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Highly Immunogenic and Totally Synthetic Lipopeptides as Self-Adjuvanting Immunocontraceptive Vaccines

Weiguang Zeng, Souravi Ghosh, Yuk Fai Lau, Lorena E. Brown, and David C. Jackson

In this study, we describe the synthesis of various lipopeptides based on the sequence of luteinizing hormone-releasing hormone (LHRH) and report on their abilities to induce Abs against this “self” hormone when inoculated into mice in the absence of additional adjuvant. The peptides consisted of a colinear CD4+ T helper cell epitope from the L chain of influenza virus hemagglutinin and LHRH, which has B cell epitopes but no T cell epitopes present in its sequence. Lipids were attached either at the N terminus or between the T cell epitope and LHRH, in the approximate center of the peptide. The lipopeptide constructs displayed different solubilities and immunological properties that depended not only on the lipid content but also on the position of attachment of the lipids. Some of these constructs were highly immunogenic, inducing high titers of Ab, which were capable of efficiently sterilizing female mice when administered in saline by s.c. or intranasal routes. The most effective vaccines were highly soluble, contained the dipalmityl-S-glyceryl cysteine moiety, and had this lipid attached at the center of the molecule. The relative ability of the lipopeptides to induce an Ab response in the absence of external adjuvant was reflected by their ability to up-regulate the surface expression of MHC class II molecules on immature dendritic cells. These results demonstrate that the composition and position within peptide vaccines of self-adjuvanting lipid groups can influence the ability to induce the maturation of dendritic cells and, in turn, the magnitude of the resulting Ab response. The Journal of Immunology, 2002, 169: 4905–4912.

A n attractive option for contraception in man and animals is the use of immunocoontraceptive vaccines. The gametes and endocrine hormones that are involved in reproductive function provide a variety of target Ags which have been studied with a view to controlling reproduction through vaccination (1). Luteinizing hormone-releasing hormone (LHRH), also known as gonadotropin releasing hormone, has the amino acid sequence EHWSYGLRP and is secreted by the hypothalamus. The hormone enters the portal system and acts on the anterior pituitary, causing the release of follicle stimulating hormone and luteinizing hormone, thereby initiating a cascade of events leading to the control of gametogenesis. Abs directed against LHRH neutralize the hormone’s activity, inhibiting the reproductive process and causing reproductive sterility (2, 3).

Use of LHRH-based vaccines in humans as an immunoncontraceptive, although theoretically possible, is unlikely, because profound behavioral modification can occur as a consequence of inhibiting the production of testosterone in males. However, a very active area of research is the use of LHRH-based vaccines in the control of hormone-dependent cancers, including breast (4) and prostate cancers (5, 6). Because the sequence of LHRH is conserved in all mammals, LHRH-based immunocontraceptives are candidates for use in the companion animal and livestock arenas. Here the concerns of behavioral modification are not of such overriding importance and the use of immunocontraception as an alternative to surgical castration has attracted a great deal of attention (5, 7–10).

Although the peptide nature of the hormone makes it an obvious candidate for epitope-based vaccine design, the major problem of poor immunogenicity in the absence of an adjuvant confronts this as it does all peptide-based vaccine candidates. Of the many adjuvants currently available, many are too toxic for use in humans and animals or are ineffective. However, lipids and lipopeptides have been shown to be capable of adjuvanting otherwise weak immunogens (11–15) and show none of the harmful side effects of other adjuvant formulations. In lipopeptide constructs, a lipid moiety with known adjuvanticity can be covalently coupled to peptide to generate a fully synthetic self-adjuvanting and potentially safe vaccine (16–19). There have been a number of successful applications of lipopeptides as immunogens to generate Ab and cellular responses (12, 17, 20), and several different lipids have been used for these purposes. Tripalmitoyl-S-glyceryl cysteine (Pam3Cys) is a synthetic version of the N-terminal moiety of Braun’s lipoprotein that spans the inner and outer membranes of Gram-negative bacteria and has been shown to be capable of stimulating virus-specific CTL responses against influenza virus-infected cells (21) and to elicit protective Abs against foot-and-mouth disease (17) when coupled to the appropriate synthetic epitopes. Recently dipalmitoyl-S-glyceryl cysteine (Pam2Cys), an analog of Pam3Cys, has been synthesized (22) and been shown to correspond to the lipid moiety of macrophage-activating lipopeptide 2 isolated from mycoplasma that lack cell walls (23–25). In contrast to Pam3Cys, Pam2Cys has only two ester-bound palmitic acids and a free amino group, which leads to improved water solubility of Pam2Cys-peptide constructs compared with Pam3Cys-peptide constructs. It has also been reported that Pam2Cys is a more potent stimulator of splenocytes (22) and macrophages (24, 25) than is Pam3Cys. The structural similarity of Pam2Cys to Pam3Cys and its enhanced macrophage-activating properties, as well as its less hydrophobic
nature, indicate that Pam2Cys could also be used as a self-adjuvanting moiety.

