CONJUGATES OF HEPATITIS B SURFACE ANTIGEN (14-32)PRE-S2 REGION WITH N-ACETYLMURAMIC ACID, N-ACETYLNORMURAMIC ACID, AND THEIR L-ALANYL-D-ISOGLUTAMINE DERIVATIVES: SYNTHESIS AND STUDIES ON IMMUNOGENICITY

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Four novel analogs of hepatitis B virus surface antigen (14-32)Pre-S2 region fragment attached covalently to N-acetylmuramic acid, N-acetylmuramyl-L-alanyl-D-isoglutamine, N-acetylnormuramic acid, and N-acetylnormuramyl-L-alanyl-D-isoglutamine were synthesized by the solid phase method. The ability of analogs to induce cellular and humoral immunity to native HBsAg was tested on rabbits. Cellular immune response occurred in vitro, and HBs antibodies were detected in all immunized animals. No additional adjuvants were used in the tests.

In continuation of our studies on protein S (refs[1-4]) and potent immunogenic hepatitis B surface antigen Pre-S2 region[5,6] we synthesized four new analogs of (14–32)Pre-S2 region covalently attached to natural immunostimulators: muramyldipeptides or their derivatives[7]. The majority of synthetic antigens require adjuvants to be used for the detection or enhancement of immunological response level. Commonly used Freund's complete adjuvant (FCA) is not only a potent immunostimulator but may also trigger undesirable side effects such as body temperature rise, arthritis, lymphatic gland enlargement, as well as disorders due to unmetabolized mineral oil entering into the composition of FCA (ref.[7]). Thus, FCA is highly harmful for higher forms of organisms[7]. Such undesirable side effects of FCA can be eliminated or reduced by the use of synthetic muramylpeptides, i.e. the mureine fragments which are the main carriers of adjuvant activity. N-Acetylmuramyl-L-alanyl-D-isoglutamine, called muramyldipeptide (MDP), is the smallest immunogenic fragment of the bacteria cell wall[7-9]. MDP and its analogs are highly active and low-toxic adjuvants, enhancing nonspecific immunity of higher organisms against bacterial and virus infections and displaying antineoplastic activity. They also enhance the effectiveness of immunizing vaccines and act synergistically with various drugs[10]. Apart from the broad immunogenic activity spectrum, muramylpeptides exhibit neuropharmacological properties affecting directly the central

nervous system, which is revealed by pyrogenic properties limiting substantially the applications of MDP (ref.[11]). The pyrogenic properties of MDP were successfully eliminated either by the synthesis of analogs in which D-isoglutamine carboxyl group was blocked as ester[9,12], or by covalent coupling with antigen[7,13].

The greatest capabilities of synthetic muramylpeptides devoid of the majority of undesirable FCA effects are anticipated as regards their employment in enhancing the effectiveness of immunizing vaccines. Currently, efforts are being made towards the application of the so-called second-generation vaccines with synthetic antigenic determinants. These determinants are devoid of harmful biological contaminants but most often they are very weakly immunogenic, requiring the co-operation of strong immunostimulators.

Several experiments have till now been reported in which MDP and its analogs were tested in mixtures, as well as in covalent coupling of adjuvant to protein antigen[7,9,14].

In the present paper, we report the solid-phase synthesis of four analogs of strongly immunogenic hepatitis B surface antigen (14–32)Pre-S2 region[15] acylated at the N-terminus with N-acetylmuramic acid (Mur), N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP), N-acetylnormuramic acid (norMur), and N-acetylnormuramyl-L--alanyl-D-isoglutamine (norMDP). The results of immunological tests are also presented.

The peptides synthesized have the following sequence:

14 20 30 32

X-Asp-Pro-Arg-Val-Arg-Gly-Leu-Tyr-Leu-Pro-Ala-Gly-Gly-Ser-Ser-Ser-Gly-Thr-Val,

where X denotes: Mur N-acetylmuramic acid, MDP N-acetylmuramyl-L-alanyl-D-isoglutamine, norMur N-acetylnormuramic acid, norMDP N-acetylnormuramyl-L-alanyl-D-isoglutamine.

