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Vaccine 38 (2020) 7409–7413

Contents lists available at ScienceDirect

Vaccine

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

Short communication

Protection against herpes simplex virus type 2 infection in a neonatal murine model using a trivalent nucleoside-modified mRNA in lipid nanoparticle vaccine

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ARTICLE INFO

Article history:
Received 21 July 2020
Received in revised form 19 September 2020
Accepted 28 September 2020
Available online 9 October 2020

Keywords:
Neonatal herpes
Maternal antibody
Neutralizing antibody
Trivalent nucleoside-modified mRNA vaccine in lipid nanoparticles
Genital herpes
Herpes simplex virus type 2

ABSTRACT

Neonatal herpes is a dreaded complication of genital herpes infection in pregnancy. We recently compared two vaccine platforms for preventing genital herpes in female mice and guinea pigs and determined that HSV-2 glycoproteins C, D and E expressed using nucleoside-modified mRNA in lipid nanoparticles provided better protection than the same antigens produced as baculovirus proteins and administered with CpG and alum. Here we evaluated mRNA and protein immunization for protection against neonatal herpes. Female mice were immunized prior to mating and newborns were infected intranasally with HSV-2. IgG binding and neutralizing antibody levels in mothers and newborns were comparable using the mRNA or protein vaccines. Both vaccines protected first and second litter newborns against disseminated infection based on virus titers in multiple organs. We conclude that both vaccines are efficacious at preventing neonatal herpes, which leaves the mRNA vaccine as our preferred candidate based on better protection against genital herpes.

1. Introduction

Neonatal herpes is an uncommon but potentially devastating infection [1]. The annual global incidence of neonatal herpes is approximately 14,000 cases, or 1 case per 10,000 births of which HSV-1 comprises 4000 and HSV-2 10,000 [1]. HSV-1 contributes more cases than HSV-2 in the Americas, Europe and Western Pacific [1]. Transmission to newborns most often occurs during labor and delivery from mothers with active genital herpes. Over half the infants with neonatal herpes have disseminated infection or encephalitis, with 60% mortality if untreated, and severe neurologic disease in two-thirds of survivors despite antiviral treatment [1,2]. Currently, no vaccine is available to prevent genital or neonatal herpes. Efforts to prevent neonatal herpes include visual inspection for lesions at delivery, cesarean section when lesions are present, and behavioral messaging to reduce risk of transmission late in pregnancy. These measures are often ineffective because of asymptomatic infection.

An effective method of protection is through maternal antibodies that develop after infection and pass transplacentally or through breast milk to newborns [3,4]. Vaccination of women is another approach to develop antibodies that can protect newborns until they are old enough to be vaccinated on their own, with influenza as an example [5]. An important issue for candidate herpes vaccines is whether maternal antibodies produced by immunization will provide sufficient protection to a newborn that is exposed to HSV either because of breakthrough infection in the pregnant woman or because of postnatal exposure. Murine models of neonatal herpes infection have addressed vaccine protection of newborn pups. Maternal immunization of female mice with an HSV-2 replicant defective live virus vaccine, dl5-29, protected newborn pups against neonatal HSV-1 and HSV-2 infection [6]. A follow up study

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https://doi.org/10.1016/j.vaccine.2020.09.079
0264-410X/

was performed by these same investigators in a collaboration with our laboratory using an HSV-2 subunit protein vaccine consisting of glycoproteins C, D and E (gC2, gD2, gE2) administered with CpG and alum as adjuvants. Our rationale for using the three immunogens in the trivalent vaccine is that antibodies to gC2 and gD2 neutralize virus, antibodies to gD2 and gE2 block cell-to-cell spread, and antibodies to gC2 and gE2 block immune evasion from antibody and complement [7–10]. The trivalent protein vaccine also provided excellent protection against HSV-1 and HSV-2 neonatal herpes [11].