In this study, we describe the synthesis of Pam3Cys- and Pam2Cys-peptide constructs based on LHRH and report on their immunogenicity and ability to sterilize female mice when inoculated in the absence of additional adjuvants. These lipopeptides consist of an epitope for CD4+ T cells and the LHRH sequence, which contains one or more epitopes for B cells. Because the LHRH sequence contains no T cell epitopes, the CD4+ T cell epitope GALNRGQKVE and a 2-fold flanking sequence contains no T cell epitopes, the CD4+ T cell epitope was designed to provide T cell help. In a previous publication from this laboratory, it was shown that a peptide composed of these two epitopes assembled as a colinear tandem construct was a potent immunogen and able to induce reproductive sterility in female mice when inoculated in CFA (27). In the present study, either Pam3Cys or Pam2Cys was attached to the peptide-based vaccine to allow delivery in the absence of additional adjuvant. The lipid-containing groups were attached at two different positions within the peptide to produce linear and branched configurations of lipopeptide. The immunogenic properties of these vaccines were determined by examining the titer and isotype of anti-LHRH Ab, and the biological effects were determined by measuring any influence on the reproductive capabilities of vaccinated mice.

Materials and Methods

Chemicals

Unless otherwise stated, chemicals were of analytical grade or its equivalent. N,N,N,N-tetramethylpiperidine, trifluoroacetic acid (TFA), O-benzotriazole-N,N,N,N-tetramethyl-uronium-hexafluorophosphate, 1-hydroxybenzotriazole, and diisopropylamine and disopropylcarbodimide were obtained from Arapep (Melbourne, Australia) and Sigma-Aldrich (Castle Hill, New South Wales, Australia). O-benzotriazole-N,N,N,N-tetramethyl-uronium-tetrafluoroborate was obtained from Bachem (Bubendorf, Switzerland). Dichloromethane (DCM) and diethylether were obtained from Merck (Kilsyth, Australia). Phenol and trisopropylsilane were obtained from Sigma-Aldrich; trinitrobenzenesulfonic acid and diaminopyridine (DMAP) were obtained from Fluka (Buchs, Switzerland); 1.8-diazabicyclo[5.4.0]undec-7-ene was obtained from Sigma-Aldrich; and palmatic acid was obtained from Fluka.

Synthesis of Pam3Cys and Pam2Cys

Pam3Cys and Pam2Cys were prepared according to the method described by Wiesmuller et al. (28) and modified by Zeng et al. (29). The synthesis of Pam2Cys was adapted from previously described methods (30,31); specifically, 3-bromopropan-1,2-diol was used instead of 3-chloro-propan-1,2-diol, and centrifugation, not filtration, was used to recover the product.

Peptide synthesis

The general procedure used in this laboratory for the synthesis of peptides has been described previously (32). To enable lipid attachment between the CD4+ T cell epitope and LHRH, F-moc-lysine(Mtt)-OH was inserted at a point between the two epitopes in the approximate center of the resin-bound peptide. Following completion of peptide synthesis, the Mtt group was removed by continual flow washing with 1% TFA in DCM over a period of 30–45 min.

Pam3Cys or Pam2Cys was then coupled to the exposed e-amino group according to the procedure described previously (29). Briefly, a 2-fold excess of Pam3Cys or Pam2Cys, O-benzotriazole-N,N,N,N-tetramethyl-uronium-tetrafluoroborate, and 1-hydroxybenzotriazole was dissolved in DCM, and a 3-fold excess of diisopropylamine was added. This solution was then added to the resin-bound peptide to generate lipopeptide. Monitoring the reaction by analytical reversed phase (RP)-HPLC, we found that quantitative palmitoylation was achieved after 16 h.

All resin-bound peptide constructs were cleaved from the solid-phase support with reagent B (88% TFA, 5% phenol, 2% triisopropylsilane and 1% water) for 2 h, and purified by RP chromatography as described previously (33). Analytical RP-HPLC was conducted using a Vydac C4 column (4.6 × 300 mm; Edwards Instruments, New South Wales, Australia) installed in a Waters (Box Hill, Victoria, Australia) HPLC system and developed at a flow rate of 1 ml/min using 0.1% TFA in H2O and 0.1% TFA in CH3CN as the limit solvent. All products presented as a single major peak on analytical RP-HPLC and had the expected mass when analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry on a Bruker BIFLEX instrument (Bruker Daltonics, Bremen, Germany) equipped with delayed ion extraction. The final quantitation of the lipopeptides was done by measuring the absorption at 280 nm, exploiting the presence of a tryptophan and a tyrosine residue in the peptide constructs (molar extinction coefficient of 6.6 × 104).

