Self and Foreign 60-Kilodalton Heat Shock Protein T Cell Epitope Peptides Serve As Immunogenic Carriers for a T Cell-Independent Sugar Antigen[1]

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Healthy individuals manifest natural T cell reactivity to epitopes of the 60-kDa heat shock protein (hsp60) of both self and bacterial origin. The present studies were done to learn whether defined peptides of hsp60 could function as T cell carrier epitopes for a poorly immunogenic T-independent capsular polysaccharide, the Vi Ag of *Salmonella typhi*. Homologous peptides were synthesized from the mouse self-hsp60 molecule (CP1m), from the closely related human hsp60 molecule (CP1h), and from the more distant *Escherichia coli* (CP1ec) and mycobacterial (CP1mt) hsp60 molecules. The peptides were conjugated to Vi and tested for their immunogenicity in BALB/c (H-2^d) and H-2 congenic mice (H-2^k and H-2^b). We now report that the self-CP1m and cross-reactive CP1h peptides were as immunogenic as was the non-cross-reactive foreign CP1ec peptide. Small amounts of the CP1 peptide, even in PBS, sufficed to induce anti-Vi Abs of the IgG1 (T-dependent) isotype in naive mice. The carrier effect was associated with the ability of the peptides to bind to APC and to induce T cell proliferation. H-2^d and H-2^k mice, but not H-2^b mice responded to CP1m/h and CP1ec. None of the mice responded to CP1mt. No signs of inflammation or autoimmune disease were detected. Thus, natural T cell autoimmunity exists and can be harnessed to provide T cell help for Ab production to a foreign bacterial molecule in a synthetic vaccine. *The Journal of Immunology,* 1995, 154: 5977–5985.

Vaccination against infectious diseases is often done by inoculating the individual with the attenuated or killed infectious agent or with Ags of the infectious agent to stimulate a protective immune response.Vaccination, however, may fail because virulent bacteria often are encapsulated in poorly immunogenic polysaccharide coats. These bacterial polysaccharides are usually T independent (T-ind)[5] and so induce mostly IgM Abs of low titer and short duration, and without immunologic memory (1). To achieve high and long-lasting titers of IgG Abs and immunologic memory, attempts have been made to provide T cell help by conjugating capsular polysaccharide molecules to protein carriers (2–4) such as tetanus, cholera, or diphtheria toxoids (2, 5). However these carriers are not ideal; besides dosage limitations and the risk of sensitization to the carrier itself, the immune response to high m.w. carrier molecules, which harbor stimulatory as well as suppressive T cell epitopes, is not always predictable (6–8). To overcome these problems, defined Th epitope peptides derived from various bacterial and viral proteins were tested and found to function as carriers in vaccine constructs with B cell epitopes (9–12). However, to trigger a strong memory response when the host confronts the virulent infectious agent, the T cell carrier epitope should be expressed along with the target B cell epitope. This fact would seem to require that a specific T cell carrier peptide be used for each vaccine. Unless, however, nature could provide a single molecule with T cell epitopes expressed in every infection, the epitopes of such a molecule might serve as universal carriers.

Consider the 60-kDa heat shock protein (hsp60), a family of molecules well recognized by the immune system (13–15). Because of their high conservation throughout

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[5] Abbreviations used in this paper: T-ind, T cell independent; hsp60, 60-kDa heat shock protein; LNC, lymph node cells; TT, tetanus toxoid; CP, control peptide; CDI, (3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride.

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evolution, a similar hsp60 molecule is present in all pathogenic and non-pathogenic microbes. Indeed, T cell clones directed against epitopes shared by hsp60 of different bacteria have been reported (16, 17). Hence, T cells specific to hsp60 are induced by many different microbes, virulent and avirulent. Hsp60 has been called the common bacterial Ag (14, 18). However, no cell in this world can apparently exist without the ability to express hsp60 when stressed (19). Mammalian hsp60 is highly homologous to the bacterial cognates, showing about 50% amino acid identity (20). Moreover, mammalian hsp60 has two properties relevant to the issue of vaccination: epitopes of the hsp60 molecule are expressed at the surface of stressed macrophages or tissue cells and self-hsp60 epitopes are natural immunogens. Heat shock, IFN-γ, or inflammation results in presentation of endogenous hsp60 epitopes on MHC class II molecules leading to the activation of hsp60-specific T cells, even in healthy individuals (21–23). Some of these T cells were reported to recognize epitopes shared by bacterial and human hsp60 molecules (24).

In other words, when bacteria are ingested by host macrophages, both the suffering bacteria (25) and the activated macrophages (22) hyper-express hsp60. Thus, hsp60 is shared by the host and its parasites, and is immunogenic, cross-reactive, and universally expressed in inflammation. Moreover, hsp60 is a prominent member of the set of self molecules for which there naturally exists autoimmunity; hsp60 is a part of the immunologic homunculus (26). These attributes led us to ask if peptides of hsp60, either self or microbial, might be able to function as T cell epitopes and provide help for a T-ind bacterial Ag in mice. We identified dominant helper peptide epitopes derived from different hsp60 cognates and show here that it is possible to exploit natural autoimmunity to a self peptide of the hsp60 molecule for efficient vaccination. A homologous, but non-cross-reactive foreign T cell epitope originating from the *Escherichia coli* hsp60 protein (GroEL) was also effective. The T-ind Ag in the vaccine was the capsular polysaccharide Vi from *Salmonella typhi*. The hsp60 peptide carrier effect did not require adjuvant, was not restricted to a single MHC-background, was achieved without the need for intentional priming, and resulted in a high and long-lasting specific IgG Ab response.