We recently reported that immunization of female mice and guinea pigs with gC2, gD2, and gE2 administered as nucleoside-modified mRNA encapsulated in lipid nanoparticles (LNP) outperformed the protein formulation in preventing genital herpes infection [7]. Immunization with nucleoside-modified mRNA-LNP has gained considerable recognition in response to COVID-19, and is a highly promising approach for infectious disease vaccine development [12–14]. The nucleoside-modified mRNA immunogens have been particularly potent in producing high titer antibody responses, likely related to potent $CD4^+$ T follicular helper cell and germinal center B cell responses [15]. Three years ago, we began transitioning from a trivalent baculovirus protein vaccine to a nucleoside-modified mRNA vaccine for prevention of genital herpes [7,8]. Here, we compared protection provided by immunization of mothers (dams) with the baculovirus protein vaccine or the nucleoside-modified mRNA vaccine against intranasal (IN) HSV-2 infection in pups.

2. Materials and methods

2.1. Mice

Protocol 805187 was approved by the University of Pennsylvania Institutional Animal Care and Use Committee following guidelines of National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*.

2.2. Vaccines

Female 6–8-week-old BALB/c mice (Charles River) were immunized intramuscularly (IM) prior to mating using trivalent mRNA-LNP, trivalent protein CpG/alum, or Poly(C) RNA-LNP (control) as immunogens [7]. The control immunogen contained 10 μg of Poly(C) RNA, while the nucleoside-modified mRNA vaccine contained 10 μg each of gC2, gD2 and gE2 at a ratio of 1 μg RNA or mRNA to 20 μg LNP. The protein vaccine contained 5 μg each of gC2, gD2 and gE2 with 50 μg CpG/mouse and 25 μg alum/μg protein. Two immunizations one month apart were performed with mRNA or Poly(C), while three immunizations two weeks apart were administered with the protein vaccine, each in a volume of 50 μl.

2.3. Breeding and infection (see graphic abstract)

Immunizations with mRNA or Poly(C) were on days −70 and −42, or with protein on days −70, −55, and −42. Two weeks after the last immunization, on day −28, females were paired with males. Postnatal day 0 (PND 0) was the day the litter was detected. Neonatal pups were infected IN with 1×10^3 PFU HSV-2 strain MS on PND 3 [9]. Three days later, brain, heart, lung, spleen, liver, kidney and colostrum (milk) were harvested. Some neonates were sacrificed to assess serum antibody levels on PND 3 or PND 6–7, and some dams were bred for a second litter.

2.4. Serological testing

Serum was simultaneously collected from dams and their pups on PND 3 or 6–7. Serum from dams was obtained by lancet piercing of a submandibular vein, while pup serum was obtained by decapitation and pooling samples from the same litter. Serum endpoint neutralizing antibody titers were determined without complement and reported as the highest dilution to reduce the number of virus plaques by ≥50% compared to PBS control [9]. Serum IgG binding (ELISA) endpoint titers for gC2, gD2 or gE2 were performed by coating ELISA plates with 50 ng of gC2, gD2 or gE2 antigen and adding serial two-fold dilutions of serum at an initial dilution of 1:500, followed by HRP-conjugated anti-mouse IgG [7]. To determine whether dams that nursed HSV-2-infected pups seroconverted, ELISA plates were coated with HSV-2 glycoprotein G (gG2) and serum was evaluated for a four-fold or greater rise in titer [16].

Nursing neonates were infected IN with 1×10^3 PFU HSV-2 MS on PND 3. Three days later, approximately 25 mg of colostrum was harvested by scooping the gelatinous contents from the excised stomach and stored at −80 °C. The colostrum was slowly thawed on ice, and 400 μl of PBS was added to each sample because of its viscosity and fatty content. The colostrum was homogenized and diluted 1:20 in PBS for neutralizing antibody assays or 1:125 for IgG gD2 ELISA.

