Abstract

The woodchuck hepatitis B virus (WHV), the closest genetically related virus of HBV, and its natural host Marmota monax constitute a well recognized animal model. The application of this model for the evaluation of immunogenicity and protection of new formulations of HBV vaccines for human use, for lamivudine-CHO-PreS/S vaccine therapy and WHV particles coupled with HBV derived woodchuck PreS/S antibodies (IC complex) studies, as shown that the PreS/S-CHO vaccine is the first human vaccine able to elicit non sterilizing protection in the woodchuck model. The very early appearance and selection of the domain B FLLA motif resistant mutant not neutralized by the antibodies produced following vaccination, has confirmed that more potent antivirals and/or multiple targeted options with possible inclusion of immune complexes should be considered.

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1. Introduction

Hepatitis B virus (HBV) infection is a relevant health problem worldwide. Estimated infected person are more than 370 million. The availability of efficient antiviral treatment is needed to ameliorate the outcome and to limit the spread of infection. The applied vaccination programs have been shown to be able to induce lower infection rates and prevalence values in the different countries. However the currently applied recombinant HBsAg vaccine has to be administered in a three-dosage schedule. Furthermore low or absence of response may be observed in immunocompromised, older and obese individuals [1,2]. The possibility of emergence of viral escape mutants has also been shown, representing an additional biological limit [3]. The use of immunodominant epitopes combination, of new adjuvants and cytokines for new formulation vaccines represents a major challenge for prevention and for

*Corresponding author. Tel.: +39-06-4990-3233; fax: +39-06-4990-2662
E-mail address: maria.rapicetta@iss.it
potentiate and regulate immunotherapeutic strategies in HBV infections treatments [4]. For such preclinical studies, the woodchuck hepatitis virus (WHV) and its natural host, the Eastern woodchuck (*Marmota monax*), constitute a useful predictive model for the similarities in viral replication and pathogenesis with HBV [5]. Vaccine and immunotherapy studies have been recently performed from our group using this model. This paper reports the comparative results following the administration of HBV vaccines with different antigen and adjuvant compositions. Furthermore an immunotherapeutic approach, derived from HBsAg vaccination, was evaluated for antiviral activity in naturally infected WHV chronic carrier woodchucks.

### 2. Animals and methods

#### 2.1. Woodchucks and sera

The studies were conducted in adult woodchucks, trapped in the mid-Atlantic region of the United States and purchased from International Animal Exchange Inc. (Ferndale, MI). The animals received human care in compliance with the guidelines of the Italian Ministry of Health and were fed with aflatoxin-free laboratory rabbit chow until death. They were maintained in isolation in the animal facility at the *Istituto Superiore di Sanità* (the Italian National Institute of Health) in Rome. The blood samples containing ethylenediaminetetraacetic acid (EDTA) at a final concentration of 10 IU/ml were centrifuged at 1500 rpm for 10 min. Plasma was recovered and stored at −80°C.

#### 2.2. Vaccines, antivirals, Immunocomplex

The used vaccines include CHO-HBsAg (preS1 and preS2 full sequences) without adjuvants and RC529 adjuvanted virosomal S/Core proteins, were kindly provided by Berna Biotech of Bern, Switzerland. Lamivudine drug was provided by GlaxoWellcome Inc. The Immunocomplex (IC) was constituted by pooled sera derived from two CHO-PreS/S vaccinated woodchucks (anti-HBs titre 2.5 mIU) mixed with WHV (10⁷ WHV-DNA copies) and incubated 1 h at 37°C.

#### 2.3. Serological assays

Anti-HBs antibodies were detected using an immuno-assay ELISA AUSAB (Abbott Laboratories 100, Abbott Park Road, Abbott Park, Illinois). Serum antibodies to WHV core antigen (anti-WHc), antibodies to WHV “e” antigen (anti-WHe) and WHV “e” antigen (WHeAg) were detected using electro-chemo-luminescence immuno-assays (Elecsys, Roche Molecular diagnostics) [6].

#### 2.4. WHV-DNA quantification and sequencing

WHV genome copies were determined using a TaqMan Real-Time PCR. Applied probes and primers and method were previously described [7]. The sensitivity of the Real-Time PCR was 50 copies per milliliter (copies/ml). Sequence analysis was carried out with ABI prism Dye terminator sequencing kit and ABI prism 7000 sequence detection system (Applied Biosystems).

### 3. Results

#### 3.1. Immunogenicity of PreS/S and S/Core HBV vaccines and protection level after WHV challenge

The comparison between CHO derived PreS/S virosomal HBV and of RC529 adjuvanted S/Core vaccination is reported in Fig. 1. The kinetics of antibody response following the administration, of 4-5 injections of vaccines and
after the intravenous inoculation of challenge WHV (w197) in vaccinated woodchucks is shown. It could be observed that sustained anti-HBs response was present soon after the administration of the first dose for CHO-PreS/S vaccine and after 2-4 injections for S/Core vaccine. Anti-HBs titres were higher after challenge, in the group of PreS/S vaccinated woodchucks in comparison with S/Core vaccinated, which, however, maintained sustained anti-Core response.