Two serine residues are present between the protein and lipid portion of Braun’s lipoprotein from which Pam3Cys is derived (28). Furthermore, the presence of serine between the lipid and peptide moieties of Pam3Cys-containing lipopeptides has been said to improve immunogenicity (34). Therefore, we also investigated the effect of serine by incorporating two residues between the peptide and lipid moieties of the Pam2Cys-containing pepti
de constructs. This was simply done by sequential addition of two serine residues to the peptide before covalent attachment of the lipid moiety.

A schematic diagram of the peptides and lipopeptides used in this study is shown in Fig. 1 and a summary of their characteristics, as conducted by analytical RP-HPLC and mass spectrometry, is presented in Table I.

Immunization protocols

Groups of five female BALB/c mice, 6–8 wk old, were inoculated at day 0 and again on day 28. For s.c. inoculations (100 μl volume per dose), lipopeptide constructs were prepared in saline and nonlipidated peptides formulated as an emulsion in an equal volume of CFA for the primary injection or IFA for the secondary inoculation. For intranasal (i.n.) inoculations, 50 μl of peptide in saline were applied to the nares of mice anesthetized with penthane for inhalation. Sera were prepared from blood taken at 4 wk after the primary inoculation and 2 wk after the secondary inoculation.

ELISAs

ELISAs were conducted on serum samples as described previously (27) using LHRH as the coating Ag. The titer of Ab was expressed as the reciprocal of the highest dilution of serum to achieve an OD of 0.2, which represents approximately five times the background binding in the absence of Ab. The isotype of Abs specific for LHRH was determined using rabbit antisera directed against mouse IgM, IgG1, IgG2a, IgG2b, IgG3, or IgA (ICN Pharmaceuticals, Costa Mesa, CA) as previously described (27).

Fertility studies

After being inoculated with peptide immunogen and following exposure to male mice, female mice were tested for their ability to drop litters. A group of mice immunized with saline in CFA was used as a control. A male mouse was introduced into a cage in which two or three female mice were kept, and male mice were rotated between each cage to expose each group of female mice to every male. Males and females were kept together for a total of 3 wk, at the end of which the males were removed and the females kept under observation.

Dendritic cell (DC) culture

DC were cultured in medium based on complete IMDM. This consisted of IMDM containing 25 mM HEPES and without α-thioglycerol or 1-glutamine (JRH Bioscience, Lenexa, KS), supplemented with 10% (v/v) heat-inactivated (56°C, 30 min) FCS (CSL, Victoria, Australia), gentamicin (24 μg/ml), glutamine (2 mM), sodium pyruvate (2 mM), penicillin (100 IU/ ml), streptomycin (180 μg/ml), and 2 ME (0.1 mM). For DC generation, complete IMDM was further supplemented with 30% supernatant from cultured NIH/3T3 cells and 5% GM-CSF in the form of a supernatant from Ag8653 cells transfected with the GM-CSF gene (DC Medium).

The culture method for immature DC was adapted from Wizner et al. (35). Splenocytes from a BALB/c mouse were seeded at 1.5 × 105 cells per 55-mm dish (Techno-Plas, South Australia, Australia) in 3 ml of DC medium and incubated at 37°C with 5% CO2. All the equipment used for culturing was pyrogen free. The medium was changed every 4 days, and all cells were returned to the dish. On day 12, both suspended and weakly adherent cells were collected by forcefully pipetting and then aspirating the medium. The procedure was repeated with 2 ml of PBS. The remaining strongly adherent cells were discarded. The collected cells were pelleted by centrifugation and resedimented into a new dish. Cells were subsequently maintained on a 4-day alternating cycle of medium change and passage. After 1 mo of continuous culturing, the floating and semiadiherent cells took on the appearance and staining characteristics of immature DC and are referred to as D1 cells. Under these passage conditions, the majority of cultured D1 cells maintain an immature phenotype characterized by an intermediate expression level of cell surface MHC class II molecules.
Flow cytometric analysis of D1 cells

D1 cells (8 × 10^4 cells per sample) were seeded in a new petri dish with 1 ml of DC medium and incubated with 0.0045 nmol lipopeptide, dissolved in complete IMDM. LPS purified from Escherichia coli serotype O111:B4 (Difco, Detroit, MI; a kind gift from Dr. E. M. Anders, Department of Microbiology and Immunology, University of Melbourne) was used at 5 µg/ml as a positive control for DC maturation. After overnight incubation, the cells were harvested and washed once with PBS with 1% FCS. To prevent nonspecific binding to FcγRII/III, the cells were preincubated with 20 µl of normal mouse serum for 5 min at room temperature. The cells were then exposed to FITC-conjugated mAb 14-4-4S (IgG2a, anti-I-Ek,d) (36) for 30 min on ice. mAb 36/1 (37) specific for the hemagglutinin of influenza virus was used as an isotype control. All Abs were used at 2.5 µg/ml. The samples were washed once with PBS containing 1% FCS and fixed with PBS containing 4% paraformaldehyde on ice for 15 min. Flow cytometry analysis was performed using a FACSort (BD Biosciences, San Jose, CA), and the data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

