Lipoamino acid based adjuvant carrier system: Enhanced immunogenicity of group A streptococcal peptide epitopes

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Experimental

Chemistry

Boc (*tert*-butoxycarbonyl)-L-amino acids and 4-methylbenzhydrylamine (MBHA) resin were purchased from Novabiochem (Läufelfingen, Switzerland). Dimethylformamide (DMF), trifluoroacetic acid (TFA), N,N-diisopropylethylamine (DIEA) and dichloromethane (DCM) (all peptide synthesis grade) were purchased from Auspep (Melbourne, Australia) and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronuim hexafluorophosphate (HBTU) was obtained from Richelieu Biotechnologies (Quebec, Canada). Electrospray MS were obtained on a Perkin-Elmer API 3000 instrument using acetonitrile-water as mobile phase. Peptides were prepared by manual solid phase peptide synthesis on a 0.5 mmol scale by using *in situ* neutralization/HBTU activation procedure for Boc chemistry as described previously (Schnölzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S.B.H. *In situ* neutralization in Boc-chemistry solid phase peptide synthesis. *Int. J. Peptide Protein Res.* **1992**, *40,* 180-193). Each synthetic cycle consisted of N^{α}-Boc removal by a 1- to 2-min treatment with neat TFA, a 1-min DMF flow wash, a 10- to 20-min coupling time with four-fold excess of preactivated Boc-amino acid in the presence of excess DIEA and a second DMF flow wash. N^{α}-Boc-amino acids were preactivated immediately before coupling with equivalent amount of HBTU (0.5 M HBTU in

DMF) in the presence of excess DIEA. After each coupling step, yields were determined by measuring residual free amine with the quantitative ninhydrin assay (Stewart, J.M.; Young, J.D. Solid Phase Peptide Syntheis, 1984, 2nd edition Pierce Chemical Company, Rockford, Illinois USA). When necessary further couplings were performed until the coupling yield reached a min 99.8%. After coupling of Gln residues, a dichloromethane flow wash was used before and after deprotection by using TFA, to prevent possible high-temperature (TFA/DMF)-catalyzed pyrrolidone formation.

Boc amino acids were used with the following side chain protection: Arg(Tos), Asp(OcHxl), Asn and Gln unprotected, Glu(OcHxl), Cys(4-MeBzl), Lys(2ClZ), Ser(Bzl) and Tyr(BrZ). Racemic LAA were synthesized using literature methods (Gibbons, A.W.; Hughes, R.A.; Szeto, A.; Charalambous, M.; Aulabaugh, A.; Mascagni, P.; Toth, I. Lipidic Peptides I. Synthesis resolution and structural elucidation of fatty amino acids and their homo- and hetero-oligomers. *Liebigs Ann. Chem.* **1990,** 1175-1183). After completion of the synthesis the peptide was removed from the resin support with the high HF method (1ml cresol, 1ml thiocresol, 20ml HF) to yield the crude peptide, which was precipitated with ice-cold ethyl ether and redissolved in 50% acetonitrile in water (25ml). The reaction mixture was lyophilized.

Synthesis of the LCP1 system: First preactivated Boc-Gly-OH was coupled to the MBHA resin (0.67 mmol/g) . The next two cycles were carried out with Boc-lipoamino acids containing 12 carbon atoms (C12: -HN-CH[$(CH_2)_9CH_3$]-CO-) which was followed sequentially by the coupling of Boc-Gly-OH, Boc-C12-OH and Boc-Lys(Boc)-OH. After deprotection of the lysine α- and ε- amino groups a four-branch system was formed by coupling of Boc-Lys(Boc)-OH to the free amino groups. After deprotection, four identical peptide chains (J8:

QAEDKVKQSREAKKQVEKALKQLEDKVQ) were synthesized directly on the branched lysine core with the appropriate protecting groups applied on the side chains of the amino acids.

Synthesis of the LCP2 system: The synthesis of the four branch lysine core was similar to that of the LCP1 system, but Boc-Lys(Fmoc)-OH was used to form the four branches. First the Boc-protecting groups were removed from the N-terminal Boc-Lys(Fmoc)-OH residues and J8 peptides were synthesized on these two branches. After coupling of the N-terminal amino acid of each J8 peptide the Boc-protecting group was removed and the free H_2N-terminal group was acetylated with a mixture of 2.5 mmol acetic anhydride and 2.5 mmol DIEA in DMF resulting in the $(Acetyl\text{-}J8)_2$ $(Fmoc)_2$ $(=Lys)_2$=Lys-C10-Gly-C10-C10-Gly-MBHA resin. After removing the Fmoc-protecting groups by mixing the peptide-resin in 20% piperidine in DMF for 20 minutes the 21 amino acid 8830 peptide (DNGKAIYERARERALQELGPC) was assembled on each of the two new branches with the appropriate protecting groups applied on the side chains of the amino acids.

