Palmitoyl Derivatives of GpMBP Epitopes: T-Cell Response and Peptidases Susceptibility†

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We report for the first time the immunoadjuvant effects of lipoconjugation of peptide antigens in an in vitro system by using CD4+ T-cells. The lipopeptides obtained by conjugating a palmitoyl moiety at the N^{α}-terminal of Gln^{74} or at the ϵ-NH_2 of Lys^{75} of GpMBP(74−85) induced increased T-cell responsiveness compared to the native nonlipidated peptide. On the other hand, lipoderivatives of GpMBP(82−98) did not increase the T-cell response, demonstrating that the superagonist inducing effect of lipoconjugation is epitope-specific. Digestion of the two native peptides with cathepsin D and L, both implicated in antigen processing, and with a complete lysosomal fraction of a EBV-transformed B cell line shows that GpMBP(74−85) is resistant to cellular proteases, while GpMBP(82−98) is easily digested by these enzymes. These results suggest that the first prerequisite for increasing the T-cell response by lipoconjugation is the stability of the native peptide to peptidases, providing an important insight into the understanding of the immunoadjuvant effect of lipoderivative antigens.

Introduction

Multiple sclerosis (MS), an inflammatory disease of the central nervous system (CNS) white matter, is considered to be immune mediated. Several observations have strengthened the hypothesis that an autoimmune T-cell response to CNS myelin protein play a role in the pathogenesis of MS lesions.[1] Among the candidate autoantigens, myelin basic protein (MBP) is the most deeply studied. Immunization of susceptible animals with MBP or with MBP peptides induces experimental autoimmune encephalomyelitis (EAE), an experimental inflammatory demyelinating disease of the CNS, mediated by encephalitogenic CD4+ T-cells.[2] EAE is considered the best experimental animal model of MS.[1] In Lewis rats immunized with GpMBP, encephalitogenic T-cells recognizing the 74−85 amino acid sequence GpMBP(74−85) dominate the immune response.[3,4] Another subdominant encephalitogenic epitope of GpMBP in Lewis rat is represented by the sequence 86−98.

There are a lot of examples in the literature demonstrating that the conjugation of a lipidic moiety to an immunodominant peptide may affect the T-cell response.[5−9] Chemically defined modification of a peptide by the built-in immunoadjuvant lipopeptide Pam_3-Cys-Ser was described as a reproducible way of stimulating cytotoxic T lymphocytes (CTL) response in vivo. Therefore these new synthetic mitogens are highly suitable compounds for the study of the early events of the immune response for structure−activity studies.[10] In the last years, synthetic peptides modified also with simple lipophilic moieties, such as a palmitoyl group (Pam), have been shown to be efficient tools in inducing specialized CTL. Their different bioactivity, compared to lipid free analogues, has been attributed to their permanence in the cell membrane and subsequent facilitated interaction with membrane receptors.[11,12] Examination of the different posttranslational modifications, which allow membrane association of proteins, indicates that even a single lipidic chain is able to anchor large proteins. For example, the N-terminal addition of an alkyl chain as small as a myristoyl group on a glycyl residue is sufficient to anchor cytosolic proteins to membranes. It has been shown that a single modification of a relatively long peptide by a lipidic amino acid resulted in the ability to reproducibly induce, without immunoadjuvant, CD4, CD8, and antibody response.[9] Lipopeptides could therefore represent useful tools for the study of immunological responses. On the other hand, very little is known on the molecular mechanism that underlies the immunological effect of lipoconjugation of peptide antigens.

It has been recently described that lipoconjugation of a HLA-A2.1-restricted HBV 1 polymerase peptide epitope dominant for human CD8+ T-cell responses increased the lifespan of functional presentation of the peptide to CD8+ cytotoxic T-cells. This effect has been linked to a

† Dedicated to Prof. Luis Moroder on the occasion of his 60th birthday.

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Table 1. Chemical Data for the Synthesized GpMBP Peptides and Lipopeptides

no.	peptides	gradients at 3 mL min^{-1} for semipreparative HPLC	yield[a] mg (%)	ESI-MS $[M + H]^+$: found (calcd)	HPLC (t_R, min)[b]
1	GpMBP(74−85)	5−45% B/50 min	84 (23)	1415 (1414.7)	13.20[c]
2	Pam-GpMBP(74−85)	20−70% B/65 min	55 (14)	1653 (1652.9)	5.65[d]
3	[Lys^{75}(Pam)]GpMBP(74−85)	25−70% B/65 min	100 (44)	1653 (1652.9)	9.90[e]
4	Met-Ser-GpMBP(74−85)	5−45% B/40 min	17 (7)	1632 (1632.8)	8.00[f]
5	Pam_3Cys-Ser-GpMBP(74−85)		110 (35)	2393 (2393.5)	25.30[g]
6	GpMBP(82−98)	10−45% B/25 min	8 (7)	1995 (1995.1)	11.42[h]
7	Pam-GpMBP(82−98)	50−80% B/30 min	13 (10)	2233 (2233.3)	6.64[i]
8	Lys(Pam)-GpMBP(82−98)	40−85% B/50 min	14 (10)	2361 (2361.4)	6.51[l]

[a] Calculated as TFA salts. [b] Analytical HPLC gradients at 1 mL min^{-1}. [c] 5−25% B in 20 min. [d] 35−55% B in 20 min. [e] 20−50% B in 20 min. [f] 5−45% B in 20 min. [g] 30−95% B in 22 min then 95% B isocratic. [h] 25−50% B in 15 min. [i] 65−85% B in 10 min. [l] 70−100% B in 10 min.

