection; a basis for excluding a peptide from consideration as a carrier sequence would be cross reactivity between the peptide and antibody to the carrier.

The worldwide use of TT prompted us to choose this protein as the model. Furthermore, a conjugate of TT and repeats of the sequence Asn-Ala-Asn-Pro (NANP), the immunodominant sequence of the major surface protein of *Plasmodium falciparum* sporozoites (*7, 8*), has already undergone clinical testing as a vaccine (*2*). The goal was to prepare a peptide bearing helper T and nonimmunogenic B cell epitopes; such a composite peptide has been described (*9*). To obtain a peptide capable of eliciting the desired T cell activity, we hydrolyzed TT with trypsin after reduction and alkylation. Peptides in the digest were separated by column chromatography and activity was monitored by in vitro T cell proliferation tests with human peripheral blood leukocytes (PBLs) and lymph node cells from mice injected with TT. Since helper T cells proliferate under these conditions, the assays provided an initial screen for appropriate peptides. A peptide from an active fraction was partially sequenced and, on the basis of the published sequence of TT, a peptide containing amino acid residues 73 to 99 (TT73–99) was synthesized.

The peptide was tested for cross reactivity with antisera against TT. Neither mouse nor human antisera against TT reacted with TT73–99 (Table 1, experiment 1, and Table 2). These data suggested that TT-specific B cells reactive with TT73–99 may not have been sensitized after TT immunization in either species and, therefore, that this potential basis for suppression would be absent. The positive T cell proliferation results in initial screening studies indicated that TT73–99 was recognized by helper T cells. To investigate this, we primed mice with TT73–99 and challenged them with $(NANP)_3TT$. Such animals produced increased titers of antibody to NANP [anti-NANP] and antibody to TT (anti-TT) (Table 1, experiment 1). The use of BALB/c mice, which are genetically unresponsive to NANP at the T cell level (*10*), and the absence of antibody cross reactivity between NANP or TT and TT73–99 indicated that priming of the helper T cell had occurred.

TT73–99 contained information for carrier function and did not cross-react with antibody against the parent protein. This peptide should not be susceptible to carrier-specific, B cell–mediated suppression. We predicted that this peptide, when linked to a B cell epitope, would not be susceptible to T cell–mediated suppression in TT-primed mice. We tested this prediction by comparing the effect of prior immunization with TT on the subsequent response to a conjugate containing either the entire protein, $(NANP)_3TT$, or a peptide with only a portion of the protein, $(NANP)_4TT73–99$.

As expected, TT priming inhibited the anti-NANP response to $(NANP)_3TT$ even though the anti-TT response was elevated in pretreated mice (epitope-specific suppression). Not only did TT priming fail to inhibit the anti-NANP response to $(NANP)_4TT73–99$, it actually resulted in the enhancement of the anti-NANP response after primary and secondary chal-

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Table 1. Circumvention of epitope suppression. In experiment 1, TT73–99 was tested for its capacity to prime helper T cells. BALB/c mice received nothing or 15 μg of TT73–99 Freund's incomplete adjuvant (FIA) subcutaneously. Later (54 days) mice received 25 μg of [Ac-Cys-$(NANP)_3]_{35}$TT, $(NANP)_3$TT/FIA, or TT/FIA. Peak responses during days 54 to 70 as determined by enzyme-linked immunosorbant assay (ELISA) (*10*) are shown. Values are geometric mean titers (*18–20*) (coefficient of variation); each group contained five mice analyzed individually. Experiment 2 shows the effects of preinjection with TT on challenge with $(NANP)_4$TT73–99 or $(NANP)_3$TT. BALB/c mice received nothing or 50 μg of TT/$Al(OH)_3$ (*19*), subcutaneously. Later (36 and 168 days) mice received 25 μg of antigen-FIA, subcutaneously. $(NANP)_4$TT73–99 was synthesized as described (*20*). Shown are primary peak responses during days 36 to 70 and secondary responses from weekly samples obtained for 3 weeks after the last injection. Values are geometric mean titers (coefficient of variation); each group had five mice assayed individually. Titers were determined as for experiment 1.

