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A novel challenge model to evaluate the efficacy of hepatitis C virus vaccines in mice

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ABSTRACT

An effective hepatitis C virus (HCV) vaccine should elicit robust humoral and cell mediated immunity (CMI). A small animal challenge model is required to assess the efficacy of vaccines which elicit CMI. In this study, HCV proteins were expressed in hepatocytes of immunocompetent mice after hydrodynamic injection of a plasmid encoding the HCV NS3/4A protein. This vector, constructed as the "challenge", was optimized for long term, specific gene expression in hepatocytes. To monitor HCV antigen expression in transfected hepatocytes, the plasmid also encoded secreted alkaline phosphatase (SEAP), which was detected in the mouse serum. The design of this novel challenge plasmid was based on studies using luciferase and SEAP as reporter molecules to examine the kinetics of the proteins expressed in hepatocytes and secreted into blood. We designed two constructs to control SEAP expression. In one construct, SEAP expression was controlled by the EMCV IRES, while in the other, a SEAP and luciferase polyprotein was cleaved by the FMDV2A proteinase. We found that SEAP expressed after FMDV2A self cleavage was more sensitive and showed a higher correlation with luciferase expressed in liver. The NS3/4A challenge model using the FMDV2A design provided a window period of 50 days to monitor changes in SEAP expression after hydrodynamic injection of DNA. In a challenge experiment, mice which received an adenovirus-based HCV vaccine showed accelerated clearance of SEAP and thus, of NS3/4A positive hepatocytes compared with a mock vaccinated group, that coincided with an increased number of $CD8^+$ lymphocytes in the liver.

1. Introduction

The development of an effective HCV vaccine is impeded by the lack of a convenient animal model [1]. Chimpanzees are the most authentic model but are unavailable due to ethical reasons and expense [2]. Several small animal models have been developed [3–11]. Transgenic mice transplanted with human liver tissue or hepatocytes are unsuitable as a challenge model due to their immunodeficiency [3,4,8] or the mismatch between the host immune system and human MHC molecules [5]. A recent mouse model engrafted with human hepatocytes and $CD34^+$ hematopoietic stem cells was developed to restore immunity in mice. Vaccination of these mice resulted in an HCV-specific T cell response, but challenge experiments were not performed [9].

Recombinant viruses encoding HCV proteins have also been used as challenge models in mice [6,7], but restrict analysis of virus titres in the ovaries or spleen to a single time point. HCV antigen-positive tumour cells which proliferate after challenge represent another model [11]. These challenge models fail to evaluate the potential of T cells to traffic to the liver, the major site of HCV replication. To overcome this, an immunocompetent mouse model which supports limited HCV replication was developed [10]. This model depends on transient expression of human CD81 and Occludin (hCD81 and hOCLN) to complement the mouse homologs of SCARB1 and CLDN1 and was used to detect HCV NAb after vaccination with a RecVV. A transgenic version of this model was recently reported to support the complete HCV replication cycle [12], although virus production was limited in animals which were not immunocompromised.

In this study, we developed a new animal model based on several considerations: first, in challenge experiments, large numbers of

Abbreviations: SEAP, secreted alkaline phosphatase; EMCV IRES, internal ribosomal entry site from encephalomyocarditis virus; FMDV, foot and mouth disease virus; SVR, sustained virological response; DAA, direct acting antiviral agent; NAb, neutralizing antibody; RecVV, recombinant vaccinia virus; IFU, infectious units; AFP, α-fetoprotein; pLuc, pLIVE-Luciferase; pLIS, pLIVE-Luciferase-IRES-SEAP; pLFS, pLIVE-Luciferase-FMDV2A-SEAP; pNIS, pLIVE-NS3/4A-IRES-SEAP; pNFS, pLive-NS3/4A-FMDV2A-SEAP; pNIS, pLive-Luciferase-IRES-SEAP.

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animals are required to generate statistically significant data, so the model should be economic and convenient; second, longitudinal studies of individual animals rather than culling different animals at a single time point are preferable; third, it should analyse the effect of CMI in liver.

2. Materials and methods

2.1. Plasmid construction

Details of plasmids are presented as supplementary data.

2.2. Cell culture

Hepa1-6 cells were grown in DMEM supplemented with 10% FCS/1% Pen/Strep and transfected with DNA in 24 well plates using Lipofectamine LTX & Plus reagent (Invitrogen). Forty-eight hours later the medium was collected for the SEAP assay and the cell pellets examined by the luciferase assay.

2.3. SEAP assay

SEAP was detected using the Phospha-light kit (Applied Biosystems) in 96 well flat bottom white microplates (Greiner BioOne). Twenty μl mouse serum or 50 μl culture supernatant were used and luminescence quantified by a Fluostar Optima microplate reader (BMG LABTECH). According to the positive control, 0.1 ng ml^{-1} SEAP in serum results in a luminescence reading of 3×10^4.

