Vaccine xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Specific memory B cell response and participation of CD4$^+$ central and effector memory T cells in mice immunized with liposome encapsulated recombinant NE protein based Hepatitis E vaccine candidate

Shruti P. Kulkarni [a], Subrat Thanapati [a], Vidya A. Arankalle [b,*], Anuradha S. Tripathy [a,*]

[a] Hepatitis Group, National Institute of Virology, Pune, 130/1, Sus Road, Pashan, Pune 411021, Maharashtra, India
[b] Interactive Research School in Health Affairs (IRSHA), Bharati Vidyapeeth Deemed University, Pune-Satara Road, Katraj-Dhankawadi, Pune 411043, Maharashtra, India

ARTICLE INFO

Article history:
Received 5 July 2016
Received in revised form 14 October 2016
Accepted 16 October 2016
Available online xxxx

Keywords:
Hepatitis E vaccine
Anamnestic response
CD4$^+$ memory T cells
Functional memory B cells

ABSTRACT

Background: Liposome encapsulated neutralizing epitope protein of Hepatitis E virus (HEV), rNEp, our Hepatitis E vaccine candidate, was shown to be immunogenic and safe in pregnant and non-pregnant mice and yielded sterilizing immunity in rhesus monkeys.
Methods: The current study in Balb/c mice assessed the levels and persistence of anti-HEV IgG antibodies by ELISA, frequencies of B, memory B, T and memory T cells by flow cytometry and HEV-specific IgG secreting memory B cells by ELISPOT till 420 days post immunization (PI) with 5 μg rNEp encapsulated in liposome based adjuvant (2 doses, 4 weeks apart). Mice immunized with a lower dose (1 μg) were assessed only for anamnestic response post booster dose.
Results: Vaccine candidate immunized mice (5 μg dose) elicited strong anti-HEV IgG response that was estimated to persist for lifetime. At day 120 PI, frequency of memory B cells was higher in immunized mice than those receiving adjuvant alone. Anti-HEV IgG titers were lower in mice immunized with 1 μg dose. A booster dose yielded a heightened antibody response in mice with both high (>800 GMT, 5 μg) and low (⩽100 GMT, 1 μg) anti-HEV IgG titers. At day 6th post booster dose, HEV-specific antibody secreting plasma cells (ASCs) were detected in 100% and 50% of mice with high and low anti-HEV IgG titers, respectively, whereas the frequencies of CD4$^+$ central and effector memory T cells were high in mice with high anti-HEV IgG titers only.
Conclusions: Taken together, the vaccine candidate effectively generates persistent and anamnestic antibody response, elicits participation of CD4$^+$ memory T cells and triggers memory B cells to differentiate into ASCs upon boosting. This approach of assessing the immunogenicity of vaccine candidate could be useful to explore the longevity of HEV-specific memory response in future HEV vaccine trials in human.

1. Introduction

Hepatitis E caused by hepatitis E virus (HEV) is a worldwide disease. The virus has four genotypes and a single serotype [1]. HEV infection mostly presents as outbreaks and sporadic cases with self-limiting hepatitis, but may lead to fulminant hepatic failure, especially in pregnant women, with ~20–25% mortality [2]. It is estimated that one third of the world's population is infected with HEV [3].

HEV infections can be prevented by reducing exposure to the virus and/or inducing immunity through vaccination. There is a need of a vaccine that would protect the high risk groups from infection and also prevent HEV transmission through asymptomatic infections [4]. An HEV genotype 1-based recombinant protein vaccine licensed only in China was protective in vaccinated individuals for at least 4.5 years [5–7].

There are conflicting reports regarding the longevity of antibody responses following exposure to HEV infection. A sero-epidemiological study conducted 30 years post hepatitis E outbreak reported detection of anti-HEV antibodies in only 4.5% of individuals [8]. Our group has demonstrated that after natural

Abbreviations: HEV, Hepatitis E Virus; rNEp, recombinant neutralizing epitope protein; PI, post immunization; VI, vaccine immunized; VI-5μgμ, mice immunized with 5g rNEp+ adjuvant; VI-1μgμ, mice immunized with 1g rNEp+ adjuvant; ASCs, antibody secreting cells; T_{CM}, T central memory; T_{EM}, T effector memory.

