Pimelautide or Trimexautide as Built-in Adjuvants Associated with an HIV-1-Derived Peptide: Synthesis and *in Vivo* Induction of Antibody and Virus-Specific Cytotoxic T-Lymphocyte-Mediated Response

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Covalent association of lipopeptidic immunostimulants is known to improve the immunogenicity of short peptides. In this paper, we describe the synthesis of four analytically pure immunogens, prepared by two different strategies, in which a hexadecameric peptide (V3) derived from the principal neutralizing domain of HIV-1 envelope glycoprotein was associated with two different murein-derived lauroyl-peptides, Pimelautide (RP 44102), or Trimexautide (RP 56142). The *in vivo* immunogenicity of these compounds was evaluated according to two different criteria: the ability to elicit a cellular-T cytotoxic (CTL response) and the ability to stimulate antibody response. Our studies show that one of our compounds (TrxSucV3) was able to efficiently induce a relevant virus-specific CTL response, while another one (PimSucV3) was able to stimulate a strong antibody response to the linked peptide, or to a co-injected protein. These results suggest that both activities rely on different structure–activity relationships and that such a chemically defined model of peptide vaccines may be used to selectively stimulate subpopulations of immunocompetent cells.

Introduction

Vaccination against infectious diseases is an attractive alternative to chemotherapy, especially in the field of viral infections. In this context, there have been recently renewed attempts to develop a new generation of recombinant subunit or synthetic peptide vaccines: these vaccines can elicit specific, humoral, and cellular immune responses, and, being defined at the molecular level, are characterizable and potentially safe. Unfortunately, subunit or peptide antigens are usually not, or only weakly, immunogenic in the absence of immunological adjuvants.

Chemical modification of peptide antigens by built-in adjuvants[1] appears as a promising way of generating, with a relatively good accessibility, totally characterized vaccines, which might generate antibody and cellular responses: a fully characterized synthetic vaccine, associating the luteinizing hormone-release hormone to the murein-derived adjuvant *N*-acetylmuramyl-*L*-alanyl-D-isoglutamine (MDP) was shown to promote an immunological castration in mice;[2] a low molecular mass synthetic vaccine against foot-and-mouth disease was described, which contained tripalmitoyl-*S*-glycerylcysteinyl serylserin (P3CSS) lipopeptide immunoadjuvant linked to peptide antigen derived from the virus protein VP1.[3] Covalent association of the same lipopeptide immunoadjuvant to a glycopeptide was proposed as a model synthetic vaccine, eliciting immune response to tumor-associated carbohydrates antigens.[4] However, real promise for peptides in terms of vaccines lies in their recently described ability to circumvent the need for active infection to stimulate virus-specific cytotoxic T-cell (CTL) response: modification by P3CSS[5] or by a simple α-aminohexadecanoic acid[6] appears to be an effective method of diverting peptides into the endogenous processing pathway, allowing association with the class I MHC molecules and presentation at the surface of the cell.

Concerted efforts are now in progress to develop new immunoadjuvants containing a lipid moiety together with a stable peptidic part, which could be introduced on peptide antigens, in order to induce *in vivo* antigen-specific antibodies and cellular responses.[7] In this context, the "desmuramyl peptidolipid" adjuvants, the lauroyl-L-alanyl-D-glutamyl-L,L-2,6-diaminopimeloylglycine (pimelautide, RP 40639), and the lauroyl-L-alanyl-D-glutamyl-L,L-2,6-diaminopimelic acid (trimexautide, RP 56142) (see ref 8 for a review) might be good candidates for a similar approach, although to our knowledge their activity in the context of covalent association has not yet been evaluated.

Both lauroyl-peptides (Figure 1) exhibit immunopotentiating activities which have been thoroughly documented: they stimulate phagocytic cells and, directly or indirectly, T lymphocytes, while little if any direct stimulation of B-lymphocytes can be evidenced. *In vivo*, these compounds exert an adjuvant effect on antibody production against various antigens and on delayed-type hypersensitivity, which has been correlated to the activation of the cell-mediated immunity. Unlike the synthetic lipopeptides derived from the N-terminus of the lipoprotein from the outer cell membrane of *Escherichia coli*, these molecules are devoid of any mitogenic effect. The lauroyl-peptides are active at parenteral doses ranging between 0.1 and 3 mg/kg. The acute toxicity of pimelautide in the mouse is very low (LD50 = 410 mg/kg iv). Although the compound is pyrogenic in the rabbit, in a study in dogs, sc injections given three times weekly for 1 month were well-tolerated up to a dose of 1 mg/kg.[8]

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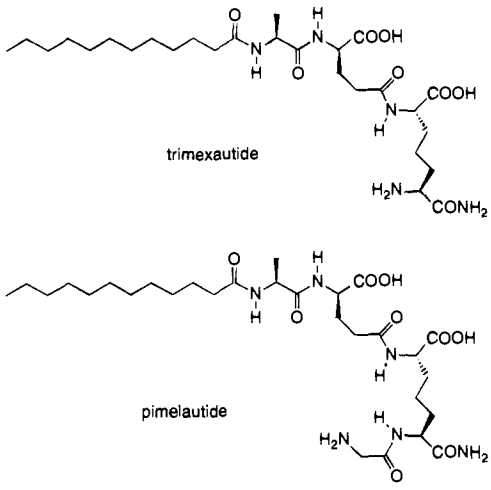


Figure 1. The lauroyl-peptide adjuvants, pimelautide and trimexautide.