Injection			Antibody titer ($\times 10^3$)					
			Primary response			Secondary response		
1	2	3	$(NANP)_{50}$	TT	TT73–99	$(NANP)_{50}$	TT	TT73–99
			Experiment 1					
	$(NANP)_3$TT		1.4 (1.4)	1.9 (1.2)	0.15 (0)			
TT73–99	$(NANP)_3$TT		18 (1.4)	26 (1.5)	0.54 (2)			
	TT		0.15 (0)	51 (1.2)	0.15 (0)			
			Experiment 2					
	$(NANP)_4$ TT73–99	$(NANP)_4$ TT73–99	3.4 (1.5)	0.37 (1.1)	1.1 (1.9)	116 (1.3)	0.15 (0)	1.3 (1.8)
TT	$(NANP)_4$ TT73–99	$(NANP)_4$ TT73–99	15 (1.7)	98 (1.2)	4.1 (1.5)	422 (1.2)	173 (1.2)	2.3 (1.7)
	$(NANP)_3$TT	$(NANP)_3$TT	21 (1.1)	151 (1.2)	0.15 (0)	179 (1.3)	878 (1.3)	0.15 (0)
TT	$(NANP)_3$TT	$(NANP)_3$TT	0.9 (1.9)	454 (1.2)	0.15 (0)	10 (1.3)	878 (1.4)	0.15 (0)

lenges (Table 1, experiment 2). Since T and B cells have been previously reported to be involved in epitope-specific suppression in mice primed with TT (*4*), the present results indicated that substitution of TT73–99 for TT prevented both of these cell types from mediating suppression. This absence of inhibition and enhanced responsiveness in carrier-primed animals provides an empirical test for the efficacy of a candidate peptide.

Thus, epitope-specific suppression can be circumvented by supplying carrier information in the form of a peptide derived from the original protein. Certain peptides have been shown to be restricted by a large variety of human class II alleles (*11*). To determine whether TT73–99 could serve as a generally recognized carrier, mice with various genetic backgrounds were injected with $(NANP)_4$TT73–99 and the anti-NANP, and sporozoite responses were measured. Although the responses were variable, each of the seven mouse strains tested produced anti-NANP and antibody to sporozoite (anti-sporozoite antibody) (Table 3). Since only $H\text{-}2^b$ mice are responsive to the NANP sequence at the T cell level, these results indicated that TT73–99 might be also generally recognized by T cells of the genetically diverse human population.

To study the applicability of this approach in humans, we obtained PBLs from 20 unrelated volunteers at F. Hoffmann–La Roche, Basel, Switzerland, and tested them in an in vitro T cell proliferation assay for reactivity with TT73–99 or TT. Although the diversity of class II alleles is not known, PBLs from 12 of 20 volunteers responded to TT73–99 (stimulation index ≥2) (Table 2). In more limited studies, T cells proliferated in response to $(NANP)_4$TT73–99 but not to $(NANP)_{50}$ or $(NANP)_3$ (*12*). We

Table 2. Immune reactivity of humans to TT and TT73–99. (**A**) T cell proliferation tests were performed with PBLs obtained from 20 volunteers after separation from whole blood (*21*). Duplicate cultures in microculture plates contained 4×10^5 cells and TT or TT73–99 (10 μg/ml), in 0.2 ml of culture medium (*22*). Four days after initiation of culture, 1 μCi of [^{3}H]thymidine was added to each culture; 24 hours later cultures were harvested and evaluated. Stimulation index is the ratio of label (counts per minute) in the culture with antigen to the label in the culture without antigen. (**B**) Sera from 55 volunteers (different from those used in the cellular analysis) who participated in phase I clinical studies analyzing the ability of interferons α and γ to act as adjuvants for $(NANP)_3$TT (*23*) were tested for reactivity both before and up to 12 weeks after the initial vaccination. (Volunteers were injected in weeks 0 and 8.) Values are geometric mean titers (coefficient of variation) determined as described (*21*). Preclinical titers are those before injection with $(NANP)_3$TT. Pre- and post-clinical anti–TT73–99 titers were equivalent to titers on plates coated with bovine serum albumin, which was used as the blocker in ELISA.