Materials and Methods

Mice

Female BALB/c (H-2^d), BALB/k (H-2^k), and BALB/b (H-2^b) mice were purchased from Harlan Olac (Bicester, UK) and were used at 6 to 8 wk of age.

Vi Ag

The Vi used for conjugation and immunization was purified from *Citrobacter freundii* WR7011 and kindly provided by Prof. John B. Robbins, National Institutes of Health, Bethesda, MA. The polysaccharide preparation contained less than 1% each of contaminating protein, nucleic acid, or LPS. The molecular size of the Vi was estimated to be 3×10^3 kDa. Vi fragments of about 45 kDa were prepared by ultrasonic disruption and were kindly provided by Dr. Dominique Schulz, Pasteur-Mérieux, Lyon, France.

Table I. *Alignment of amino acid residues shared by self and foreign CP1 homologues*

Peptide	
CP1m	**N**[a] **E D Q K I G I E I I K R A L K I**
CP1h	**N E D Q K I G I E I I K R T L K I**
CP1ec	**N E D Q** N V **G I** K V A L **R A** M E A
CP1mt	E G **D** E A T **G** A N **I** V **K** V **A L** E A

[a] Bold letters indicate amino acids identical to the mouse homologue of CP1m.

Peptides and proteins

Peptides were prepared with an automated multiple peptide synthesizer (Abimed model AMS 422, Langenfeld, Germany) using the company's protocols for *N*-α-fluorenylmethoxycarbonyl (F-moc) synthesis or were prepared manually by a standard solid phase method (27) employing either *N*-α-F-moc or *N*-α-t-butyloxycarbonyl (t-Boc) strategies. Crude products were purified by reversed phase HPLC on a semi-preparative C_8 column (Lichrosorb RP-8, 7 μm, 250 $\times$ 10 mm, Merck, Darmstadt, Germany). 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 (v/v). The purity of the single peptide products was ascertained by analytic reversed phase HPLC and amino acid analysis. Peptide CP1h corresponds to positions 458–474 of the human hsp60 molecule (Table I). CP1m, CP1mt, and CP1ec represent, respectively, the mouse, the mycobacterial, and the *E. coli* variants of the human CP1 sequence (Table I). Peptide AcR (VIVELIPSTSSAV), a generous gift of Prof. Edna Mozes (Weizmann Institute of Science, Rehovot, Israel), corresponds to positions 259–271 of the murine acetylcholine receptor α-chain, and is a known T cell epitope in BALB/c mice (28). Peptide CP3 (PALDSLTPANED) is derived from the sequence of human hsp60 corresponding to residues 449–460. Peptide SerRes (LRGGGVC GPAGPAGTVCS) originates from the sea urchin chemoattractant resact carrying a C-terminal serine-extension. Tetanus toxoid (TT) was kindly donated by Prof. Ruth Arnon (Weizmann Institute of Science), and OVA was obtained from Sigma Chemical Co. (St. Louis, MO).

Vi conjugation to peptides and proteins

Equal amounts (w/w) of Vi and peptide or protein were dissolved in a minimal volume of double distilled water and incubated at room temperature at pH 6 in the presence of two weight equivalents of water-soluble diimide, i.e., (3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (CDI) (Aldrich, Milwaukee, WI). After 2 h, the same amount of CDI was added to the reaction mixture and the incubation continued for 12 more hours. Unconjugated reaction components were eliminated by dialysis against double distilled water. The peptide density in the conjugate was determined by amino acid analysis and the sugar content estimated by Fourier transformed infrared spectroscopy according to the procedure described by Stone and Szu (29) yielding a sugar recovery of 40 to 60% after the conjugation procedure. The polysaccharide/peptide, or polysaccharide/protein ratio of the different preparations varied from 0.5 to 2.9.

Immunization

The mice were immunized s.c. twice, 4 wk apart, with Vi alone or with the different Vi conjugates emulsified in IFA unless otherwise noted. Serum samples were collected 12 days after each injection, and later.