favored internalization and cytoplasm delivery in APC, resulting in a prolonged expression of conformationally correct MHC−peptide complexes.[13,14]

In the context of developing more stable molecules, cyclic analogues of GpMBP(74−85), which maintain the biological function of the original peptide, were previously designed, synthesized, and evaluated for activity in the EAE system.[15] The authors demonstrated that the cyclic conformation of the immunodominant peptide elicits a response in pharmacological quantities thanks to the increased resistance to degradation in comparison with the native sequence.[16]

In this study, we designed and synthesized a series of lipopeptides of the two different immunodominant peptide epitopes for CD4+ T-cells in Lewis rats, i.e., GpMBP (74−85)[4] and GpMBP (82−98), to investigate the T-cell response in an in vitro system. Our results demonstrate, for the first time, that lipoconjugation can also increase the responsiveness of CD4+ T-cells. In addition, we show that the biological effect on T-cell response can be different among different peptide antigens. The two different effects are correlated to the specific susceptibility of the peptide antigens to peptidases.

Results and Discussion

Chemistry. As synthetic lipopeptides have been demonstrated to have immunoadjuvant properties both in vitro and in vivo,[9,17] we synthesized two different wild-type immunodominant MBP epitopes in Lewis rats, GpMBP(74−85) [QKSQRSQDENPV, (**1**)] and GpMBP-(82−98) [ENPVVHFFKNIVTPRTP, (**6**)], and several derived lipid-bound peptides to investigate the T-cell response (Table 1).

Lipopeptides were synthesized by introducing a palmitoyl moiety linked through an amide bond in the N-terminal position or on a Lys residue in different positions.

The palmitoyl moiety was introduced in the two immunodominant MBP epitopes in Lewis rat, GpMBP-(74−85) (**1**) and GpMBP(82−98) (**6**). The syntheses were performed by SPPS following the Fmoc/*t*Bu strategy, in the standard synthetic protocol described in the general procedure. GpMBP(74−85) and its lipoderivatives were synthesized as peptide amides on a TentaGel S RAM resin. The lipopeptide **2** was synthesized by introducing the palmitoyl residue in the N-terminal position of **1**, anchored to the resin, using the pentafluorophenyl ester of palmitic acid (Pam-OPfp). Lipopeptide **3**, bearing a N^{ϵ}-palmitoyllysine residue, was prepared using Fmoc-Lys(Pam)-OH during the SPPS. Pam-OPfp was also used for the synthesis of the building-block Fmoc-Lys-(Pam)-OH.

The lipopeptide Pam_3Cys-Ser-GpMBP(74−85) (**5**), containing the built-in immunoadjuvant lipopeptide Pam_3Cys-Ser, was synthesized by coupling Pam-Cys-(CH_2-CHOH-CH_2OH)-OH to the resin-linked peptide Ser-GpMBP(74−85). The two additional palmitoyl residues were introduced, still in solid phase, on the hydroxyl functions of the *S*-(2,3-dihydroxypropyl) present on the side chain of Cys, by recirculation of palmitoyl chloride, as previously described.[18,20] As control peptide, we synthesized Met-Ser-GpMBP(74−85) (**4**), elongating the wild-type sequence **1** with Met and Ser at the N-terminus.

In the case of GpMBP(82−98) (**6**), the corresponding lipopeptides were synthesized as acids on a Fmoc-Pro-NovaSyn-TGA resin. Lipopeptides **7** and **8** were synthesized introducing, during solid-phase peptide synthesis (SPPS) of **6**, a palmitoyl moiety (by Pam-OPfp) and a N^{ϵ}-palmitoyllysine residue [by Fmoc-Lys(Pam)-OH], respectively, in the N-terminal position. All the compounds were purified by RP-HPLC and characterized by ESI-MS and analytical HPLC (Table 1).

Immunology

In Vitro Effects of Lipoconjugation of GpMBP-(74−85) by a Palmitoyl Moiety on T-Cell Response. Spleen cells were isolated from Lewis rats immunized with GpMBP(74−85) at day 12 after immunization and tested in a proliferation assay to evaluate the response to the wild-type and lipid-bound peptides. In vitro proliferation of spleen cells from Lewis rats immunized with **1** was more vigorous and started at lower antigen concentrations with lipopeptides **2** and **3**, corresponding to the immunodominant epitope GpMBP(74−85) (Figure 1). Similar results were obtained by using spleen cells from animals immunized with the lipopeptides, without significant differences in the ranking of agonist activity of the peptides (**2** > **3** > **1**) (data not shown).

