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Neutralizing antibodies and broad, functional T cell immune response following immunization with hepatitis C virus proteins-based vaccine formulation

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ABSTRACT

HCV is a worldwide health problem despite the recent advances in the development of more effective therapies. No preventive vaccine is available against this pathogen. However, non-sterilizing immunity has been demonstrated and supports the potential success of HCV vaccines. Induction of cross-neutralizing antibodies and T cell responses targeting several conserved epitopes, have been related to hepatitis C virus (HCV) clearance. Therefore, in this work, the immunogenicity of a preparation (MixprotHC) based on protein variants of HCV Core, E1, E2 and NS3 was evaluated in mice and monkeys. IgG from MixprotHC immunized mice and monkeys neutralized the infectivity of heterologous HCVcc. Moreover, strong CD4+ and CD8+ T cells proliferative and IFN-γ secretion responses were elicited against HCV proteins. Remarkably, immunization with MixprotHC induced control of viremia in a surrogate challenge model in mice. These results suggest that MixprotHC might constitute an effective immunogen against HCV in humans with potential for reducing the likelihood of immune escape and viral persistence.

1. Introduction

Hepatitis C virus (HCV) infection is a worldwide health problem [1]. More than 80% of acutely infected individuals progress to a persistent infection state that frequently results in cirrhosis and hepatocellular carcinoma [2]. There is not preventive vaccine and therapeutic options available have important limitations [3,4].

Non-sterilizing protective immunity exists, supporting the idea that viral persistence may be prevented by vaccination [5]. However, heterogeneity and genetic variability of HCV is a major obstacle for developing a HCV vaccine [6]. Early, strong, and multi-specific T-cell responses, targeting a large number of epitopes of HCV proteins are associated with clearing viremia and avoiding viral escape [7,8]. Epitopes related to cell mediated immune response are found in all HCV proteins, although Core and NS3 are considered the most relevant antigens since they are highly conserved among virus isolates, contrariwise to E1 and E2 [9,10].

Besides a multi-specific cellular immune response, induction of vigorous and cross-reactive neutralizing antibodies is assumed to be necessary for an effective HCV vaccine [11,12]. E1 and E2 are envelope glycoproteins, which play important roles in HCV attachment and entry into target cells, and constitute the main target of neutralizing antibodies [13]. Vaccination with recombinant E1 and/or E2 proteins has shown to partially protect chimpanzees from HCV infectious challenge or ameliorate the outcome of infection [11,14,15].

Several immunization approaches have been evaluated against HCV [9,11,16–19]. Generally, vaccine candidates have targeted several HCV antigens and they frequently have focused on cellular immune responses [9,18]. In this regard, immunogenic hierarchy in T cell immune response against hepatitis C antigens has been described after HCV polyprotein vaccination [20]. Some vaccine candidates have demonstrated to be immunogenic and safe, although their clinical impact has not been significant yet [21,22].

In the present work, immunogenicity of MixprotHC formulation, based on the mix in a particular amount and ratio of recombinant protein variants of Core, E1, E2 and NS3 antigens formulated in

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Alum, was evaluated in mice and monkeys. The rationale behind this preparation is based on the desired balance of humoral and cellular immune response against a broad spectrum of HCV antigens, including epitopes conserved among viral isolates, taking into account a possible immunogenic hierarchy among HCV proteins. Our study provides important proof-of-concept that it is possible to induce neutralizing antibodies and broad functional T cell responses against HCV in BALB/c mice and African green monkeys vaccinated with MixprotHC formulation.

2. Materials and methods

2.1. Antigens

HCV protein variants Co.120 (Co) [23], E1.340 [24], E2.680 [25], and NS3 [26] encompassing amino acids 1–120, 192–340, 384–680 and 1192–1457 of the HCV polyprotein from a genotype 1b Cuban isolate, respectively, have been described before. The HCV recombinant antigens were prepared as a single large batch for the immunization schedules and had an endotoxin level under 0.1 endotoxin units per mL.

Concanavalin A (ConA, Sigma–Aldrich, USA, 5 μg/mL) was used as positive control in cellular immune response evaluation.

2.2. Immunogenicity studies

Animals were purchased from Centro para la Producción de Animales de Laboratorio (Havana City, Cuba). Female BALB/c mice (H-2d) mice, 6–8 weeks old, 18–20 g of weight, and African green monkeys (*Cercophitecus aethiops* sabaeus), males, 3–7 kg body weight and 4–7 years old, were used for immunogenicity studies. The housing, maintenance, care and ethics for analysis of animals were in compliance with institutional guidelines.