Serology

Vi Ab levels were determined by ELISA. To attach the negatively charged Vi to the polystyrene plates, the polysaccharide was mixed with

methylated BSA (Sigma) dissolved in PBS. Microwell plates were coated for 3 h at 37°C with 2.5 μg Vi/well. After washing the plates with PBS containing 0.33% Brij 35 (Sigma), nonspecific binding was blocked with a solution of PBS and 1% skimmed milk. One hundred-microliter aliquots of diluted test sera and of a reference serum were added to the plates and incubated for 1 h at 37°C. The reference serum, a hyperimmune burro antiserum, containing 550 μg/ml Vi Ab, was prepared by multiple i.v. injections of formalin-fixed *S. typhi* Ty2 (30) and was kindly provided by Drs. J. B. Robbins and S. C. Szu, National Institutes of Health, Bethesda, MA. Goat anti-mouse IgG $F(ab')_2$-alkaline phosphatase conjugate and rabbit anti-horse IgG $F(ab')_2$ enzyme conjugate (Sigma), diluted 1/5000, were used to detect Vi-specific IgG Abs in the mouse or the reference sera. Different IgG subclasses of polysaccharide-specific Abs were detected by the use of biotinylated rabbit anti-mouse subclass-specific antiserum, a generous gift of Prof. Klaus Rajewsky, University of Cologne, Cologne, Germany, and streptavidin-conjugated alkaline phosphatase. Bound Abs were visualized by the addition of a substrate solution containing 0.6 mg/ml of *p*-nitrophenylphosphate (Sigma) in diethanolamine-H_2O, pH 9.8. The enzyme reaction was stopped after 20 min by the addition of 10 μl 5 N NaOH to each well and the OD was read at 405 nm. The results are shown either as the OD or as the percentage of binding of the test serum to the reference serum (see above). Quantitation of Vi-specific Abs in test sera were determined by RIA as described before (31).

T cell proliferative responses

Proliferation assays of inguinal lymph node cells (LNC) were performed 10 days after s.c. immunization of naive mice with 20 μg of peptide emulsified in IFA into the hind footpads. The LNC (2×10^5/well) were cultured in the presence of different peptides using a range of Ag concentrations for 5 days as described (32). Results are expressed as [^{3}H]*d*-TdR uptake, cpm.

N-terminal biotinylation of peptides

Resin-bound peptide (Wang-resin, Nova biochem, CaBiochem AG; Laufelingen, Switzerland), 11 mg, was suspended in a minimal volume of *N*-methyl-2-pyrollidone (NMP). Biotin-*N*-hydroxysuccinimide (Sigma), 15 μmol, and 15 μmol diisopropylethylamine were added. After 16 h, the reaction products were washed with *N*-methyl-2-pyrollidone, methanol, and ether. The biotinylated peptide was deprotected and cleaved from the resin with a cleavage mixture containing 5% triethylsilan (Fluka Chemicals, Buchs, Switzerland), 5% water, and 90% trifluoroacetic acid. The cleaved peptide was precipitated with ice-cold, peroxide-free ether and the pellet was dissolved in water and subsequently lyophilized. The degree of biotinylation was estimated by HPLC and by an optical test based on binding of 2(4-hydroxyazobenzene) benzoic acid to biotin (33). Peptide CP1m was biotinylated by 60%, CP1h by 80% and CP1mt by 100%.

Binding of biotinylated peptides to cells

Binding experiments were performed with BALB/c splenic adherent cells as described (34). Briefly, spleen cells (10^8) were suspended in complete medium supplemented with 10% (v/v) FCS and were incubated in petri dishes at 37°C for 1 h. Thereafter, nonadherent cells were removed and the plates were washed with cold RPMI 1640 and placed on ice. Adherent cells were collected using a rubber policeman and 10^6/sample were incubated for 20 h with the biotinylated peptide (25 μM) in PBS containing 0.1% BSA, at 37°C. The cells were washed twice at 4°C with PBS/BSA. Phycoerythrin-streptavidin (Jackson ImmunoResearch Laboratories, Avondale, PA) was added (0.5 μg/100 μl cell suspension) and the reaction mixture was incubated for 20 min at 4°C. After washing the cells were analyzed by flow cytometry using a FACScan (Becton Dickinson, Mountain View, CA). At least 5000 cells were examined in each analysis.

Statistical analysis

Data were analyzed for significance using the Mann-Whitney Rank-Sum test.

Results

Identification of hsp60 T cell epitope peptides

We identified several dominant T cell epitopes from the sequence of the human and the mouse hsp60 molecule by immunizing BALB/c mice with mixtures of overlapping peptides encompassing the entire sequence of the human and the mouse hsp60 protein. Here we present data obtained for the CP1 ($hsp60_{458-474}$) peptide, which was one of the most immunogenic.

We immunized BALB/c mice with peptides CP1m of the self-mouse or with CP1h of the related human hsp60 sequences, or with the bacterial peptides CP1mt or CP1ec of the mycobacterial or *E. coli* hsp60 homologues, respectively (Table I). Figure 1, *A* and *B*, illustrates that both CP1h and CP1m induced strong lymph node proliferative responses that were immunologically cross-reactive. The CP1mt peptide was not immunogenic (Fig. 1*C*) and the CP1ec peptide was immunogenic, but not cross-reactive with any of the homologous peptides (Fig. 1*D*). The non-homologous control peptide, AcR 259–271, did not elicit a proliferative response in any of the CP1-immunized mice.

The immunogenicity of the self-mouse CP1m and the human CP1h peptides and the lack of immunogenicity of the mycobacterial CP1mt peptide were reflected in peptide-binding studies. Figure 2, *a* and *b*, demonstrates a high degree of peptide binding of CP1h and CP1m; more than 70% of BALB/c splenic adherent APC were labeled after 20-h incubation with the biotinylated peptides. The observed binding, confirmed by non-biotinylated peptide inhibition assays, appeared to be restricted to MHC class II molecules analyzed by inhibition studies with anti-class I- and anti-class II-specific Abs (data not shown). The CP1mt peptide did not bind to BALB/c APCs (Fig. 2*c*).