Results

Solubility properties of the lipopeptides

Visual inspection (Fig. 1) of the different lipopeptide preparations showed that they differed markedly in their solubilities. Better solubility was most evident in those cases where lipid was attached through the ε-amino group of a lysine residue, Lys, situated between the two epitopes at the approximate center of the molecule ([T]-Lys(Pam3Cys)-[B] or [T]-Lys(Pam2Cys)-[B]); in the case of branched constructs, the centrally located lysine residue to which the lipid is attached is denoted in italics, Lys. In some cases (Pam2Cys-Ser-Ser-[T]-[B] and Pam3Cys-Ser-Ser-[T]-[B]), two serine residues (Ser-Ser) were added between the peptide and lipid moiety. This was also done in the case of the branched construct [T]-Lys(Pam2Cys-Ser-Ser)-[B], in which the two serine residues were added to the ε-amino group of the central lysine residue before the lipid moiety was attached. The photographic insert demonstrates the solubility differences of left, the branched construct [T]-Lys(Pam2Cys-Ser-Ser)-[B], and right, the linear construct Pam2Cys-Ser-Ser-[T]-[B]. Solutions are ~1 mg/ml and are in saline.

Immunogenicity of lipopeptides

The three lipopeptides, Pam2Cys-Ser-Ser-[T]-[B], [T]-Lys(Pam3Cys)-[B], and [T]-Lys(Pam2Cys)-[B], when administered s.c. in saline, induced high levels of anti-LHRH Ab. In fact, Ab titers induced after two doses of these lipopeptides were similar to those obtained with [T]-[B] or [T]-Lys-[B] when they were administered in CFA (Fig. 2). The titers of anti-LHRH Abs in sera of mice that had received Pam2Cys-[T]-[B] or Pam3Cys-Ser-Ser-[T]-[B] may be slightly lower. The two soluble branched lipopeptides [T]-Lys
(Pam3Cys)-[B] and [T]-Lys(Pam2Cys)-[B] induced 10- to 100-fold higher levels of anti-LHRH Ab following the primary inoculation than did the other less soluble lipopeptide constructs. Two groups of five mice receiving [T]-[B], which was simply admixed with Pam3Cys-Ser-(Lys)₄ in the ratio 1:1 or 1:5, did not elicit significant levels of anti-LHRH Ab, a finding that contrasts with other results reported using Pam3Cys-Ser-(Lys)₄ (38) as an externally added adjuvant.

Ab titers and the fertility status of animals were determined following the second dose of vaccine. The results (Table II) indicate that none of the mice that received either of the two branched soluble lipopeptide constructs [T]-Lys(Pam3Cys)-[B] or [T]-Lys(Pam2Cys)-[B], which were administered in saline, or the two nonlipidated constructs, [T]-[B] or [T]-Lys-[B], which were administered in CFA, became pregnant. Two animals from the groups that received Pam2Cys-[T]-[B] or Pam3Cys-Ser-Ser-[T]-[B] did drop litters 2 wk after the second dose of vaccine and one mouse from the group that received Pam2Cys-Ser-Ser-[T]-[B] also delivered a litter. All members of control groups, i.e., mice that received saline in CFA or the peptide [T]-[B] coadmixed with Pam3Cys-S-(Lys)₄, dropped litters.

Ab levels were followed up to 7 mo after the second dose of peptide vaccine; the titers of anti-LHRH Ab present in lipopeptide-primed mice and also in those mice that were primed with nonlipidated peptide administered in CFA decreased between 4- and 20-fold over a 26 wk period. Three months following the secondary inoculation of vaccine, a second fertility study yielded similar results to the 2-wk postimmunization trial. Mice that had received the soluble lipopeptides [T]-Lys(Pam3Cys)-[B] or [T]-Lys(Pam2Cys)-[B] in saline or the nonlipidated [T]-[B] and [T]-Lys-[B] in CFA were still infertile.

**Pam2Cys is a more potent adjuvant than Pam3Cys**

The results presented in Fig. 2 and Table II indicate that the two branched lipopeptides [T]-Lys(Pam3Cys)-[B] and [T]-Lys(Pam2Cys)-[B] not only were more soluble but also elicited higher Ab titers, particularly in the primary Ab response, than the linear immunogens Pam2Cys-[T]-[B],