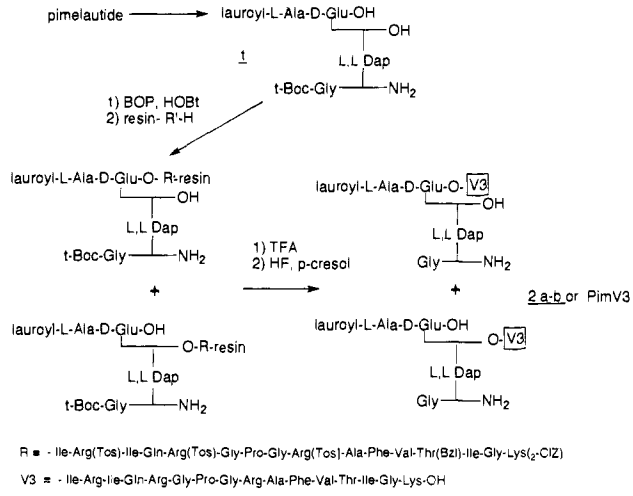
Here, we describe the synthesis and *in vivo* immunogenicity of three different constructs, associating a lauroylpeptide adjuvant and a peptide antigen; this peptide was selected from the third hypervariable domain of the HIV-1 envelope glycoprotein gp 120, the "V3 loop" (residues 303–335), which contains the principal binding site for neutralizing antibodies. We have selected as model antigen a 16-amino acids sequence (residues 312–327), herein referred as the V3 peptide, which, beside this crucially important B-cell epitope, contains at least one cytotoxic T-cell epitope, identified in the H-2^d restricted model within the sequence 319–327, and at least one T-helper epitope.[9]

Chemistry

Trimexautide and pimelautide are obtained by chemical coupling of lauric acid to a biologically inactive tetrapeptide:[10] their chemical stability is compatible with all the solvents and reagents of conventional peptide chemistry. Once introduced as built-in adjuvant in a peptide antigen, the good stability in storage, preexisting in both moieties of the construct, is likely to be preserved. Last but not least, the lipophilicity of their unique lauroyl chain is counterbalanced by the polarizable and ionizable groups of the peptide chain, conferring to this series of compounds an excellent water solubility, which facilitates their purification, characterization, sterilization by simple filtration of the solutions, and handling in biological tests.

Both lauroyl-peptides have three functional groups available for the formation of an amide bound with the peptide antigen: the α-carboxylic functions of the D-glutamic acid and of the diaminopimelamic acid and the α-amino function of the glycine residue (pimelautide), or of the diaminopimelamic acid residue (trimexautide). The lauroyl-peptide could theoretically be introduced in many functional groups on the hexadecapeptide antigen; however, as the side chains had to be preserved in order to retain the full antigenicity of the peptide, only the extremities were available for modification by the immunoadjuvant. As simplicity of manufacture is also an important practical criterion for vaccines, we decided to synthesize N-terminally modified peptides, which could be obtained by solid-phase synthesis using standard protocols and reagents.

Scheme 1. Strategy A: Synthesis of PimV3 (**2a–b**).



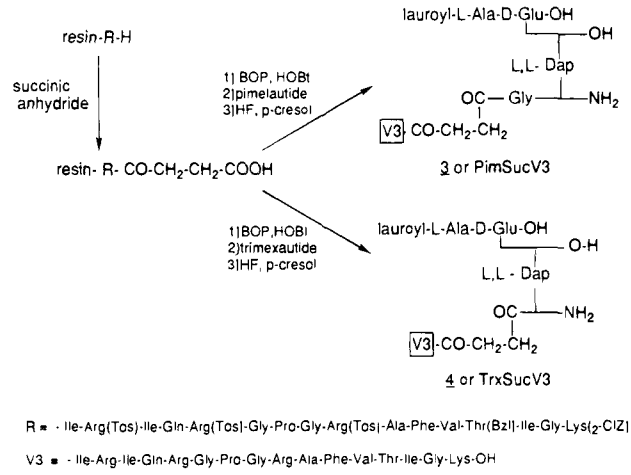
The resin-bound, protected hexadecapeptide antigen was built up by conventional stepwise solid-phase peptide method[11] by using N^{α}-*t*-Boc amino acids with benzyl-type side chain protections for trifunctional amino acids on a phenyl(acetamidomethyl)polystyrene resin. After deprotection by 50% TFA in dichloromethane of the N-terminal function of the hexadecapeptide and neutralization by diisopropylethylamine, the lauroyl-peptide adjuvants were introduced according to two possible strategies.

Strategy A. Activation and coupling of one of the two carboxylic functions of the lauroyl-peptide after protection of its amino function: The N^{α}-*t*-Boc-protected pimelautide was activated with the 1-(benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate in presence of 1-hydroxybenzotriazole and diisopropylethylamine (BOP/HOBt).[2] Using 1 equiv of activating reagents per lauroyl-peptide molecule, we formed the relatively stable hydroxybenzotriazole ester, compatible with long coupling times and a small excess of the electrophilic component. After final deprotection and cleavage, this strategy yielded a mixture of two regioisomers (compounds **2a–b** or Pim-V3), which were not separated before immunization of the animals (Scheme 1). As expected, this compound was soluble in water. After dissolution in water at a concentration of 5 mg/mL, and sterilizing filtration on a cellulose acetate membrane, the concentration found in the soluble fraction was 84% of the initial peptide content.