Peptide purification was done on a Waters HPLC system (Model 600 controller, 490 E UV detector, 60 F pump) using a preparative Vydac protein & peptide C18 column (2 cm x 25 cm). HPLC grade acetonitrile and water were filtered through a membrane filter and degassed with helium prior to use. All HPLC solvents contained 0.1 % trifluoroacetic acid. Separation was achieved with a solvent gradient beginning with 0 % acetonitrile, increasing constantly to 90% acetonitrile for 40 minutes and staying at this concentration for 10 minutes at a constant flow of 12 ml per min. Compounds were detected at 230 nm. The lipoamino acids were racemic, hence the HPLC fractions of the major product, whose intensities were greater than

half of the maximum absorption were collected. The products showed single bands on SDS Gels at the expected molecular weights.

Analytical reversed-phase HPLC was performed on a Shimadzu instrument (LC-10AT Liquid Chromatograph, SCL-10A System Controller, SPD-6A UV Detector and a SIL-6b Auto injector with a SCL-6B System Controller) using Vydac C-18 column (5 μm, 0.46 cm x 25 cm). Separation was achieved with a solvent gradient beginning with 0 % acetonitrile, increasing constantly to 90% acetonitrile for 30 minute at a constant flow of 1 ml per min. Compounds were detected at 214 nm.

SDS gel electrophoresis was carried out on a Mighty Small SE 245 Unit (Hoefer Scientific Instruments, San Francisco) and an Appliqeue Power Supply (Illkrich, France) using Sigma myoglobin fragments as SDS molecular weight markers. The developed gels showed that the compounds had bands around the expected molecular weights in agreement with the markers. The mass spectrum of product **LCP1** (calcd molecular weight: 14158.45) multiple charged ions: $(M+7H^+)/7$: 2023.1 (calcd 2023.6), $(M+8H^+)/8$: 1771.3 (calcd 1770.8), $(M+11H^+)/11$: 1288.9 (calcd 1288.1), $(M+12H^+)/12$: 1179.7 (calcd 1180.8), $(M+13H^+)/13$: 1088.7 (calcd 1090.1), $(M+14H^+)/14$: 1011.5 (calcd 1012.3), $(M+15H^+)/15$: 943.8 (calcd 944.9) $(M+16H^+)/16$: 885.4 (calcd 885.9) $(M+17H^+)/17$: 833.8 (calcd 833.8) $(M+18H^+)/18$: 788.3 (calcd 787.6), $(M+19H^+)/19$: 746.4 (calcd 746.2). The mass spectrum of product **LCP2** (calcd: 12373.11) multiple charged ions: $(M+9H^+)/9$:1375.4 (calcd 1375.8), $(M+10H^+)/10$: 1238.2 (calcd 1238.3), $(M+11H^+)/11$: 1125 (calcd 1125.8), $(M+12H^+)/12$: 1031.4 (calcd 1032.1), $(M+14H^+)/14$: 884 (calcd: 884.8), $(M+15H^+)/15$:825.7 (calcd 825.9), (M+16H+)/16: 773.4 (calcd 774.3). Amino acid analysis (Cohen, S.A.; Michaud, D.P. Synthesis of a Fluorescent Derivatizing Reagent, 6-Aminoquinolyl-N-Hydroxysuccinimidyl Carbamate, and Its Application

for the Analysis of Hydrolysate Amino Acids via High-Performance Liquid Chromatography. *Anal. Biochem.* **1993,** *211,* 279-87) of **LCP1**: Asx 7.52 (8), Glx 33.83 (36), Ser 3.49 (4), Arg 4 (4), Gly 2.13 (2), Ala 12.00 (12), Val 11.71 (12), Leu 8.17 (8), Lys 32.52 (31). Amino acid analysis of **LCP2**: Asx 7.12 (8), Glx 27.81 (26), Cys not determined (2), Ser 1.86 (2), Arg 8.41 (8), Gly 6.32 (6), Ala 12.00 (12), Pro 1.75 (2), Tyr 1.75 (2), Val 5.73 (6), Ile 1.76 (2), Leu 8.43 (8), Lys 17.60 (19).