**FIGURE 2.** Immunogenicity of peptide immunogens. All lipopeptides were administered s.c. in saline for both primary and secondary inoculations. The two nonlipidated peptides [T]-Lys-[B] and [T]-[B] were administered in CFA for the primary (○) and in IFA for the secondary (●) doses of vaccine. For administration of [T]-[B] admixed with Pam3Cys-S-(Lys)₄, peptide was dissolved in saline and mixed with the lipopeptide in 1:1 or 1:5 molar ratio. The dose of peptide immunogens used was 20 nmol. In all cases, the control groups of animals received saline emulsified in CFA for priming and saline emulsified in IFA for the secondary inoculation.
Pam3Cys-Ser-Ser-[T]-[B], and Pam2Cys-Ser-Ser-[T]-[B]. The results presented in Fig. 2 and Table II also indicate that Pam2Cys-containing peptides may be better immunogens than Pam3Cys-containing peptides. To examine this further, we investigated the effect of decreasing the dose on the immunogenicity of [T]-Lys(Pam3Cys)-[B] and [T]-Lys(Pam2Cys)-[B]. The results of the Ab titer determinations (Table III) is suggestive of a trend toward somewhat higher Ab titers elicited by [T]-Lys(Pam2Cys)-[B], but a striking difference is observed in the mating trial; 1 of 5 and 0 of 5 mice receiving 10 and 1 nmol [T]-Lys(Pam2Cys)-[B], respectively, dropped litters, whereas 3 of 5 and 5 of 5 mice receiving [T]-Lys(Pam3Cys)-[B] at these doses dropped litters. The effect of including two additional serine residues into the Pam2Cys-containing immunogens had little effect on the fertility status of animals, although there was some improvement in the Ab titers that were generated following the second dose (1 and 10 nmol of [T]-Lys(Pam2Cys-Ser-Ser)-[B]).

**i.n. immunization with Pam2Cys-peptide induces strong specific systemic Ab responses**

It has been reported previously that immunization with Pam3Cys-containing peptides by the mucosal (oral or i.n.) route induces strong mucosal and/or systemic immune responses (33, 39, 40). In the present series of experiments, we compared the immunogenicity of the linear Pam2Cys-Ser-Ser-[T]-[B] and branched [T]-Lys(Pam2Cys-Ser-Ser)-[B] peptides following inoculation in saline by the i.n. route. The same vaccines were also inoculated by the s.c. route, and the systemic anti-LHRH Ab responses were measured. The solution used for inoculation of [T]-Lys(Pam2Cys-Ser-Ser)-[B] was clear and the one for Pam2Cys-Ser-Ser-[T]-[B] was opalescent, indicating solubility differences between the two preparations.

Following two i.n. inoculations, each of the vaccines induced similar titers of serum anti-LHRH Abs, which were slightly lower than those induced following s.c. inoculation (Table IV). The more soluble [T]-Lys(Pam2Cys-Ser-Ser)-[B] induced significantly higher levels of anti-LHRH Ab 4 wk after a single dose than did the less soluble Pam2Cys-Ser-Ser-[T]-[B] (p = 0.00007); in fact, this was similar to the result obtained following s.c. inoculation. The results of the fertility trial showed that two i.n. inoculations of [T]-Lys(Pam2Cys-Ser-Ser)-[B] prevented all mice from becoming pregnant in contrast to those animals receiving Pam2Cys-Ser-Ser-[T]-[B] i.n., in which 3 of 5 mice dropped litters.

A comparison of the longevity of the responses induced by the two constructs when administered by the two different routes is also shown in Table IV. Twenty-six weeks following the second dose of vaccine, the levels of Ab in all mice had dropped below those observed 2 wk after the second dose. However, the decrease in anti-LHRH Ab in the group that received [T]-Lys(Pam2Cys-Ser-Ser)-[B] s.c. was much less apparent, again indicating the immunological superiority of the configuration in which Pam2Cys-Ser-Ser is attached at the center of the molecule.

We also determined the titers of individual Ab isotypes that were directed toward LHRH and obtained from animals following two s.c. or i.n. doses of the soluble lipopeptide [T]-Lys(Pam2Cys-Ser-Ser)-[B] (Fig. 3). i.n. inoculation appeared to induce higher levels of IgG3, IgG2b, and possibly IgM than did s.c. inoculation, even though the amount of total Ig induced by i.n. inoculation was less.

**Exposure of DC to peptides and lipopeptides induces different levels of cell surface MHC class II molecules**

The priming of naive CD4+ T cells in secondary lymphoid organs by DC is preceded by maturation of DC upon exposure to Ag. This maturation is characterized by up-regulation of MHC products and costimulatory molecules on the DC surface. Therefore, we determined whether the various peptides and lipopeptides could differentially activate DC in an attempt to explain the different immunogenic properties of these vaccine candidates. The results of experiments (Fig. 4) in which a line of immature DC, D1 cells, were exposed to peptides, stained for surface expression of MHC...