Strategy B. Succinylation of the N^{α}-deprotected peptidyl-resin, activation of the newly introduced carboxylic function with the BOP/HOBt reagents, and coupling to the free amino group of the fully unprotected lauroyl-peptide (Scheme 2): After final deprotection and cleavage by hydrogen fluoride, this strategy yielded unambiguous compounds **3** (PimSucV3) or **4** (TrxSucV3), depending on the introduced lauroyl-peptide (pimelautide or trimexautide, respectively).

Despite extensive washings of the activated peptidyl resin before introduction of the lauroyl-peptide, 8% of a compound containing two pimelautide molecules per molecule of peptide antigen was identified as a side product during the synthesis of **3**, and 4% of a compound containing two trimexautide during the synthesis of **4**, indicating either the persistence of adsorbed activating reagents on the peptidyl resin or a transfer of the activation to one of the carboxylic functions of the

Scheme 2. Strategy B: Synthesis of PimSucV3 (**3**) and TrxSucV3 (**4**)



lauroyl-peptide by transesterification, responsible for the coupling of a second lauroyl-peptide molecule (see Figure 2). Both side products were eliminated by purification using reversed-phase high-pressure liquid chromatography on a C4 support. Compounds **3** and **4** were also soluble in water; after dissolution in water at a concentration of 5 mg/mL and sterilizing filtration on a cellulose acetate membrane, the concentrations found in the soluble fraction were 98% and 88% of the initial peptide contents, respectively.

The different constructs were checked for homogeneity by HPLC on an ion-exchange support in the presence of an organic modifier or by RP-HPLC on a support with cyanopropyl modifying groups (Figure 3). Purified peptides had the expected amino acid composition, as determined by amino acid analysis after total acid hydrolysis. The expected molecular weight, determined by Plasma Desorption Mass Spectrometry, was observed.

Biological Tests

Antibody Response to the V3 Peptide. An evaluation of the antibody responses to the V3 peptide generated after immunization of BALB/c mice (five mice per condition) with compounds **2a–b**, **3**, **4**, and the unmodified peptide V3, in water solution, was performed. As a reference, we also immunized mice with the peptide V3 emulsified in FCA or IFA for the boosts. Figure **4** represents the results of these experiments. Sera were collected on days 0, 31, 52, and 97 and pooled. After the third injection, the best results were obtained when PimSucV3 (**3**) was used as immunogen; the antibody response obtained was even stronger than when using CFA as adjuvant for the first immunization. The antibody response obtained after immunization with PimV3 or TrxSucV3 was comparable with the antibody response obtained using the V3 peptide in water solution, indicating a loss of the adjuvant properties leading to antibody production.

***In Vivo* Induction of a Virus-Specific Cytotoxic T-Cell Response.**[8] Early detectable, MHC class I-restricted CTL, capable of specifically lysing endogenously expressing antigen target cells (i.e. P815{H-2^d,DBA/2} cells, infected by a vaccinia virus recombinant for the *env* gene: Vac-*env*), could be generated in BALB/c (H-2^d) mice immunized with Vac-*env*. As already described, the V3 peptide,[6] although able to sensitive P815 cells for lysis by Vac-*env*-primed CTL, was unable to induce CTL *in vivo*.

In the following experiments, we evaluated the efficiency of *in vivo* priming according to two criteria: a short delay before detection of cytotoxic activity (before the 18th day of culture of the splenocytes, i.e., after only two *in vitro* stimulations) and the ability of the CTL to lyse specifically virus-infected target cells.

A representative experiment is shown in Figure 5. A strong priming of CTL able to lyse peptide-coated and Vac-*env*-infected target cells could be detected in Balb/c mice immunized sc with 50 nmol of TrxSucV3 (**4**) in PBS, followed by a boost injection 3 weeks later under the same conditions. After two *in vitro* stimulations of the splenocytes obtained from the immunized mice, a strong CTL response was observed that was able to lyse peptide-coated and (Vac-*env*) virus-infected target cells. No cytotoxic activity found when testing the same target cells infected with a control (Vac-*gag*) virus (Figure 5). The CTL lines were class I-restricted because they did not lyse allogeneic EL4{H-2^b,C57BL/6} target cells, in contrast to the histocompatible P815{H-2^d,DBA/2} (not shown). Neither of these target cell lines expressed class II MHC molecules. In addition, the CTL lines obtained in this experiment were sensitive to treatment with anti-CD8 plus complement, whereas treatment with anti-CD4 had no effect (not shown). Thus, effector cells induced by the compound **4** (TrxSucV3) were clearly class I-restricted CD8+ CTL.

In these conditions, we could detect CTL specific for virus-infected cells in 10 out of 12 mice immunized with TrxSucV3 (**4**). Under the same conditions, we could detect CTL in two out of four mice immunized with PimV3 **2a–b** and in two out of four mice immunized with PimSucV3 **3**.

***In Vivo* Adjuvanticity.** The *in vivo* adjuvanticities of our compounds were tested with bovine serum albumin (BSA) as model protein antigen. Groups of mice were immunized twice with 100 μg of bovine serum albumine alone or admixed with 100 μg of the compounds **2a–b**, **3**, or **4** in water solution. The BSA-specific immune response, determined on day 28, could be markedly enhanced by addition of PimSucV3 (**3**) to the antigen (Figure 6). Although still able to enhance the response to BSA, both other compounds (PimV3 (**2a–b**) and TrxSucV3 (**4**)) were clearly less efficient in this test.