2.5. Virus titers in organs

Neonatal brain, lung, liver, spleen, and kidney were placed in 300 μl of DMEM containing 5% fetal bovine serum and vancomycin (25 μg/ml). The organs were minced with scissors, homogenized, and 10-fold serial dilutions added to Vero cells to determine virus titers by plaque assay [9].

2.6. Statistical analysis

Significance tests were two-sided and were calculated by the Mann-Whitney test using GraphPad Prism version 8.3, or Fisher's Exact test.

3. Results

3.1. HSV-2 IN dosing determination

Newborn pups were infected IN on PND 3 with 1×10^2, 1×10^3 or 1×10^4 PFU of HSV-2. The LD_{50} was calculated as 15 PFU/mouse (Fig. 1A). 1×10^3 PFU (67 LD_{50}) was selected for subsequent neonatal infections.

3.2. Serological testing

Pups from first litters of immunized dams were infected IN with 1×10^3 HSV-2 on PND 3 and colostrum was collected on PND 6. Pups born to dams immunized with trivalent mRNA-LNP or trivalent protein CpG/alum had gD2 IgG detected in their colostrum by ELISA (binding antibodies), while pups in the Poly(C) group had no gD2 IgG (Fig. 1B). The mean gD2 endpoint titer for pups in the mRNA group was 1:3667 and in the protein group was 1:2615 ($P = 0.1074$). Siblings had gD2 IgG titers that were either identical or varied by ≤four-fold, suggesting that siblings acquired comparable levels of antibodies from their mothers. Neutralizing antibody titers were also measured in colostrum. Neutralizing antibody was absent in Poly(C) and protein pups, but was somewhat higher in the mRNA pups in that 2 of 8 neonates had neutralizing antibody

