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Studies on Peptides. CLXVI.[1,2] Solid-Phase Syntheses and Immunological Properties of Fragment Peptides Related to Human Hepatitis B Virus Surface Antigen (HBsAg) and Its Pre-S2 Gene

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Eight fragment peptides of 25 residues each and one 26-residue fragment, covering the entire 226 amino acid sequence of human hepatitis surface antigen (HBsAg, subtype ayw), were synthesized by the solid-phase method and partially purified by gel-filtration. In addition, two fragment peptides (26- and 29-residue peptides) which cover the 55 amino acid sequence encoded in the pre-S2 gene were also synthesized and partially purified. These partially purified peptides were submitted to immunological screening. In the *in vitro* enzyme linked immunoassay (ELISA), a 26-residue pre-S2 gene peptide (positions 1—26) exhibited relatively high reactivity against anti-HBsAg antisera. In the *in vivo* assay, three fragment peptides (positions 51—75, 101—125, and pre-S2 1—26), with relatively high hydrophilicity were shown to be immunogenic in rabbit without coupling to a carrier protein.

Keywords——synthetic vaccine; hepatitis B virus antigen; pre-S gene; solid-phase peptide synthesis; HBsAg antigenic site; Hope, Woods prediction; hydrophilicity value

Recently, several of virus surface antigenic proteins have been sequenced by the rapid development of deoxyribonucleic acid (DNA) cloning and sequencing technology. Such achievements have paved the way to produce various vaccines by recombinant DNA procedures and in addition, led to the idea of utilizing synthetic peptides for vaccination, since protein segments are capable of eliciting anti-protein immune response.[3]

Of various vaccines, the vaccine for human hepatitis B (hHB) virus seems to be of particular importance. The surface antigen (HBsAg) isolated from human blood of chronic carriers was the only available source for vaccine preparation, since the virus can not be grown in culture.[4] At present, the entire amino acid sequences of the major envelope constituent of hHB virus (S-protein)[5] and its precursor (pre-S1 and pre-S2 gene) detected as two minor constituents (L and M)[6] are known (Fig. 1), and biosynthetic vaccines prepared by using *E. coli*,[7] yeast,[8] and another organism[9] are under intensive investigation in clinical trials.

The synthetic approach vaccination may solve several major problems with in the present vaccines against viral diseases, such as safety and specificity for evolving new virus strains. In this approach, it is important to find antigenic regions within the sequence of the target surface protein through which the biological activity can be neutralized. It has been suggested that only a limited number of the potential antigenic sites are important for the

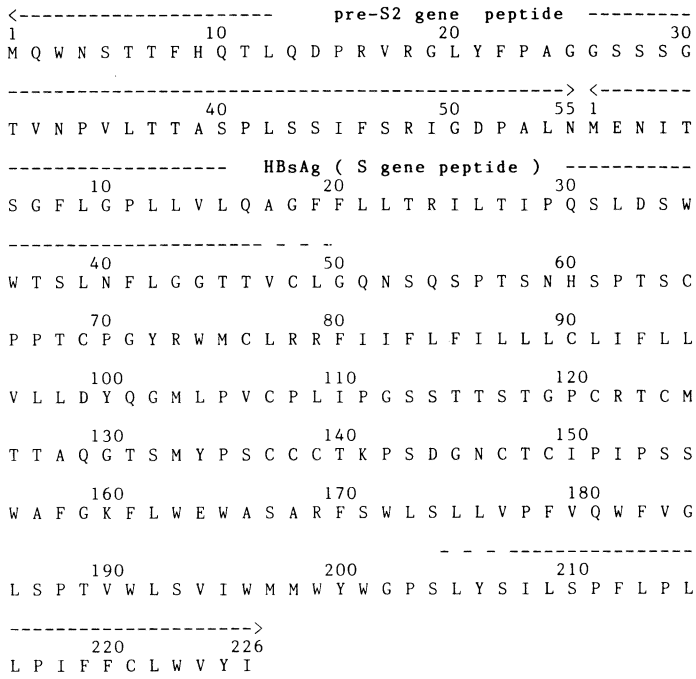

Fig. 1. Amino Acid Sequences of HBsAg (Subtype ayw) and Its Pre-S2 Gene Peptide

immunogenicity.[3] However, at the present time, there is no general principle to predict such possible antigenic determinants. It is still unclear whether proteins possess a large number and variety of antigenic determinants including three-dimensional elements.[10]

