RECOMBINANT VACCINIA VIRUS EXPRESSING PRE-S/S PROTEIN OF DUCK HEPATITIS B VIRUS AND ITS PRELIMINARY USE FOR TREATMENT OF PERSISTENT INFECTION

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Summary. – The envelope (Pre-S/S) gene of duck hepatitis B virus (DHBV) was amplified by polymerase chain reaction (PCR) and cloned into plasmid pGJP5, under the control of vaccinia virus promoter $P_{7.5}$. By recombination in cell culture, and screened in human TK$^-$ 143 cells in the presence of 5-bromouracil deoxyriboside (5-BUdR), a recombinant vaccinia virus, bearing the envelope gene of DHBV (pGDHBV-5) which could replicate in cell cultures was constructed. DHBV surface antigen (DHBsAg) was detected in pGDHBV-5-infected cell lysate by dot enzyme immunoassay (EIA). After multiple-site intradermal injections of pGDHBV-5, DHBsAg could be detected in the serum of immunized adult ducks. This indicated that the recombinant virus replicated and expresed DHBsAg in ducks. The recombinant virus was used as a therapeutic vaccine to immunize persistently DHBV-infected ducks. After immunization, a transient significant decrease of serum DHBsAg was observed.

Key words: duck hepatitis B virus; envelope protein; recombinant vaccinia virus; vaccine; persistent infection

Introduction

DHBV belongs to the *Hepadnaviridae* family, in which human hepatitis B virus (HBV) is the prototype. One of the characteristics of hepadnavirus infection is that viruses tend to persist, especially when hosts were infected at an early age. Marion (1988) has reviewed the use of animal models for studies of HBV, and we (Wen *et al.*, 1996) have recently reviewed the use of DHBV-infected animal models for experimental study of HBV therapeutic vaccines. In previous studies, we have used synthetic peptide mimicking the Pre-S epitope of DHBV cross-linked to tetanus toxoid as therapeutic vaccine (Wen *et al.*, 1992), and we have shown that solid matrix-anti-DHBs-DHBsAg complex was effective for clearance of DHBV DNA and DHBsAg in persistently infected ducks (Wen *et al.*, 1994). In this study, we present data on construction of recombinant vaccinia virus harbouring DHBV Pre-S/S gene, which could express DHBV Pre-S/S protein in ducks. Preliminary result of using this recombinant virus as a live therapeutic vaccine to treat persistently DHBV-infected ducks is presented.

Materials and Methods

Virus, antiserum, plasmids and bacterium. DHBV 5.2, a plasmid containing a cloned head-to-tail dimer of DHBV DNA, was kindly provided by Dr. W. Mason. Rabbit anti-DHB serum was raised in our laboratory and its titer assayed by ELISA was 1:600. *E. coli* (HB101) and vaccinia virus (Tiantan strain, provided by National Institute of Biological Products, Beijing) were used for transfection and infection, respectively. Plasmid pGJP-5,

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Abbreviations: 5-BUdR = 5-bromouracil deoxyriboside; DHBV = duck hepatitis B virus; DHBsAg = DHBV surface antigen; EIA = enzyme immunoassay; DMEM = Dulbecco's modification of Eagle's Medium; HBV = human hepatitis B virus; PCR = polymerase chain reaction

constructed by Wu *et al.* (1987) and containing a polylinker, the 7.5 K promoter and the thymidine kinase gene of vaccinia virus, was employed.

Cell culture. Vero cells were used for propagation of vaccinia virus. The culture medium used was the Dulbeco's modification of Eagle's Medium (DMEM) with 10% of foetal calf serum. Human TK⁻ 143 cells (kindly provided by Dr. B. Moss) were used for screening of recombinant vaccinia virus. The culture medium used was DMEM with 5% of foetal calf serum and 50 μg/ml 5-BUdR.

PCR amplification of DHBV Pre-S/S gene. A primer pair was used for amplification of DHBV Pre-S/S gene. Sense primer: nt 781-803, 5'-CAATCAAGAGCTCATTTATGATG-3' (for cloning purpose, the original sequence 5'-CAATCAACATCACATTTATGATG-3' was changed in 3 nucleotides as underlined); antisense primer: nt 1807-1787, 5'-GTCAAAGTGAATTCTTATTCC-3'. Purified DHBV 5.2 DNA was used as template and PCR was caried out in 30 cycles (94°C for 1 min, 37°C for 2 mins, 72°C for 3 mins with extension at 72°C for 10 mins). The amplified fragment was shown as a 1012 bp band by agarose gel electrophoresis.