* Corresponding authors.
E-mail addresses: varankalle@yahoo.com (V.A. Arankalle), anuradhastripathy@hotmail.com (A.S. Tripathy).

http://dx.doi.org/10.1016/j.vaccine.2016.10.046
0264-410X/

infection, anti-HEV IgG is long lasting and probably protective against further infection [9]. Strong T cell responses have been associated with the control of hepatitis E [10,11]. Persistence of HEV genotype 3-specific memory T cell responses has been reported for >1.5 years post infection [12].

Studies conducted in non-human primates were mainly restricted to antibody response and did not evaluate T cell immunity. Several such studies showed that immunity induced by primary HEV infection could protect against homologous as well as heterologous virus challenge [13–16]. Importantly, passive transfer of high titred anti-HEV positive plasma provided protection from HEV infection, documenting role of antibodies in protection [17]. Anti-HEV IgG antibodies persisted for 7 years in experimentally infected rhesus monkeys and were estimated to persist from 6.9 to 84.3 years. Even low levels of anti-HEV IgG antibodies were protective against re-infection [15]. Contrary to this, a complete loss of serological evidence and lack of immunological memory upon challenge in infant rhesus monkeys with and without maternally transmitted anti-HEV antibodies 3 and 7 years post primary infection with HEV [18] suggests that immunologic correlates of protection are not defined in hepatitis E.

As a part of vaccine development, we evaluated different preparations and identified liposome encapsulated recombinant neutralizing epitope protein of HEV (rNEp) as the vaccine candidate [19–23]. This candidate was shown to be immunogenic and safe in pregnant and non-pregnant mice and yielded sterilizing immunity in rhesus monkeys [22, unpublished data]. In an attempt to link these observations with immune parameters, the present study has assessed the persistence of (a) anti-HEV IgG antibodies, (b) B, memory B, T and memory T cells and HEV-specific IgG secreting memory B cells in mice post immunization with liposome encapsulated rNEp.

2. Materials and methods

2.1. NE antigen (rNEp)

The neutralizing epitopes (NE) region of the open reading frame 2 of genotype 1 of HEV was cloned in pET-15b vector (Novagen, Germany) earlier in our lab [19]. rNEp was expressed in bacterial system and purified by affinity chromatography using Probond Nickel-chelating resin (Invitrogen, Carlsbad, USA) [22].

2.2. Preparation of vaccine formulation

The vaccine candidate consisted of rNEp encapsulated in a cationic liposome-based adjuvant [22]. The adjuvant was prepared using hydrogenated soy phosphatidylcholine (Avanti polar lipids, Alabama, USA), cholesterol and stearylamine (Sigma, St. Louis, MO, USA). 5 μg rNEp and adjuvant were mixed in a ratio of 1:200 (w/w) and lyophilized. 1 μg rNEp+ adjuvant was used for a lower dose preparation. The dose was reconstituted in sterile PBS before administration.

2.3. Immunization of mice and bleeding

The study was approved by the Institutional Animal Ethics Committee and the experiments were conducted in accordance with the guidelines. Anti-HEV IgG negative female Balb/c mice (42–56 days old) were immunized intramuscularly on days 0 and 28 with the vaccine candidate/adjuvant alone (n = 4–12 mice/group/time point). For memory B cell response experiments, vaccine candidate immunized mice (VI) administered with 5 μg dose (VI-5 μg) (n = 6) were given a booster dose at day 180 post immunization (PI) (when IgG titers were high), while those administered with 1 μg dose (VI-1 μg) (n = 6) were boosted at day 120 PI (when IgG titers were low). Unimmunized mice (n = 4–6 mice/time point) were used (1) as negative control and (2) to assess the effect of age on immune responses. Longitudinal serum samples were collected by retro-orbital bleeding before and at pre-defined time points after immunization.