The peptides were synthesized by stepwise coupling of Boc-amino acids to the growing peptide chain on Merrifield resin. Coupling reactions were mediated by dicyclohexylcarbodiimide (DCC) or by DCC in the presence of N-hydroxybenzotriazole (HOBt) (ref.[16]). The details of the synthesis are presented in the experimental section. 1-α-O-Benzyl-4,6-O-benzylidene-N-acetylmuramic acid, 1-α-O-benzyl-4,6--O-benzylidene-N-acetylmuramyl-L-alanyl-D-isoglutamine, 1-α-O-benzyl-4,6-O-benzylidene-N-acetylnormuramic acid, and 1-α-O-benzyl-4,6-O-benzylidene-N-acetylnormuramyl-L-alanyl-D-isoglutamine residues were introduced by DCC/HOBt coupling. The completeness of each coupling reaction was monitored by the ninhydrin test[17]. Recoupling was performed if the test was positive. The peptide derivatives were cleaved from the resin, and all protecting groups were removed with liquid HF at 0°C in the presence of anisole[18]. After lyophilization, crude products were desalted and purified as previously described[6,19]. The final products were homogeneous on TLC and revealed the expected ratios of amino acids. The details have been given in Table I.

TABLE I

Data of the synthesis and physico-chemical characteristic of antigen fragments

Peptide	$[\alpha]_D^{20}$ 1M AcOH	R_F A B C	Amino acid analysis						Yield of peptide, %		Formula (M.w.)	Calculated/Found		
			Ala Asp	Gly Leu	Pro Arg	Ser Tyr	Thr Val	Glu NH_3	crude[a]	purified		%C	%H	%N
Mur-(14-32)Pre-S2	−78·3°	0·28	1·00	4·01	1·98	2·88	0·98	—			$C_{92}H_{150}N_{26}O_{34}$	51·0	7·0	16·8
	(*c* 0·23)	0·20	0·97	2·03	1·94	1·03	2·04	—	93·3	28·1	(2 164)	51·1	6·9	16·7
		0·81												
MDP-(14-32)Pre-S2	−53·8°	0·27	2·00	4·06	1·94	2·89	0·97	0·94			$C_{100}H_{163}N_{29}O_{37}$	50·8	6·9	17·2
	(*c* 0·36)	0·20	0·96	2·04	1·93	1·02	1·99	1·09	91·3	36·1	(2 363)	50·7	6·9	17·0
		0·82												
norMur-(14-32)Pre-S2	−70·8°	0·23	1·00	4·01	1·96	2·89	0·87	—			$C_{91}H_{148}N_{26}O_{34}$	50·8	6·9	16·9
	(*c* 0·42)	0·20	0·97	1·98	1·94	0·97	2·04	—	93·7	27·6	(2 150)	50·7	6·8	16·8
		0·78												
norMDP-(14-32)Pre-S2	−57·7°	0·21	2·00	4·04	2·02	2·88	0·96	0·98			$C_{99}H_{161}N_{29}O_{37}$	50·6	6·9	16·9
	(*c* 0·27)	0·20	1·04	1·98	1·98	0·98	1·96	1·03	86·1	21·9	(2 349)	50·5	6·8	16·8
		0·78												

[a] Based on the initial valine content of the resin.

Rabbits were immunized with four analogs: Mur-(14–32)Pre-S2, MDP-(14–32) Pre-S2, norMur-(14–32)Pre-S2, norMDP-(14–32)Pre-S2, and for comparison with a mixture of free peptide with muramyl-L-alanyl-D-isoglutamine or normuramyl--L-alanyl-D-isoglutamine. The results of humoral anti-HBs(Pre-S2) and cellular response in animals immunized with the (14–32)Pre-S2 region analog with the application of synthetic immunomodulators are presented in Tables II and III. Cellular immunity to HBsAg(Pre-S2), evaluated by the in vitro migration inhibition test (MIT) using peripheral blood leucocytes, occurred in all immunized animals. The results of the migration inhibition test in the presence of a control antigen (normal human serum – NHS) and prior to immunization were negative.