Carrier effect of CP1 conjugates

To evaluate if peptide CP1 could engage T cell help, we conjugated CP1 homologues to the capsular polysaccharide Vi of *S. typhi*, immunized BALB/c mice with the Vi-peptide conjugates or with underivatized Vi, 2 μg, and analyzed IgG anti-Vi Abs. Figure 3*A* shows the results using whole Vi (m.w. $\approx 3 \times 10^6$) and Figure 3*B* shows the results using Vi fragments (m.w. $\approx 5 \times 10^4$) in the assay. As expected, mice immunized with Vi alone developed negligible amounts of Vi-specific IgG Abs. In contrast, Vi conjugated to CP1h, CP1m, or CP1ec induced IgG anti-Vi Abs. The Vi-CP1mt conjugate did not induce a carrier effect, just as CP1mt did not induce a proliferative T cell response. Thus the self-CP1m peptide, the cross-reactive CP1h peptide, and the non-cross-reactive CP1ec peptide each appeared to provide T cell help to the Vi Ab response. Further aspects of this carrier effect were studied using the CP1h peptide.

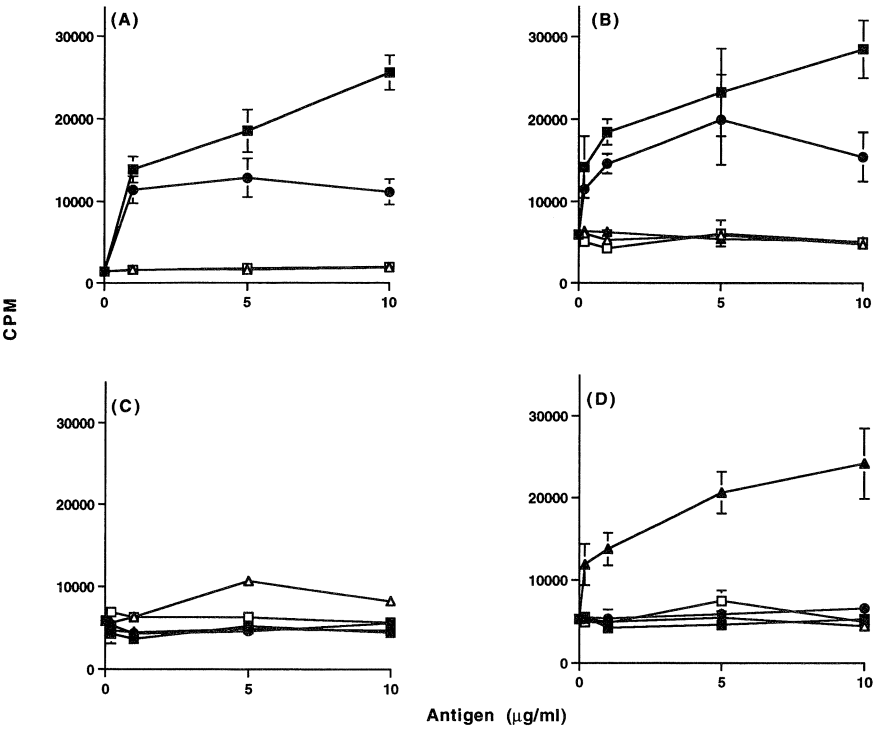

FIGURE 1. Proliferative response of LNC after immunization with CP1 homologues. Groups of three BALB/c mice were immunized s.c. with 20 μg of peptide CP1h (*A*), CP1m (*B*), CP1mt (*C*), or CP1ec (*D*), emulsified in IFA. LNC were prepared 10 days later and assayed for specific proliferation to peptide CP1h (●), CP1m (■), CP1mt (△), CP1ec (▲), or AcR259–271 (□) for 96 h in the indicated concentrations. These results are representative of the four experiments performed. Each assay was done in quadruplicate for which the SD values are indicated in the figure.

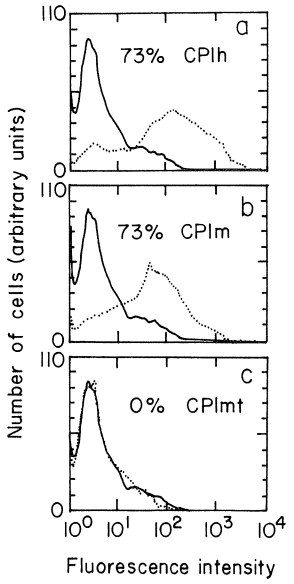

FIGURE 2. Binding of biotinylated CP1 homologues to splenic adherent cells. Splenic adherent cells of BALB/c mice were incubated with the biotinylated CP1h (*a*), CP1m (*b*), or CP1mt (*c*) for 20 h. Cells were incubated with (– – –) or without 25 μM biotinylated peptide (——). The cell-surface-associated peptides were revealed with a phycoerythrin-streptavidin amplification system and cell staining analyzed by flow cytometry. The percent of bound peptide is indicated in each panel. The data of the binding assay are representative of five experiments.

