Demonstration of the Immunogenicity of Hepatitis B Core Antigen in a Hepatitis B e Antigen Polypeptide (P19)

By K. TAKAHASHI, M. IMAI, M. NOMURA, A. OINUMA, A. MACHIDA, G. FUNATSU, Y. MIYAKAWA AND M. MAYUMI*

Immunology Division, Jichi Medical School, Hepatitis Division, The Tokyo Metropolitan Institute of Medical Science, Faculty of Agriculture, Kyushu University, and the Third Department of Internal Medicine, University of Tokyo, Japan

(Accepted 21 July 1981)

SUMMARY

The core of Dane particles, the presently accepted hepatitis B virus nucleocapsid, contains two polypeptides (P19 and P45) with the antigenicity of hepatitis B e antigen (HBeAg). The antigenicity of hepatitis B core antigen (HBcAg) was not detectable in either of them by the conventional in vitro assay methods, despite the fact that both of these polypeptides were derived from the core of Dane particles. When a rabbit had been immunized with the purified preparation of P19 emulsified in complete Freund's adjuvant, however, humoral antibody against HBcAg was produced in addition to the antibody against HBeAg. Amino acid analysis of P19 disclosed a high content of arginine (12.9%), leucine (11.9%), serine (10.3%) and proline (10.2%). The amino acid composition of P19 was found to be strikingly similar to the composition of the 183 amino acid sequence deduced from the sequence of hepatitis B virus DNA which has been presumed to be encoding HBcAg. We conclude that both HBeAg and HBcAg are antigenic determinants borne by the major polypeptide (P19) constituting the core of Dane particles.

INTRODUCTION

To date, three different antigens have been identified which occur in the serum of persons infected with hepatitis B virus (HBV). They are hepatitis B surface antigen (HBsAg), hepatitis B core antigen (HBcAg) and hepatitis B e antigen (HBeAg) (Blumberg et al., 1965; Almeida et al., 1971; Magnius & Espmark 1972). HBsAg is borne by 20 nm spherical and tubular particles, and constitutes the outer coat of Dane particles, the presently accepted HBV (Dane et al., 1970). HBcAg exists on the core of Dane particles, and HBeAg is a protein separate from the other particulate antigens. The presence of HBeAg in the serum correlates with HBcAg as an antigenic marker of Dane particles (Takahashi et al., 1976a), and a high reactivity for HBsAg-associated DNA polymerase which is contained in the core of Dane particles (Nordenfelt & Kjellen, 1975; Imai et al., 1976). Indeed, such observations have provided a solid basis for the correlation of HBeAg and infectivity for hepatitis B both in perinatal and needlestick exposure cases (Okada et al., 1976; Alter et al., 1976).

The association of HBeAg and Dane particles was substantiated more convincingly by the recent demonstration of two polypeptides with the antigenicity of HBeAg (P19 and P45) derived from the core of Dane particles (Takahashi et al., 1979) and also from the core particles in the nucleus of infected hepatocytes (Yoshizawa et al., 1979). Despite an intimate association of HBeAg and HBcAg, the antigenicity of HBcAg was not demonstrable on either of these polypeptides by the conventional in vitro assay methods.
The present paper gives an account of the relationship of HBeAg and HBcAg by demonstrating the immunogenicity of HBcAg in P19. The relation of HBcAg and HBeAg was further strengthened by the finding that the amino acid composition of P19 closely resembled that of the 183 amino acid residues deduced from the nucleotide sequence on HBV DNA presumed to be encoding HBcAg (Pasek et al., 1979).

METHODS

Serological tests. HBcAg and antibody to HBcAg (anti-HBc) were determined by immune adherence haemagglutination (IAHA) method (Tsuda et al., 1975). HBeAg and corresponding antibody, anti-HBe, were tested by passive haemagglutination (PHA) method (Takahashi et al., 1977). Antibodies were also tested by immunodiffusion in a gel consisting of 0.9% agarose dissolved in a solution containing 0.01 M-tris, 0.1 M-NaCl, 2% dextran T-500, 2% polyethylene glycol 6000, 0.1% NaN₃, and 0.074% EDTA-Na₂ which was adjusted to pH 7.6 by adding NaOH.

Isolation of hepatitis B core particles. Dane particles were purified from 30 l of plasma containing HBsAg of a subtype adr and HBeAg by the method described previously (Takahashi et al., 1976b). In essence, Dane particles were isolated by a succession of steps involving pelleting, floating centrifugation and rate-zonal centrifugation. Purified Dane particles were incubated in the presence of 1% Nonidet P40 and 0.1% 2-mercaptoethanol at 37 °C for 2 h, and their cores were exposed. The preparation of core particles thus obtained (3 ml) showed a HBcAg IAHA titre of 212.