Successively, we tested the in vitro activity of lipopeptides on the proliferative response of long-term CD4+ T-cell lines (TCL) generated from spleen cells of Lewis rats immunized with entire MBP and maintained in culture by periodic restimulation with the whole protein. The large majority of TCL, obtained from Lewis rats using entire MBP, proliferated to the immunodominant peptide **1**. When the proliferative response to lipoderivatives was compared, it turned out that the lipid bound analogues exerted an increased stimulatory

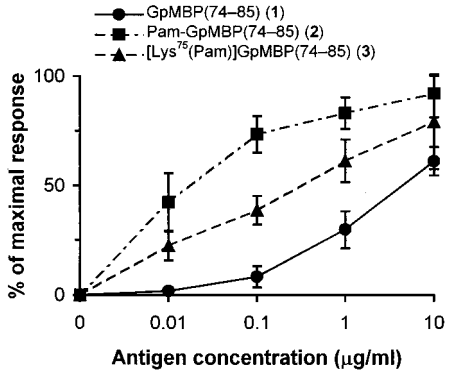

Figure 1. Proliferative response of spleen cells from Lewis rats immunized with GpMBP(74−85) to different concentrations of the wild-type peptide **1** and its lipoderivatives **2** and **3**. Data are expressed as a percentage of maximal response. Each point represents the mean value ± standard error of five different experiments. Maximal response ranged from 4841 to 28095 cpm.

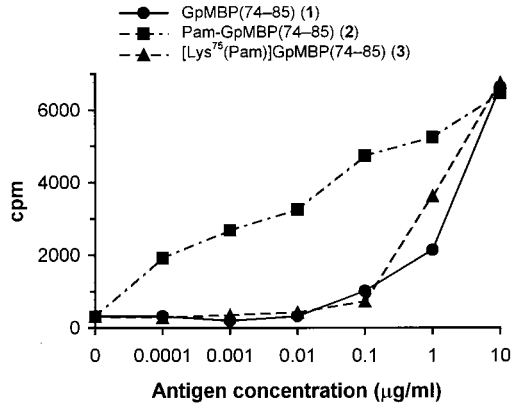

Figure 2. Proliferative response of a TCL generated from spleen cells of Lewis rats immunized with MBP to different concentrations of the wild-type peptide **1** and its lipoderivatives **2** and **3**. Data show a representative experiment out of three carried out with different TCLs, and they are expressed as cpm. Background proliferation was 315 cpm.

activity compared to the lipid free peptide. Proliferation started at lower antigen concentrations, and the response was overall more intense (Figure 2).

To rule out the possibility that this superagonist activity of the lipopeptides was due to an unspecific mitogenic activity, the proliferative response of a TCL specific for a different antigen, i.e., protein purified derivative (PPD) of *Mycobacterium tubercolosis*, was tested to these antigens. No response was observed to the lipid-conjugated peptides, suggesting that the stimulatory activity of lipid-bound MBP peptides was specific for MBP-reactive T-cells (Figure 3).

Finally, we could demonstrate that the increased responsiveness of T-cells to the lipopeptide required a stable coupling of the two components. The proliferative response of TCL to **1** was not increased when an equimolar amount of N^{ϵ}-palmitoyllysine was added during the proliferation assay (data not shown).

In Vitro Effects of Lipoconjugation of GpMBP-(74−85) by Pam_3Cys-Ser on T-Cell Response. It was previously reported[10] that Pam_3Cys-Ser covalently linked to synthetic peptides in the N-terminal position exerted potent immunoadjuvant activity. We decided to test Pam_3Cys-Ser-GpMBP(74−85) (**5**) and the corresponding

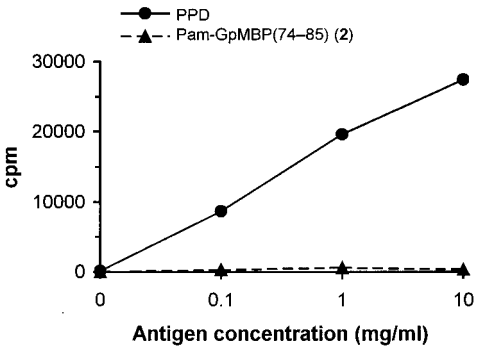

Figure 3. Proliferative response of a TCL specific for PPD and tested to different concentrations of PPD itself and to the lipopeptide Pam-GpMBP(74−85). Data show a representative experiment out of three, carried out with different TCLs, and they are expressed as cpm. Background proliferation was 782 cpm.

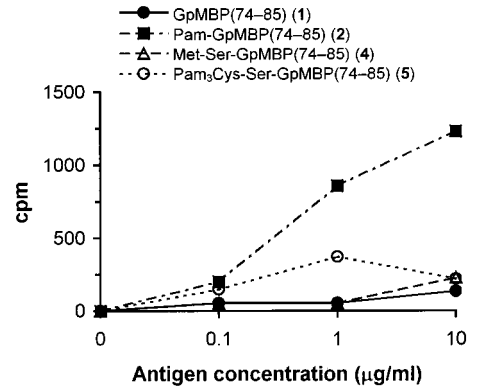

Figure 4. Proliferative response of a TCL specific for GpMBP-(74−85) and tested to different concentrations of GpMBP(74−85), Met-Ser-GpMBP(74−85), and their lipoderivatives. Data show a representative experiment out of three, carried out with different TCLs, and they are expressed as cpm. Background proliferation was 53 cpm.

control peptide Met-Ser-GpMBP(74−85) (**4**). We chose to introduce a Met residue in order to mimic the thioether function present in the Pam_3Cys moiety.

From the analysis of the proliferative response of a TCL to increasing concentrations of the tested peptides, we could observe that also the lipopeptide **5** induced a higher response compared to the lipid-free analogue **4** (Figure 4).

This effect was detected only at low peptide concentration. The low solubility of lipopeptide **5** may account for the lack of immunoadjuvant activity at higher antigen concentration.