Dose response

Figure 4 shows the titration curves of IgG Abs obtained by immunizing BALB/c mice with either 2.5 μg or 0.25 μg of unconjugated Vi or with Vi-CP1h conjugate. Vi alone or Vi conjugated to a control peptide (CP3) were poorly immunogenic. Vi-CP1h, in contrast, was immunogenic even at a dose of 0.25 μg of Vi. Note that the amount of CP1h peptide in this conjugate was only about 0.17 μg/animal. Thus, helper T lymphocytes specific for CP1h can be activated by tiny amounts of the peptide without the mice having been primed intentionally with the free peptide or with the whole hsp60 protein.

CP1h compared with carrier proteins

Experiments were done to compare the carrier effect induced by peptide CP1h to that elicited by conventional large carrier proteins. Figure 5 shows the primary and the secondary Vi-specific IgG responses induced by Vi conjugated to CP1h, OVA, or TT. All three constructs were effective, but the Vi-CP1h conjugate induced a significantly higher titer than did the Vi-TT conjugate in both the primary and secondary Ab responses ($p < 0.026$).

Table II shows the amounts of anti-Vi Abs in the pooled sera of mice immunized and boosted with Vi alone or with Vi-CP1h. The findings are compared with the published results of Szu et al., (5) which were done by using the same anti-Vi standard Ab. Compared with Vi alone, the Vi-CP1h conjugate induced more than a 60-fold higher Ab

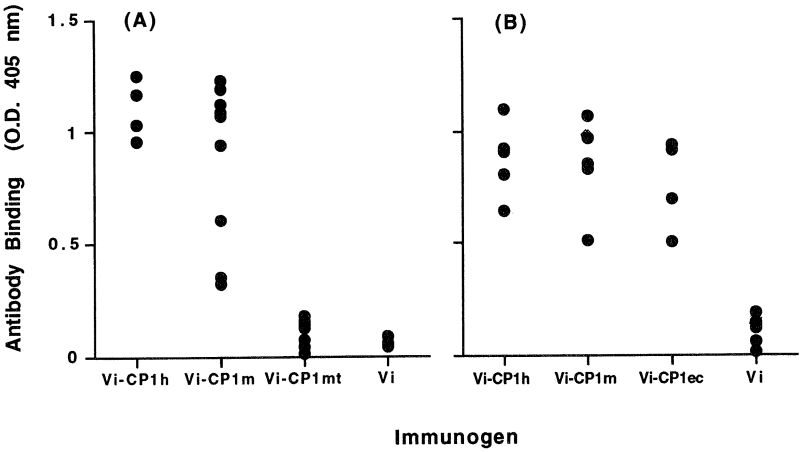

FIGURE 3. CP1 homologues conjugated to Vi provide help. Groups of four to eight BALB/c mice were immunized with 2 μg Vi conjugated to the indicated CP1 peptides or to Vi alone. Ags were administered s.c. emulsified in IFA. Twelve days after a booster immunization, serum samples were collected, and Vi-specific IgG Abs were measured by ELISA. Results obtained in two different experiments using Vi (*A*) or Vi fragments (*B*) in the ELISA assay are presented for individual mice. Serum samples were tested at a dilution of 1/800. Similar results were obtained in the five experiments done.

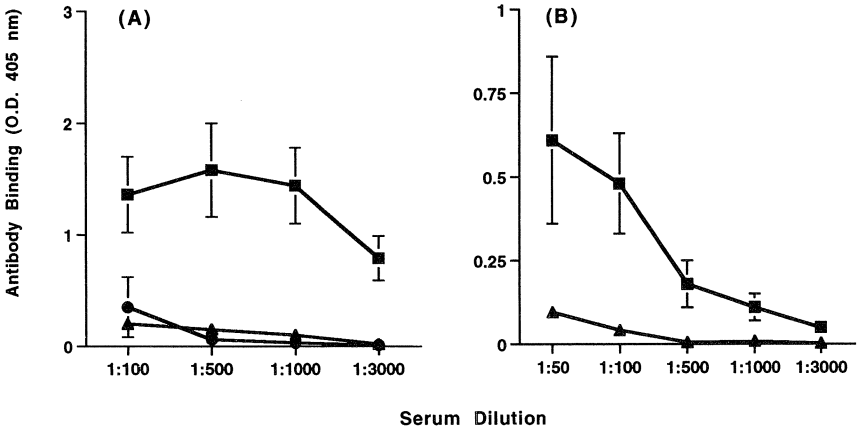

FIGURE 4. The CP1h-carrier effect at different doses. Three groups of four BALB/c mice were immunized s.c. with 2.5 μg Vi (*A*) or with 0.25 μg Vi (*B*) underivatized (▲), or conjugated to peptide CP1h (■), or CP3h (●), emulsified in IFA. Twelve days after a booster injection with a repeat dose of the Ag, sera were taken and the Vi-specific IgG response determined by ELISA. The amount of injected, conjugated peptide CP1h was 1.7 μg (*A*), or 0.17 μg (*B*). The data of the ELISA assay are representative of five experiments that were done. SD values are indicated in the graph.

level, reaching Vi Ab concentrations of nearly 20 μg/ml. For comparison, the highest reported Vi-specific Ab concentration produced by a conjugate of Vi and TT contained about fivefold less specific Ab.