Currently, several research groups[11] are engaging in specifying crucial regions for hHB virus immune response, though unanimous answers have not yet been reached. Mostly, their interest has been focused on hydrophilic regions of the surface antigen (HBsAg), *i.e.*, some attention to the N-terminal portion (positions around 2—80)[11] and mostly to the most hydrophilic middle portion of HBsAg (positions around 95—160),[12] predicted by Hopp and Woods principle.[13] In 1984, Neurath *et al.*[14] added an interesting observation that a hydrophilic region of the pre-S2 gene peptide elicited relatively high immune response, and this region was synthetically examined.[15]

In order to obtain basic data for preparation of synthetic vaccines in future, we have synthesized eight fragment peptides of 25 residues each and one 26-residue fragment which cover the entire 226 amino acid sequence of HBsAg, and in addition, two fragment peptides (26- and 29-residue peptides) which cover the 55 amino acid sequence (positions pre-S2 1—55) encoded in the pre-S gene. These peptides were synthesized by the Boc-based solid-phase method[16] and partially purified by gel-filtration. Then, the eleven peptides were submitted to the *in vitro* and *in vivo* immunological screening tests.

Materials and Methods

Resins, Amino Acid Derivatives and Reagents——The chloromethylated polystyrene resin crosslinked with 2% divinylbenzene (Cl content 2 mmol/g) and three Boc-amino acids, Boc-Thr(Bzl)-OH, Boc-His(Tos)-OH[17] and Boc-Tyr(Cl_2-Bzl)-OH,[18] were purchased from Protein Research Inc. (Mino, Osaka). Z(OMe)-amino acids were prepared according to Weygand and Hunger.[19] A part of the resin was converted to the corresponding hydroxymethylated or bromomethylated resin by the known methods.[16] Reagents (analytical grade) were purchased

from Nakarai Chemical Co. Solvents were freshly distilled.

Solid Phase Peptide Synthesis——Each peptide was synthesized manually according to the program of Blake and Li.[20] In order to eliminate disulfide exchange reactions, each Cys residue was replaced by Ala. Except for the three Boc-amino acids mentioned above, Z(OMe)-amino acids were preferentially employed, including the following derivatives, *i.e.*, Glu(OBzl), Asp(OBzl), Ser(Bzl), Lys(Z), Arg(Mts),[21] Trp(Mts)[22] and Met(O).[23] As examined previously[24] and recognized by others,[25] the Z(OMe) group is cleaved more easily and steadily by TFA than the Boc group, reducing the possibility of deleted peptide formation. Except for two amino acids, the respective C-terminal amino acids were esterified onto the chloromethylated polystyrene-resin by the standard method.[16] The hydroxymethyl resin was employed for introduction of the C-terminal Met (O) residue and the bromomethyl resin for the Asn residue. The content of the first esterified amino acid (0.3 to 0.5 mmol/g) served as a basis of calculation in the subsequent chain elongation reaction. The symmetrical anhydride method[20] was employed to condense amino acids, except for Gln, Asn and Pro. The former two were introduced by the Np method[26] as usual. The Pro residue was introduced as a dipeptide unit, Z (OMe)-X-Pro-OH (X = desired amino acid), since quantitative condensation of X onto Pro is often difficult to achieve on the solid support, owing to its poor sensitivity to the ninhydrin test.[27] Throughout the synthesis, double coupling was occasionally performed until the resin became negative to ninhydrin.