Conclusion

This work demonstrates the feasibility of synthesizing analytically pure compounds corresponding to covalent associations between the desmuramyl peptidolipid adjuvants pimelautide or trimexautide and a peptide antigen. We took advantage of the structural features of the pimelautide and trimexautide, a relatively low lipidic charge, the existence of ionized functions at physiological pH, and the absence of the acid-sensitive osidic moiety found in other murein-derived immunoadjuvants (such as MDP), which allowed us to synthesize at low cost readily soluble molecules that were easy to handle during chemical workup and biological trials.

The immunogenicity of these compounds was evaluated according to two different criteria: the ability to elicit *in vivo* a CTL response and the ability to stimulate

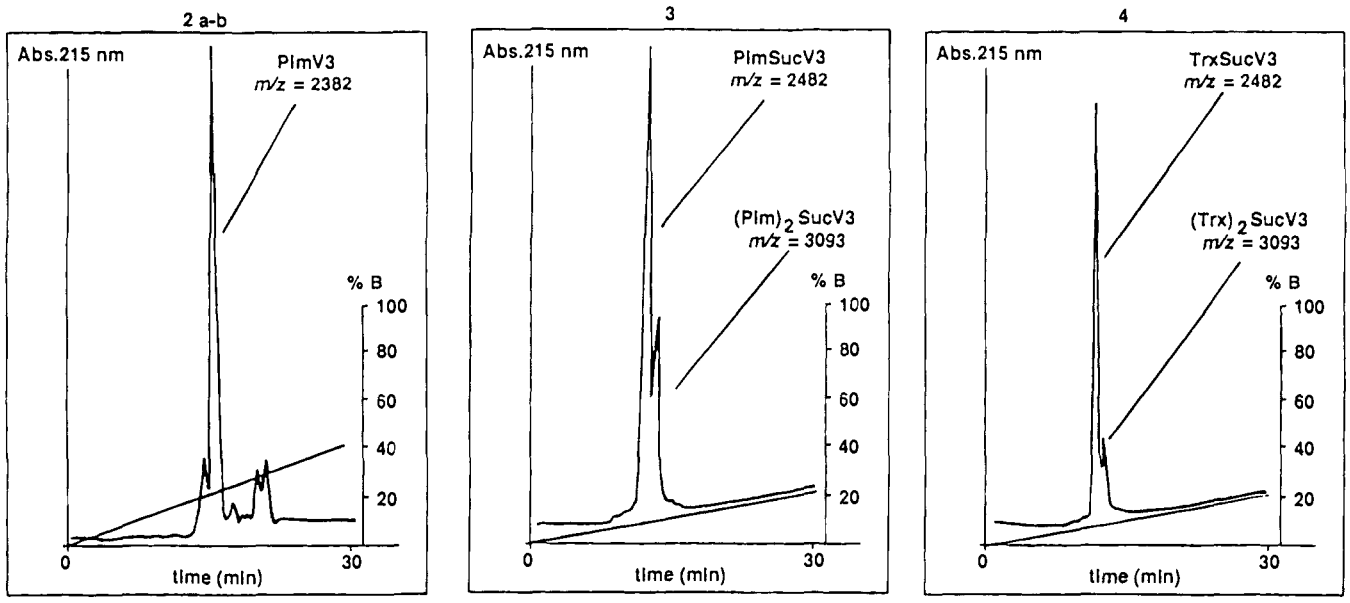


Figure 2. Ion-exchange HPLC of crude compounds **2a–b**, **3**, and **4** after HF cleavage. Solvent composition: (A) 5 mM phosphate buffer pH 3/acetonitrile, 50/50, v/v; (B) 400 mM NaCl, 5 mM phosphate buffer pH 3/acetonitrile, 50/50, v/v, on a Sulfoethyl Aspartimide (Nest-group) 250 × 4.6 mm column. The different peaks were collected and analyzed by plasma desorption mass spectrometry. Available molecular weight assignments (displayed on the chromatograms) show that introduction of lipopeptides containing unprotected carboxylic function on an activated peptidyl-resin leads to less than 10% of side products resulting of the incorporation of two lipopeptides $(Pim)_2SucV3$ and $(Trx)_2SucV3$, respectively.

antibody response. Our studies show that the compound TrxSucV3 (**4**) is able to efficiently induce a physiological, relevant, virus-specific CTL response, which does not correlate with a strong activity when evaluated in terms of antibody response, neither toward the linked peptide antigen nor toward a co-injected protein antigen (bovine serum albumine). Conversely, the compound PimSucV3 (**3**) was able to stimulate a strong antibody response to the linked peptide and to a co-injected protein, while this compound appeared less efficient in inducing a CTL response.

These results suggest that both activities rely on different structure–activity relationships and that such chemically defined models of peptide vaccines may be used to selectively stimulate subpopulations of immunocomponent cells. A detailed study of the T-helper cell response and of the cytokine production profile following immunization with these compounds is now in progress.

Experimental Section

Abbreviations: DCC, *N,N'*-dicyclohexylcarbodiimide; DCM, dichloromethane; DIEA, diisopropylethylamine; DMEM, Dubelcco's modified Eagle medium; DMF, *N,N*-dimethylformamide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; HOBt, 1-hydroxybenzotriazole; NMP, *N*-methylpyrrolidinone; TFA, trifluoroacetic acid; ELISA, enzyme-linked immunosorbent assay; Dap, 2,6-diaminopimelic acid; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; HF, hydrogen fluoride.