However, considering that **2**, the simplest N-terminal palmitoyl derivative of the immunodominant epitope GpMBP(74−85), induced the strongest proliferative response, it can be suggested that the more difficult synthesis of **5** does not offer significant advantages in this experimental context.

In Vitro Effects of Lipoconjugation of GpMBP-(82−98) by a Palmitoyl Moiety on T-Cell Response. To assess whether the superagonist activity of the lipoderivatives was epitope-specific, we investigated the in vitro effects of lipoconjugation on a different immunodominant epitope. Spleen cells were isolated from animals immunized with the dominant peptide GpMBP-(82−98) at day 12 after immunization and tested in a

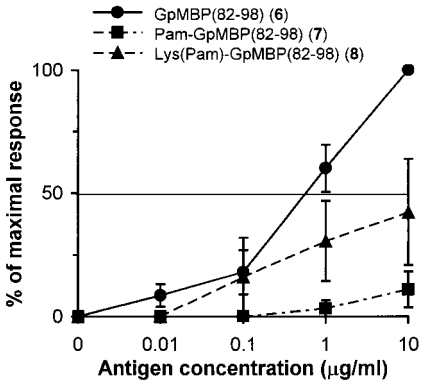

Figure 5. Proliferative response of spleen cells from Lewis rats immunized with GpMBP(82−98) to different concentrations of the wild-type peptide **4** and its lipoderivatives **5** and **6**. Data are expressed as a percentage of maximal response. Each point represents the mean value ± standard error of three different experiments. Maximal response ranged from 4510 to 63022 cpm.

proliferation assay to evaluate the response to the wild-type and lipid-bound peptides. Differently from the immunodominant peptide **1**, in vitro proliferation of spleen cells from Lewis rats immunized with **6** was more vigorous and started at lower antigen concentrations using the wild-type peptide in comparison to the lipopeptides **7** and **8**. No lipoderivative of the wild-type peptide **6** increased the T-cell response (Figure 5).

Cleavage of GpMBP Peptides by Cathepsin D, Cathepsin L, and Lysosomal Fractions. Very recently, the possible factors that may be critical for CTL induction by using a dipalmitoylated lipopeptide consisting of an ovalbumin helper T-cell epitope covalently linked to an influenza virus CTL epitope were described. Antigen processing of lipopeptide appears to be required for T-cell induction since there was virtually no in vitro binding of lipopeptide to purified major histocompatibility complex (MHC) molecules. A major portion of lipopeptide immunogenicity was due to its particular nature inasmuch as CTL induction in mice correlated with insoluble lipopeptide constructs, whereas more soluble analogues were significantly less immunogenic. Immunohistological analysis of tissue from immunized animals revealed that lipopeptide migration from the subcutaneous injection site to the spleen could be detected as early as 1 h after immunization, and cell-associated lipopeptide was observed on macrophages and dendritic cells, implicating both cell populations in the processing and presentation of lipopeptide particles to CTLs.[14]

On the basis of these findings, in order to try to explain the effect of lipoconjugation of the two immunodominant MBP peptides, we decided to test their possible processing pathways, such as their stability to cellular proteases. In vitro digestion of the two MBP epitopes for Lewis rats allowed us to determine their stability. Cathepsin D, an aspartate protease, and cathepsin L, a cysteine protease, both known to be involved in antigen processing,[21] as well as a lysosomal fraction of B-lymphoblastoid cell lines (B-LCL),[22] were used. Digestion mixtures were analyzed by LC-MS. Several cleavage sites for the different cathepsins and an asparaginyl endopeptidase,[23] all destroying the MHC binding region, in addition to exopeptidases (amino and

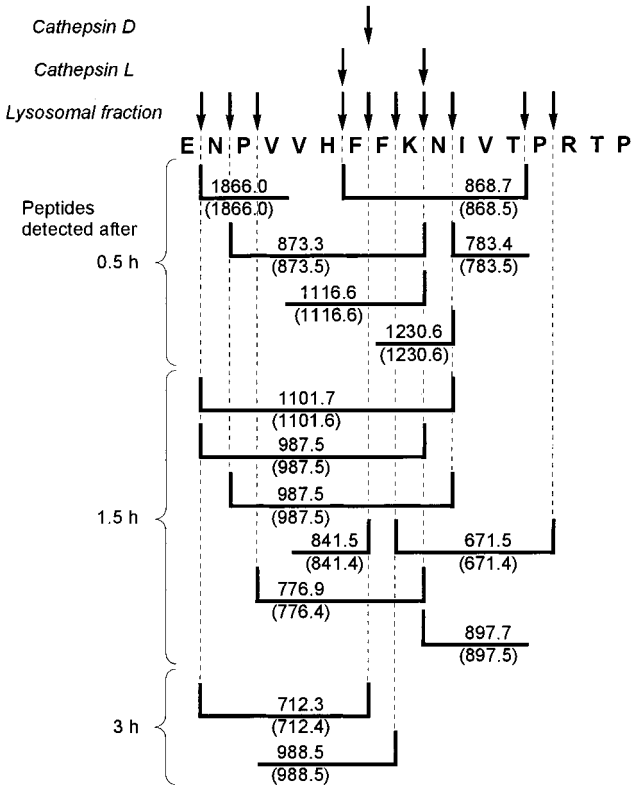

Figure 6. Cathepsin D and L and lysosomal fraction cleavage sites (↓) of GpMBP(82−98) and fragments generated by digestion with lysosomal fraction, as detected by LC-MS analysis ($[M + H]^+$ found; calculated values in parentheses).

