The technology described here allows the chemical synthesis of vaccines requiring correctly folded epitopes and that contain difficult or long peptide sequences. The final self-adjuvanting product promotes strong humoral and/or cell-mediated immunity. A module containing common components of the vaccine (T helper cell epitope and the adjuvanting lipid moiety S-[2,3-bis(palmitoyloxy)propyl]cysteine) was assembled to enable a plug and play approach to vaccine assembly. The inclusion within the module of a chemical group with chemical properties complementary and orthogonal to a chemical group present in the target epitope allowed chemoselective ligation of the two vaccine components. The heat-stable enterotoxin of enterotoxigenic Escherichia coli that requires strict conformational integrity for biological activity and the reproductive hormone luteinizing hormone-releasing hormone were used as the target epitopes for the antibody vaccines. An epitope from the acid polymerase of influenza virus was used to assemble a CD8+ T cell vaccine. Evaluation of each vaccine candidate in animals demonstrated the feasibility of the approach and that the type of immune response required, viz. antibody or cytotoxic T lymphocyte, dictates the nature of the chemical linkage between the module and target epitope. The use of a thioether bond between the module and target epitope had little or no adverse effect on antibody responses, whereas the use of a disulfide bond between the module and target epitope almost completely abrogated the antibody response. In contrast, better cytotoxic T lymphocyte responses were obtained when a disulfide bond was used.

Synthetic lipopeptides containing S-[2,3-bis(palmitoyloxy)propyl]cysteine (Pam2Cys) and incorporating a CD4+ T (Th1) cell epitope and either a B cell or CD8+ T cell epitope are capable of inducing both humoral and cellular immunity in experimental animals and humans (for reviews, see Refs. 1–4). The self-adjuvanting properties of lipopeptides abolish the need for additional, potentially toxic adjuvants in vaccine preparations because the lipid moiety Pam2Cys provides the required stimulatory signals to the immune system through engagement of toll-like receptors present on dendritic cells. This form of vaccine candidate is capable not only of inducing humoral and cell-mediated immunity depending on the nature of the target epitope(s) but also mucosal immunity if administered intranasally, a property that potentially removes the requirement for needle vaccine delivery (5–9).

The lipid moiety Pam2Cys can be attached to the N terminus of the peptide, giving the vaccine construct a linear orientation, or attached to the ε-amino group of the intervening lysine residue between the Th1 epitope and the target epitope, yielding a branched configuration. Branched lipopeptides demonstrate enhanced immunogenicity over their linear counterparts, a property that has been attributed to their superior stimulation of dendritic cells and improved solubility (9). Non-lipidated branched constructs also display greater resistance to proteolytic degradation (10). In addition to study of the influence of the geometry of the construct on the immunogenicity of peptide vaccines, the strict requirement for the toll-like receptor-2 agonist activity of Pam2Cys has also been demonstrated in a recently study (11). Furthermore, the addition of amino acid spacers between the lipid group and the epitopes of interest also contributes to the immunogenicity of the lipid component with serine or arginine spacers shown to be most effective (12).

Conventionally, a peptide vaccine comprising a Th1 epitope and a target epitope construct is synthesized in toto; the peptide sequence is synthesized contiguously before attachment of the lipid moiety. In most cases, this method of synthesis is simple and yields a product of reasonable quality. Occasionally, however, the peptide sequence may pose difficulties during synthesis, or the target epitope possesses a particular conformation that is necessary for the induction of antibodies capable of reacting with the native antigen. Such “conformational determinants” are difficult to fold correctly when part of long contiguous sequences that contain Th1 epitope sequences unrelated to the sequence that has to be folded correctly.

To address these problems, we have developed a modular approach based on an earlier concept (13) that consists of the separate assembly of the lipidated self-adjuvanting module consisting of a Th1 cell epitope to which Pam2Cys is covalently attached (Pam2Cys-Th1) and the target epitope. Orthogonal chemical groups with complementary reactivities incorporated into each component allow the subsequent chemoselective attachment.
ligation of the two components to yield the lipitated peptide vaccine (see Fig. 1).

