Protection of Woodchucks from Infection with Woodchuck Hepatitis Virus by Immunization with Recombinant Core Protein

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(Accepted 14 April 1989)

SUMMARY

Woodchucks were immunized with recombinant woodchuck hepatitis virus (WHV) core antigen (WHcAg) to investigate whether such immunization protects against WHV infection. The C gene was cloned into a pUC12 vector and expressed in Escherichia coli. Core particles purified by sucrose and CsCl gradient centrifugation had a buoyant density of 1.37 g/ml which corresponded to the density of WHcAg particles present in chronically infected liver. Two animals immunized with the recombinant antigen developed high antibody titres and were protected against infection after challenge with WHV. The surface antigen (WHsAg) and WHV DNA were not detected in the sera of immunized animals after challenge and these animals did not develop anti-WHs. Three control animals developed a typical WHV infection. The protection from WHV infection may depend not on the presence of antibodies against the core protein but on a cellular immune response to WHcAg.

INTRODUCTION

Vaccination with hepatitis B virus (HBV) surface antigen (HBsAg) or woodchuck hepatitis virus (WHV) surface antigen (WHsAg) protects against HBV or WHV infection (Szmuness et al., 1981; Cote et al., 1986). Antibodies against HBsAg (anti-HBs) neutralize HBV; this was shown by passive immunization with anti-HBs that could prevent infection after exposure to the virus (Beasley et al., 1983). The core protein of HBV (HBcAg) is highly immunogenic producing good cellular and humoral immune responses (Milich & McLachlan, 1986; Milich et al., 1987a, 1988). In addition HBcAg-primed T cells have a helper function, at least in humoral immune responses to envelope proteins of HBV after immunization with the complete virus (Milich et al., 1987b). In the natural course of HBV infection, a T cell response to the core protein follows immediately after a T cell response to preS2 and shortly after antibodies to HBcAg, i.e. anti-HBc of the IgM class can be detected (Vento et al., 1987). Anti-HBc is always present at the onset of disease and reaches a high concentration in sera of patients chronically infected with HBV, but these antibodies do not protect. Anti-HBc passively transmitted to newborns from mothers who are chronic carriers of HBV do not protect these babies from HBV infection (Beasley et al., 1977), indicating that these antibodies do not neutralize the virus.

Murray et al. (1984, 1987) and Iwarson et al. (1985) demonstrated that immunization of chimpanzees with the core protein of HBV provided complete or partial protection from HBV infection. In Iwarson's study, three chimpanzees were completely protected. After challenge with HBV, anti-HBc and antibody to e antigen (anti-HBe) titres increased, but only one animal seroconverted to anti-HBs. In Murray's studies, two of four immunized chimpanzees showed a low level of HBV replication after challenge; HBsAg was detectable in sera for 2 to 3 weeks and these chimpanzees later developed anti-HBs. At present it is not known what the requirements are for complete protection. The incomplete protection may be due to a low immune response in the animals vaccinated without adjuvant.

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This study was carried out to test whether immunization with WHcAg protects woodchucks from WHV infection. We used recombinant WHcAg instead of using the natural form from chronically infected liver. Recombinant WHc protein also assembles to form core particles similar to native core particles, which can be easily purified in large amounts. HBcAg has been shown to be highly immunogenic even without adjuvant (Iwarson et al., 1985) and might be used as an alternative vaccine against HBV infection e.g. in non-responders or low responders to HBsAg. We decided to use an immunization protocol with Freund's adjuvant to achieve a high immune response. Further studies will be carried out with other adjuvants which may be used in humans.

METHODS

Woodchucks. In 1985 a woodchuck colony was established in our laboratory, and breeding began in 1986. Three woodchucks included in our study (MW 301, female; MW 302, male; MW 303, male) were born in our colony at the Gesellschaft für Strahlen und Umweltforschung, Munich, F.R.G. At the beginning of the study, the animals were 10 months old. Two additional control animals (NW 518, NW 519) were trapped in New York State (North Eastern Wildlife, Ithaca, N.Y., U.S.A.).

Expression of WHc antigen in clone pUCWc-XP in Escherichia coli. The WHc gene without its preC region, derived from the clone PW8 (Ogston et al., 1982) was kindly provided by Dr J. Summers, Albuquerque, N.M., U.S.A. and was cloned into a pUC12 vector under the control of a lacUV5 promoter. The WHc gene was cloned into pUC12 at its XhoI site at nucleotide (nt) 2029 and at its PstI site at nt 3051 (Galibert et al., 1982) (Fig. 1). The expressed product of clone pUCWc-XP has a deletion of three amino-terminal amino acids of the native WHc gene and contains nine additional amino acids derived from the β-galactosidase of the vector at its amino terminus. The carboxy terminus of WHcAg is complete. E. coli strain JM109 was transformed with the recombinant plasmid pUCWc-XP and 24 clones were tested by restriction analysis.