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**Table II. Anti-LHRH antibody titers and incidence of pregnancy following inoculation with peptide constructs**

<table>
<thead>
<tr>
<th>Inoculuma</th>
<th>Mean Anti-LHRH Titer (log_{10}) at the Indicated Times Following the Second Dose of Vaccineb</th>
<th>Incidence of Pregnancy 2 wk after 2nd dose</th>
<th>13 wk after 2nd dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pam2Cys-[T]-[B]</td>
<td>4.24 ± 0.60 3.38 ± 0.18 3.34 ± 0.97 3.18 ± 0.63 3.16 ± 0.53</td>
<td>2/5</td>
<td>3/5</td>
</tr>
<tr>
<td>Pam3Cys-Ser-Ser-[T]-[B]</td>
<td>3.36 ± 0.23 3.12 ± 0.16 3.04 ± 0.24 2.78 ± 0.19 2.75 ± 0.23</td>
<td>2/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Pam2Cys-Ser-Ser-[T]-[B]</td>
<td>4.78 ± 0.18 3.96 ± 0.10 3.80 ± 0.16 3.52 ± 0.25 3.48 ± 0.25</td>
<td>1/5</td>
<td>2/5</td>
</tr>
<tr>
<td>[T]-Lys(Pam3Cys)-[B]</td>
<td>4.48 ± 0.62 4.18 ± 0.43 4.06 ± 0.38 3.86 ± 0.54 3.75 ± 0.48</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>[T]-Lys(Pam2Cys)-[B]</td>
<td>4.68 ± 0.40 3.96 ± 0.34 3.94 ± 0.38 3.78 ± 0.21 3.70 ± 0.29</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>[T]-[B]</td>
<td>4.92 ± 0.32 4.32 ± 0.32 4.28 ± 0.32 4.06 ± 0.36 3.98 ± 0.35</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>[T]-Lys-[B]</td>
<td>4.70 ± 0.18 4.36 ± 0.15 4.24 ± 0.16 4.12 ± 0.20 3.82 ± 0.08</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>[T]-[B] plus Pam3Cys-Ser-Lys(1:5 admixture)</td>
<td>&lt;2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Saline</td>
<td>&lt;2</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

| a The peptides [T]-[B] and [T]-Lys-[B] were each administered in CFA, and saline was also administered in CFA as a control. All other peptide constructs were administered in saline. The dose of each peptide was 20 nmol, and all inocula were administered s.c. |
| b Titers represent the geometric means of groups of five female BALB/c mice. |

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**Table III. Anti-LHRH antibody titers and fertility status of mice inoculated with different doses of peptide vaccines**

<table>
<thead>
<tr>
<th>Inoculuma</th>
<th>Mean Anti-LHRH Ab Titer (log_{10}) 2 wk Following Second Dose</th>
<th>Pregnancy Statusb (no. of animals in each group that dropped litters)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[T]-Lys(Pam3Cys)-[B] 10 nmol</td>
<td>3.76 ± 0.36</td>
<td>3/5</td>
</tr>
<tr>
<td>[T]-Lys(Pam3Cys)-[B] 1 nmol</td>
<td>3.22 ± 0.51</td>
<td>5/5</td>
</tr>
<tr>
<td>[T]-Lys(Pam2Cys)-[B] 10 nmol</td>
<td>4.22 ± 0.33</td>
<td>1/5</td>
</tr>
<tr>
<td>[T]-Lys(Pam2Cys-Ser-Ser)-[B] 1 nmol</td>
<td>3.61 ± 1.18</td>
<td>0/5</td>
</tr>
<tr>
<td>[T]-Lys(Pam2Cys-Ser-Ser)-[B] 10 nmol</td>
<td>4.64 ± 0.23</td>
<td>0/5</td>
</tr>
<tr>
<td>[T]-Lys(Pam2Cys-Ser-Ser)-[B] 1 nmol</td>
<td>3.92 ± 0.65</td>
<td>1/5</td>
</tr>
<tr>
<td>[T]-[B] in CFA 10 nmol</td>
<td>4.72 ± 0.21</td>
<td>1/5</td>
</tr>
<tr>
<td>[T]-[B] in CFA 1 nmol</td>
<td>3.56 ± 0.22</td>
<td>3/5</td>
</tr>
<tr>
<td>Saline in CFA</td>
<td>&lt;2</td>
<td>5/5</td>
</tr>
</tbody>
</table>

| a Lipopeptides were administered in saline and the nonlipidated peptide [T]-[B] and saline controls were inoculated in CFA for the primary inoculation and IFA for the secondary inoculation. All vaccines were administered by the s.c. route. |
| b Fertility experiments were initiated 2 wk after the second dose of vaccine. |
class II molecules, and then analyzed by flow cytometry demonstrated that [T]-Lys(Pam2Cys-Ser-Ser)-[B] was the most effective and Pam2Cys-[T]-[B] was the least effective in causing maturation of DC. The ability of [T]-Lys(Pam2Cys-Ser-Ser)-[B] to up-regulate class II expression approached that of bacterial LPS, and Pam2Cys-[T]-[B] was the least effective in causing maturation of DC. The nonlipidated peptide was unable to induce maturation of D1 cells at a rate greater than 26%, which is the rate that occurs spontaneously in culture. The ability of the lipopeptides to induce the maturation of D1 cells was concentration dependent (data not shown). The relative abilities of these lipopeptides to induce maturation of D1 cells directly reflected their ability to induce Ab, providing a possible mechanism for differences in immunogenicity.