Purification of P19. Cores of Dane particles were incubated in the presence of 1% 2-mercaptoethanol and 1% SDS at 37 °C for 2 h in order to break them into constituent polypeptides, P19 and P45. As shown in Fig. 1, P19 was isolated by polyacrylamide gel electrophoresis in a Hoefer SE 500 apparatus (Hoeffer Scientific Instruments, San Francisco, Ca., U.S.A.) between two glass plates (120 × 140 mm) standing vertically 1.5 mm apart, following the method described elsewhere (Takahashi et al., 1979). Thus, 3 ml of a solution of P19 with an A₂₈₀ value of 0.4 and a HBeAg titre of 210 were obtained.

Immunization. A male white rabbit weighing 2.5 kg was inoculated with 1 ml of the preparation of P19 emulsified in 2 ml complete Freund’s adjuvant in hind footpads and intracutaneously. The rabbit was boosted with the same amount of similarly treated P19 2 weeks later. The serum was harvested 2 and 3 weeks after the last inoculation and tested for anti-HBc and anti-HBe by haemagglutination methods.

Amino acid analysis. A preparation of P19 was freed of NaCl, SDS and glycine by gel filtration on Sephadex G-25, and an amount corresponding to an A₂₈₀ value of 0.3 was lyophilized. The sample was treated with 0.05% 2-mercaptoethanol and 6 M-HCl at 105 °C for 24 h in a depressurized condition, and then subjected to analysis in a Nihon Denshi JLC-6AH autoaminoanalyser. Cysteine and tryptophan were not measurable by this method.

RESULTS

Immunogenicity of P19

A rabbit immunized against P19 raised both anti-HBc and anti-HBe with haemagglutination titres of 2⁵ and 2⁶ at 2 weeks after the last inoculation, and the titres increased up to 2¹¹ and 2¹⁰ respectively, after an additional week. When the rabbit antisera was tested by Ouchterlony immunodiffusion, the presence of both anti-HBc and anti-HBe was clearly visualized (Fig. 2).
P19

**Fig. 1.** SDS–polyacrylamide gel electrophoresis of polypeptides derived from cores of Dane particles. When cores of Dane particles were treated with 2-mercaptoethanol and SDS, two polypeptides, P19 and P45, were liberated (a). P19 was separated from P45 by electrophoresis (b), and used for immunization and amino acid analysis.

**Fig. 2.** Ouchterlony double-immunodiffusion analysis of a rabbit antiserum raised against P19. (a) Rabbit anti-P19; (b) HBsAg-positive serum (anti-HBc and anti-HBe also present); (c) HBsAg-positive serum (anti-HBc and HBeAg also present); (d) purified hepatitis B core particles. Two precipitin lines are seen which can be identified as HBeAg line [between (a) and (c), also (b) and (c)], and HBcAg line [between (a) and (d), also (c) and (d)].

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<th>Mol. %</th>
<th>Moles</th>
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* Amino acid composition of HBeAg polypeptide deduced from the nucleotide sequence described by Pasek et al. (1979).
† Molecular number of lysine was estimated at 2 in P19.
‡ Cysteine and tryptophan were not measurable by the method employed, and were assumed to be equal in number to those in the HBcAg polypeptide deduced from HBV DNA.
Amino acid composition of P19

Amino acid composition of P19 is given in Table 1. P19 was found to be enriched in respect of arginine (12.9%), leucine (11.9%), serine (10.3%) and proline (10.2%). Using the amino acid composition of P19 as a guide, we looked for a nucleotide sequence along HBV DNA which would produce a polypeptide with composition and size comparable to those of P19. We found that the gene presumed by Pasek *et al.* (1979) to be encoding HBcAg would produce a polypeptide strikingly similar to P19. The amino acid composition of the putative HBcAg polypeptide is listed for comparison in Table 1. The molecular size of the HBcAg polypeptide deduced from the 549 base pairs coding 183 amino acids was calculated to be 21042, considerably greater than the molecular size of P19 (19000) estimated from its migration position on SDS–polyacrylamide gel electrophoresis (Takahashi *et al.*, 1979).

**DISCUSSION**

It has been deduced that both HBcAg and HBeAg should be contained in the core of Dane particles, because a rabbit, when immunized with purified Dane particles, raised anti-HBe in addition to anti-HBs and anti-HBc (Takahashi *et al.*, 1980). When the core of Dane particles was treated with 2-mercaptoethanol and SDS, two major polypeptides designated P19 and P45 were obtained, both of which had high HBcAg activity (Takahashi *et al.*, 1979). The antigenicity of HBcAg was not detectable, however, on either P19 or P45 by the conventional *in vitro* assay methods, and any further approach to locate HBeAg and HBcAg on the same molecular entity did not seem to be possible in spite of the close interrelation of these two antigenic activities.