Deprotection and Partial Purification——Each peptide resin (2.0 g) was treated with 1 M TFMSA-thioanisole/TFA[28] (10 ml) in the presence of *m*-cresol (100 eq) and ethanedithiol (50 eq) in an ice-bath for 120 min, then the resin was removed by filtration. The filtrate was concentrated *in vacuo* at a bath temperature below 30 °C. Dry ether was added to precipitate the product. Ether was removed by decantation and 10% AcOH or TFA was added, depending on the solubility of each product. The residues soluble in 1 N AcOH or 50% AcOH, were treated with Amberlite IR-4B (acetate form, approximately 3 g) for 30 min. After filtration, each filtrate was applied to a column of Sephadex G-25 (3.5 × 120 cm), which was eluted with the respective solvents. The fractions corresponding to the front main peak (monitored by ultraviolet (UV) absorption measurement at 230 or 275 nm) were collected and the solvent was removed by lyophilization or evaporation. In the latter case, the residue was treated with H_2O to obtain a powder. The deprotected peptides insoluble in the above two solvents, were precipitated from dimethyl sulfoxide (DMSO) with MeOH to obtain a powder.

Immunoassay——*In vitro* and *in vivo* assays were conducted. In the former enzyme-linked immunosorbent assay (ELISA),[29] peroxidase-conjugated mouse monoclonal or guinea-pig polyclonal antibody against HBsAg was employed. In the latter assay, each fragment peptide, without conjugation to any carrier, was emulsified in complete Freunds's adjuvant and administered subcutaneously to a group of two rabbits at multiple sites [200 μg + 100 μg (after 2 weeks) + 50 μg (after 5 weeks)]. Appearance of anti-HBsAg-activity was measured by using the Ausab RIA kit (Dainabot, Japan).[30]

Results and Discussion

Eleven fragment peptides were obtained by the Boc-based solid phase method, followed by deprotection with 1 M TFMSA-thioanisole/TFA and subsequent gel-filtration or precipitation as stated above; yields were 45—64%, based on the first amino acid loaded on the resin. Amino acid ratios in 6 N HCl hydrolysates of these partially purified peptides are listed in Table I. For screening, we decided to submit these partially purified peptides to bioassays.

HBsAg is a hydrophobic peptide. As predicted, several fragments are insoluble in H_2O, as listed in Table I. Thus, we were able to submit only the following 4 fragments of HBsAg and two fragments of Pre-S2 gene to the *in vitro* assay; 2 (26—50), 3 (51—75), 5 (101—125), 6 (126—150), 10 (pre-S2 1—26) and 11 (pre-S2 27—55). Other fragments, especially the 3 fragments near the C-terminal portion of HBsAg, 7 (151—175), 8 (176—200) and 9 (201—226), are insoluble even in DMF. Of the fragments tested, only pre-S2 fragment (No. 10) exhibited significantly higher reactivity than the others against both antibodies employed (Fig. 2).

For the *in vivo* assay, all synthetic peptides, including water-insoluble fragments, were emulsified in the adjuvant and administered to rabbits (two for each sample). After 6 weeks of immunization, blood samples were taken and their anti-HBsAg activities were measured by the Ausab method. Of the fragments tested, pre-S2 gene peptide (No. 10) was shown to be immunogenic for one rabbit, inducing antibodies with relatively high activity, as seen in the above *in vitro* assay. In addition, in this *in vivo* assay, two fragments, No. 3 (51—75) and No. 5 (101—125), induced antibodies with relatively higher titer than other HBsAg fragments, as

TABLE I. Amino Acid Ratios in 6 N HCl (24 h) Hydrolysates of Synthetic HBsAg(ayw) and Pre-S2 Fragment Peptides

