SYNTHESIS OF HEPATITIS B SURFACE ANTIGEN PRE-S2 REGION FRAGMENTS AND STUDIES ON THEIR IMMUNOGENICITY

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Dedicated to the memory of Dr Karel Bláha.

Two peptide fragments of hepatitis B surface antigen pre-S2 region were synthesized by the solid phase method. The peptides were purified by gel filtration or ion-exchange chromatography on Sephadex SP-C-25. Both peptides induced a cellular and humoral immune response in rabbits. The results showed that fragment 14—22 of pre-S2 region contains one of the antigenic determinants.

The most important immunological response to hepatitis B virus (HBV) is directed towards antigenic determinants of the surface of the virus particle, hepatitis B surface antigen (HBsAg). Several regions of this antigen have been reproduced by synthesis and shown to induce antibodies recognizing the virus[1]. Hepatitis B virus and hepatitis B surface antigen particles, contain three forms of hepatitis B envelope proteins with a shared sequence of 226 amino acids[2,3]. The major constituent of the envelope or S-protein consists of 226 amino acid residues[4]. The "middle" protein has an additional 55 amino acids at the N-terminal and (pre-S2 region)[2,3]. The "large" protein has all of the amino acids of the "middle" protein with additional 108–119 N-terminal amino acids (depending on the antigenic subtype) (pre-S1 region)[2,3]. Antibodies specific for pre-S determinants, are elicited during hepatitis B infection and by immunization with HBV "middle" and "large" proteins[3]. It is known that selected pre-S antigenic determinants can be mimicked with high fidelity by synthetic peptide analogues that are immunogenic without any carriers[5]. It was also shown that pre-S2 determinants are significantly more immunogenic as compared with determinants of protein S (ref.[6]). All these findings prompted us to synthesize and evaluate some immunogenic properties of the fragments of the "middle" protein.

It is well known that hydrophilic regions are exposed on the surface of the HBV and HBsAg particles[7]. In continuation of our studies[8–10] we now describe the synthesis and some immunological properties of two fragments of the pre-S2 region.

The sequence of the first peptide corresponds to positions 14 through 32 of the "middle" protein. This peptide, composed of 19 amino acid residues, which represents one of the hydrophilic domains of the pre-S2 region has been previously obtained by others[11] and served as a reference. It is also important that the sequence of this fragment occurs in different subtypes of HBV. The peptide *II* was designed to check if a small fragment of compound *I* which contains only the most hydrophilic domain would still have immunogenic properties.

$$\text{Asp}^{14}\text{-Pro-Arg-Val-Arg-Gly-Leu-Tyr-Leu-Pro-Ala-Gly-Gly-Ser-Ser-}$$
$$\text{-Ser-Gly-Thr-Val}^{32}$$

I

$$\text{Asp}^{14}\text{-Pro-Arg-Val-Arg-Gly-Leu-Tyr-Leu}^{22}$$

II

Both peptides were synthesized by stepwise coupling of Boc-amino acids to the growing peptide chain on Merrifield resin. Coupling reactions were mediated by dicyclohexylcarbodiimide or by DCC/HOBt method. The completeness of each coupling reaction was monitored by the Kaiser test[12]. The first amino acid was coupled to the extent of only 40% ($\sim 0{\cdot}3$ mmol g^{-1}) as determined by amino acid analysis, to avoid incomplete coupling steps in the next stages of the synthesis. The side chain group of Asp, Tyr, Ser and Thr were protected with the benzyl group. The guanidine group of Arg was transformed into tosyl derivative. Prior to each coupling the Boc-peptide resin was deprotected with 1M solution of HCl in acetic acid and neutralized with 10% triethylamine in dichoromethane. After completing synthesis, the peptides were cleaved from the resin by treatment with liquid HF at 0°C in the presence of anisole. Crude peptides were purified by means of gel filtration on Sephadex G-25 and LH-20 or by ion-exchange chromatography on Sephadex SP-C-25. The final products were homogeneous on TLC and revealed the expected ratios of amino acids.

The results of cellular (MIT) and humoral [anti-HBs(pre-S2)] response to the native HBsAg in rabbits immunized with synthetic peptides are shown in Tables I and II. The inhibition of leucocyte migration in the presence of HBsAg (pre-S2) occurred in all immunized animals, but not in the control of non-immunized rabbits. There was no response in vitro to normal human serum. After immunization with peptides adsorbed on alum gel, no HBsAg antibodies were detected. Anti-HBs (pre-S2) were present in all serum samples from the same rabbits immunized with peptides emulsified with Freund's complete adjuvant (FCA). The level of antibodies induced by peptide *II* was higher than that obtained for peptide *I*. The cellular response was similar for both peptides. These results could indicate that peptide *II*,

composed of 9 amino acid residues, which represents the most hydrophilic fragment of peptide *I*, contains one of the antigenic determinants of the pre-S2 region.