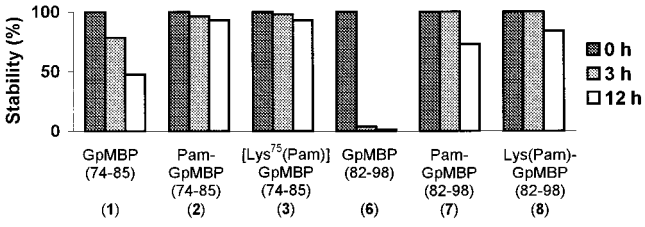

Figure 7. Degradation of the peptides **1**−**3** and **6**−**8** after different incubation times with lysosomal fraction.

carboxypeptidases) could be identified in **6** (Figure 6), while the epitope **1** remained remarkably stable to lysosomal proteases (Figure 7).

Degradation studies on the peptides **2**, **3**, **7**, and **8** showed that the lipopeptides were substantially stable to the lysosomal peptidases (Figure 7).

Experimental Section

Dichloromethane (DCM) was freshly distilled from P_2O_5. *N*,*N*-Dimethylformamide (DMF), previously stored over 4 Å molecular sieves, was used for all couplings, 9-fluorenylmethoxycarbonyl (Fmoc) cleavage, and washing steps. Piperidine was distilled from KOH. Flash column chromatography (FCC) was carried out on SiO_2 (ICN; 32−63 μm, 60 Å). Thin-layer chromatography (TLC) was carried out on SiO_2 (Merck; 60 Å F_{254}), and spots were located with UV light (254 and 366 nm), Fluram (Fluka; fluorescamine), ninhydrin, or a solution of *p*-anisaldehyde (ethanol/sulfuric acid/acetic acid/*p*-anisaldehyde, 90:3:1:2). HPLC-grade CH_3CN was purchased from Carlo Erba (Italy). All other chemicals were commercially pure compounds and were used as received. Melting points were determined with a Büchi 510 apparatus and are uncorrected.

^1H and ^{13}C NMR spectra were recorded on a Varian Gemini 200 spectrometer. Chemical shifts are reported in ppm relative

to $CHCl_3$ fixed at 7.26 ppm for the ^1H spectra and at 77.0 ppm for the ^{13}C spectra; coupling constants (J) are reported in hertz. Assignments were aided by heteronuclear correlated spectroscopy (HETCOR) experiments. IR spectra were recorded on a Perkin-Elmer model 881 spectrometer for KBr pellets. Elemental analyses were performed on a Perkin-Elmer 240 C elemental analyzer.

Resins were purchased from Rapp Polymere (Tübingen, Germany) or Novabiochem (Bubendorf, Switzerland). Fmoc-protected amino acids were purchased from Novabiochem and *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) from PerSeptive Biosystems (Framingham, MA). Semipreparative purifications of peptides were performed on a Vydac ODS 218TP1010 (250 × 10 mm) column using a Beckman System Gold gradient apparatus equipped with a diode array detector. Analytical reversed phase high-pressure liquid chromatography (RP-HPLC) was used to determine the purity of the fractions, using a Vydac ODS 218TP54 (250 × 4.6 mm) column. The solvent systems used were as follows: A [0.1% trifluoroacetic acid (TFA) in H_2O] and B (0.1% TFA in CH_3CN). The flow rates were 1 mL/min for analytical HPLC and 3 mL/min for semipreparative HPLC, with the linear gradients indicated in Table 1. Electrospray ionization-mass spectroscopy ESI-MS was carried out on a Micromass Model VG Quattro apparatus. Proteolytic products were characterized by RP-HPLC/ESI-MS using a Vydac ODS 218TP5215 (150 × 2.1 mm) column connected to a TSQ 700 mass spectrometer (Finnigan MAT, San Jose, CA).

General Procedure for Continuous-Flow Solid-Phase Peptide Synthesis. All the peptides (Table 1) were synthesized by the continuous-flow solid-phase method on a semiautomatic apparatus (NovaSyn Gem Synthesizer) following the Fmoc/*t*Bu strategy. The resin TentaGel S RAM was used for the syntheses of peptides **1**−**5** as amide derivatives, while Fmoc-Pro-NovaSyn-TGA was used for the syntheses of peptides **6**−**8** as acids. Fmoc-protected amino acids were used in a 2.5-fold excess and activated with HATU/NMM in DMF. Acylation end points were determined by checking that the absorbance at 597 nm, due to the release of an anionic dye (acid Violet 17) from the protonated resin-bound amino groups, did not change by more than 0.002 absorbance units over 10 min after a recirculation time of 30 min. Cleavage of the Fmoc group was accomplished with 20% piperidine in DMF. Deprotection reactions were followed by monitoring at 365 nm the resulting dibenzofulvene-piperidine adduct. On completion of the synthesis, the resin was washed with DCM and ether and dried in vacuo. Peptide cleavage from the resins and deprotection of the amino acid side chains was carried out with TFA/thioanisole (95:5) for 30 min at 0 °C and 2 h at room temperature. The resin was filtered off and washed with TFA, and the filtrate was partially evaporated. The crude products were precipitated with diethyl ether, collected by centrifugation, suspended or dissolved in H_2O, and lyophilized. The peptides were purified by semipreparative HPLC. Fractions containing homogeneous material as monitored by HPLC were combined and lyophilized. Characterization of the products was performed using analytical HPLC and ESI-MS spectrometry. The analytical data are reported in Table 1.