No.	1	2	3	4	5	6	7	8	9	10	11
Positions	1—25 (HBsAg)	26—50	51—75	76—100	101—125	126—150	151—175	176—200	201—226	1—26 (Pre-S2	27—55 peptide)
Residue	(25)	(25)	(25)	(25)	(25)	(25)	(25)	(25)	(26)	(26)	(29)
Asp	0.97 (1)	2.05 (2)	1.74 (2)	1.00 (1)		2.08 (2)				2.24 (2)	2.06 (3)
Thr	1.70 (2)	3.65 (4)	3.30 (3)		3.60 (4)	6.83 (5)		0.86 (1)		3.14 (3)	3.47 (3)
Ser	0.98 (1)	2.75 (3)	4.81 (5)		2.29 (3)	5.12 (3)	3.28 (5)	1.94 (2)	2.88 (3)	0.94 (1)	5.09 (7)
Glu	2.03 (2)	0.93 (1)	2.32 (2)		0.96 (1)	1.85 (1)	0.83 (1)	0.56 (1)		3.20 (3)	
Pro	1.09 (1)	0.95 (1)	5.98 (5)		4.09 (4)	3.63 (2)	0.39 (2)	2.35 (2)	4.04 (4)	2.27 (2)	3.39 (3)
Gly	3.46 (3)	3.00 (3)	1.00 (1)		2.72 (3)	1.98 (2)	0.89 (1)	1.08 (1)	0.90 (1)	2.98 (3)	1.91 (2)
Ala	1.16 (1)	1.04 (1)	2.33 (2)	1.68 (2)	2.91 (3)	12.82 (6)	3.45 (3)		1.27 (1)	1.00 (1)	2.16 (2)
Val	0.95 (1)	0.97 (1)		0.68 (1)	0.80 (1)			3.55 (5)	0.95 (1)	1.00 (1)	2.11 (2)
Met	0.40 (1)		0.77 (1)		1.70 (2)	1.99 (1)		2.03 (2)		0.90 (1)	
Ile	2.00 (2)	0.82 (1)		1.87 (4)[a]	0.68 (1)	1.00 (1)	0.15 (1)	0.44 (1)	3.00 (3)		1.82 (2)
Leu	6.15 (6)	4.98 (5)		5.85 (10)	1.82 (2)		2.80 (3)	3.02 (3)	6.23 (6)	2.44 (2)	2.92 (3)
Tyr			1.03 (1)	0.95 (1)		1.93 (1)		1.00 (1)	1.74 (2)	1.00 (1)	
Phe	3.10 (3)	0.96 (1)		2.10 (4)			3.00 (3)	1.59 (2)	2.92 (3)	1.95 (2)	1.00 (1)
Trp		— (1)	0.43 (1)				0.93 (4)	1.45 (4)	— (2)	0.44 (1)	
Lys						1.12 (1)	0.99 (1)				
His			1.47 (1)							0.86 (1)	
Arg	0.94 (1)		0.85 (1)	2.25 (2)	1.00 (1)		1.19 (1)			2.30 (2)	0.78 (1)
Solubility	−	−	+	−	+	+	−	−	−	−	+

a) Low recovery was due to incomplete hydrolysis.

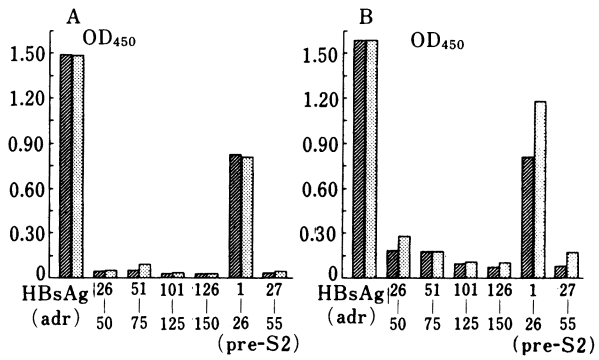

Fig. 2. ELISA of Synthetic Peptides

Each sample was assayed twice. ▨, first; ▩, second. A, conjugate: monoclonal antibody (mouse). B, conjugate: polyclonal antibody (guinea pig).

shown in Fig. 3. In these three cases, the maximal responses were observed after 5 weeks' immunization and the titers decreased slowly during the following two weeks.