N^{α}-*tert*-butyloxycarbonyl (*N*-Boc) protected amino acids were purchased from Propeptide (Vert-Le-Petit, France); *N-t*-Boc-Gly-phenylacetamidomethyl resin (0.824 mmol/g) was from Applied Biosystems (Foster City, CA). DIEA, NMP, and DCC were from Aldrich (Beerse, Belgium); TFA, acetic anhydride, and HOBt were from Jansen (Geel, Belgium). BOP was from Richelieu Biotechnologies (S^{te}-Hyancinthe, Quebec, Canada), HF was from Matheson (Secaucus, NJ), and acetonitrile (HPLC grade) was from Prolabo (Paris, France).

The lauroyl-peptide adjuvants pimelautide (RP 40 639) and trimexautide (RP 56 142) were kindly provided by Dr. Pierre Laduron (Rhône-Poulenc, France). The V3 peptide was obtained as previously described.[6]

The vaccinia virus recombinant for the *env* gene of HIV-1-BRU (Vac-*env*) was kindly provided by Dr. M. P. Kiény (Transgene Strasbourg, France).

Amino Acid Analysis. The synthetic lipopeptides were characterized by amino acid analysis after acid hydrolysis (6 N HCl, 110 °C, 24 h in the presence of phenol under reduced pressure) on an amino acid analyzer Beckman model 7300. The standard 2,6-diaminonopimelic acid used as a reference for amino acid quantification was kindly provided by Dr. D. Blanot (URA CNRS 1131, Orsay, France).

Solubility Determination. The lyophilized peptides were put in distilled water at a concentration of 5 mg/mL, sonicated during 30 s, and heated at 37 °C for 5 min. A titrated solution of norleucine was added as internal standard, and the solutions were filtered on Nalgène cellulose acetate membrane sterilizing units (porosity: 0.2 μM). Aliquots from both filtered and nonfiltered solutions were evaporated to dryness and hydrolyzed under acidic conditions, and the amino acids were quantified as above. The ratios [peptide]/[norleucine] were used to determine the peptide concentration changes after filtration.

Plasma Desorption Mass Spectrometry. Spectra were recorded on a BIO-ION 20, (Uppsala, Sweden). Approximately 10 μg of peptide were deposited onto a nitrocellulose-backed aluminized foil. The spectra were accumulated during 10^6 fission events (about 10 min).

After purification by reversed-phase HPLC on a Vydac C4 7μ 300 Å (9 × 250 mm) column, the peptides were checked for purity by analytical reversed-phase HPLC using a cyanopropyl-modified support Nucleosil 5CN 5μ (4.6 × 250 mm) (Macherey-Nagel, Düren, Germany), using a H_2O/TFA 0.05%/acetonitrile gradient solvent system, and by ion exchange HPLC on a sulfoethyl aspartimide SCX column (4.6 × 100 mm) (Nest group - C.I.L, Ste-Foy-La-Grande, France), using an ionic strength gradient solvent composition from buffer A (acetonitrile 50% in 5 mM pH 3 phosphate buffer) to buffer B (NaCl 0.4 M in A).

Synthesis. 1. Synthesis of the Resine-Bound Hexadecapeptide H-Ile-Arg(Tos)-Ile-Gln-Arg(Tos)-Gly-Pro-Gly-Arg(Tos)-Ala-Phe-Val-Thr(Bzl)-Ile-Gly-Lys(2-ClZ)-resin. The protected peptidyl-resin was built up by using the conventional solid-phase "Boc-benzyl strategy" on a *N-t*-Boc-Gly-phenylacetamidomethyl alcohol resin (Applied Biosystems, Foster City, CA). The washings and coupling steps were performed with an automatic Applied Biosystem 430A peptide synthesizer. Side chain protections were as follows: arginine