Construction of recombinant plasmid DHBV Pre-S/S. The amplified DHBV Pre-S/S gene fragment was cloned into vector pGJP-5 at the *Eco*RI and *Sac*I restriction sites (Fig. 1). After

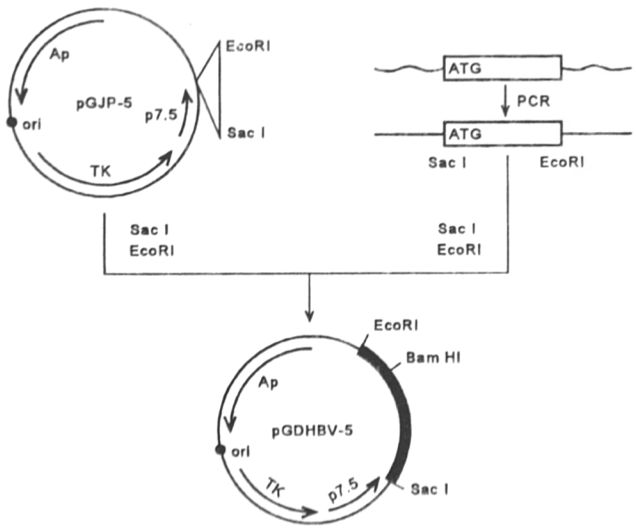

Fig. 1
Diagram of construction of pGDHBV-5

ligation and transformation of *E. coli* HB101, recombinant clones with insert of DHBV Pre-S/S were screened and identified. One clone (pGDHBV-5) was used for construction of recombinant vaccinia virus (Maniatis *et al.*, 1982).

In vivo construction and screening of recombinant vaccinia virus. Vero cells were first infected with vaccinia virus (2 hrs) and then transfected with purified pGHBV-5 DNA (48 hrs) with the aim to produce a recombinant vaccinia virus. TK⁻ recombinant vaccinia virus plaques were screened and picked under the selection of 5-BUdR (Weir *et al.*, 1982).

Persistent infection with DHBV. One-day-old ducklings were experimentally infected intraperitoneally with 100 μl of DHBV-positive serum and persistent DHBV infection was identified by assaying both DHBV DNA and DHBsAg in their serum for at last 4 weeks after infection (Wen *et al.*, 1994).

Dot enzyme immunoassay (Dot-EIA) of DHBsAg. As reported by Zhang *et al.* (1988), 5 μl of sera was spotted on nitrocellulose filters, fixed, incubated with rabbit anti-DHB serum, and detected with peroxidase-labelled *Staphylococcus aureus* protein A or by the ECL system (Amersham).

Results

Construction of recombinant vaccinia virus bearing DHBV Pre-S/S gene

After infection with vaccinia virus and transfection with purified pDHBV-5 plasmid DNA, Vero cells were further incubated for 48 hrs and the TK⁻ recombinant vaccinia virus was selected under the pressure of 50 μg/ml 5-BUdR. Ten plaques were picked and each was separately inoculated onto human TK⁻ 143 cells. The supernatant and cell lysates of all virus recombinants were assayed, among which three were DHBsAg-positive, but DHBsAg could not be detected in the supernatant. Two recombinants were subjected to a second round of plaque purification, where the infected cell lysate of one recombinant showed a stronger DHBsAg expression. This virus recombinant was designated pGDHBV-5 recombinant vaccinia virus and was used in further studies.

Expression of DHBV Pre-S/S antigen by pGDHBV-5 recombinant vaccinia virus in ducks

Two healthy four-week-old ducks were inoculated each with 0.5 ml of the recombinant vaccinia virus at a concentration of 5×10^7 PFU/ml. The recombinant virus was inoculated intradermally at 4 sites on the back of the animals and re-inoculated after 4 weeks. Serum samples were collected and assayed for DHBsAg after 4 and 8 weeks, respectively. The DHBsAg expression in one inoculated duck serum is shown in Fig. 2.

Immunization of persistently DHBV-infected ducks with pGDBHV-5 recombinant vaccinia virus

Sixteen persistently DHBV-infected four-week-old ducks were divided into 3 groups. Group A (6 ducks) was immunized with pGDHBV-5 recombinant vaccinia virus (2.5×10^7 PFU per duck); group B (6 ducks) was immunized with vaccinia virus (2.5×10^7 PFU per duck); group

C (4 ducks) was immunized with phosphate-buffered saline. The immunization was intradermal as described above. No change in DBHsAg in group C was observed during after the immunization. The results with groups A and B are listed in Table 1. Immediately after each immunization with the recombinant virus, serum DHBsAg level increased due to expression of DHBsAg by the recombinant virus. However, significant drop of serum DHBsAg level was seen 6 weeks after the immunization in majority of persistently infected ducks. This decrease of DHBsAg was not long lasting.