Hexadecanoic Acid Pentafluorophenyl Ester [Pam-OPfp]. Pam-OPfp was synthesized, following the procedure of Kisfaludy et al.[24] for the synthesis of the pentafluorophenyl ester of amino acids, by adding, at 0 °C, dicyclohexylcarbodiimide (DCC, 3.1 g, 15 mmol) in dioxane (20 mL) to a stirred solution of pentafluorophenol (2.76 g, 15 mmol) and hexadecanoic acid (3.85 g, 15 mmol) in DMF (8 mL) and dioxane (20 mL). The mixture was stirred overnight at room temperature, then it was filtered, and the solution was evaporated to dryness. The oily residue, triturated with hexane, gave a white, crystalline product (5.85 g, 92%): mp 43−44 °C; IR (KBr) 1775 (CO), 1516 (aromatic C−C) cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (t, 3 H, CH_3-$(CH_2)_{14}$), 1.26 (br m, 24 H, CH_3-$(CH_2)_{12}$), 1.77 (m, 2 H, β-H_2), 2.65 (t, 2 H, α-H_2). Anal. ($C_{22}H_{31}O_2F_5$) C, H.

***N*$^{\alpha}$-Fmoc-*N*$^{\epsilon}$-hexadecanoyllysine [Fmoc-Lys(Pam)-OH].** The ϵ-amino group of Fmoc-Lys(Boc)-OH (5 g, 10.7 mmol) was deprotected with 25% TFA in DCM (250 mL) for 30 min at 0 °C and 2 h at room temperature. The solution was evaporated and the residue lyophilized. To a solution of the deprotected amino acid in DMF (265 mL), brought to pH 8 with *N*-methylmorpholine (NMM, 4 mL), were added at 0 °C Pam-OPfp (4.5 g, 10.7 mmol), 1-hydroxy-7-azabenzotriazole (1.45 g, 10.7 mmol), and NMM (4 mL, 36 mmol). The reaction mixture was stirred at 0 °C for 30 min and then at room temperature for 24 h. The solvent was evaporated and the oily residue dissolved in $CHCl_3$/MeOH and washed with 0.1 M HCl. The organic layer was dried over Na_2SO_4, filtered, and evaporated and the residue triturated with diethyl ether to give the crystalline Fmoc-Lys(Pam)-OH (3.016 g, 47%): mp 138−139 °C; TLC R_F 0.69 (CH_2Cl_2/MeOH 5:1); IR (KBr) 1738 (acid CO), 1692 (urethane CO), 1638 (amide I) cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (t, 3 H, CH_3-$(CH_2)_{14}$), 1.0−1.8 (br m, 30 H, CH_3-$(CH_2)_{12}$ + β- to δ-H_2), 2.03 (pseudo t, 2 H, CH_3-$(CH_2)_{13}$−CH_2), 3.05 (m, 2 H, ϵ-H_2), 3.9 (m, 1 H, α-H), 4.26 (m, 3 H, CH_2−O + fluorenyl 9-H), 5.78 (br m, 2 H, 2 × NH), 7.36 (m, 4 H, fluorenyl 2-H + 3-H + 6-H + 7-H), 7.60 (d, 2 H, fluorenyl 1-H + 8-H), 7.75 (d, 2 H, fluorenyl 4-H + 5-H). Anal. C, H, N.

***S*-(2,3-Dihydroxypropyl)-*N*-hexadecanoylcysteine [Pam-Cys(CH_2-CHOH-CH_2OH)-OH].** The synthesis was performed following the previously described pathway.[20] Yield 80%; mp 70−72 °C; TLC R_F 0.48 ($CHCl_3$/MeOH/H_2O 16:6:1); IR (KBr) 1650 (acid CO), 1640 (amide I), 1520 (amide II) cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.88 (t, 3 H, CH_3-$(CH_2)_{14}$), 1.25 (s, 24 H, CH_3-$(CH_2)_{12}$), 1.64 (m, 2 H, CH_3-$(CH_2)_{12}$-CH_2), 2.11 (pseudo t, 2 H, CH_3-$(CH_2)_{13}$-CH_2), 2.3−3.0 (m, 4 H, Cys β-H_2 + S-CH_2CHOH), 3.33 (d, 2 H, CH_2OH), 3.54 (m, 1 H, C*H*OH), 4.20 (m, 1 H, Cys α-H), 7.7 (d, 1 H, NH).

H-Gln-Lys-Ser-Gln-Arg-Ser-Gln-Asp-Glu-Asn-Pro-Val-NH_2 [GpMBP(74−85)] (1) and Pam-Gln-Lys-Ser-Gln-Arg-Ser-Gln-Asp-Glu-Asn-Pro-Val-NH_2 [Pam-GpMBP(74−85)] (2). These peptides were synthesized starting from 1 g of resin (0.21 mmol) and following the General Procedure. For peptide **2**, the palmitoyl moiety was introduced at the end of the synthesis by means of Pam-OPfp, dissolved in DCM in a 2.5-fold excess (0.222 g, 0.525 mmol) in the presence of 1-hydroxybenzotriazole (0.072 g, 0.525 mmol) and NMM (0.087 mL, 0.788 mmol).