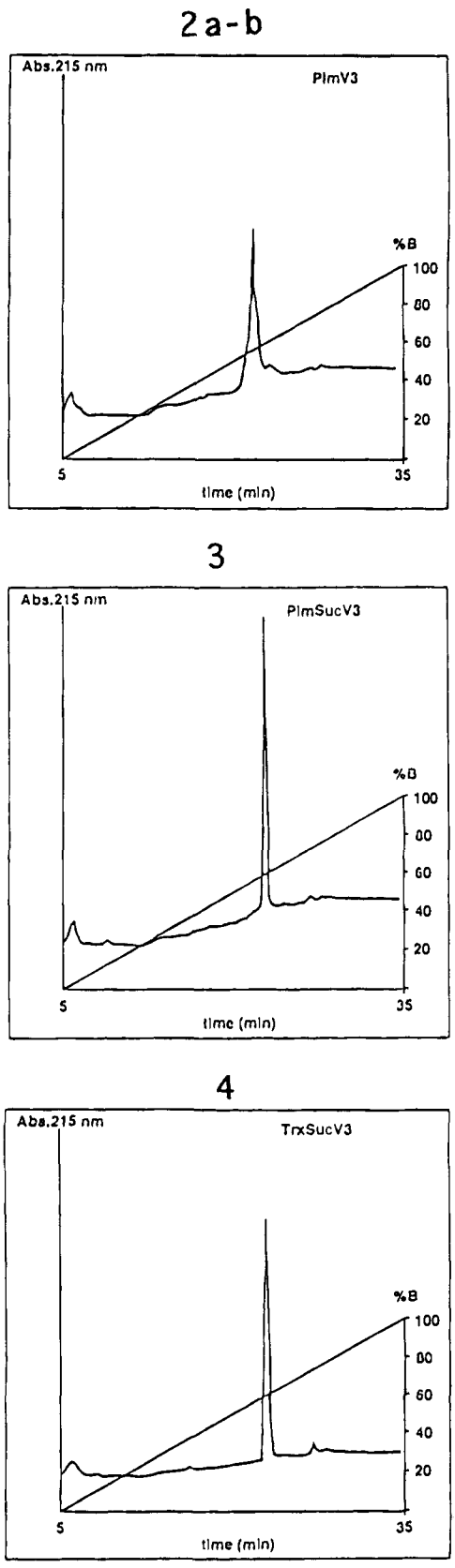


Figure 3. Analytical RP-HPLC of the purified compounds **2a–b**, **3**, and **4**. After purification on a Vydac C4 7 μm 300 Å column (9 × 250 mm) column, the lipopeptides were analyzed on a Nucleosil CN 5 μm, 300 Å column (4.6 × 250 mm). Solvent composition: (A) H_2O/TFA 0.05%; (B) acetonitrile/H_2O/TFA, 80/20/0.05%. Compound **2a–b** (PimV3) contains a mixture of two regioisomers (Scheme 1); as expected, analysis of this compound shows a broad shoulder.

(tosyl), lysine(N^{ϵ}-2-chlorobenzyloxycarbonyl), threonine (benzyl). A 4-fold excess of amino acid was used for couplings. *N,N'*-Dicyclohexylcarbodiimide/1-hydroxybenzotriazole (1:1, 2 mmol each) in NMP was used for couplings.

2. N^2-*N*-[*N*-Lauroyl-L-Ala)-γ-D-Glu-OH)-N^6-{*N*-*t*-Boc-Gly)-L,L-Dap-OH (*N*-*t*-Boc Pimelautide, 1). 230 mg (0.37 mmol) of pimelautide were dissolved in 1 M $HNaCO_3$ aqueous solution (10 mL) together with 10 mL of 0.1 M di-*tert*-butyldicarbonate in tertiobutanol. The pH was adjusted to 9 with 1 M Na_2CO_3 aqueous solution. After 2 h stirring, 230 mL of di-*tert*-butyl dicarbonate were added and the solution was stirred for 72 h. Then the solution was acidified to pH 3 with 1 M HCl and extracted with DCM (2 × 25 mL). The organic layer was dried on Na_2SO_4 and evaporated under reduced pressure yielding a crystalline residue. Yield: 230 mg, 86% Ninhydrin test: negative. Amino acid analysis: Glu, 1.0 (1), Gly 0.99 (1), Ala 0.99 (1), Dap 1.02 (1).

3. N^2-(*N*-{*N*-Lauroyl-L-Ala}-γ-D-Glu-Ile-Arg-Ile-Gln-Arg-Gly-Pro-Gly-Arg-Ala-Phe-Val-Thr-Ile-Gly-Lys-OH)-N^6-Gly)-L,L-Dap-OH + N^2-(*N*-{*N*-Lauroyl-L-Ala)-γ-D-Glu-OH)-N^6-Gly-L,L-Dap-Ile-Arg-Ile-Gln-Arg-Gly-Pro-Gly-Arg-Ala-Phe-Val-Thr-Ile-Gly-Lys-OH (PimV3, 2a–b). *N*-*t*-Boc pimelautide 1 (145 mg, 0.2 mmol) was dissolved in NMP (6 mL) together with 88 mg of BOP, 27 mg of HOBt, and 100 mL of DIEA. The mixture was added to 350 mg (0.1 mmol) of the previously synthesized, N^{α}-deprotected resin-bound peptide. After 7 h of stirring, the resin was washed and dried. Hydrogen fluoride final deprotection and cleavage from the resin was performed in a Teflon-Kel F apparatus (Asti, Courbevoie, France), performing a 90 min treatment with HF/*p*-cresol/*p*-thiocresol 10:0.75:0.25, at 0 °C. The crude peptide was extracted with 5% acetic acid and purified by reversed-phase preparative HPLC, performed on Vydac C4 7μ 300 Å column (9 × 250 mm), with an acetonitrile–water–0.1% trifluoroacetic acid solvent system, using a 90 min gradient from 12% to 60% acetonitrile. Amino acid analysis: Thr 0.96 (1), Glu 1.97 (2), Pro 1.06 (1), Gly 3.54 (4), Ala 1.94 (2), Val 1.02 (1), Ile 2.88 (3), Phe 1.07 (1), Lys 0.96 (1), Arg 3.04 (3), Dap 0.99 (1). MS: *m/z* obs 2382 (expected 2380).

4. Succinylation of the Resin-Bound Hexadecapeptide. The N^{α}-succinyl-Ile-Arg(Tos)-Ile-Gln-Arg(Tos)-Gly-Pro-Gly-Arg(Tos)-Ala-Phe-Val-Thr(Bzl)-Ile-Gly-Lys(2-ClZ)-resin was obtained as follows: 600 mg (0.167 mmol) of the N^{α}-*t*-Boc peptidyl-resin obtained as described above was deprotected using a 50% TFA solution in DCM, washed with DCM, and then reacted with 6 mL of a 0.14 M solution of succinic anhydride in NMP. After double coupling (2 × 20 min), the succinylation was complete and the resin was washed twice with NMP (2 × 20 mL) and twice with DCM (2 × 20 mL). The Ninhydrin test was negative.