A. Cellular

Antigen	Stimulation index (no. of volunteers)
TT73–99	<2 (8), 2 (3), 3 (2), 4 (3), 5 (1), 8 (1), 14 (1), 36 (1)
TT	<2 (1), 3 (1), 10–30 (5), 40–60 (5), 70–100 (4), >100 (4)

B. Humoral

Antigen	Antibody titer ($\times 10^3$)	
	Preclinical	Postclinical
TT73–99	1.2 (1.1)	1.3 (1.1)
TT	44 (1.3)	141 (1.2)

Table 3. $(NANP)_4$TT73–99 elicits anti-sporozoite antibody in genetically diverse mice. Mice (two to three per strain) received 25 μg of $(NANP)_4$TT73–99 in Freund's complete adjuvant and FIA subcutaneously on days 1 and 32, respectively. Responses were assayed for 3 weeks after boost; peak geometric mean titers are shown (coefficients of variation ranged between 1.2 and 1.6). Anti-$(NANP)_{50}$ titers were determined as described in Table 1. Anti-sporozoite titers were determined by indirect immunofluorescence as described (*21*), except that fluoresceinated antibody to mouse immunoglobulin was used. Normal mouse sera had anti-$(NANP)_{50}$ titers ≤150 and anti-sporozoite titers <10.

Strain	H-2 haplotype	Anti-$(NANP)_{50}$ titer ($\times 10^2$)	Anti-sporozoite titer
B10.RIII	r	59	28
C57B1/10	b	14,305	115,852
B10.s	s	366	1,280
B10.BR	k	915	20,480
B10.M	f	9.4	10
B10.G	q	2,288	40,960
B10.D2	d	1,448	20,480

conclude that TT73–99 can be recognized by T cells when the peptide also contains the NANP sequence.

In addition to the method for peptide selection we used, peptides could also be initially selected on the basis of models that predict T cell epitopes (*13*). Effective sequences must not necessarily be dominant, but they should be capable of using previously primed helper T cells to obtain the desired antibody response without interference from suppressive cells and be generally recognized by the human population. It will be important to establish the degree to which helper and suppressor epitopes on a protein of interest coincide and the influence of haplotype on these activities. For various proteins examined, these epitopes are distinct (*6*), although for L-tyrosine-*p*-azobenzenearsonate they are the same or very similar (*14*). A peptide from ovalbumin (ovalbumin 229–276), but not the parent protein containing this sequence, can prime cytotoxic T cells in vivo to recognize transfected cells that endogenously produce ovalbumin (*15*). Therefore, it appears that the presence of a sequence in a vaccine may not in itself guarantee its desired function.

Optimal effectiveness cannot necessarily be expected for a vaccine that uses T cell sequences distinct from those of the pathogen to be neutralized or destroyed, especially in those cases where T cells provide an important effector function in protection. Nevertheless, when humoral immunity constitutes an important effector mechanism, a vaccine capable of initiating the production of high concentrations of protective antibody can be extremely effective; this has been shown in malaria studies in infected mice preimmunized with synthetic peptide protein conjugates (*16*). The mouse immunogenicity studies reported here use Freund's adjuvant, a formulation considered unacceptable for human use. The development of new vaccine candidates and adjuvants (*17*) is important for the success of new vaccines. Since the amount and quality of antibody can influence protection, combinations of effective T cell epitopes and adjuvants will have to be defined. In this regard, humans who had low proliferative responses to TT73–99 might produce low antibody responses to $(NANP)_4$TT73–99, as exemplified by B10.RIII, B10.S, and B10.M mice; this amount of response would probably be insufficient for protection. It may be necessary to use larger peptides to ensure responsiveness in a higher proportion of the population, although with larger peptides one risks including a suppression-inducing epitope.

In conclusion, our results with peptide TT73–99 show that it is possible to take advantage of T helper cell priming and eliminate the potential disadvantage of epitope suppression stemming from prior immunization to the carrier. In addition, although the diversity of restriction elements for the T cell responses to TT73–99 is not known, this sequence from a widely used vaccine may serve as a general carrier. These findings suggest the possibility for the identification of peptide carriers from other current vaccines and for their use alone or in combination in the development of engineered vaccines.

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