To analyze antibody and CTL responses, two types of target epitopes were used in this study. 1) Antibody responses were studied using two target epitopes. (i) Human ST (STh) is an enterotoxin produced by enterotoxigenic strains of Escherichia coli and has the sequence NSSNYC<sup>C</sup>E<sup>LC</sup>10<sup>C</sup>C<sup>1</sup>NPAC<sup>15</sup>TGC<sup>18</sup>Y. The molecule contains 6 cysteine residues, which form three intramolecular disulfide bridges (Cys<sup>6</sup>-Cys<sup>11</sup>, Cys<sup>7</sup>-Cys<sup>15</sup>, and Cys<sup>10</sup>-Cys<sup>18</sup>) (14). This toxin is secreted by pathogenic strains of E. coli and causes diarrhea especially among children in developing countries. Antibodies directed to ST can neutralize the toxin and prevent the occurrence of diarrhea (15), although ST itself is not immunogenic. (ii) Gonadotrophin-releasing hormone also known as luteinizing hormone-releasing hormone (LHRH) with the sequence HWSYGLRPG is secreted by the hypothalamus and contains 6 cysteine residues, which form three intramolecular disulfide bridges (Cys<sup>6</sup>-Cys<sup>11</sup>, Cys<sup>7</sup>-Cys<sup>15</sup>, and Cys<sup>10</sup>-Cys<sup>18</sup>) (14). This toxin is secreted by pathogenic strains of E. coli and causes diarrhea especially among children in developing countries. Antibodies directed to ST can neutralize the toxin and prevent the occurrence of diarrhea (15), although ST itself is not immunogenic.

2) CTL responses elicited by a module-based lipopeptide vaccine were studied by using a branched lipopeptide vaccine candidate derived from the fusion protein of the Morbillivirus canine distemper virus and which demonstrates promiscuity between different MHC class II molecules from the same and different species; (i) T<sub>FLU</sub>, which is derived from the light chain of influenza virus hemagglutinin, has the sequence ALNNRFQ-IGKVELKS (21), and is an effective T<sub>HL</sub> epitope in BALB/c mice; and (ii) T<sub>OVAT</sub>, which is derived from ovalbumin, has the sequence ISQAVHAAHAEINEAGR (22), and is an effective CD4<sup>+</sup> T<sub>H</sub> epitope in C57BL/6 mice.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—All reagents, unless otherwise stated, were of analytical grade or its equivalent. Dichloromethane and N,N<sup>2</sup>-dimethylformamide, and palmitic acid were from Merck. O-Benzotriazole-N,N,N<sup>2</sup>,N<sup>2</sup>-tetramethyluronium hexafluorophosphate was obtained from Novabiochem, and 1-hydroxy-1,1,3,3-tetramethyltetrazolium was from CEM Corp. (Matthews, NC). Dicyclohexylcarbodiimide and trifluoroacetic acid (TFA) were from Fluka (Buchs, Switzerland). 1,8-Diaza-bicyclo-[5.4.0]undec-7-ene, diisopropylethylamine, triisopropylsilane, and diisopropylethylamine were obtained from Sigma-Aldrich. 2,4,6-Trinitrobenzenesulfonic acid was from Fluka (Buchs, Switzerland). Diethyl ether was from Merck, and phenol was obtained from BHD Laboratory Supplies (Poole, UK). Fmoc amino acids were obtained from either Auspep (Melbourne, Australia) or Merck. Boc-aminooxyacetic acid was obtained from Fluka (Buchs, Switzerland).

**Peptide Synthesis**—Peptides, lipopeptides, and lipopeptide modules were synthesized, purified, and characterized in house using Fmoc chemistry throughout as described elsewhere (5, 11, 13); the specific protocols used are detailed below.

**Synthesis of Lipidated Modules**—The lipopeptide vaccines used in this laboratory comprise three components: a T<sub>H</sub> epitope, the target epitope, and Pam2Cys, which assumes a branch within the overall lipopeptide structure (9) (see Fig. 1). This basic branched structure was maintained in the current study by building Pam2Cys into the T<sub>H</sub> epitope, and the whole was then ligated to the separately synthesized target epitope.

Following synthesis of T<sub>H</sub> epitopes T<sub>MV</sub>, T<sub>FLU</sub>, and T<sub>OVAT</sub>, Dde-Lys(Fmoc)-OH was coupled to the exposed N-terminal amino group, the Fmoc group present on the α amino group of this N-terminal lysine residue was removed, and the peptide was lipidated as described previously (9). Once lipidation was complete, a Boc-protecting group was attached to the exposed amino group of the Pam2Cys group by coupling di-tert-butyl dicarbonate. The 1-[4,4-dimethyl-2,6-dioxocyclohex-1-ylide]-ethyl (Dde) group on the α amino group of the N-terminal lysine residue was then removed by treatment with 2% hydrazine hydrate (Fisons, Homebush, Australia) in N,N<sup>2</sup>-dimethylformamide for 10 min. Boc-aminooxyacetic acid was then coupled to the exposed α amino group. This procedure led to the production of Modules 1, 2, and 3, each of which contained an aminooxy group at the N terminus.