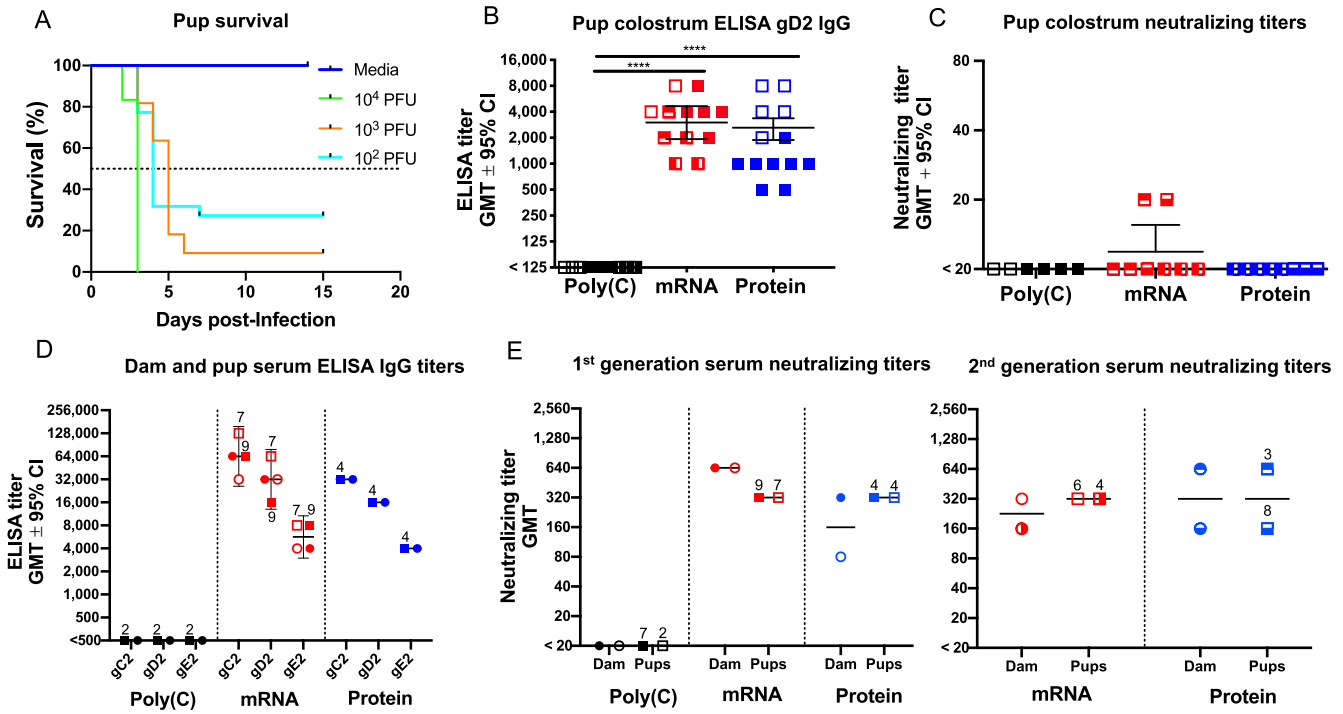

Fig. 1. Survival curves, colostrum and serum antibody titers. (A) Pups born to unimmunized dams were infected IN on PND 3 with media (n = 4 from one litter) or HSV-2 at 10^4 PFU (n = 6 from three litters); 10^3 PFU (n = 11 from two litters); or 10^2 PFU (n = 22 from four litters). (B) Pup colostrum gD2 IgG ELISA titers on PND 6 in neonates born to dams immunized with Poly(C) RNA, mRNA or protein (n = 10 pups for Poly(C) from three litters, n = 12 for trivalent mRNA from four litters, and n = 13 for trivalent protein from two litters). P values comparing Poly(C) RNA with trivalent mRNA or trivalent protein were calculated by the two-tailed Mann-Whitney test; ****p < 0.0001. Comparing mRNA with protein, P = 0.4667. (C) Neutralizing antibody titers in colostrum on PND 6 (n = 6 for Poly(C) RNA from two litters, n = 8 for trivalent mRNA from three litters, and n = 8 for trivalent protein from two litters). (D) Dam and pup serum ELISA gC2, gD2 and gE2 IgG. (E) HSV-2 serum neutralizing endpoint titers in PND 3 and PND 6–7 litters and their dams. The left panel is dams and their first-generation litters, while the right panel is dams and their second-generation litters. In (B-E) circles represent dams and squares pups. In (B and C) each symbol represents an individual animal and pups from the same litter are identified by the same filled, open or partially-filled symbol. Litter size in (D and E) is noted above the pup symbols. Blood from pups of the same litter were pooled.

titers above the limit of detection of 1:20 compared to 0 of 8 in the protein group (Fig. 1C).

We measured IgG titers to gC2, gD2 and gE2 in serum from dams and their first litters on PND 3 or PND 6–7. One dam immunized with Poly(C) and her two pups had no detectable gC2, gD2 or gE2 IgG. In the mRNA group, two dams and their seven or nine pups had IgG antibodies to gC2, gD2 and gE2 that were either the same titer or within one or two dilutions of each other. In the protein group, one dam and her four pups had IgG antibodies to gC2, gD2 and gE2 that were identical in the dam and pups (Fig. 1D). IgG titers were generally higher in the mRNA than protein groups, but the small number of mother-pup pairs precluded statistical analysis.