Discussion

Ducks have been extensively used as an animal model for HBV, because they are easy to handle, inexpensive and can be used in large numbers. Infection of newly hatched

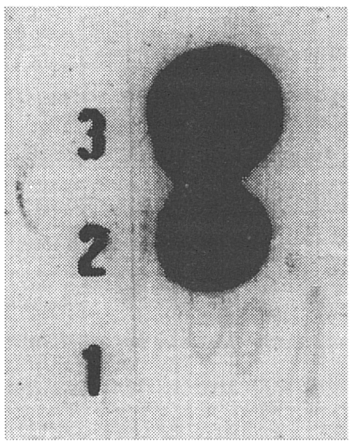

Fig. 2
Expression of DHBsAg by pGDHBV-5 recombinant vaccinia virus in ducks
Five µl of duck serum was spotted onto nitrocellulose filter and reacted with rabbit anti-DHBs serum followed by dot-EIA for detection of DHBsAg. (1) Duck serum before immunization; (2) Duck serum collected at 4 weeks after the first immunization with pGDHBV-5; (3) Duck serum collected at 8 weeks after the first immunization with pGDHBV-5 (this duck was re-immunized with pGDHBV-5 after 4 weeks).

Table 1. Serum DHBsAg in persistently infected ducks after immunization with recombinant vaccinia virus and vaccinia virus

	Serum DHBsAg[a]											
	pGHBV-5[b]						vaccinia[c]					
Duck No.	1	2	3	4	5	6	7	8	9	10	11	12
Before immunization	++	++	++	++	++	++	++	++	++	++	++	++
4 weeks[d]	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	++
8 weeks[d]	+++	±	–	–	–	–	+	±	±	++	++	++
12 weeks[d]	+++	+++	+++	+++	+++	+++	+++	++	++	++	+++	++
16 weeks[d]	±	–	–	+	++	–	–	++	+	++	+++	+
19 weeks[d]	++	+++	+++	++	++	++	ND	ND	ND	ND	ND	ND

[a]Dot EIA: (+++) very strongly stained spot; (++) strongly stained spot; (+) faintly stained spot; (±) spot barely seen; (–) no staining.
[b]Ducks immunized with pGDHBV-5 recombinant vaccinia virus.
[c]Ducks immunized with vaccinia virus.
[d]Serum samples collected at the indicated weeks after the first immunization. On the whole, ducks were immunized twice (at the first and eight week).
ND = not done.

ducklings simulates perinatal infection of humans, and the persistence of DHBV in ducks makes it an excellent model for studying therapeutic vaccines for hepadnaviruses. Smith *et al.* (1983) reported expression of HBsAg gene in recombinant vaccinia virus, and Wang *et al.* (1986, 1990) successfully used this system to express different constructs of HBV envelope genes. Since a modified recombinant vaccinia virus has the potential of developing into a live vaccine, it may also be used for therapeutic purposes. In this study, we successfully constructed a recombinant vaccinia virus (pGDHBV-5) expressing the envelope proteins of DHBV, and for the first time we showed that this recombinant virus expressed DHBsAg, which was detected in duck serum for at least 6 weeks. Since the expression of DHBsAg is related to replication of the recombinant virus, it is presumed that pGDHBV-5 could replicate probably to a limited extent, in ducks. A future study of replication of pGDHBV-5 vaccinia virus in avian cell cultures and a long follow-up study of virus replication in ducks will reveal the biological characteristics of this recombinant virus.

Preliminary immunization study of persistently DHB V-infected ducks showed that non-specific immunization with vaccinia virus led to slight decrease of serum DHBsAg. Immunization with the recombinant vaccinia virus resulted in significant decrease of serum DHBsAg. However, this effect was only transient, as after 11 weeks, persistently-infected ducks had again a high level of serum DHBsAg. The transient dcrease of serum DHBsAg could be due to the neutralizing effect of anti-DHBs serum induced in the immunized ducks. However, due to the presence of serum DHBsAg, the antibody response could not be detected. Another possibility is that the recombinant vaccinia virus presented DHBsAg through an altered pathway to the antigen-presenting cells of the DHBV-infected hosts, resulting in cell-mediated immune response and secretion of cytokines, which down-regulated the expression of viral antigen or inhibited the replication of DHBV. Studies employing this recombinant virus will provide interesting information on use of a live therapeutic virus vaccine for treatment of viral persistent infection.

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