For production of Modules 4 and 5, Boc-Cys(Trt)-OH was coupled to the exposed α amino group of lipidated peptides T<sub>FLU</sub> and T<sub>OVAT</sub> thereby providing an N-terminal thiol group for subsequent ligation to target epitopes. Lipidated modules were cleaved from the resin and purified according to the conditions described elsewhere (11).

**Synthesis and Assembly of Folded ST**—The N-terminal Asn residue of ST was omitted to expose Ser at the N terminus, which then allows oxidation to an aldehyde group (see below and Fig. 3). Orthogonal, pairwise protection of cysteine residues (see Fig. 3A) was used to ensure formation of the correct disulfide bond assignments within the ST sequence (23). Trityl (Trt)-protecting groups used at Cys<sup>5</sup> and Cys<sup>10</sup> were removed during the TFA cleavage procedure, and subsequent air oxidation catalyzed by 2,2'-dithiodipyridine was then performed for formation of the Cys<sup>5</sup>-Cys<sup>10</sup> disulfide bond. The Cys<sup>6</sup>-Cys<sup>14</sup> disulfide bond was formed by iodine-mediated deprotection and concomitant oxidation of the acetamidomethyl-protected cysteine residues used at these positions. Cysteine residues at positions 9 and 17 were protected with the tert-butyl group, and formation of the Cys<sup>9</sup>-Cys<sup>17</sup> disulfide bond was carried out in 5% dimethyl sulfoxide (DMSO) in TFA. The final disulfide assignments are shown in Fig. 3B.

**Ligation of Module and Target Epitopes Using Oxime Bond Formation**—ST, LHRH, and PA target epitopes were modified at their N termini to include a serine residue. Following purification of these target epitopes, an aldehyde functional group (CHO) was generated by oxidation of the N-terminal serine with sodium periodate as described previously (24). Vaccines based on these targets were then assembled by chemoselective
**Self-adjuvanting Synthetic Vaccines**

ligation between the aldehyde group and the aminoxy group present on the Pam2Cys-T_H module (Fig. 2A).

**ST Vaccine**—A 1.2-fold molar excess of purified CHO-ST (2.10 μmol; 4 mg) was dissolved in 800 μl of 50% ACN, 50% double distilled H₂O, and 0.1% TFA (pH 2) and then exposed to Module 1 (1.75 μmol; 4.8 mg) (see Fig. 3C). The reaction mixture was held at room temperature for 2 h, and the ligation progress was monitored by analytical HPLC until the reaction was complete. The final product, ST-oxime-Pam2Cys-T̂FLU, was isolated by semipreparative HPLC and had a mass of 4,623 Da (expected mass, 4,623 Da) and a yield of 5.1 mg.

**LHRH Vaccines**—Purified LHRH containing an aldehyde group at its N terminus (1.15 μmol; 1.45 mg) was dissolved with either lipidated Pam2Cys-T₄ Modules 1, 2, or 3 (0.575 μmol; 1.66 mg) in 400 μl of 50% ACN, 50% double distilled H₂O, and 0.1% TFA (pH 2). The reaction was carried out for 2 h at room temperature with CHO-LHRH, and the products LHRH-oxime-Pam2Cys-T₄MV, LHRH-oxime-Pam2Cys-T₄FLU, and LHRH-oxime-Pam2Cys-T₄OVA were isolated (see Fig. 2A).