HBsAg antibodies were detected in all immunized rabbits. The level of antibodies induced by the mixture of free peptide with N-acetylmuramyl-L-alanyl-D-isoglutamine was higher than that obtained for the mixture with N-acetylnormuramyl-L--alanyl-D-isoglutamine. Humoral response observed in animals immunized with (14–32)Pre-S2 fragment bonded covalently to N-acetylmuramyl-L-alanyl-D-isoglutamine and to muramic acid was higher than that obtained after immunization

TABLE II

Humoral anti-HBs(Pre-S2) response to the native antigen in rabbits immunized with synthetic peptides

Peptide	Rabbit No.	Antibody titer[a] (EIA)
(14-32)Pre-S2 + MDP	1	1 : 500
	2	2 : 200
(14-32)Pre-S2 + norMDP	1	1 : 100
	2	1 : 50
Mur-(14-32)Pre-S2	1	1 : 100
	2	1 : 500
MDP-(14-32)Pre-S2	1	1 : 200
	2	1 : 200
nor-Mur-(14-32)Pre-S2	1	1 : 100
	2	1 : 10
norMDP-(14-32)Pre-S2	1	1 : 10
	2	1 : 200

[a] Highest serum dilution at which the result was 3·1 times higher than that of negative test (prior to immunization); ETA enzyme immuno assay.

with this fragment coupled to N-acetylnormuramyl-L-alanyl-D-isoglutamine or N-acetylnormuramic acid.

Immunogenicity of (14–32)Pre-S2 region fragment adsorbed on alum gel was tested previously. Antibodies were present in animals immunized with peptides emulsified with Freund's complete adjuvant (FCA). The results were reported previously[6].

The results demonstrated indicate immunogenic capability of synthetic analogs of muramic acid or MDP, enabling immunological response to be obtained with no necessity to apply Freund's adjuvant inducing undesirable side effects and disorders caused by unmetabolized mineral oil.

By the combination of synthetic, relatively simple and highly immunogenic low molecular muramylpeptides with synthetic antigenic determinants devoid of harmful biological contaminants, new prospects in designing effective and harmless immunizing vaccines are established.

TABLE III

Migration inhibiton of rabbit peripheral blood leucocytes in the presence of HBsAg(Pre-S2)

Peptide	Rabbit No.	Mean % migration inhibition	
		HBsAg	NHS[a]
(14-32)Pre-S2 + MDP	1	28·0	9·0
	2	83·5	2·5
(14-32)Pre-S2 + norMDP	1	71·0	2·0
	2	27·0	10·5
Mur-(14-32)Pre-S2[b]	1	62·0	0·0
	2	46·5	6·0
MDP-(14-32)Pre-S2[b]	1	31·3	0·0
	2	27·0	9·0
norMur-(14-32)Pre-S2[b]	1	23·0	8·5
	2	72·0	4·0
norMDP(-14-32)Pre-S2[b]	1	39·0	0·0
	2	48·5	7·0
Control		8·5	4·0

[a] NHS normal human serum. [b] (14-32)Pre-S2 fragment bonded covalently to MDP, muramic acid, norMDP, and normuramic acid.

EXPERIMENTAL

Starting materials: N^{α}-butyloxycarbonyl-amino acids, trifluoroacetic acid (TFA), dicyclohexylcarbodiimide (DCC), triethylamine (TEA), acetic acid, dimethylformamide (DMF), methylene chloride (CH_2Cl_2), and methanol were purchased from Fluka AG (Switzerland). All solvents and reagents used for solid-phase synthesis were of analytical grade and were redistilled before use. Chloromethylated resin (copolystyrene–1% divinylbenzene, 0·75 mmol of Cl/g of resin) Bio-Beads SX1 was obtained from Bio-Rad Laboratories (U.S.A.). Sephadex G-15, G-25 and Sephadex LH-20 were supplied by Pharmacia (Sweden). TLC was carried out on silica plates (Merck) and the spots were visualized by iodine or ninhydrin. The following solvent systems were used: A, 1-butanol–pyridine–acetic acid–water (51 : 12 : 12 : 25, v/v); B, 1-butanol–pyridine–acetic acid–ethyl acetate (1 : 1 : 1 : 1, v/v); C, ethanol–0·1M pyridine–0·1M acetic acid (3 : 1 : 1, v/v). Solutions containing 10—50 μg of the sample were applied to the plates and chromatograms were developed for a minimum length of 10 cm. In all cases, unless otherwise specified, single symmetrical spots were observed for purified materials. For amino acid analysis, the peptides (approximately 0·5 mg) were hydrolyzed with constantly boiling hydrochloric acid (400 μl) containing phenol (20 μl) in evacuated sealed ampoules for 18 h at 110°C. The analyses were performed on a Mikrotechna type AAA 881 analyser. The optical rotations were measured with a Perkin–Elmer model 141 polarimeter with an accuracy of 0·01°.