Groups of ten mice were intramuscularly (im) injected into quadriceps muscle with the MixprotHC, a mixture of 0.1 μg Co.120, 16.7 μg E1.340, 16.7 μg E2.680 as described before [16] and 10 μg NS3, formulated in Aluminum hydroxide (Alum) (0.5 mg Al^{3+} for 20 μg of antigen), at weeks 0, 2, 4, 8 and 12. Blood samples were collected two weeks after the last immunization from retro-orbital sinus and sera were analyzed for neutralizing antibodies. Five animals per group were euthanized after the final blood collection and spleens were taken for proliferative response analysis.

Four monkeys per group were inoculated by im injection with MixprotHC in the same amount and proportion used in mice, or Alum. Monkeys were immunized five times at months 0, 1, 2, 4 and 5. To evaluate neutralizing antibodies and lymphoproliferative as wells as IFN-γ ELISPOT responses, sera and peripheral blood mononuclear cells (PBMCs) were isolated 30 days after the last immunization.

2.3. CFSE based proliferation assay

T-cell proliferation assays were carried out essentially as described elsewhere [27]. Cells were cultured at 2.5×10^5 cells/well, in triplicate with medium alone or 8 μg/mL of Core, E1, E2 and NS3 protein. The cells from mice were stained with anti-mouse CD4 allophycocyanin (APC) and anti-mouse CD8 phycoerythrin (PE) (eBioscience, USA). The samples were acquired on a Flow cytometer (Partec, Germany) and then analyzed using Summit V4.0 (Build 2060 DakoCytomation, USA). Data are expressed as stimulation index (SI). The stimulation index was calculated by the following equation: SI = (% $CFSE^{low}$ cells after stimulus/% $CFSE^{low}$ unstimulated cells) × 100. We considered a positive result to be SI ≥ 3.

2.4. Evaluation of neutralizing antibody response against HCV

For the evaluation of the neutralizing capacity of HCV-specific antibodies, total immunoglobulins were purified by Protein A-Sepharose 4 Fast flow (Amersham Biosciences, UK). Neutralization assay using HCVcc, genotype 1a–2a and purified antibodies (50 μg/mL), was performed essentially as described before [28]. The immunostaining was carried out with E1-specific antibody and fluorophore-taged antibody (anti-mouse Alexa 488). Foci forming units were counted in an Axiophot 2 microscope. Results are shown as % of HCVcc infectivity = (foci forming units in the presence of antibodies/foci forming units in the absence of antibodies) × 100. A sample was considered positive if at least a 50% reduction in the infectivity of HCVcc was observed in the presence of antibodies, compared to the absence of antibodies.

2.5. ELISPOT

The ELISPOT assay for detection of interferon-γ (IFN-γ)-releasing cells, using protein stimulation, was performed essentially as previously described [29]. Spot counting was performed in a stereomicroscope (Carl Zeiss, Germany). The results were expressed as mean number of spot-forming cells (SFC) × 10^6 PBMC, after subtraction of number of IFN-γ-producing cells of non-stimulated condition. The positive results were considered as average spot number at least twice greater than the average spot number obtained in the negative-control wells and at least 40 spots per million of cells.

2.6. Surrogate challenge model

Two weeks after the last immunization, five mice per group were challenged with 1×10^6 pfu (plaque-forming units) of vaccinia virus vvRE (expressing HCV structural proteins) or WR (Western reserve, negative control vaccinia virus), by intraperitoneal injection, as previously described [16]. Mice were sacrificed 5 days after challenge. Results are expressed as logarithm ($\log_{10}$) of virus titer in ovaries of mice. A positive response was considered for animals with viral loads at least one $\log_{10}$ less than mean vvRE viral titer detected in the control group mice and at least one $\log_{10}$ less than average viral titer detected in mice challenged with WR control virus.

2.7. Statistical methods

GraphPad Prism version 4.00 statistical software (GraphPad Software, San Diego, CA, USA.) was used to carry out statistical analysis. For comparison of two groups Mann–Whitney tests for nonparametric analyses were used. To compare three or more groups the Kruskal-Wallis test with Dunn's multiple comparisons as a post test was used for nonparametric analysis. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Antibodies in mice immune sera vaccinated with MixprotHC neutralize HCVcc

The results presented in Fig. 1 showed neutralization activity (49% of infectivity in Huh-7 cells) when HCVcc were previously incubated with purified IgG from pooled sera of mice immunized with MixprotHC. However, IgG purified from the control group showed lack of neutralization activity (Fig. 1).