2.4. Luciferase assay

To detect luciferase expression *in vitro*, pelleted cells were resuspended in 100 μl medium, transferred to the Greiner microtitre plate and 100 μl D-luciferin K+ (PerkinElmer) solution (600 μg ml^{-1}) added and read with the Fluostar Optima microplate reader. *In vivo* luciferase expression was measured by Xenogen IVIS 100 (PerkinElmer) after injection of D-luciferin K+ (PerkinElmer), 3 mg/20 g mouse andluminescence quantified by Igor Pro 4.09A software.

2.5. Mice

Experiments were approved by the Animal Ethics Committees of the University of Adelaide and the Women and Children's Hospital network. Female C57Bl/6 mice were purchased from the University Laboratory Animal Services. All interventions except hydrodynamic (HD) injection were performed under isofluorane anaesthesia and blood samples obtained by retro-orbital bleed. Mice were 12 weeks old at the time of HD injection.

2.6. Hydrodynamic injection

HD injection followed the described protocol (Mirus). Mice were injected with 20 μg plasmid DNA in TransIT-QR HD delivery solution (Mirus) in a volume of 1/10 body weight. Any mouse which failed to receive the full volume was excluded from further analysis.

2.7. Cryosection staining

Mouse livers were embedded in OCT compound (Tissu-Tek), then frozen in liquid nitrogen. For immunofluorescence, acetone-fixed cryosections were stained by sequential incubations/washes in pooled anti-HCV-positive human serum (1:50), Alexa fluor 555-conjugated goat anti-human IgG antibody (1:200) for 1 h at 37 °C, followed by Prolong Gold antifade reagent DAPI (Life Technologies). NS3 positive cells were counted by ImageJ 1.46R software (NIH). For immunohistochemistry, the fixed cryosections were incubated in 0.5% H_2O_2 in methanol to inactivate endogenous peroxidase, before staining. After washing, the sections were incubated with HRP-conjugated goat anti-human IgG and visualized with diaminobenzidine (DAB) (Abcam) followed by hematoxylin staining.

2.8. Immunization and challenge

Eight week old C57Bl/6 female mice were injected subcutaneously with 5×10^7 IFU recombinant adenovirus vaccine in 100 μl saline and boosted 2 weeks later with the same dose. The protected group was vaccinated with adenovirus encoding HCV-E1E2NS3 (genotype 1b, codon optimised NS3) and the unprotected group with adenovirus encoding HCV-CE1E2-GFP (genotype 1a). All mice were challenged with 20 μg pNFS (encoding wild type NS3/4A genotype 1b [13]) by HD injection 2 weeks after boosting.

2.9. Lymphocyte trafficking

Two groups of C57BL/6 female mice were vaccinated as described above. A third group represented an unvaccinated control group. The protected and unprotected groups were challenged with 50 μg pNFS while the control group was challenged with 50 μg pLive (p-empty). Hepatic lymphocytes were harvested 3 days after challenge as described [14]. The cells were blocked with mouse γ globulin, stained for surface markers, CD3-PercP-Cy5.5 (eBioscience), CD8-FITC (BD Biosciences) and CD4-eFluor 450 (eBioscience), andanalysed on a FACS Canto II (Becton Dickinson).

2.10. Statistical analyses

Graph construction, the coefficient of correlation (R^2) calculation, *t*-test and *F*-test were performed using OriginPro 8.0. All correlations given an R^2 value reached statistical significance ($p < 0.01$, *F*-test). For the challenge experiments, the effect of Group over Time was assessed using a linear mixed effects model with subject treated as a random factor. The outcome measure was log transformed prior to analysis in order to meet the distributional assumptions of a linear mixed effect model. The data were transformed back to the original scale prior to reporting. Statistical analyses were completed using SAS 9.3 (SAS Institute Inc).

3. Results

3.1. In vitro expression of luciferase and SEAP from pLIS and pLFS

We constructed a series of plasmids, based on pLive (Fig. 1A). Protein expression is controlled by the mouse albumin promoter/α-fetoprotein enhancer ensuring hepatocyte-specific expression [15]. Introns 1 and 2 were included to optimize expression [16]. SEAP expression is controlled by the EMCV IRES or encoded as a fusion protein, luciferase-FMDV2A-SEAP (Fig. 1A; pLIS and pLFS respectively). The FMDV2A protease was designed to cleave and release SEAP cotranslationally [17].

Transfection of Hepa1-6 cells with pLIS or pLFS produced similar luciferase levels, but pLFS expressed 10-fold higher levels of SEAP (Fig. 1B, left), as cap-dependent translation is more efficient than EMCV IRES-directed initiation [18]. The luminescence reading of SEAP and luciferase activity was integrated in one figure (Fig. 1B, left) as the SEAP luminescence reading from pLFS in 50 μl of supernatant was coincidentally similar to the luminescence reading from the cell lysate. We then examined the correlation between intracellular luciferase and SEAP in the culture supernatant and calculated