It was found in the present systematic syntheses of HBsAg and its pre-S2 gene peptide fragments, that most of the segments are poorly soluble in water, and unexpectedly even in DMF. The surface protein produced by human hepatitis-B virus is indeed a unique molecule, as regards its solubility. We plotted the average hydrophilicity value (A) for each fragment [A = sum of hydrophilicity value of constituent amino acid residues/residue number]. As shown in Fig. 4, the above three fragments with less hydrophobic, but not entirely hydrophilic nature, induced relatively higher immune responses. The above *in vivo* assay results seem to be consistent with earlier observations[12] based on the Hopp and Woods profile.[13] It seems noteworthy that one fragment related to pre-S2 gene, No. 10 (corresponding to the peptide in ref. 14) was immunologically active in both *in vitro* and *in vivo* assays and produced a higher

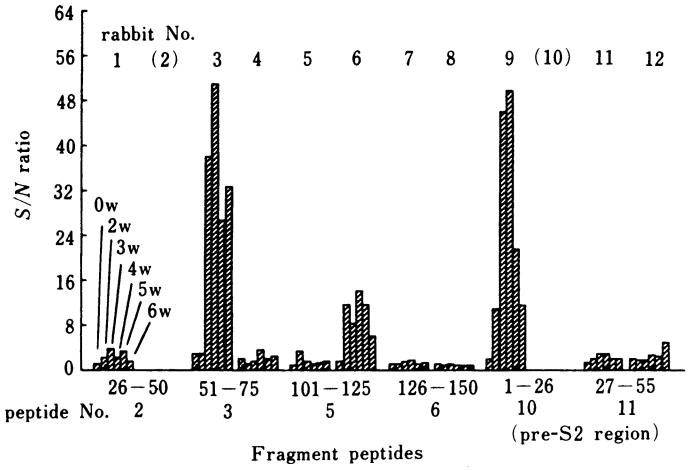

Fig. 3. *In Vivo* Assay of Synthetic Peptides

Two rabbits were used for each sample. No. 2 and No. 10 rabbits died.

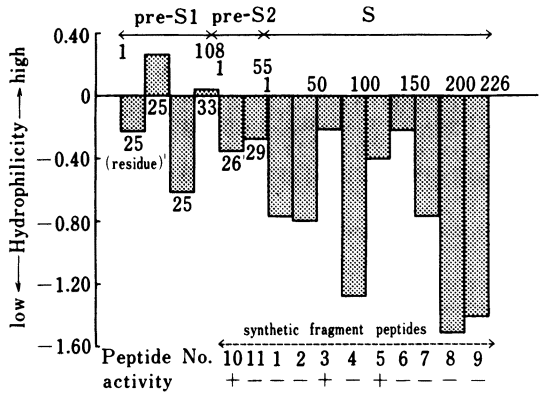

Fig. 4. Average Hydrophilicity Values of Synthetic peptide Fragments

immune response than the other pre-S2 gene fragment, No. 11, though their average hydrophilicity values are nearly the same.

The immune responses we observed here were not longlasting ones. Our fragment peptides tested are too small to be involved in helper function[31] for antibody synthesis. Thus, we preliminarily conjugated the above four fragments, Nos. 2, 3, 6, and 10, onto the reduced form of bovine serum albumin (BSA) with the aid of 2,4-dinitrophenyl-*p*-(β-nitrovinyl)benzoate.[32] The BSA cojugate thus obtained (approximately 2.4 molecules each per BSA) were tested *in vivo*, but the titer we obtained was nearly equivalent to that produced by No. 10 alone. Too small quantities attached to BSA might be the reason for such a poor response. From a safety viewpoint, it would be essential to find non-toxic carriers, instead of BSA, for production of synthetic vaccines. We intend to concentrate our efforts along this line.

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References and Notes

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