EXPERIMENTAL

N,N-Dimethylformamide (DMF) was distilled under reduced pressure; triethylamine (NEt_3) was distilled from ninhydrin. Other sovents and reagents were of analytical grade. Thin-layer chromatography was carried out on silica plates (Merck), and the spots were visualized by ninhydrin or iodine. The following solvent systems were used; A, 1-butanol–acetic acid–water–pyridine (5 : 1 : 4 : 5, v/v), B, 1-butanol–acetic acid–water (4 : 1 : 5, v/v, upper phase), C, ethanol–0·1M acetic acid–0·1M pyridine (3 : 1 : 1, v/v). Solutions containing 10—50 μg of the sample were applied to the plates and the chromatograms were developed for a minimum length

TABLE I

Migration inhibition of rabbit peripheral blood leucocytes in the presence of HBsAg pre-S2

Peptide	Rabbit No.	Mean % migration inhibition	
		HBsAg	NHS[a]
II	1	56·0	0
	2	53·1	2
I	1	49·0	3·5
	2	64·3	14·0
control	1	13·0	2·1
	2	7·6	10·0

[a] Normal human serum.

TABLE II

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

Peptide	Rabbit No.	Antibody titer[a]
II	1	1 : 80
	2	1 : 160
I	1	1 : 80
	2	1 : 40

[a] The highest serum dilution to yield the positive result of ELISA.

of 10 cm. In all cases, unless otherwise specified, single symmetrical spots were observed for purified materials. For amino acid analysis the peptides (0·5 mg) were hydrolysed 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 typ AAA 881 analyser. The optical rotations were measured with a Hilger–Watts polarimeter with an accuracy of 0·01°.

Boc-Asp(OBzl)-Pro-Arg(Tos)-Val-Arg(Tos)-Gly-Leu-Tyr(OBzl)-Leu-Pro-Ala-Gly-Gly--Ser(OBzl)-Ser(OBzl)-Ser(OBzl)-Gly-Thr(OBzl)-Val-resin (*III*)

Boc-Val-resin was prepared from chloromethylated resin (Bio-Rad, Bio-Beads Sx1, 0·75 mmol . . Cl g^{-1}) by the Gisin method[14]. The substitution level was 0·3 mmol of valine per 1 g resin. A 3·333 g (1·0 mmol) sample of this resin was subjected as previously described[15–17] to eighteen cycles of deprotection, neutralization and coupling. On completion of the synthesis the protected peptide resin *III* (5·81 g, 97·9%) was obtained.

Boc-Asp(OBzl)-Pro-Arg(Tos)-Val-Arg(Tos)-Gly-Leu-Tyr(OBzl)-Leu-resin (*IV*)

Chloromethylated resin was esterified with Boc-Leu to a load of 0·32 mmol g^{-1} as described for Boc-Val-resin. Then a 3·13 g (1·0 mmol) sample of this resin was subjected to eight cycles of deprotection, neutralization and coupling to obtain protected peptide resin *IV* (4·44 g, 91·7%).

Asp-Pro-Arg-Val-Arg-Gly-Leu-Tyr-Leu-Pro-Ala-Gly-Gly-Ser-Ser-Ser-Gly-Thr-Val (*I*)

Protected peptide resin *III* (1·78 g; 0·3 mmol) was treated with 10 ml of liquid HF at 0°C for 60 min in the presence of 1 ml of anisole. 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 × 25 ml of diethyl ether, degassed and lyophilized to give crude peptide (590 mg, 99·0%). This product (179 mg) was purified on a column (120 × 2·5 cm) of Sephadex G-25 eluted with aqueous acetic acid (10%) with a flow rate of 5·5 ml h^{-1}. The eluate was fractioned and monitored for absorbance at 254 nm. The fractions comprising the major peak were pooled and lyophilized, and the residue (64·3 mg) was further subjected to gel filtration on a Sephadex LH-20 column (110 × 2·0 cm) eluted with aqueous acetic acid (10%) with a flow rate of 6 ml h^{-1}. The peptide was eluted as a single peak. Lyophilization of the pertinent fractions gave peptide *I*. Yield 29 mg (16·1%). $[\alpha]_D^{21}$ −94·6° (*c* 0·5, 1M-AcOH), TLC: R_F 0·50 (A), R_F 0·06 (B). Amino acid analysis: Thr 0·97, Ser 2·89, Asp 0·99, Pro 2·04, Gly 4·11, Ala 1·00, Val 2·02, Leu 2·04, Tyr 0·96, Arg 2·06.