After induction of the WHc gene expression with 1 mM-isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h, bacteria in a 1 l culture were pelleted, resuspended in 20 mM-Tris-HCl pH 8.0 and incubated with 70 mM-EDTA and 5 mg/ml lysozyme for 30 min at 37°C. NP40 (0.2%) and NaCl to a final concentration of 150 mM were added and incubation was continued for 30 min. After an ultrasonic treatment of 5 min, the lysate was centrifuged for 1 h, the supernatant was layered onto a two-step sucrose gradient (30 % and 15 % w/v) and centrifuged at 40 000 r.p.m. for 4 h in a TST 41.14 rotor (Kontron). The pellet was resuspended in CsCl (density 1.2 g/ml) and loaded onto a discontinuous CsCl gradient (density 1.25 to 1.4 g/ml). After centrifugation for 70 h at 38 000 r.p.m. in a TST 41.14 rotor, 250 μl fractions were collected. The content of WHcAg was measured by a WHc-ELISA developed in our laboratory (see below).

Immunization protocols. Woodchucks MW 301 and MW 302 were each inoculated subcutaneously (s.c.) four times with 100 μg recombinant WHcAg emulsified in incomplete Freund's adjuvant at days 0, 20, 34 and 70, in four sites. Ten days after the last immunization these and control animals (MW 303, NW 518 and NW 519) were challenged intravenously (i.v.) with 20 μl WHV-positive woodchuck serum from a chronic carrier positive for WHsAg (titre 10−3) and WHV DNA (105 particles). After immunization and challenge, serum samples were taken weekly. To compare the immune response to WHcAg of woodchucks to that of other animals three rabbits were immunized four times [at day 1, day 14 and 43, s.c.; day 54, i.v.] at four sites with recombinant WHcAg (rabbit R1, 10 μg/inoculation; rabbit R2, 5 μg/inoculation; rabbit R3, 100 μg/inoculation). After development of anti-WHc, rabbit R1 was inoculated in addition with WHV-positive woodchuck serum, to investigate whether the amount of WHsAg in this inoculum could stimulate the production of anti-WHs.

Serology
WHsAg, WHcAg and anti-WHs were tested by ELISAs established in our laboratory (Schlipkötter et al., 1989). Anti-WHc was tested by a modified ELISA as described by Ponzetto et al. (1985).

WHcAg ELISA. The wells of microtiter plates were coated with 50 μl of anti-WHs IgG (rabbit) diluted 400-fold in 0.1 M-carbonate buffer pH 9.4, and incubated overnight at 4°C. Wells were washed three times with 0.05% Tween 20 in phosphate-buffered saline (PBS). To block non-specific reactions, 100 μl 1% gelatine and 2% negative woodchuck serum were added to the wells for 2 h at 37°C. The wells were washed again and 50 μl of sera to be tested (10-fold dilution in PBS) were added and incubated at 37°C for 2 h. Plates were washed again and incubated with peroxidase-labelled anti-WHs [100-fold dilution with 2% negative woodchuck serum and 2% bovine serum albumin (BSA)] at 37°C for 2 h. After a final washing, 100 μl of substrate [1 mg/ml o-phenylenediamine, 1 μl/ml H2O2 in 0.1 M-phosphate buffer pH 6.0] was added for 15 min. The reaction was stopped with 100 μl 0.5 M-H2SO4 and the absorbance was measured at 495 nm.

WHcAg ELISA. The wells of microtiter plates were coated with 50 μl of anti-WHc (IgG preparation of serum from a WHV carrier) at a 1000-fold dilution in 0.1 M-carbonate buffer, incubated overnight at 4°C and washed with PBS-Tween 20. Blocking of excess binding sites was carried out as described above. After washing 50 μl
RESULTS