2.4. Serology

The serum samples (n = 12/time point) were screened to detect anti-HEV IgG antibodies by an in-house ELISA [24]. The end point of serum anti-HEV IgG levels was determined as previously described [19,21].

2.5. Preparation of splenocytes suspension, staining and flow cytometry

Mice (n = 4/group) from unimmunized, adjuvant and candidate vaccine immunized (VI-5 μg) groups were sacrificed at 1, 7, 14, 30, 120 and 180 days PI. Mice in all groups were age-matched and their age at these time points was 84, 91, 98, 112, 196 and 252 days respectively. Spleens were harvested aseptically and single cell suspensions of splenocytes were prepared. 1×10^6 splenocytes were stained with anti-mouse antibodies: CD45R/B220-PerCP-Cy5.5, IgG1-APC, CD4-APC-H7, CD8-APC-H7, CD44-APC and CD62L-PE (BD Biosciences, CA, USA). Samples were acquired in BD FACS Aria II flow cytometer.

B ($CD45R/B220^+$), Th ($CD4^+$) and Tc ($CD8^+$) cells were gated from splenocytes. Memory B ($CD45R/B220^+IgG^+$), Th ($CD4^+$ $CD44^{high}$) and Tc ($CD8^+$ $CD44^{high}$) cells were gated from their parent cells. Splenocytes of mice (VI-5 μg and VI-1 μg) were stained for memory B, T central memory (T_{CM}) ($CD4/8^+$ $CD44^{high}$ $CD62L^{high}$) and T effector memory (T_{EM}) ($CD4/8^+$ $CD44^{high}$ $CD62L^{low}$) on 6th day post booster dose as shown in supplementary Fig. 1.

Mice immunized with 1 μg dose were assessed for anamnestic B cell response and memory T cell subsets post booster dose only.

2.6. Memory B cell responses by ex vivo ELISPOT

Mice from VI-5 μg and VI-1 μg groups were stimulated *in vivo* by administering a booster dose when anti-HEV IgG titers were high (>800) and low (⩽100) (n = 6/group). The assay was performed as previously reported with some modifications [25]. Briefly, 96-well Elispot plates (MSIPS 4510, Millipore Bedford, MA, USA) were coated with 10 μg/ml rNEp at 4 °C overnight. The plates were washed with PBS containing 0.5% (v/v) Tween-20 and blocked with FBS at 37 °C for 3–4 h. Spleens were harvested on 6th day post booster dose and 0.8×10^6 splenocytes were added to the wells in triplicates. Splenocytes incubated with lipopolysaccharide (10 μg/mL) (Sigma, St Louis, MO, USA) or medium alone served as controls. After incubation at 37 °C for 24 h, 1 μg/ml biotin-labeled anti-IgG1 antibody was added and kept at RT for 3 h. Subsequently, the plate was developed as described previously [25]. The spots were counted on ELISPOT reader (AID, Strassberg, Germany) and the results were expressed as HEV-specific antibody secreting cells (ASCs) per 10^6 splenocytes. A positive result was defined when ASCs were observed in at least 2 replicate test wells and were twice the numbers observed in the negative control.

2.7. Software and statistical analyses

All statistical analyses were performed with SPSS 20 software (SPSS Inc., Chicago, IL, USA). Intergroup comparisons were done using a non-parametric Mann–Whitney *U* test. Logarithmic curve was fitted to the anti-HEV IgG data to estimate the antibody persistence. Flow cytometry data were analyzed using FACS Diva

Please cite this article in press as: Kulkarni SP et al. Specific memory B cell response and participation of $CD4^+$ central and effector memory T cells in mice immunized with liposome encapsulated recombinant NE protein based Hepatitis E vaccine candidate. Vaccine (2016), http://dx.doi.org/10.1016/j.vaccine.2016.10.046