**Ligation of Module and Target Epitopes Using Thioether Bond Formation**—The target epitopes LHRH and PA(224–236) were modified by coupling bromoacetic acid (Aldrich) to the exposed N-terminal amino group of the fully protected peptide while still attached to the solid support using symmetric anhydride chemistry. The bromoacetylated LHRH (Br-CH₂CO-PA) and Module 5, the lipidated modular protein Cys-LHRH or Cys-PA was dissolved in 50% ACN, 50% double distilled H₂O, and 0.1% TFA to which a 0.5-fold molar excess of 2,2'-dithiodipyridine (Fluka) dissolved in 100% ACN was added. The reaction was allowed to proceed in a light-proof container for 2 h at room temperature, and the final product, LHRH-thioether-Pam2Cys-T₄FLU, was then isolated. Under similar conditions and using either BrCH₂CO-LHRH or BrCH₂CO-PA and Module 5, the lipidated modular products LHRH-thioether-Pam2Cys-T₄OVA and PA-thioether-Pam2Cys-T₄OVA were isolated (see Fig. 2B).

**Ligation of Module and Target Epitopes Using Disulfide Bond Formation**—The target epitopes LHRH and PA(224–236) were modified by coupling bromoacetic acid (Aldrich) in the exposed N-terminal amino group of the fully protected peptide while still attached to the solid support using symmetric anhydride chemistry. The bromoacetylated LHRH (Br-CH₂CO-LHRH) and PA(224–236) (Br-CH₂CO-PA) were then cleaved extended to include a cysteine residue at the N terminus while still attached to the solid phase support, and at the same time, the side chain-protecting groups were removed. The lyophilized peptide powder Cys-LHRH or Cys-PA was dissolved in 50% ACN, 50% double distilled H₂O, and 0.1% TFA to which a 0.5-fold molar excess of 2,2'-dithiodipyridine (Fluka) dissolved in 100% ACN was added. The reaction was held for 30 min at room temperature following which the thiopyridylcysteinyl-LHRH (tpCys-LHRH) or thiopyridylcysteinyl-PA (tpCys-PA) was identified and isolated using reverse phase HPLC and mass spectrometry (see Fig. 2C). The vaccine candidates LHRH-disulfide-Pam2Cys-T₄OVA and PA-disulfide-Pam2Cys-T₄OVA were obtained using tpCys-LHRH or tpCys-PA and Module 5 under similar conditions. The molecular masses of modules and the final vaccine products are shown in Table 1.

**Mice and Inoculation Protocols**—BALB/c and C57BL/6 mice were bred and housed in the animal facility at the Department of Microbiology and Immunology, The University of Melbourne, Parkville, Australia. For antibody response studies, groups of five 6–8-week-old female BALB/c mice were inoculated subcutaneously in the base of the tail with 20 nmol of peptide-based immunogen delivered in saline (100 μl) on day 0 and again on day 21. Sera were prepared from blood taken at 3 weeks following primary inoculation and 10 days following the secondary inoculation. For CTL response studies, groups of three 6–8-week-old female C57BL/6 mice were anesthetized with methoxyflurane and inoculated intranasally with 25 nmol of the peptide immunogens delivered in saline (50 μl) or infected intranasally with 10⁶ pfu of influenza virus A/HK-X-31 (HKX-31, H3N2) in 50 μl of phosphate-buffered saline (PBS).

**Enzyme-linked Immunosorbent Assays (ELISAs)**—ELISAs were conducted on serum samples as described previously (25) using either LHRH or ST as coating antigen. The antibody titer was determined by expressing the reciprocal logarithmic dilution achieving an optical density of 0.2, which represents ~5 times the background binding in the absence of anti-LHRH or anti-ST antibody.

**Fertility Studies**—Fertility studies were carried out as described previously (18). Briefly, female mice were examined for their ability to drop litters following inoculation with peptide immunogens and exposure to untreated male mice. A male mouse was introduced to either two or three female mice 2 weeks after females had received the second dose of vaccine. Males were rotated between each group of females to expose each female to every male. Males and females were kept together for a total of 3 weeks at the end of which time males were removed and the females were kept under observation. A group of female mice inoculated with saline was used as a control.

**Cell Preparations**—Spleen and lung lymphocyte populations were recovered from mice 7 days following inoculation. Single cell suspensions were prepared from perfused lungs following enzymatic digestion with collagenase A (2 mg/ml; Roche Applied Science) or spleens by passing through a mesh sieve.
Treatment with Tris-ammonium chloride (7.4% (w/v) ammonium chloride (Ajax Chemicals) and 2.06% (w/v) Tris) was used to lyse red blood cells. Cell counts were performed using a hemocytometer, and cell concentrations were adjusted to 1 × 10⁷ cells/ml with RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; Invitrogen), 1 mM sodium pyruvate, 2 mM L-glutamine, 55 μM 2-mercaptoethanol, 12 mg/ml gentamycin, 100 units/ml penicillin, and 100 μg/ml streptomycin (all supplements were from Invitrogen) hereafter referred to as RF10.