Serum neutralizing titers were collected on PND 3 or PND 6–7 from six dams (2 in Poly(C), 2 in mRNA and 2 in protein groups) and their first litter of pups, and from four dams (2 in mRNA and 2 in protein group) and their second litter of pups (Fig. 1E). The average interval between first and second litters was 80 days for mRNA and 49 days for protein pups. One dam was evaluated over two generations of pups (denoted by an open circle in the mRNA group, Fig. 1E), while the other nine dams were assessed at the time of either their first or second litter. Eight of the 10 litters of pups were uninfected at the time serum was collected, while serum from two second generation litters born to dams immunized with trivalent protein was harvested three days post-infection at the same time that organs were harvested. These two dams did not subsequently seroconvert to HSV-2 despite nursing infected pups for three days. No neutralizing antibodies were detected in the Poly(C) dams or their pups. Neutralizing antibody titers in the dams did not differ significantly between mothers having their first or second litter or between mothers immunized with mRNA or protein (Fig. 1E). Neutralizing antibody titers in dams and their pups were identical in 4 mother-pup pairs, varied by 2-fold in 3 pairs (higher in dams than pups in 2 of 3), or 4-fold in one pair (higher in pups). The higher ELISA IgG and neutralizing titers in some newborns than dams likely represent the combined contribution of transplacental and colostrum antibodies [6,17]. The need to dilute the colostrum with PBS because of its viscosity and fatty content makes it difficult to compare colostrum titers with serum titers. We conclude that high titers of neutralizing antibodies are detected in neonates during first and second litters providing evidence of durable transplacental and/or milk transfer of antibodies from dams to their pups.

3.3. Virus titers in neonatal tissues

Neonates were infected IN on PND 3 with 1×10^3 PFU HSV-2. Brains, lungs, livers, spleens and kidneys were harvested three days later (Fig. 2A). Most organs from neonates in the Poly(C) group were positive for virus, while very few were positive in the mRNA or protein groups. The few positive samples were from brains in the mRNA and protein groups (Fig. 2A and Table 1).

Second litter pups born to four previously immunized dams were evaluated for neonatal herpes. Three of the dams had nursed first litter pups between PND 3 and 6 that were infected IN with HSV-2; however, none of these dams seroconverted to HSV-2 as determined by ELISA IgG antibodies to gG2. Therefore, protection in pups of the second-generation litters is because of maternal immunization and not maternal HSV-2 infection. In the second-generation litters, all organs from two pups in the Poly(C) group

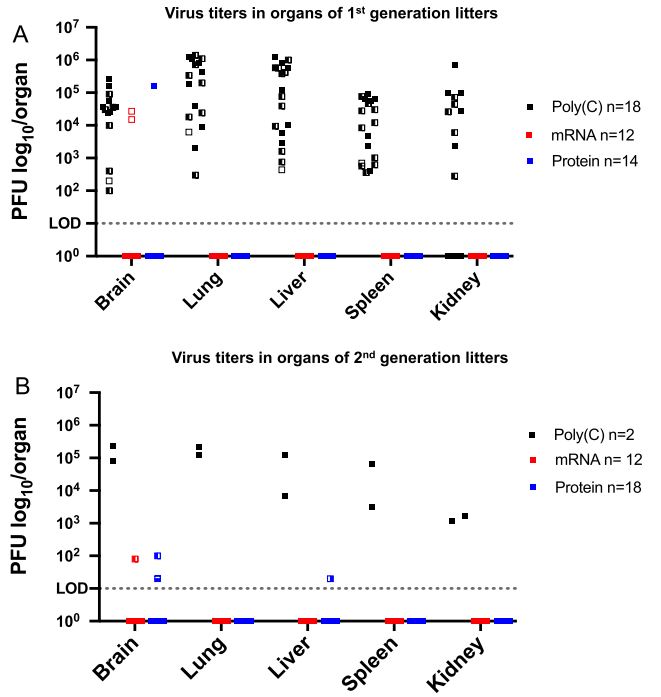

Fig. 2. Organ virus titers of first and second litters on post-infection day 3. (A) First generation pups; n = 3 litters for Poly(C), n = 3 for mRNA, and n = 2 for protein. (B) Second generation pups; n = 1 litter for Poly(C), n = 3 for mRNA, and n = 3 for protein. Each symbol represents an individual organ from one pup. Pups with positive virus titers from the same litter are identified using the same filled, open or partially-filled symbol.

Table 1
Summary of organs positive for HSV-2.