Asp-Pro-Arg-Val-Arg-Gly-Leu-Tyr-Leu (*II*)

Treatment of the protected nonapeptide resin *IV* (1·452 g, 0·3 mmol) as detailed for *I* gave the crude peptide *II*; yield 308 mg (95·8%). This product (100 mg) was purified on a column (120 × × 2·5 cm) of Sephadex G-15 eluted with aqueous acetic acid (2·0%) with a flow rate of 5·5 ml . . h^{-1}. The eluate was fractioned and monitored for absorbance at 254 nm. The fractions comprising the major peak were pooled and lyophilized, and the residue (61·7 mg) was further subjected for ion-exchange chromatography on a column (2 × 10 cm) of Sephadex SP-C-25 equilibrated with 0·05M ammonium acetate, pH 4·2. After application of the sample, isocratic elution was carried out with the same buffer. The fractions comprising the major peak were pooled and lyophilized, dissolved in water and lyophilized again to give peptide *II*. Yield 50·6 mg (50·6%). $[\alpha]_D^{21}$ −75·1° (*c* 0·5, 1M-AcOH), TLC: R_F 0·57 (A), R_F 0·50 (C). Amino acid analysis: Asp 0·99, Pro 0·98, Gly 1·00, Val 1·00, Leu 2·04, Tyr 0·95, Arg 2·06.

Immunization Procedures

Rabbits in groups of two were first immunized with both fragments 14—22 and 14—32 adsorbed on the alum gel and then with the same peptides emulsified with Freund's complete adjuvant (FCA). Intradermal injection of 100 μg of peptide adsorbed on the alum gel were given six times every 18 days and next the same six doses of peptides emulsified with FCA were injected twice. Two control rabbits received alum gel and then FCA in saline according to the same scheme. Simultaneously, serum samples were collected for humoral immunity tests.

Rabbit sera were evaluated for anti-HBs (pre-S2) antibodies in a solid phase ELISA 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.[13]) using peripheral blood leucocytes from rabbits. In this test the same sample of antigen was used as for 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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REFERENCES

1. Neurath A. R., Kent S. B. H. in the book: *Immunochemistry of Viruses. The Basis for Serodiagnosis and Vaccines* (M. H. V. Van Regenmortel and A. R. Neurath, Eds), pp. 405—414. Elsevier, Amsterdam 1985.
2. Neurath A. R., Kent S. B. H., Strcik N., Taylor P., Stevens C. E.: Nature *315*, 154 (1985).
3. Neurath A. R., Strick N., Kent S. B. H., Offensperger W., Wahl S., Christman J. K., Acs G.: J. Virol. Meth. *12*, 185 (1985).
4. Galibert F., Mandart E., Fitoussi F., Tiollais P., Charnay P.: Nature *281*, 646 (1979).
5. Neurath A. R., Kent S. B. H., Strick N., Stark D., Sproul P.: J. Med. Virol. *17*, 119 (1985).
6. Neurath A. R., Kent S. B. H., Parker K., Prince A. M., Strick N., Brotman B., Sproul P.: Vaccine *4*, 35 (1986).
7. Bull H. B., Breese I.: Arch. Biochem. Biophys. *101*, 665 (1975).
8. Lammek B., Kupryszewski G., Ragnarsson U., Świderska H., Jastrzębska M., Nowosławski A. in the book: *Peptides 1986* (D. Theodoropoulos, Ed.), p. 459. de Gruyter, Berlin 1987.
9. Lammek B., Kupryszewski G., Ragnarsson U., Jastrzębska M., Świderska H., Nowosławski A.: Pol. J. Chem. in press.
10. Lammek B., Świderska H., Jastrzębska M., Nowosławski A.: Pol. J. Chem., in press.
11. Okamoto H., Imai M., Usuda S., Tanaka E., Tachibana K., Mishiro S., Machida A., Nakamura T., Miyakawa Y., Mayumi M.: J. Immunol. *134*, 1212 (1985).
12. Kaiser E., Colescott R. L., Bossinger C. D., Cook B. I.: Anal. Biochem. *34*, 595 (1970).
13. Falk E., Zabriskie J. B.: *In vitro Methods in Cell-mediated Imunity*, p. 301. Academic Press, New York 1971.
14. Gisin B. F.: Helv. Chim. Acta *56*, 1475 (1973).
15. Merrified R. B.: J. Am. Chem. Soc. *85*, 2149 (1963).
16. Manning M.: J. Am. Chem. Soc. *90*, 1348 (1968).
17. Lammek B., Neugebauer W., Perkowska D., Kupryszewski G.: Pol. J. Chem. *52*, 757 (1978).