Intracellular Cytokine Staining for IFNγ—Cells from the spleen or lung were incubated for 5 h in 96-well U-bottomed plates (1 × 10⁶ cells/well) in 200 μl of RF10 containing 1 μl/ml GolgiPlug (BD Biosciences) and 25 units/ml recombinant human IL-2 (Roche Applied Science) in the presence or absence of 1 μg/ml PA(224–236) or the control peptide NP(366–374) (sequence ASNENMETM) from the nucleoprotein of influenza virus. Cells were washed in FACs buffer (PBS containing 1% FCS and 5 mM EDTA) before staining with peridinin chlorophyll protein-conjugated anti-CD8a-Cy5.5 (BD Pharmingen) on ice for 30 min. Following two washes, cells were fixed and permeabilized using reagents supplied in the Cytoperm/Cytofix kit (BD Biosciences) according to the manufacturer’s instructions. After a further two washes, cells were stained for 30 min on ice with fluorescein isothiocyanate (FITC)-conjugated anti-IFNγ antibody (BD Pharmingen). After two additional washes, cells were resuspended in FACs buffer and analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Immunocytometry Systems), and data were analyzed using FlowJo software (version 4.6.2; Tree Star Inc., Ashland, OR).

In Vivo CTL Assay—Analysis of in vivo CTL activity was performed according to the method of Coles et al. (26). Target cells were prepared using splenocytes from naïve C57BL/6 mice that were treated with Vybrant® DiD cell labeling solution (Molecular Probes, Eugene, OR) and then pulsed either with the unlabeled peptide NP(366–374) or the peptide epitope PA(224–236) at a final concentration of 5 μg/ml or with medium alone at 37 °C for 60 min. Control cells that were exposed to medium only were also prepared. The three populations of cells were then labeled with low (100 nM), medium (1 μM), or high (5 μM) concentrations of 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes), respectively. A population (5 × 10⁶ cells in 200 μl) comprising equal numbers of these differentially labeled cell populations was then administered intravenously on day 7 to groups of three female C57BL/6 mice that had been previously inoculated intranasally with either 25 nmol of contiguously synthesized lipopeptide or modular lipopeptides prepared as described above in saline, saline only, or 10⁴ pfu of X-31 live influenza virus. Spleens were removed 16 h later and analyzed for the presence of CFSEhigh, CFSEmedium, and CFSElow cells by flow cytometry. Specific lysis of the two cell populations was calculated by determining the ratio of non-peptide-pulsed/peptide-pulsed target cells in vaccinated or virus-challenged mice compared with animals that received saline.

Statistical Analyses—All p values were calculated using a one-way analysis of variance with a 95% confidence interval using the Tukey test algorithm for post-test analyses.

RESULTS

The lipopeptide vaccines used in our laboratory comprise three components: a T₄ H epitope, the target epitope, and Pam2Cys, which assumes a branch within the overall lipopeptide structure (9). This basic branched structure was maintained in the current study by building Pam2Cys into the T₄ H epitope (Fig. 1, A–C), and the whole was then ligated to the separately synthesized target epitope (Fig. 1D). Judicious use of appropriate heterobifunctional X and R groups introduced into the module and target epitope, respectively, allowed a variety of chemical linkages to be used to couple the two vaccine components (Fig. 2).

Antibody Responses Elicited by Modular Vaccine Candidate Based on Conformationally Intact, Synthetic Analog of STH—STh is an 18-mer peptide and is non-immunogenic when administered alone (15). Previous reports have indicated that when chemically coupled or genetically fused to a protein carrier the ST-protein conjugate is able to elicit anti-ST antibody, and in some instances, the antiserum is able to neutralize the toxic activity of ST. In this study, we manufactured an analog of ST by chemical synthesis, omitting the N-terminal asparagine residue to expose a serine residue at this position and also replacing the C-terminal tyrosine residue with phenylalanine. We then folded the molecule into the correct conformation through specific disulfide assignments between the cysteine residues (Fig. 3, A and B) and determined its biological integrity in a suckling mouse assay (27). The results of this experiment (data not shown) indicated that the toxicity of the synthetic, folded STh analog was comparable with that of native STh, indicating the high fidelity of the synthesis and the folding process we used. The N-terminal serine residue of this totally synthetic and folded ST molecule was then oxidized to produce an N-terminal aldehyde group (Fig. 2B), which was ligated to a lipidated T₄ H module using oxime chemistry (Figs. 2A and 3C).