5. N^2-(*N*-{*N*-Lauroyl-L-Ala}-g-D-Glu-OH)-N^6-(*N*-{succinyl-Ile-Arg-Ile-Gln-Arg-Gly-Pro-Gly-Arg-Ala-Phe-Val-Thr-Ile-Gly-Lys-OH}Gly)-L,L-Dap-OH (PimSucV3, 3). BOP (221 mg, 0.5 mmol) was dissolved in NMP (5 mL) together with 71 mg (0.5 mmol) of HOBt and 200 mL (1.25 mmol) of DIEA. The mixture was added to the washed succinyl-resin-bound hexadecapeptide, stirred for 10 min, and then washed twice with DMF (2 × 20 mL) and twice with DCM (2 × 20 mL). A solution of 285 mg (0.5 mmol) of pimelautide in NMP (10 mL) was added to the previously activated resin. After overnight stirring, the resin was washed twice with NMP (20 mL), twice with DMF (20 mL), and twice with DCM (20 mL). A deactivation of any remaining activated ester was achieved using 10 mL of H_2O/DMF/DIEA, 4/5/1, v/v. HF deprotection, cleavage, and RP-HPLC purification were performed as described above. Amino acid analysis: Thr 0.95 (1), Glu 1.83 (2), Pro 1.17 (1), Gly 3.99 (4), Ala 1.88 (2), Val 1.09 (1), Ile 2.90 (3), Phe 1.08 (1), Lys 0.93 (1), Arg 3.12 (3), Dap 0.93 (1). MS: *m/z* obs 2482 (expected 2480).

6. N^2-(*N*-{*N*-Lauroyl-L-Ala}-g-D-Glu-OH)-N^6-(succinyl-Ile-Arg-Ile-Gln-Arg-Gly-Pro-Gly-Arg-Ala-Phe-Val-Thr-Ile-Gly-Lys-OH)-L,L-Dap-OH (TrxSucV3, 4). Trimexautide (315 mg) in NMP (10 mL) was added to the succinyl-resin-bound hexadecapeptide activated as above. HF deprotection, cleavage, and RP-HPLC purification were performed as described above. Amino acid analysis: Thr 0.97 (1), Glu 2.11

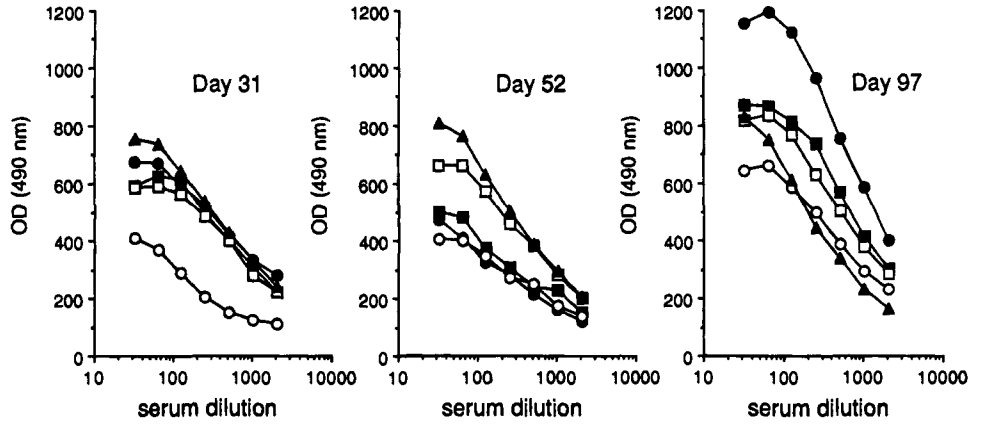


Figure 4. IgG antibody production to the V3 peptide. Groups of five Balb/c mice (age 6–8 weeks) were immunized sc on days 0, 17, 40, and 90 with 50 nmol of PimSucV3 (**3**) (closed circles), TrxSucV3 (**4**) (closed squares), PimV3 (**2a–b**) (open squares), V3 peptide alone (open circles), or in presence of complete freund adjuvant (closed triangles). Sera were collected on days 0, 31, 52, and 97 and pooled. Specific anti-peptide V3 Ig-G contents were measured by ELISA.

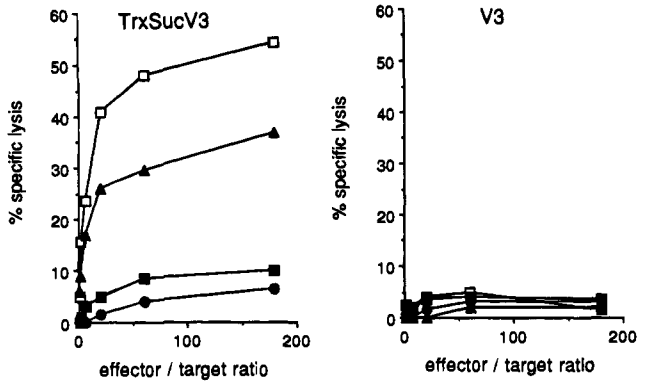


Figure 5. Cytolytic activity of cell lines derived from BALB/c (H-2^d) mice: animals were primed sc with 50 nmol of TrxSucV3 (**4**) (left panel) in saline, or 50 nmol of V3 peptide alone (right panel). Three weeks later, they were boosted in identical conditions. Fifteen days after the last injection, spleens were removed for *in vitro* lymphocyte stimulation. Cell lines were tested for their capacity to lyse P815 target cells pretreated with the V3 peptide (open squares), P815 target cells infected with Vac-*env* (closed triangles) or with a virus expressing an irrelevant protein (Vac-*gag*, closed squares), or in culture medium alone (closed circles).