Carrier peptide CP1h works without adjuvants

To test whether Vi-CP1h vaccination requires an adjuvant, we immunized BALB/c mice with Vi alone or with Vi-CP1h in IFA, in PBS, or in CFA. Figure 6 shows that by 12 days after the first inoculation, Vi-CP1h in all three antigenic preparations gave rise to higher anti-Vi Ab levels than did the unconjugated Vi. The secondary anti-Vi immune responses were also effective in IFA, PBS, or CFA. Although CFA may have induced a somewhat greater effect, it is notable that Vi-CP1h was immunogenic, even in PBS.

IgG subclasses

Experiments were done to learn if the CP1h carrier peptide induces an isotype switch to IgG1 as do large carrier proteins conjugated to capsular polysaccharides (35, 36). Figure 7 shows that Vi injected alone elicited an immune response restricted mainly to the IgG3 subclass (*B*), whereas the Vi-CP1h conjugate shifted the subclass predominance clearly to IgG1 (*A*).

Effects of H-2

The above results were obtained in BALB/c mice. To investigate the effect of H-2, we studied the response of H-2 congenic mice: BALB/c (H-2^d), BALB/k (H-2^k), and BALB/b (H-2^b). Figure 8 shows that the CP1h and CP1m peptides were effective in enhancing the immune response to Vi in BALB/c and BALB/k mice, but not in BALB/b mice. The proliferative responses to the peptides and the binding of the peptide to splenic adherent cells manifested the same H-2 effects (not shown). Thus, the self CP1m and the cross-reactive CP1h peptides were effective T cell epitopes in the H-2^d and H-2^k, but not in the H-2^b genotype.

Discussion

The results presented here show that the peptides CP1h, CP1m, and CP1ec from the sequence of the hsp60 family could be used as carrier determinants to convert a poorly

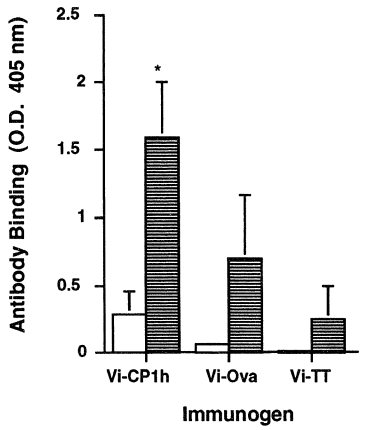

FIGURE 5. Comparison of the carrier effect provided by CP1h and protein molecules. Four to five BALB/c mice/group were injected s.c. with 2.5 μg of Vi conjugated to CP1h, Ova, or TT emulsified in IFA. Blood samples were taken 12 days after the first immunization (□) and after a booster injection 4 wk later (▤). Vi-specific IgG Abs were measured by ELISA. Serum samples were tested in a dilution of 1/500. *Significantly higher than the Vi-Ab level induced by Vi-TT ($p < 0.026$). Similar results were obtained in a repeat experiment.

Table II. *Anti-Vi Ab levels after immunization with different Vi vaccines*

Vaccine[a]	Anti-Vi Ab Concentration (μg/ml)
Vi (unconjugated)	0.297[b]
Vi-CP1h	19.50[b]
Vi-TT	4.18[c]

[a] Four BALB/c mice/group were immunized s.c. with 2.5 μg underivatized Vi or Vi conjugated to CP1h or to TT in IFA. The results shown for Vi-TT were published by Szu et al. (5).
[b] Vi-specific Ab concentration of pooled serum samples after one booster immunization.
[c] Mean Vi-specific Ab concentration after two booster immunizations.

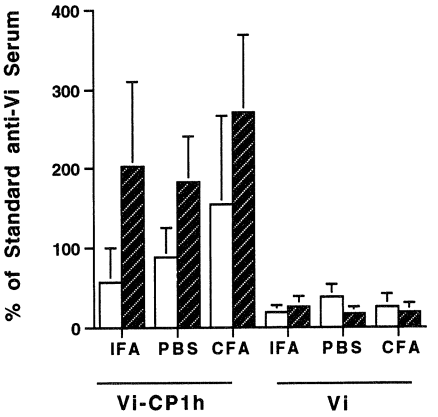

FIGURE 6. Adjuvant-free carrier effect of CP1h. Four BALB/c mice/group were immunized s.c. with 2 μg Vi or Vi conjugated to CP1h. The Ags were administered in PBS or emulsified in CFA or IFA. Sera of mice were collected 12 days after the first immunization (□) and 12 days after a boost (▨). The presence of anti-Vi IgG Abs was determined by ELISA. Specific Ab levels are expressed as the percentage of a standard burro anti-Vi serum applied at a dilution of 1/24,000. The test BALB/c serum samples were assayed at a dilution of 1/200. Similar results were obtained in a repeat experiment.