BALB/c mice were inoculated with the vaccine candidate ST-oxime-Pam2Cys-T₄ MV both subcutaneously and intranasally on days 1 and 21. Sera were prepared from blood obtained on days 21 and 31, and ELISAs were performed to determine the antibody response using ST as the coating antigen. The results (Fig. 4) demonstrated that anti-ST antibody was elicited following administration by either route of inoculation and that the antibody levels were significantly higher than those obtained when non-lipidated ST alone was administered in saline. These results demonstrate the feasibility and efficacy of using a modular approach for the assembly of totally synthetic self-adjuvanting vaccines when correctly folded B cell epitopes are required.

Comparison of Immunogenicity of Vaccine Candidates Assembled Using Contiguous Synthesis or Modular Approaches—Our previous studies (9, 12, 28) have demonstrated that contiguously synthesized branched lipopeptide vaccine candidates based on LHRH are able to induce strong anti-LHRH antibody responses and that these antibodies exert a biological effect by rendering mice reproductive sterile. These successful vaccine candidates are based on the orientation N-C-N-C of the target epitope and the T₄ H epitope, respectively. The orientation of the vaccine epitopes assembled using a modular approach, how-
ever, is C-N-N-C (e.g., see Fig. 2). We therefore compared the efficacies of contiguously synthesized and modular LHRRH-based vaccine candidates assembled using different chemistries to determine whether these parameters affect vaccine efficacy.

Three different modular vaccine candidates based on the same TH epitope, T_FLU, but attached to LHRRH by (i) a thioether bond (LHRRH-thioether-Pam2Cys-T_FLU), (ii) an oxime bond (LHRH-oxime-Pam2Cys-T_FLU), or (iii) a disulfide bond (LHRH-disulfide-Pam2Cys-T_FLU) were prepared (Fig. 2) and administered to BALB/c mice. The resulting antibody responses were compared with those elicited by conventionally, contiguously synthesized T_FLU-Pam2Cys-LHRRH.

The results (Fig. 5, upper panel) indicated that two doses of vaccine LHRRH-thioether-Pam2Cys-T_FLU and LHRRH-oxime-Pam2Cys-T_FLU induced strong anti-LHRRH antibody responses that are comparable with those elicited by T_FLU-Pam2Cys-LHRRH. However, little or no antibody responses were detected in those mice that received two doses of LHRRH-disulfide-Pam2Cys-T_FLU. The results of the fertility study correlate well with the level of antibody obtained as no pups were produced in groups of mice that received LHRRH-thioether-Pam2Cys-T_FLU, LHRRH-oxime-Pam2Cys-T_FLU, or T_FLU-Pam2Cys-LHRRH. In contrast, three of five mice from the group that received LHRRH-disulfide-Pam2Cys-T_FLU dropped litters. All mice that received saline only dropped litters.

We also determined whether the results we obtained in BALB/c mice could be repeated in a different mouse strain using an appropriate TH cell epitope in place of T_FLU. Using TOVA, we assembled three vaccine candidates utilizing three different chemical linkages (Fig. 2) and compared responses in C57BL/6 mice with those obtained with TOVA-Pam2Cys-LHRRH. The results (Fig. 5, lower panel) show that LHRH-thioether-Pam2Cys-TOVA elicited a specific anti-LHRRH antibody response that was as strong as the contiguously synthesized construct TOVA-Pam2Cys-LHRRH. Again, the modular construct prepared through the formation of disulfide bond formation LHRH-disulfide-Pam2Cys-T_OVA elicited weak or no antibody responses, but in contrast to BALB/c mice, significantly lower antibody responses were obtained in mice inoculated with LHRRH-oxime-Pam2Cys-T_OVA, which was prepared through oxime bond formation. Again, the results of mating studies correlated well with the antibody levels observed.