In the present paper, we report finding anti-HBc in the serum of a rabbit immunized against P19, thereby demonstrating the association of HBcAg and HBeAg with P19. A polypeptide from the core of Dane particles with a molecular size of about 19000 has been repeatedly reported (Budkowska *et al.*, 1977; Hruska & Robinson 1977; Neurath *et al.*, 1978). Antigenicity and immunogenicity of such a polypeptide were not described, however. The observation of Hruska & Robinson (1977), who inoculated animals with their core polypeptides without any anti-HBc response, stands at substantial variance with the present results. Our rabbit, when immunized with P19 emulsified in complete Freund's adjuvant, raised anti-HBc with a titre comparable to that of anti-HBe, albeit P19 did not show any activity of HBcAg detectable by *in vitro* assay methods (Takahashi *et al.*, 1979; Yoshizawa *et al.*, 1979). It is not clear why neither P19 nor P45 showed antigenicity of HBcAg by *in vitro* methods. It can be speculated that both of these polypeptides might bear only one HBcAg determinant which would escape detection by the conventional assay methods that required the availability of more than two antigenic determinants on the molecule to be tested. Alternatively, the denaturation of core protein during the preparation of P19 and P45 might have preferentially affected the expression of HBcAg on these polypeptides.

Application of the recombinant gene technology has shown that the DNA of HBV is composed of 2654 base pairs (Pasek *et al.*, 1979). The nucleotide sequence encoding the major polypeptide of HBsAg (Peterson *et al.*, 1978) was successfully identified on HBV DNA which consisted of 226 amino acids with a molecular size of 25398 (Valenzuela *et al.*, 1979). Another HBV-related polypeptide bearing HBcAg activity was produced by *Escherichia coli* containing recombinant DNA. Without any solid evidence, this polypeptide was presumed to be coded by a 546 nucleotide sequence which would produce a molecule composed of 183 amino acids with a molecular size of 21042 (Pasek *et al.*, 1979). No attempts have been made to locate HBeAg on HBV DNA.

When we looked along HBV DNA for a polypeptide with molecular size and amino acid composition comparable with P19, we found that the putative HBcAg polypeptide of Pasek *et al.* (1979) would produce a polypeptide strikingly similar to P19. The molecular size of P19
HBeAg polypeptide with HBc immunogenicity

calculated on the basis of its amino acid composition assuming the content of lysine to be 2 molecules and those of cysteine and tryptophan to be 4 molecules each, as in the case of the putative HBeAg polypeptide, was 20079 composed of 177 amino acids. In view of the close similarity of size and composition, there seems to remain little doubt about P19 being identical to the putative HBeAg polypeptide of Pasek et al. (1979). Although the calculated molecular size of P19 turned out to be somewhat greater than 19000 estimated from its migration position on SDS–polyacrylamide gel electrophoresis, such a discrepancy is not unprecedented. The major polypeptide of HBsAg (P22) was initially estimated to have a molecular size of 22000, and proved to be a polypeptide of 25398 molecular size on the basis of the nucleotide sequence encoding it (Valenzuela et al., 1979).

It is not yet certain how P19 is aligned in the core of Dane particles. Since the intact core exhibits the antigenicity of HBeAg, but not of HBeAg, it can be reasonably assumed that P19 would be assembled so as to bring its portion with HBeAg activity onto the surface and that with HBeAg activity inside. Pinpointing the antigenicity of HBcAg and HBeAg on P19 will add more to the understanding of chemical structure of the core of Dane particles.

To this end, we have purified HBeAg from the serum of symptom-free carriers of HBV and isolated a polypeptide bearing the antigenicity of HBeAg (Takahashi et al., 1980). The molecular size of this polypeptide was estimated to be 15500 by SDS–polyacrylamide gel electrophoretic analysis. The core of Dane particles, when peeled off its surface by treatment with Pronase and then disruption by 2-mercaptopethanol and SDS, also gave a polypeptide of 15500 mol. size. We suggest that P19 loses its HBcAg portion to appear into the circulation as P15.5. If such were the case, the amino acids representing HBcAg would be readily identified by subtracting the amino acids composing P15.5 from those composing P19, and looking for the nucleotide sequence on HBV DNA that would be able to account for this difference.

We thank the Japanese Red Cross Blood Center for Blood units of symptom-free carriers of HBV. This work was supported in part by grants from the Japanese Ministry of Health and Welfare and Tokyo Metropolitan Government.

REFERENCES


(Received 1 May 1981)