The WHc gene was cloned into a pUC12 vector to express WHcAg in E. coli (Fig. 1). This construct, which was called pUCWc-XP, expressed the WHc-gene as a β-galactosidase fusion protein with nine additional heterologous amino acids. This construct eliminates major structural changes of WHcAg and probably allows the core particle to form. Fig. 2 shows a Western blot and silver staining of a lysate of IPTG-induced bacteria containing pUC12 (lanes 1 and 3 respectively) and the same for a lysate of induced bacteria containing pUCWc-XP (lanes 2 and 4). Incubation of the blotted proteins with rabbit anti-WHc serum reveals a band of 22K expressed in the bacterial clone containing the plasmid pUCWc-XP. The expression of pUCWc-XP was found to be optimal after an induction time of 2 h. For purification of core proteins from E. coli, several different lysing solutions and purification methods (e.g. gel chromatography) were tested. However the best result was achieved by clearing the bacterial lysate by a low-speed centrifugation and loading the supernatant onto a sucrose gradient (15 and 30%) as described above. After centrifugation, a significant amount of WHcAg was in the pellet which suggests that WHcAg forms a particle. These core particles were purified further by CsCl gradient centrifugation, giving fractions which were tested by ELISA and Western blot analysis (Fig. 3). The WHcAg particles from E. coli had a buoyant density of 1.37 g/ml, corresponding to the density of WHcAg particles analysed from chronically infected liver (data not shown). On the top of the gradient there was a second region of WHcAg activity, which may result from soluble WHcAg. The pure WHcAg-positive fractions were pooled (fractions 10 to 14) (Fig. 3), dialysed and stored at -70 °C. The purity of WHcAg was determined by silver staining. A single band of 22K was visible after PAGE and silver staining (Fig. 3), which accounts for 90% of the absorption measured in a densitometer. In the last lane a Western blot of the pooled
Fig. 1. The WHc gene was cloned into the pUC12 vector at its XhoII restriction site at nt 2029. The second insertion site was at PstI at nt 3051. The upper part of the figure shows the open reading frames of the WHV genome.

Fig. 2. The expression of WHcAg in E. coli: Western blot of pUC12, lane 1; Western blot of pUCWc-XP, lane 2; silver staining of pUC12, lane 3; silver staining of pUCWc-XP, lane 4.
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Fig. 3. (a) Determination of WHcAg by ELISA in the CsCl fractions in a 10-fold dilution. The peak fractions were tested at a 1000-fold dilution for a more accurate determination of the peak. CsCl density (A): A_{405} for 10-fold dilution (■); A_{405} for 1000-fold dilution (●). The corresponding silver staining of the peak fractions is given in (b). In the last lane a Western blot with anti-WHc of the pooled fractions (10 to 14) is shown.

fractions with anti-WHc is shown. Approximately 1 mg of purified core protein was obtained from the 1 l culture, as determined by a protein assay (Bio-Rad).

Two woodchucks (MW 301 and MW 302) without markers of previous WHV infection were immunized four times (at days 0, 20, 34 and 70) with the recombinant core protein of WHV in incomplete Freund’s adjuvant (Fig. 4); both animals developed anti-WHc after the first immunization. The serum sample drawn at day 20 after the first immunization had an anti-WHc titre of approx. 10^{-3} in both animals; anti-WHc titres increased in MW 301 up to 10^{-5} and in MW 302 to 10^{-4}, 8 days after the third immunization. These titres correspond to those of anti-WHc in chronic carrier woodchucks. The two immunized animals were challenged with 20 μl WHV-containing serum 10 days after the third booster. At the same time three control animals were infected with the same serum. No signs of WHV infection were found in the serum of the immunized animals and WHsAg ELISA and WHV DNA spot hybridization remained negative over 17 weeks. All control animals developed viraemia. Fig. 4 shows representative results from one control animal (MW 303). WHsAg and WHV DNA were detected 17 to 49 and 24 to 40 days
Fig. 4. Determination of WHsAg (■) and anti-WHc (○) ELISA titres for sera of woodchucks MW 301 (a) and MW 302 (b) after immunization and challenge and of woodchuck MW 303 (c) after experimental infection. Arrows indicate times of immunizations and challenge. Panels (d) and (e) show the determination of anti-WHs and WHV DNA respectively for woodchuck MW 303.

post-infection (p.i.) respectively. The first serum positive for anti-WHc was obtained 25 days after inoculation and for anti-WHs was detected first at 85 days p.i. The woodchucks immunized with WHcAg remained anti-WHs-negative (Fig. 4). A biopsy taken at day 44 p.i. at the end of the viraemia of one control animal (WC 303) contained replicative forms of WHV DNA (Fig. 5) but no evidence of replication was found in biopsies of the immunized woodchucks (WC 301 and 302). For comparison with the immune responses of woodchucks, three rabbits immunized with different concentrations of core protein developed anti-WHc after the first immunization with recombinant core protein and titres reached approx. $10^{-3}$ by 21
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Fig. 5. Southern blot of WHV DNA in liver biopsies at day 44 p.i. of immunized woodchucks MW 301 and MW 302, and one control animal, MW 303. Lanes 1, undigested DNA; lanes 2, EcoRI-digested DNA. Molecular size markers are indicated in kb.

days after immunization. In order to investigate whether i.v. inoculation of WHV-positive serum in rabbits stimulates an anti-WHs response, we inoculated rabbit R1 with 20 µl WHV-positive woodchuck serum. Neither WHsAg nor WHV DNA was detectable in the serum but anti-WHs was detected (titre of 10⁻³) 3 weeks after inoculation of WHV.