**Lipopeptide-based Modular Constructs Induce CD8+ T Cells That Secrete γ-Interferon and Are Cytotoxic**—The results described above demonstrated that totally synthetic vaccines assembled using a modular approach are capable of eliciting high titers of antibodies comparable with those elicited by contiguously synthesized lipopeptides and that attention must be paid to the nature of the ligation chemistry used to assemble the vaccine. Our results showed that modular vaccines assembled using a thioether bond demonstrate the least adverse effect on the ability to induce antibodies. It was therefore of interest to determine whether this methodology could be applied to modular lipopeptides designed to elicit CD8+ T cell responses. The CTL epitope chosen for this study was PA(224–236), which is derived from the acid polymerase of influenza virus. Three PA(224–236)-based vaccine candidates were assembled using the modular technology (Fig. 2), and their ability to induce CD8+ T cell responses was investigated in C57BL/6 mice. As controls, one group of mice was challenged with influenza virus HKX-31, and another group received the contiguously synthesized branched lipopeptide TOVA-Pam2Cys-PA. It has been shown that such branched lipopeptide-based vaccines

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**FIGURE 1.** Schematic representation of overall assembly of branched lipopeptide-based vaccine candidate using modular approach. A TH cell epitope was assembled using conventional solid phase synthesis and Fmoc chemistry. The protected peptide was then lipidated while still attached to the solid phase by stepwise addition of the lipid moiety to the ε-amino group of the lysine residue, which is attached to the N terminus of the peptide. Appropriate chemical modification of the ε-amino group of this lysine then provided a reactive group, R, for ligation to the complementary reactive group X at the N terminus of the target epitope. For Module 1, R = aminooxyacetyl and TH = T_FLU; for Module 2, R = aminooxyacetyl and TH = T_FLU; for Module 3, R = aminooxyacetyl and TH = T_FLU; for Module 4, R = Cys and TH = T_FLU; and for Module 5, R = Cys and TH = T_OVA, all attached by a Cys or AOA, aminooxyacetic acid.
are able to elicit strong CTL responses, reduce viral loads (7, 8, 20, 29), and protect mice from lethal viral challenge (30).

Seven days following inoculation, PA(224–236)-specific responses were measured in the lungs of all animals by using an IFN-γ intracellular staining assay. The results (Fig. 6, upper panel) show that PA-disulfide-Pam2Cys-TOVA induced a strong PA(224–236)-specific CD8 T cell response, which was similar in magnitude to the response observed following viral infection (p > 0.05). Furthermore, PA-disulfide-Pam2Cys-TOVA induced greater numbers of PA(224–236)-specific IFN-γ + CD8 + cells than did PA-thioether-Pam2Cys-TOVA, PA-oxime-Pam2Cys-TOVA, or the contiguously assembled lipopeptide construct TOVA-Pam2Cys-PA. Little or no PA(224–236)-specific response was detected in mice that received non-lipidated PA-based constructs (data not shown).

In vivo effects of the CD8 + T cells elicited were determined by using a cytotoxicity assay. The results (Fig. 6, lower panel) showed that inoculation with virus or lipopeptides induced responses that led to efficient removal of PA epitope-pulsed target cells in vivo. The order of the efficiency of in vivo cytolysis was as follows: virus > PA-disulfide-Pam2Cys-TOVA = TOVA-Pam2Cys-PA > PA-thioether-Pam2Cys-TOVA or PA-oxime-Pam2Cys-TOVA. This hierarchy is similar to that observed in the intracellular staining assay with the exception that inoculation with whole virus demonstrated greater cytolysis than that observed with the modular construct PA-disulfide-Pam2Cys-TOVA, a result presumably due to the presence in virus of multiple epitopes capable of eliciting CD8 + T cells in these mice.
Self-Adjuvanting Synthetic Vaccines

FIGURE 5. Evaluation of immunogenicity of vaccines prepared using different chemistries to ligate lipidated T$_h$ epitope modules to LHRH-based target epitope. Groups of mice were inoculated either with saline, a conventionally synthesized lipopeptide vaccine, or modular lipopeptide vaccines assembled using thioether, disulfide, or oxime chemistry. Antibody titers in sera obtained in the primary response (open symbols) and secondary response (closed symbols) were determined by ELISA using LHRH as the coating antigen. The numbers associated with each of the data sets refer to the incidence of litters dropped by female BALB/c (upper panel) or C57BL/6 (lower panel) mice assessed by a fertility trial. Upper panel, module based on the T$_h$ epitope T$_{FLU}$; Lower panel, module based on the T$_h$ epitope T$_{OVA}$.