H-Gln-Lys(Pam)-Ser-Gln-Arg-Ser-Gln-Asp-Glu-Asn-Pro-Val-NH_2 {[Lys^{75}(Pam)]GpMBP(74−85)} (3). The peptide was synthesized starting from 0.5 g of resin (0.12 mmol) following the general procedure. The introduction of the lipophilic moiety was performed by using a 2.5-fold excess of Fmoc-Lys(Pam)-OH in DMF (HATU/NMM activation) as for the other protected amino acids.

H-Met-Ser-Gln-Lys-Ser-Gln-Arg-Ser-Gln-Asp-Glu-Asn-Pro-Val-NH_2 [Met-Ser-GpMBP(74−85)] (4). The peptide was synthesized starting from 0.5 g of resin (0.12 mmol) following the general procedure.

Pam-Cys[CH_2-CH(O−Pam)-CH_2O−Pam]-Ser-Gln-Lys-Ser-Gln-Arg-Ser-Gln-Asp-Glu-Asn-Pro-Val-NH_2 [Pam$_3$Cys-Ser-GpMBP(74−85)] (5). The peptide Ser-GpMBP(74−85) was synthesized starting from 0.5 g of resin (0.12 mmol) following the General Procedure. The lipophilic built-in immunoadjuvant Pam-Cys[CH_2-CHOH-CH_2OH]-OH was introduced at the end of the synthesis, in the N-terminal position of the resin-linked peptide in a 2.5-fold excess by *endo*-*N*-hydroxy-5-norbornene-2,3-dicarboximide/*N*,*N*′-diisopropylcarbodiimide activation in DMF/DCM (1:1). Two additional palmitoyl residues were introduced, in solid phase, on the hydroxyl functions of 2,3-dihydroxypropyl group present on the side chain of the Cys residue. Palmitoyl chloride (1.2 mmol) in pyridine/DCM (1:1) was recirculated on the resin for 14 h. After treatment with DMF and 20% piperidine in DMF, the resin was washed with DMF and DCM and the peptide cleaved from the resin as described in the general procedure. The peptide was used with no further purification.

H-Glu-Asn-Pro-Val-Val-His-Phe-Phe-Lys-Asn-Ile-Val-Thr-Pro-Arg-Thr-Pro-OH [GpMBP(82−98)] (6). The peptide was synthesized starting from 0.5 g of resin (0.05 mmol) following the general procedure. Deprotection of Thr^{97} was

performed with a 5 mL/min piperidine flow for a period shorter than usual to avoid the formation of the corresponding diketopiperazine.

Pam-Glu-Asn-Pro-Val-Val-His-Phe-Phe-Lys-Asn-Ile-Val-Thr-Pro-Arg-Thr-Pro-OH [Pam-GpMBP(82−98)] (7). The peptide was synthesized starting from 0.5 g of resin (0.05 mmol) following the general procedure. The palmitoyl moiety was introduced at the end of the synthesis by means of Pam-OPfp, dissolved in DCM/DMF in a 5-fold excess (0.106 g, 0.25 mmol) in the presence of HATU (0.095 g, 0.25 mmol) and NMM (0.042 mL, 0.38 mmol).

H-Lys(Pam)-Glu-Asn-Pro-Val-Val-His-Phe-Phe-Lys-Asn-Ile-Val-Thr-Pro-Arg-Thr-Pro-OH [Lys(Pam)-GpMBP(82−98)] (8). The peptide was synthesized starting from 0.5 g of resin (0.05 mmol) following the general procedure. The introduction of the lipophilic moiety was performed by using a 2.5-fold excess of Fmoc-Lys(Pam)-OH in DMF (HATU/NMM activation), as for the other protected amino acids.

Immunization. Female Lewis rats were immunized intradermally in the hind foot pads with 200 μg of MBP(74−85) or MBP(82−98) peptide emulsified in incomplete Freund's adjuvant (IFA, Sigma, Germany) supplemented with 300 μg of *Mycobacterium tubercolosis* H37 RA (DIFCO, Detroit, MI).

In Vitro Lymphocyte Proliferation. Spleens from immunized Lewis rats were removed aseptically from rats at day 12 after immunization. Single cell suspensions of spleen were prepared by passage of spleens through a stainless steel mesh and tested in a proliferation assay to evaluate the response to the immunizing peptides and to its lipoderivatives. Proliferative response of spleen cells was evaluated by ^{3}H-thymidine incorporation in the last 8 h of culture. Incorporated radioactivity was measured in a scintillation counter (Microbeta Plus, Wallac, Finland). Results are expressed as the mean counts per min (cpm).

Digestion of the Peptides with Cathepsin D. Digestion mixtures were prepared with 10 μg of each peptide [10 μL of 1 mg/mL solution in water, 1% dimethyl sulfoxide, (DMSO)] and 0.1 μg of bovine cathepsin D (Sigma) in 1 N citrate buffer pH 5.0 (10 μL). Each mixture was incubated at 37 °C for 40 min and then frozen. The digestion was followed by HPLC using a Vydac C18 column (5 μm, 150 × 2.1 mm, 0.2 mL/min flow rate) with a gradient of 0−90% B in 30 min for GpMBP-(82−98) and for lipoderivatives of GpMBP(74−85) and a gradient of 0−80% B in 30 min for GpMBP(74−85). For lipoderivatives of GpMBP(82−98) a Vydac C4 column (5 μm, 150 × 2.1 mm, 0.2 mL/min flow rate) with a gradient of 20−100% B in 30 min was used.