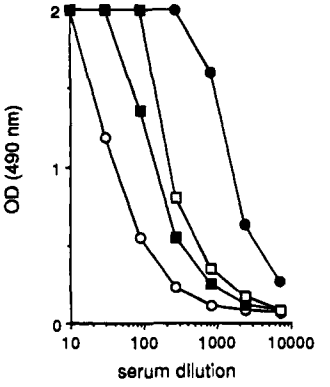


Figure 6. Adjuvanticity of the lipopeptides. Groups of five Balb/c mice (age 6–8 weeks) were immunized sc on days 1 and 14 with 100 μg of bovine serum albumine alone (open circles) or in combination with 100 μg of PimSucV3 (compound **3**, closed circles), TrxSucV3 (compound **4**, closed squares), or PimV3 (compound **2a–b**, open squares). Animals were bled on day 28, on which the sera of each group were pooled. Sera were tested for their BSA specific Ig content by ELISA. The data represents mean values obtained from triplicate experiments.

(2), Pro 0.95 (1), Gly 3.08 (3), Ala 1.99 (2), Val 0.95 (1), Ile 2.85 (3), Phe 1.05 (1), Lys 0.98 (1), Arg 2.95 (3), Dap 1.05 (1). Plasma desorption mass spectrum: *m/z* obs 2423 (expected 2425).

Biological Tests. ELISA. 96-well plates (Nunc) were incubated overnight at room temperature with 200 μL of a 10 μg/mL solution of the V3 peptide in a 0.05 M carbonate/bicarbonate buffer pH 9.6 and blocked afterward with 300 μL of a 2% ULTROSER HY solution (for cell culture, IBF, LKB, Villeneuve-la-Garenne) in 1.8% NaCl/0.01 M PBS, pH 7.2, during 1 h at room temperature. After washing (1.8% NaCl/0.01 M PBS pH 7.2/0.1% Tween-20), dilutions of sera in 2% ULTROSER HY/1.8% NaCl/0.01 M PBS, pH 7.2, were put in duplicate wells and incubated for 2 h at 37 °C; the presence of antibodies was revealed after multiple washings by adding 200 μL of 1/10 000 goat-anti mice IgG g-chain specific or anti IgM μ-chain specific (Sigma, St. Louis, MO). After incubation (2 h at 37 °C) and washings, a substrate solution (citrate buffer/H_2O_2/*o*-phenylenediamine dihydrochloride) was added. After a 30 min incubation protected from light at room temperature, the reaction was stopped using 100 μL of H_2SO_4 (4 N). Absorbance was recorded at 492 nm using a microplate reader (Dynatech). Titers were determined as the inverse of the maximal dilution giving a 1 absorbance unit signal.

Generation of Cytolytic T-Lymphocytes. Splenocytes were cultured in 24-well plates at 5 × 10^6 cells per well in 2 mL of DMEM supplemented with 100 units/mL penicillin, 100 μg/mL, streptomycin, 2 mM L-glutamin, nonessential amino acids, 1 mM sodium pyruvate, 10 mM HEPES, 2.5 mg/mL amphotericin B, 50 μM 2-mercaptoethanol, and 10% heat-inactivated FCS (Flow Laboratories, Irvine, Scotland) (culture medium) containing 3–5 μM V3 peptide. Additional *in vitro* restimulations were performed weekly by mixing 5 × 10^5 effector cells with 5 × 10^6 irradiated (4000 rads) syngeneic spleen cells in 2 mL per well of culture medium containing synthetic peptide and supplemented with 5% of IL-2 containing supernatant prepared as described.[13] The Mab RL172.4 (anti-CD4)[14] and AD4 (anti-CD8)[15] and guinea pig serum as source of complement were used to deplete lymphocyte subpopulations *in vitro*.

Cytotoxicity Assays. P815 (H-2^d, DBA/2) or EL4 (H-2^b, C57BL/6) cells [(1–2) × 10^6] in 200 μL of culture medium were labeled with 100 μCi $Na_2{}^{51}CrO_4$ (CEA, Gif sur Yvette, France) for 1 h at 37 °C. After two washings, 3000 labeled targets and serial dilutions of effector cells were incubated in 200 μL of culture medium in round-bottomed microtiter plates. The V3 peptides were included in the appropriate assays at 3 μM. In the case of virus-infected targets, cells were incubated for 90 min at 37 °C with 10 PFU per cell of recombinant vaccinia virus as described.[16] The cytotoxicity assay was terminated after a 4 h incubation at 37 °C, and 100 μL of supernatant were harvested and specific lysis was determined as % specific lysis = 100 × (experimental − spontaneous release)/(half total ^{51}Cr incorporated − spontaneous release). In all experiments,

spontaneous release in the absence of effector cells was <20% of maximum release.

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