Immunogen	First litter	Second litter	Total
Poly(C)	82/90 (91.1%)*	10/10 (100%)*	92/100 (92%)*
mRNA	2/60 (3.3%)	1/60 (1.7%)	3/120 (2.5%)
Protein	1/70 (1.4%)	3/90 (3.3%)	4/160 (2.5%)

* P<0.0001 comparing Poly(C) with mRNA or protein for first litter, second litter and total.

were positive, while very few were positive in the mRNA or protein groups (Fig. 2B and Table 1). Trivalent mRNA and trivalent protein were significantly different from Poly(C) RNA in first litters, second litters and overall (P < 0.0001 calculated by two-tailed Fisher's Exact test), but not significantly different from one-another (Table 1).

4. Discussion

A goal of the current study was to assess whether the trivalent nucleoside mRNA-LNP vaccine is efficacious in preventing neonatal herpes, a feared complication of genital herpes. IgG ELISA antibodies and neutralizing antibody titers in mice immunized with trivalent mRNA or trivalent protein were similar to antibody titers in their newborns, and neutralizing antibodies were durable from first to second pregnancy after an interbirth interval of 80 days for mRNA and 49 days for protein. First litter pups were born approximately 40 days after the final immunization with mRNA or protein; therefore, immunity was durable in protecting second litter pups for at least 120 days for mRNA and 89 days for protein. We did not evaluate whether the pup antibody titers were derived transplacentally or from colostrum, although both are likely important contributors [6,17]. In addition to neutralizing antibodies, antibody dependent cellular cytotoxicity (ADCC) protects against HSV infection in newborn mice and humans [3,4,18,19]. Neonatal mice do not have effective ADCC effector cells by PND 3; therefore, vaccine protection was more likely mediated by neutralizing antibodies based on the age we infected and harvested organs [19]. The mRNA and protein vaccines were both highly efficacious in preventing disseminated infection to multiple organs over 2 generations of litters, while the Poly(C) control provided no protection. The mRNA vaccine protected 27/30 (90.0%) brains after IN infection over two generations, while the protein vaccine protected 29/32 (90.6%), and both vaccines prevented disseminated infection to lung, liver, spleen and kidney, except for a single pup in the protein group that had liver infection. Preventing disseminated infection and infection of the developing brain is an important goal for a vaccine in humans [4,20].

Several vaccine candidates have now been evaluated in the mouse neonatal herpes model, including replication-defective live virus, single-cycle live attenuated virus, trivalent protein, and in this report, trivalent mRNA [6,11,19]. Each vaccine provided strong protection, suggesting that more than one approach is likely to be effective. The mRNA vaccine outperformed the protein vaccine in preventing genital herpes in mice and guinea pigs [7]. Based on comparable protection by mRNA and protein vaccines against neonatal herpes reported here, we consider the nucleoside-modified mRNA vaccine to be our lead candidate for human trials.

CRediT authorship contribution statement

Philip C. LaTourette: Investigation, Project administration, Methodology, Data curation, Writing - original draft. **Sita Awasthi:** Conceptualization, Investigation, Methodology, Validation, Formal analysis, Supervision, Project administration. **Angela Desmond:** Methodology, Investigation. **Norbert Pardi:** Resources. **Gary H. Cohen:** Resources, Writing - review & editing. **Drew Weissman:** Resources, Writing - review & editing. **Harvey M. Friedman:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: HMF, SA, GHC and DW are inventors on patents held by the University of Pennsylvania for protein (HMF, SA) and mRNA (HMF, SA, GHC, DW) vaccines for genital herpes. NP is also named on a patent describing use of nucleoside-modified mRNA-LNP as a vaccine platform. The authors have disclosed their interests fully to the University of Pennsylvania, and have in place an approved plan for managing any potential conflicts arising from licensing of the patents.

Acknowledgements

HMF is the recipient of RO1 AI139618 from the National Institutes of Health, USA. HMF, GHC and DW are recipients of a grant from BioNTech SE, Germany. AD is a postdoctoral fellow supported by T32 AI118684 from the National Institutes of Health, USA (HMF, PI). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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