Discussion

Previous reports have shown that, when coupled covalently to a peptide, Pam3Cys endows self-adjuvanting properties, enabling humoral (17, 38) and cellular responses (21, 38) to be elicited. Pam2Cys, an analog of Pam3Cys that has been isolated from mycoplasma, has also been reported to be capable of activating macrophages and splenocytes (22, 24, 25). The ability to chemically synthesize vaccines provides opportunities to incorporate immunostimulatory lipids at various positions within immunogens, allowing the final structure to be tuned to give the best or most appropriate immune response. In this study, we describe the assembly of a variety of lipopeptide immunogens composed of a CD4+ T cell epitope, the self-peptide LHRH, which includes one or more B cell epitopes, and Pam3Cys or Pam2Cys.

We found that the solubility of the resulting vaccine was greatly improved by placing lipids in the approximate center of the peptide immunogen between the T cell epitope and LHRH instead of at the more usual position at the N terminus. A clear solution in saline at the concentration required for inoculation could easily be obtained with these branched structures. In contrast, the immunogens in which the lipid was coupled at the N terminus were less soluble, giving a cloudy or opalescent solution in saline. Investigation of the Ab responses and subsequent fertility trials indicated that the water-soluble lipopeptides induced higher Ab titers 4 wk after the primary inoculation and were also more efficient in preventing pregnancy than were the less soluble lipopeptides where lipid was attached to the N terminus. A water-soluble self-adjuvanting vaccine has clear advantages over partially soluble or insoluble material, allowing for simplification of the manufacturing process and more accurate metering of dose.

Investigations into the effects of varying the lipopeptide dose indicated that Pam2Cys-containing lipopeptides are better immunogens than are Pam3Cys-containing peptides. This may correlate with the finding that Pam2Cys is a more potent macrophage and splenocyte stimulator than Pam3Cys (22, 24, 25). In the present study, we found that insertion of two serine residues between the lipid moiety and the peptide sequence increased the potency of the resulting Pam2Cys-containing immunogens. Two serine residues are present in the original Braun’s lipoprotein sequence found in bacterial cell walls from which Pam3Cys is derived (28) and were reported in early publications (17, 34) to be important for activity of Pam3Cys-containing peptide constructs. Later studies also demonstrated that insertion of Ser-ε-Ser instead of Ser-Ser markedly decreased immunogenicity (41). However, serine residues are absent in naturally occurring Pam2Cys-containing structures including macrophage-activating lipopeptide 2 (25) and the splenocyte-stimulating lipopeptides initially investigated (22). In this study, where lipid is attached to the N terminus, the two serine residues could be acting either as an inert spacer between the lipid and the peptide sequence or as an extension of the T helper cell epitope and perhaps modulating immunological activity. In those cases where lipid is coupled to the ε-amino group of a lysine residue at the center of the molecule, the two serine residues are probably

![FIGURE 3](http://www.jimmunol.org/)

Isotypes of anti-LHRH Abs present during secondary Ab responses following inoculation with [T]-Lys(Pam2Cys-Ser-Ser)-[B]. Mice were bled 2 wk after receiving the second dose of the lipopeptide vaccine administered in saline either s.c. (□) or i.n. (□) in saline.
Inflammatory cytokines and chemokine production from this binding event is transmitted via Toll-like receptors and acts as a spacer. Experiments where the two serine residues are replaced by an inert spacer such as 6-aminohexanoic acid may resolve the question of whether these serine residues per se are important. To determine a mechanism for the enhanced immunogenicity of particular lipid-containing constructs, we considered the known ability of Pam3Cys and Pam2Cys to activate macrophages. It is now understood that macrophages can be stimulated by microbial products that bind to cell surface receptors; the signal resulting from this binding event is transmitted via Toll-like receptors and results in the production of pro-inflammatory cytokines and chemokines. These receptors are also present in populations of DC (for review, see Ref. 42) and, when engaged, transmit signals for cellular maturation and migration as well as for the production of molecules required for efficient Ag presentation.

The various synthetic lipopeptide vaccines used in this study were found to induce the up-regulation of class II MHC molecules on the surface of DCs. For each group, 8 × 10^6 cells were exposed to 4.5 fmol of lipopeptide and incubated overnight. The cells were collected and the MHC class II molecule expression was determined by flow cytometry after staining with FITC-conjugated anti-I-E^k,d mAb. Thirty thousand D1 cells were analyzed. The result is representative of four independent experiments. The result obtained with the nonlipidated peptide is equivalent to that obtained with medium alone, indicating a spontaneous maturation rate of 26%.