DISCUSSION

The results of our studies show that it is feasible to prepare lipidated peptide vaccine candidates using a modular approach in which thioether, disulfide, or oxime bond formation is used to ligate the two components of the vaccine. Use of a thioether bond to conjugate the two components has the advantage of simpler chemistry and somewhat higher yield of product, whereas disulfide and oxime bond formation requires an additional step for manufacture. Oxime bond formation, however, does have the advantage that the presence of the thiol group present in cysteine residues that may occur in the peptide sequence do not lead to unwanted side products. This is an especially important consideration in the case of vaccine targets such as ST that contain one or more cysteine residues that are essential for structural integrity of the antigen.

Our results also show that due consideration must be given to the particular combination of T$_h$ cell epitope and ligation chemistry used because they can affect the resulting immunogenicity. For example, the results with the LHRH vaccine candidates indicated that use of a thioether linkage between T$_h$ and LHRH had little or no negative impact on immunogenicity. In contrast, significantly lower antibody titers were obtained when the combination of oxime linkage and T$_{OVA}$ was used compared with the combination of oxime linkage with T$_{FLU}$, whereas T$_{OVA}$ in combination with LHRH through thioether linkage provided satisfactory antibody titers. Furthermore, the modular LHRH vaccines containing either T$_{FLU}$ or T$_{OVA}$ assembled using a disulfide bond linkage elicited very little antibody. The results with PA(224–236) epitope-based vaccines showed that although all lipopeptides were able to induce a PA(224–236)-specific IFN$^+$ CD8$^+$ T cell response there was a definite hierarchy (disulfide > contiguous > oxime = thioether) in the response elicited.

Our finding that the type of chemical bond used to ligate the module and target epitope differentially affects the induction of antibodies or CD8$^+$ T cells may reflect the different antigen processing requirements of the two arms of acquired immune responses. Vaccine candidates assembled using thioether or oxime bonds were able to elicit antibodies but were less immunogenic when it came to cell-mediated responses possibly because these physiologically unusual covalent bonds are more
resistant to degradation by the enzymes involved in antigen processing.

With the exception of an M2e-based vaccine (31), all of the contiguously synthesized branched lipopeptide vaccine candidates that we have constructed were assembled with the \((T_{14} \text{ epitope})-(\text{target epitope}) \text{ orientation N-C-N-C} (9)\). In the case of LHRH and PA, this allowed the C-terminal carboxamide or carboxyl function, respectively, of these epitopes to be free, a feature that is important for their biological activity. In the case of M2e, the orientation of the epitopes in the lipopeptide vaccine was designed to be \((\text{target epitope})-(T_{14} \text{ epitope}) \text{ N-C-N-C}\), which allowed the N terminus of M2e to be free, a configuration that improves the induction of biologically active antibodies (31). The orientation of the vaccine epitopes assembled using the modular approach, however, is C-N-N-C (e.g. see Fig. 2). Peptide-based vaccines with this geometry cannot easily be assembled by contiguous peptide synthesis but when using the modular approach present no difficulty because modification at the N terminus of a peptide is more facile than modification of the C terminus. The results of the biological studies demonstrated that modular lipopeptides assembled in the C-N-N-C orientation are just as effective as N-C-N-C branched lipopeptides when the chemical linkage used is a thiether bond for antibody-inducing lipopeptide or a disulfide bond for CTL-inducing lipopeptide.

The modular approach described here has several advantages over the use of contiguous assembly of synthetic peptide-based vaccines. (i) Most importantly, the modular approach allows target epitopes with a prerequisite for tertiary structure to be successfully incorporated into vaccines in those cases where \textit{in toto} synthesis may not be possible. (ii) Because each peptide component is shorter, fidelity of synthesis is improved, leading to better yields and quality of product especially in the case of long and/or difficult sequences. (iii) Modules containing a promiscuous T_{14} epitope allow a “plug and play” approach to vaccine assembly.

As the potential of synthetic lipopeptide-based vaccines is realized in the clinic, the ability to implement their large scale manufacture with increased product quality assurance is essential for the realization of full commercial development. In this study, we have described the use of multiple chemoselective ligation methods to develop a modular approach for the manufacture of synthetic lipopeptide vaccines. We have also demonstrated that such an approach can produce vaccines capable of inducing both humoral and cellular immune responses, paving the way for development of synthetic lipopeptide-based vaccines for the pharmaceutical industry.

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