immunogenic, T-ind polysaccharide Ag into a T cell-dependent immunogen. The carrier effect was reflected in a high titer of polysaccharide-specific Abs predominantly of the IgG1 mouse isotype. The Abs persisted for at least 380 days after a primary immunization, with a booster immunization given on day 225 (not shown). Relatively small amounts of immunogen, containing as little as 0.17 μg of peptide, sufficed to trigger the IgG Ab response. In the absence of T cell help, immunization with bacterial carbohydrates usually induces Abs of the IgM and the rare IgG3 classes (35). The ability of the hsp60 peptides to function as T cell epitopes in the vaccine conjugate was associated with the binding of the peptides to APC and with the ability of the peptides to induce T cell proliferative responses subsequent to immunization with higher doses of the peptide, 20 μg, in IFA. MHC restriction to H-2^d and H-2^k, but not to H-2^b, was observed using congenic mice.

The hsp60 peptide CP1h of the human sequence, which differs from the mouse CP1m sequence by one amino acid of the 17 (Table I), was totally cross-reactive with the mouse peptide. Mapping of the minimal amino acid sequence of CP1h suggests that the single sequence difference between CP1m and CP1h four residues from the carboxyl terminus is actually outside of the T cell epitope (S. Könen-Waisman, M. Fridken, and I. R. Cohen, unpublished observations). Thus, from the T cell point of view, CP1m and CP1h appear to be identical.The nine amino acid difference between CP1m and CP1ec produced diverse T cell epitopes. The fact that both CP1m and CP1ec share H-2 restriction suggests that these analogous peptides might share MHC class II docking motifs; but that possibility needs to be tested experimentally. The mycobacterial hsp60 peptide CP1mt was very poorly immunogenic in the three H-2 congenic strains of mice.

Note that the T cell carrier effects of CP1h/CP1m and CP1ec did not require priming of the mice with the peptides by the experimentalist. The IgG Abs were detectable within 2 wk after a single inoculation of as little as 0.25 μg of conjugate. Moreover, IgG Abs could be elicited even using PBS or IFA as vehicles for immunization. The ability of a soluble immunogen to induce IgG Abs when administered in PBS is a functional indication that priming has already taken place. Thus, naive BALB/c mice seem to be naturally primed for Th cells responsive to CP1m/CP1h and to CP1ec. Priming to CP1ec is likely to have occurred naturally in the course of the prolonged and recurrent contact of the mice with intestinal flora, including *E. coli* or

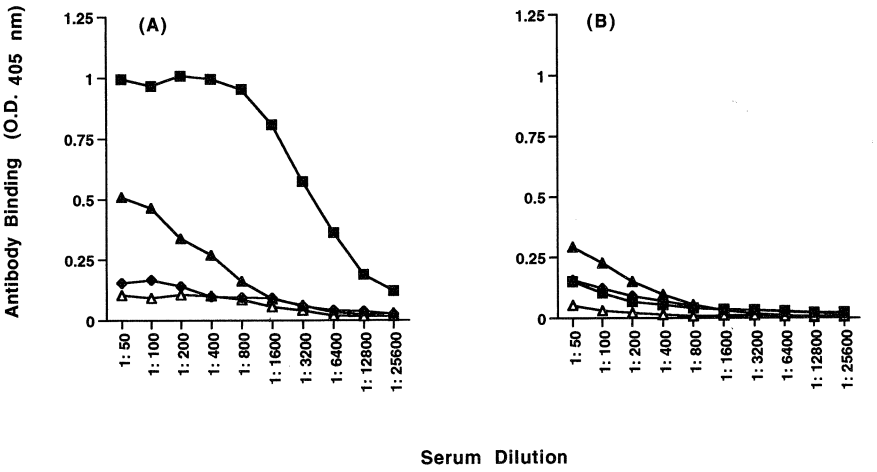

FIGURE 7. Conjugation of CP1h to Vi shifts the IgG subclass of anti-Vi Abs. Sera of BALB/c mice immunized s.c. with Vi-CP1h (*A*) or Vi alone (*B*) emulsified in IFA were collected 12 days after a booster injection with the same Ag preparations. Individual serum samples were analyzed for anti-Vi IgG Abs by the use of biotinylated IgG1 (■), IgG2a (♦), IgG2b (△), and IgG3 (▲)-specific antiserum in ELISA assays. Sera were used as pools from four to five mice. Similar effects were obtained in the three experiments that were done.