The ability of peptide and lipopeptide in up-regulating the expression of MHC class II molecules on the surface of DCs. For each group, 10^4 cells were exposed to 4.5 fmol of lipopeptide and incubated overnight. The cells were collected and the MHC class II molecule expression was determined by flow cytometry after staining with FITC-conjugated anti-I-E^k,d mAb. Thirty thousand D1 cells were analyzed. The result is representative of four independent experiments. The result obtained with the nonlipidated peptide is equivalent to that obtained with medium alone, indicating a spontaneous maturation rate of 26%.

The lipopeptides can trigger an immune response in the absence of additional adjuvant and can therefore be delivered by nonparenteral routes. Therefore, we investigated the Ab response following i.n. inoculation of Pam2Cys-containing peptides. Previous reports have shown that oral or i.n. inoculation of Pam3Cys-peptides induces strong mucosal and/or systemic responses (33, 39, 40). The results obtained in this study showed that i.n. inoculation of [T]-Lys(Pam2Cys-Ser-Ser)-[B] or Pam2Cys-Ser-Ser-[T]-[B] induced lower titers of systemic anti-LHRH Ab than those induced by inoculation by the s.c. route and also that the isotype profiles of immunoglobulins were different. i.n. inoculation of the soluble lipopeptide [T]-Lys(Pam2Cys-Ser-Ser)-[B] induced higher levels of IgG2b and IgG3 but lower levels of IgG1 and IgG2a compared with s.c. immunization. This may indicate that the two routes of immunization result in the induction of somewhat different subsets of T cells providing help for Ab production, which may, in part, be due to the different populations of DCs encountered at different sites. It may also reflect the preference that DCs have for molecules with unusual geometries (44).

With the possible exception of Pam3Cys-Ser-Ser-[T]-[B], the secondary Ab titers elicited by each of the other immunogens were comparable. Nevertheless, only in those cases where the branched lipopeptides [T]-Lys(Pam3Cys)-[B] and [T]-Lys(Pam2Cys)-[B] were used was reproductive sterility achieved. In the cases of branched lipopeptides, the Ab titers observed in the primary response were higher than those achieved with the linear forms of the lipopeptides. Comparisons of the Ab titers induced during primary and secondary Ab responses of successful (i.e., branched lipopeptide [T]-Lys(Pam3Cys)-[B] and [T]-Lys(Pam2Cys)-[B]) and unsuccessful (i.e., linear lipopeptide Pam2Cys-[T]-[B] or Pam3Cys-Ser-Ser-[T]-[B]) vaccines suggested that there may be a correlation between infertility and primary Ab response. However, we have previously shown (27) that litters are still produced by animals in which primary Ab titers are similar to the secondary Ab titers of infertile animals, indicating that it is not a simple matter of Ab titer determining fertility or otherwise. Furthermore, the avidities of Abs generated in these two situations are comparable (27). These observations suggest that the success or otherwise of antifertility vaccines based on LHRH is related to effects other than quantitative (titer) or qualitative (affinity) properties of the Ab; contributing factors could include the length of time for which the endocrine system is bathed in Ab. For these reasons, it seems to be advantageous to use those antifertility vaccines that not only induce high Ab titers but induce high Ab titers as quickly as possible. Of course, this property applies to most vaccines.

The efficacy of the antifertility vaccines was also examined following i.n. administration. i.n. inoculation of the water-soluble peptide construct [T]-Lys(Pam2Cys-Ser-Ser)-[B] induced significantly higher anti-LHRH Ab titers 4 wk after the first dose of vaccine than did Pam2Cys-Ser-Ser-[T]-[B]. Fertility trials conducted with these mice demonstrated that only i.n. inoculation with [T]-Lys(Pam2Cys-Ser-Ser)-[B] was able to totally inhibit fertility. Although similar Ab titers were apparent in both groups of mice following the second dose of Ag, high titers of Ab were elicited only during the primary response to [T]-Lys(Pam2Cys-Ser-Ser)-[B]. These results indicate that i.n. administration of epitope-based lipopeptides can produce very high titers of Ab that exert a biological effect.

Taken together, the measurements of Ab titers and the results of the fertility trials demonstrate that placement of self-adjuvanting lipid moieties between the B cell epitope and the T helper epitope,
at the approximate center of a totally synthetic peptide vaccine, increases the solubility and the immunogenicity of the vaccine. This improved immunogenicity can be further improved by the introduction of two serine residues between the lipid and the peptide portion of these branched peptide vaccines. The finding that incorporation of lipid, self-adjuvanting moieties into different positions of peptide-based vaccines profoundly alters physical, immunogenic, and biological properties provides another strategy for successful vaccine design.

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References