DISCUSSION

In recent years the immune response to proteins of the nucleocapsid of different viruses have been studied in detail. Cytotoxic T cells against nucleoproteins have been identified, e.g. those of influenza A, human immunodeficiency and rabies viruses (McMichael et al., 1986; Walker et al., 1987; Celis et al., 1988) and they may be important in eliminating virus after infection. Cytotoxic T cells can destroy infected cells expressing viral proteins or short peptides on their cell membranes and the humoral response to envelope proteins then neutralize infectious free virus particles. Complete or partial protection from infection by immunization with core protein has been demonstrated for the rabies virus and HBV. In vaccination studies of rabies virus, it has also been shown that vaccination with the nucleocapsid alone protected against lethal peripheral challenge with homologous or heterologous virus strains (Dietzschold et al., 1987); this indicates that the core protein which has a more conserved amino acid sequence than envelope proteins of different strains may be important for vaccination against viruses with marked heterogeneity of envelope proteins (e.g. human immunodeficiency virus).

In our experiments we demonstrated WHV core protein immunogenicity which protects against challenge with WHV. Expression of WHc in E. coli yielded core proteins of WHV which
formed core particles similar in density to core particles of chronically infected hepatocytes (M. Roggendorf, unpublished results). A similar assembly of HBV core particles has been described previously (Cohen & Richmond, 1982). The core particles from E. coli were easy to purify and are highly immunogenic in rabbits and woodchucks. After one immunization with recombinant WHcAg, the sera of two animals (MW 301 and MW 302) had titres of $10^{-3}$ which increased up to $10^{-5}$ after three immunizations; comparable titres were obtained in rabbits. Similar titres of anti-WHc were found in chronic carriers of WHV. After challenge with WHV, neither of the two immunized woodchucks showed signs of WHV infection and produced no anti-WHs. We included a 'challenge' experiment with a rabbit primed with core proteins; this animal was found positive for anti-WHs, indicating that our test system is sensitive and that the woodchucks most probably did not produce anti-WHs. This interesting finding of possible WHc priming in rabbits will be followed in a separate study. Southern blotting of DNA extracted from biopsies at day 44 after challenge showed the absence of replicative forms of WHV DNA in the immunized animals. Three control animals developed typical WHV infections. The negative results of biopsies from the immunized animals does not completely exclude WHV replication, as it could have occurred at a low level in other parts of the liver.

Immunity to WHV infection independent of anti-WHs can be achieved by immunizing with WHcAg. There are two possible explanations for this protective effect. The first is that HBcAg/HBeAg-specific T helper cells can elicit anti-envelope antibodies that neutralize viruses (Milich et al., 1987b) and this phenomenon could explain the ability of HBc or WHc vaccination to protect against HBV/WHV infection or at least against liver disease. Although our animals did not appear to develop anti-WHs, it is possible that our assay system is not sensitive enough to detect low levels of anti-WHs; in the studies by Iwarson et al. (1985) and Murray et al. (1984, 1987) only three of seven animals seroconverted to anti-WHs after challenge. Another explanation which would need to be proved experimentally is that after immunization with WHcAg, T helper cell activity for cytotoxic T cell precursors is induced. After challenge with WHV some liver cells are infected and express complete core proteins or peptides on their membrane, as described for HBcAg (Uy et al., 1986; Sylvan et al., 1987; Kiyosawa et al., 1988). Primed cytotoxic T cells may recognize these proteins and destroy infected hepatocytes or stop viral replication before maturation of the complete virus, which leads to infection of other hepatocytes. Cytotoxic T cells directed against core protein have been identified in patients with chronic hepatitis B (Mondelli et al., 1982; Eddleston et al., 1983; Naumov et al., 1984). Both of these mechanisms may work together to eliminate WHV at an early phase after infection.

At present, studies are in progress to characterize cytotoxic T cell responses of woodchucks after immunization with core protein using vaccinia virus recombinants in an autologous system.

This study was generously supported by the Deutsche Forschungsgemeinschaft (Ro 687/2-1).

REFERENCES


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(Received 4 January 1989)