Digestion of the Peptides with Cathepsin L. Digestion mixtures were prepared with 10 μg of each peptide (10 μL of 1 mg/mL solution in water, 1% DMSO) and 0.1 μg of human cathepsin L (kindly provided by Dr. E. Weber, Institute of Biochemistry, University of Halle, Germany) in 0.2 M citrate buffer pH 5.5 containing 1.2 mg/mL dithiothreitol (DTT, Cleland's reagent) and 0.2 mM pepstatin (10 μL). Each mixture was incubated at 37 °C for 2 h, then frozen, and followed by HPLC with the methods reported for cathepsin D.

Digestion of the Peptides with a Lysosomal Fraction. Digestion mixtures were prepared with 10 μg of each peptide (10 μl of 1 mg/mL solution in water, 1% DMSO), 10 μL of 0.15 M phosphate buffer pH 5.4, 1.2 mg/mL DTT, and 1 μL of a lysosomal fraction of the EBV-transformed B cell line BSM.[22] Each system was incubated at 37 °C for 3 h, then frozen, and followed by HPLC with the methods reported for cathepsin D.

Conclusions

It has been recently demonstrated that a single modification of a relatively long peptide by a lipophilic amino acid with a long hydrocarbon chain, results in the ability to reproducibly induce, without immunoadjuvant, a relevant virus-specific CTL response in vivo, in the absence of an immunoadjuvant.[11] However, the mechanism is not well understood.

Here we show that the synthetic lipopeptides **2** and **3** of the immunodominant epitope in Lewis rat, GpMBP-(74−85), obtained by SPPS through the conjugation of a lipophilic moiety (palmitoyl) at the terminal N^{α} of Gln^{74} and at the ϵ-NH_2 of Lys^{75}, respectively, increased the T-cell responsiveness compared to the native non-lipidated analogue. On the other hand, lipoconjugation of the different MBP immunodominant peptide GpMBP-(82−98) does not result in increased T-cell response. In addition, the data obtained by the cathepsin cleavage of the two immunodominant peptides suggest that the first prerequisite for increasing the T-cell response by a lipopeptide is the stability of the immunodominant wild-type peptide to peptidases.

This is the first demonstration of immunoadjuvant effects of lipoconjugation in an in vitro system, by using CD4+ T-cells.

Three different hypotheses can be formulated to explain the increased T-cell responsiveness to the lipid-bound peptides:

(i) A costimulatory activity of the lipophilic moiety can be envisioned. A recent study[8] showing that lipoproteins can provide costimulatory signals to CD4+ and CD8+ T lymphocytes to produce pro-inflammatory cytokines would support this hypothesis. Due to the different effect of lipoconjugation of the two GpMBP epitopes, this hypothesis is, however, unlikely.

(ii) An increased affinity to the MHC molecule and/or T-cell receptor of responding T-cells can be responsible for the lipopeptides' biological activity. In this case, the lipoderivatives of the two immunodominant peptides should bind the surface MHC molecule, and the different effect of lipidation could be explained either by a different susceptibility of the TCR of the T-cell clones specific for the two different epitopes or by a different binding kinetic of the lipopeptides to Ia and Ir, the MHC class II molecules involved in the recognition of GpMBP-(74−85) and GpMBP(82−98), respectively. This hypothesis is not consistent with previous results showing an impaired binding of lipoderivatives to MHC molecule.[14]

(iii) A more favorable antigen presentation could explain the lipopeptide superagonist activity. Several pieces of evidences indicate that lipoconjugation may favor the interaction of the peptide antigen with cell membrane. In this context, fluorescence resonance energy transfer (FRET) experiments of the fluorescence-labeled lipopeptide AMCA-ωAud-GpMBP(74−85) with covesicles made by DPPC/AMCA-ωAud-GpMBP(74−85)/phospholipids bearing a quencher on the alkyl chain (100:5:1) demonstrated a high efficiency (76%) and a small AMCA-BODIPY distance (41 Å). The high FRET efficiency is in agreement with AMCA localized near the quencher BODIPY, and it can be explained only if the AMCA-labeled lipopeptide penetrates the phospholipid bilayer. These data support the hypothesis that the presence of the lipophilic moiety facilitates the interaction with the cell membrane.[25] On the other hand, our data demonstrate that lipidation induces the two immunodominant peptides a comparable stability to peptidases, with respect to their wild-type sequences. Therefore, we must hypothesize that the lipophilic moiety, inducing both the lipopeptides to cross the cell membrane bypassing the binding to the cell surface MHC, is then removed before the antigens enter the

lysosomal compartment. Here the cathepsins will explicate their function, before the peptides bind to the nascent MHC molecules for presentation on the cell surface. In this context, there are several evidences that the intracellular processing is fundamental for peptide antigen recognition.[26] It is evident that such a mechanism would be advantageous for an epitope stable to peptidases such as GpMBP(74−85), but not for GpMBP-(82−98), which contains several cleavage sites for cathepsins and other peptidases.

In conclusion, we formulate a hypothesis on the mechanism of immunoadjuvanticity of lipopeptides, by which the lipidic moiety bound to the examined peptides would affect the T-cell response. This includes intracellular processing of the lipopeptide antigens and accounts for the epitope specificity of the effects of lipoconjugation.

These findings can provide a first insight into the understanding of the immunoadjuvant effect of lipoderivative antigens.

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