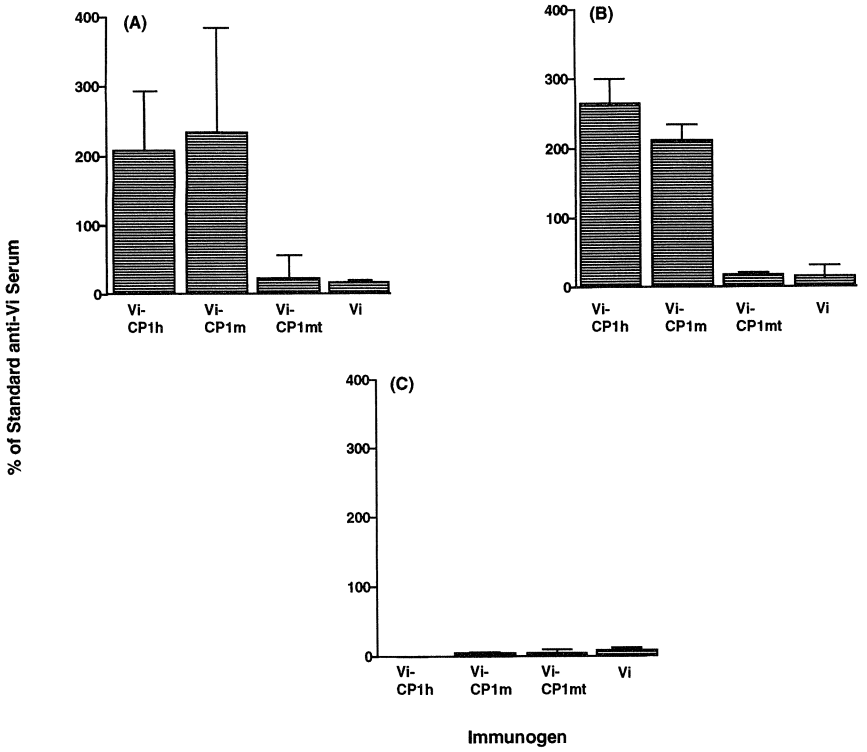

FIGURE 8. The carrier effect of CP1h and CP1m in MHC-congenic mice. Groups of five BALB/c (H-2^d) (*A*), BALB/k (H-2^k) (*B*), and BALB/b (H-2^b) (*C*) mice were immunized s.c. with 2 μg Vi or the indicated peptide-Vi conjugates emulsified in IFA. Serum samples were collected 12 days after a booster injection and tested for anti-Vi IgG Abs by ELISA. Sugar-specific Ab levels are expressed as the percent of a standard anti-Vi serum at a dilution of 1/24,000. The test results were obtained at a serum dilution of 1/800. The data of the ELISA assay are representative of three experiments.

related bacteria. This hypothesis could be tested by comparing the responses of germfree or Ag-free mice to the responses of the conventional mice observed here.

The natural priming of the mice to the self-CP1m/CP1h T cell epitope is intriguing. It is known that the healthy immune system contains T cells reactive to a variety of self-epitopes (26), including epitopes of hsp60 (23, 24). The present study, which documents T cell autoimmunity to a self-sequence of hsp60 not known to be shared with homologous bacterial sequences, differs from the earlier report of Munk et al. (24) of natural T cell autoimmunity to sequences of hsp60 shared by humans with mycobacteria. Thus, natural autoimmunity may arise in the absence of a known mimicry between a self and a foreign epitope. In any case, the present results confirm the functional existence of hsp60 epitopes in the immunologic homunculus, the immune system's picture of the self (26). If natural autoimmunity can be put to work as shown here, then natural autoimmunity exists in fact and not merely in theory.

We are currently trying to express the entire mouse hsp60 molecule free of contamination with any fusion protein to test whether the CP1m sequence is processed and presented naturally upon administration of intact self-hsp60. However, even if CP1m were found to be a cryptic epitope (37), the fact remains that mice appear to be primed naturally to a self epitope in the CP1m sequence.

The source of this priming could be either through positive selection of specific T cells in the thymus to hsp60 expressed there, or through peripheral expansion of specific T cells by contact with self CP1m or with an unknown cross-reactive epitope. Obviously, both processes could occur: initial positive selection centrally and subsequent peripheral expansion.

The existence of natural T cell immunity to the CP1m epitope has functional implications, both for our understanding of the regulation of natural autoimmunity and for the design of vaccines. Autoimmunity to a peptide epitope of the hsp60 molecule, designated p277, has been implicated in the spontaneous type I diabetes of NOD mice: a proliferative T cell response to p277 appears spontaneously during the phase of insulitis preceding overt diabetes, clones of T cells reactive to p277 adoptively transfer hyperglycemia and insulitis, and down-regulation of the T cell response to p277 by T cell vaccination or peptide vaccination can arrest the diabetogenic autoimmune process (32, 38, 39). The CP1m/CP1h peptide is adjacent to p277 and overlaps with it by three carboxyl terminal residues, yet T cell autoimmunity to CP1m/CP1h was not observed upon clinical and histologic examination to be associated with diabetes, insulitis or inflammation in any organ of the mice (data not shown). Even mice immunized with CP1h in CFA appeared free of pathogenic findings. Thus the natural autoimmunity to CP1m/CP1h expressed by Th cells and the T cell proliferative response induced by administering higher doses of peptide were not pathogenic. The contrast between diabetogenic autoimmunity to p277 in NOD mice and the benign autoimmunity to CP1m/CP1h reported here indicates that the biologic outcome of T cell autoimmunity to different epitopes in a single self-molecule can vary significantly. It would be important, obviously, to elucidate the rules, genetic and immunologic, that determine whether an autoimmune response is noxious or harmless. The results reported here raise the question of whether benign autoimmunity might be harnessed to prepare an effective, all-purpose T cell-dependent vaccine to important, but poorly immunogenic, Ags.

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