Fig. 1. Kinetics of antibody response in vaccinated woodchucks. Arrows indicate intramuscular vaccine administrations (respectively 50 µg and 20 µg antigen content). Virus challenge (Ch) was performed with w197 inoculum containing $10^8$ g eq/dose.

Fig. 2 reports the viral and antibody markers, present in both groups, after WHV challenge. The anti-HBe response was observed earlier in S/Core vaccinated animals. It was also observed before the viral challenge. This could be explained by the presence of small amounts of truncated forms of Core antigen that were able to elicit anti-HBe response. In CHO-PreS/S vaccinated animals no signs of viral markers WHeAg and WHV-DNA were observed. In the contrast, in S/Core vaccinated woodchuck a lower protection level was present. HBeAg was detected in one out of three animal and WHV-DNA in all animal, however at titres up to up to 5 logs lower in respect to the control.
Fig. 2. WHV-DNA copies, WHAg and antibody markers in vaccinated woodchucks after viral challenge.

3.2. Therapeutic studies: lamivudine/PreS/S vaccine and PreS/S Ab/WHV immunocomplex (IC) administration in WHV chronic carrier woodchucks

In Fig. 3 are reported the obtained results in terms of HBeAg and WHV-DNA in the animal groups treated with lamivudine/CHO-PreS/S vaccine (panel A) and immunocomplex (IC). In the panel A, two log decrease in titre of WHV-DNA was observed in the first 2 weeks of lamivudine/CHO-PreS/S vaccine. Early detection of resistant mutants FLLA motif A566T in B domain at week 2 was followed by the increase of viremia (see exemplified animal in Fig. 3). In the panel B is shown the level (copies/ml) of WHAg and WHV-DNA in WHV chronic carrier woodchucks administered with an immunocomplex formed by viral particle and anti-PreS/S antibodies. The preliminary results clearly indicate the reduction of viral replication following the administration of the immunocomplex as shown by the 4-5 log WHV-DNA decrease in titre and seroconversion to anti WHAg.
Fig. 3. Panel A. Lamivudine PreS/S vaccine administration in woodchuck w2988. WHV-DNA kinetics and humoral response (anti-HBs measurements). Top figure: lamivudine was orally administered dissolved in orange juice, at daily dose of 100 mg/kg body weight, for 40 weeks. Panel B. Immunogenic complex (IC) administration in WHV chronically infected woodchucks. IC was intravenously administered. Serum aliquot containing 2.5 mIU anti-HBs was mixed with equal aliquots of serum containing 10^7 WHV-DNA/50 µl incubated 1 h at 37°C before injection.

4. Discussion

The research efforts on HBV vaccines are particularly focused on the improvement of the immunogenicity properties to avoid the possible occurrence of low response and to shorten the injection schedule. This is of particular relevance for immunization campaigns in low developed countries.

This paper reports the results of vaccination studies performed with the application of WHV/Marmota monax preclinical model. The comparison of data from the application of different formulations of HBV vaccines for human use has shown the usefulness of the model for the evaluation of heterologous vaccines. Furthermore it was shown that the CHO-PreS/S vaccine was able to induce, in comparison with the S/Core vaccine, more precocious and higher levels of anti-HBs. The application of the heterologous challenge constituted by the standardised w197 inoculum [6] indicated the induction of various protection levels elicited by vaccines with different antigen compositions.

The improvement of post-vaccinal immune responses is of crucial importance for immuno-therapeutic applications. The administration, in therapeutic vaccinations of a vaccine based on yeast derived “S” protein alone, has failed to trigger antiviral status. Others immunomodulatory strategies are needed. Even if it has been shown that combinations therapy might be more beneficial and effective than the application of the antiviral nucleoside analogs
alone, in decreasing the level of viral replication, it has resulted, up to now, ineffective in eradicating the infection. The development of drug resistant mutants have also an important impact.

In a recent vaccine-therapeutic study from our group [7], in which lamivudine and PreS/S vaccine was applied, we have observed the rapid emergence of lamivudine resistant mutants that were not neutralised by the elicited post-vaccinal antibodies. The failure of antiviral activity following the selection of B FLLA motif domain resistant and not neutralized viral mutants is an important finding that further support the need of more potent antivirals. Furthermore multiple targeted options should be considered.

Our recent preliminary experiment aimed at evaluating, in WHV naturally infected chronic carriers woodchucks, the antiviral activity of the immune complex (IC) composed by anti-PreS/S antibodies and WHV particles, showed a significant decrease (4-5 logs) in viral load in a part of administered woodchucks. These results are in accordance with the data of pilot studies in humans, in which immune complexes were administered in HBeAg positive patients leading to the decrease of HBV-DNA and HBeAg titres and to anti-HBe seroconversion [8]. The results are also in agreement with of other antiviral drug-immunomodulators combination studies performed in woodchucks [9].

In conclusion, it was demonstrated that WHV/woodchuck model can be used for HBV vaccine studies, resulting the PreS/S-CHO vaccine the first human vaccine able to elicit non sterilizing protection in the woodchuck model. The level of the anti-HBs production seems to have a crucial role in neutralizing and protective immunity. The WHV/Marmota monax system could provide a well characterized model for preclinical evaluation of combined therapeutic approaches for prevention and treatment of HBV chronic disease.

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References