Synthesis of the Peptides

The peptides were synthesized by the solid-phase procedure[20]. Chloromethylated resin was esterified with Boc-Val to a load of 0·32 mmol/g according to Gisin[21]. In all cases, the Boc group was used for the protection of α-amino groups throughout the synthesis with the following side-chain protecting groups: Asp(OBzl), Arg(Tos), Ser(Bzl), Tyr(Bzl) and Thr(Bzl). The synthesis of the fully protected peptide resin was carried out according to standard procedures involving: (i) 30 min deprotection steps using 33% TFA in the presence of anisole (2%) in CH_2Cl_2; (ii) neutralizations with 10% TEA in CH_2Cl_2 (twice); (iii) couplings in CH_2Cl_2 or DMF–CH_2Cl_2 (1 : 1) performed by the use of 1·0M DCC in CH_2Cl_2 for 90 or 120 min.

1-α-O-Benzyl-4,6-O-benzylidene-N-acetylmuramic acid, 1-α-O-benzyl-4,6-O-benzylidene-N-acetylnormuramic acid, 1-α-O-benzyl-4,6-O-benzylidene-N-acetylmuramyl-N-alanyl-D-isoglutamine, and 1-α-O-benzyl-4,6-O-benzylidene-N-acetylnormuramyl-L-alanyl-D-isoglutamine were introduced with 1 eq. of DCC and HOBt, all in 4-fold excess (twice). The completeness to each coupling reaction was monitored by the ninhydrin test. The final protected peptides were cleaved from the resin, and all protecting groups were removed with anhydrous redistilled liquid HF (0 °C for 1 h) containing 10% anisole as scavenger. After removal of HF in vacuo the resin was washed with 5 × 20 ml of 10% acetic acid. The combined washings were extracted with 3 × 20 ml of diethyl ether with ethyl acetate (1 : 1), degassed and lyophilized (twice) to give crude peptide. The free peptides were desalted on a column (160 × 1·8 cm) of Sephadex LH-20 and purified by gel filtration on Sephadex G-15 or G-25 as previously described[6,19]. The eluate was fractioned and monitored for absorbance at 254 nm. The fractions comprising the major peak were collected and lyophilized to obtain pure products. The data on the synthesis and the properties of the resulting analogs are summarized in Table I.

N-Acetylmuramic and N-acetylnormuramic acids, N-acetylmuramyl-L-alanyl-D-isoglutamine, and N-acetylnormuramyl-L-alanyl-D-isoglutamine were obtained by the method described in literature[22]. Protected muramic and normuramic acids, as well as protected MDP and norMDP with free carboxyl groups in the D-isoglutamine residue facilitating the acylation of N-terminal HBV (14-32)Pre-S2 region fragment were obtained by the method of mild alkaline hydrolysis of

respective benzyl esters of protected derivatives. Fully deblocked N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) and N-acetylnormuramyl-L-alanyl-D-isoglutamine (norMDP) used in the mixture with (14-32)Pre-S2 fragment were obtained by the method previously described[23].

Immunization Procedures

Animals were immunized with the unbound fragment of (14-32)Pre-S2 region in the mixture of N-acetylmuramyl-L-alanyl-D-isoglutamine or N-acetylnormuramyl-L-alanyl-D-isoglutamine, and with the (14-32)Pre-S2 fragment bonded covalently to N-acetylmuramic acid, N-acetylnormuramic acid, N-acetylmuramyl-L-alanyl-D-isoglutamine, and N-acetylnormuramyl-L-alanyl-D-isoglutamine. Two rabbits were immunized with either peptide. Doses of 100 μg of free peptide with 5 μg of MDP or norMDP (solution in 0·14M NaCl) were injected intradermally into several places three times at 20 or 30 days intervals. The peptides emulsified with adjuvant were used in doses of 200 μg to immunize the animals according to the same scheme.

Rabbit sera were evaluated for anti-HBs(Pre-S2) antibodies in a solid phase enzyme immuno assay (ETA) using solid phase HBsAg(Pre-S2) positive and peroxidase labelled antibodies of goat anti-rabbit immunoglobulines (Ig). Cellular immunity to native HBsAg(Pre-S2) positive was evaluated by the in vitro migration inhibition test (MIT) (ref.[24]) using peripheral blood leucocytes from rabbits. In this test the same sample of antigen was used as for the detection of antibodies. Normal human serum (NHS) was used as a control antigen. Inhibition of migration greater than 20% was considered as a positive result of MIT.

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