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Effects of immunization of pregnant guinea pigs with guinea pig cytomegalovirus glycoprotein B on viral spread in the placenta

Kaede Hashimoto[a,b], Souichi Yamada[a], Harutaka Katano[c], Saki Fukuchi[a,b], Yuko Sato[c], Minami Kato[a], Toyofumi Yamaguchi[d], Kohji Moriishi[b], Naoki Inoue[a,*]

[a] Department of Virology I, National Institute of Infectious Diseases, Tokyo, Japan
[b] Department of Microbiology, School of Medicine, University of Yamanashi, Yamanashi, Japan
[c] Department of Pathology, National Institute of Infectious Diseases, Tokyo, Japan
[d] Department of Biosciences, Teikyo University of Science, Yamanashi, Japan

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ABSTRACT

Background: Cytomegalovirus (CMV) is the most common cause of congenital virus infection. Infection of guinea pigs with guinea pig CMV (GPCMV) can provide a useful model for the analysis of its pathogenesis as well as for the evaluation of vaccines. Although glycoprotein B (gB) vaccines have been reported to reduce the incidence and mortality of congenital infection in human clinical trials and guinea pig animal models, the mechanisms of protection remain unclear.
Methods: To understand the gB vaccine protection mechanisms, we analyzed the spread of challenged viruses in the placentas and fetuses of guinea pig dams immunized with recombinant adenoviruses expressing GPCMV gB and β-galactosidase, rAd-gB and rAd-LacZ, respectively.
Results: Mean body weight of the fetuses in the dams immunized with rAd-LacZ followed by GPCMV challenge 3 weeks after immunization was 78% of that observed for dams immunized with rAd-gB. Under conditions in which congenital infection occurred in 75% of fetuses in rAd-LacZ-immunized dams, only 13% of fetuses in rAd-gB-immunized dams were congenitally infected. The placentas were infected less frequently in the gB-immunized animals. In the placentas of the rAd-LacZ- and rAd-gB-immunized animals, CMV early antigens were detected mainly in the spongiotrophoblast layer. Focal localization of viral antigens in the spongiotrophoblast layer suggests cell-to-cell viral spread in the placenta. In spite of a similar level of antibodies against gB and avidity indices among fetuses in each gB-immunized dam, congenital infection was sometimes observed in a littermate fetus. In such infected fetuses, CMV spread to most organs.
Conclusions: Our results suggest that antibodies against gB protected against infection mainly at the interface of the placenta rather than from the placenta to the fetus. The development of strategies to block cell-to-cell viral spread in the placenta is, therefore, required for effective protection against congenital CMV infection.

1. Introduction

Human cytomegalovirus (HCMV) is the most common cause of congenital virus infection. Congenital infection occurs in 0.2–1% of all births, and causes birth defects and developmental abnormalities, including sensorineural hearing loss (SNHL) and developmental delay [1–3]. Since one of the major routes of transmission to pregnant mothers is suggested to be *via* the excretions of their own children [4,5], development of a vaccine is the only effective way for protection against primary HCMV infection. Indeed, a review panel from the Institute of Medicine indicated that the development of a vaccine against HCMV, particularly with the aim of preventing primary infection in pregnant women, was of the highest priority among those for infectious diseases other than HIV [6]. As one of the promising approaches, purified glycoprotein B (gB) protein in combination with the MF59 adjuvant was used for a phase 2 clinical trial on CMV-seronegative women who had recently delivered a child and had intention of having another, and this subunit gB vaccine protocol demonstrated 50% efficacy against primary infection [7]. Although such results are encouraging, further studies are required to improve the efficacy and rapid waning of protection.

Animal models are generally valuable in gaining a better understanding of pathogenesis as well as in developing therapeutics for

* Corresponding author at: Laboratory of Herpesviruses, Department of Virology I, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Tel.: +81 3 4582 2663; fax: +81 3 5285 1180.
E-mail address: ninoue@nih.go.jp (N. Inoue).

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infectious diseases. In contrast to murine and rat CMVs, guinea pig CMV (GPCMV) crosses the placenta and causes infection *in utero*. Importantly, congenital GPCMV infection causes diseases similar to congenital HCMV diseases, such as IUGR and labyrinthitis [8–11]. Previous studies using a guinea pig model demonstrated that congenital infection and mortality in pups were reduced by the administration of anti-gB antibodies [12] or by immunization with gB in the form of a DNA or purified subunit vaccine [13,14]. In the placenta, GPCMV-induced histopathological lesions with viral antigens were localized at the transitional zone between the capillarized labyrinth and the noncapillarized interlobium [15]. However, the mechanism by which gB immunization inhibits such viral spread in the placenta and fetus remains unclear. In this study, to better understand the mechanism, we analyzed the spread of viruses in the placentas and fetuses of gB-immunized dams after virus challenge.

2. Materials and methods

2.1. Cells and viruses

Guinea pig lung fibroblasts (GPL, ATCC) were initially cultured in F-12 medium supplemented with 10% fetal bovine serum (FBS) and subsequently, after infection with GPCMV, in F-12 medium supplemented with 2% FBS. GPL cells were infected with a GPCMV (strain 22122) stock purchased from ATCC. Salivary glands (SGs) of a guinea pig (Hartley strain) infected with the original GPCMV stock were recovered, minced, sonicated briefly, and then centrifuged to remove debris. The supernatant (SG-P0) was used for the infection of GPL cells, and viral stocks were prepared after propagation of the cell-free virus 5 times in GPL cells (SG-P5). Virus stocks were concentrated by ultracentrifugation (82,000 × *g* for 2 h) in a 20% sucrose step gradient. Infectious units (IUs) of the stocks were determined by immunostaining of GPL cells infected with the diluted stocks in 12- or 24-well plates and cultured for 2–3 days as described previously [16].

A recombinant GPCMV expressing red fluorescent protein (RFP) was prepared as follows: The sequence region from position 4244 to 8013 (positions are based on Ref. [17]) of GPCMV (SG-P5) was replaced with a 1.8-kb DNA fragment covering the TurboRFP gene under the control of the CMV IE promoter (Evorgen JSC, Russia) by homologous recombination in GPL cells. The RFP-expressing GPCMV candidates were then plaque-purified several times in GPL cells. One of the candidates, GPCMV-RFP(4A), was used for neutralization assay.

2.2. Recombinant adenoviral vectors

The gene encoding the extracellular domain (amino acids 1–674) of GPCMV gB (rAd-gB) and the LacZ gene encoding β-galactosidase were cloned into a pENTR-3C vector and then into pAd/CMV/V5/DEST by using the LR recombinase system (Invitrogen), resulting in pAd-gB and pAd-LacZ, respectively. Recombinant adenoviruses, rAd-gB and rAd-LacZ, were recovered by transfection of 293A cells with pAd-gB and with pAd-LacZ, respectively, amplified, and purified by centrifugation through two CsCl step gradients as described previously [18].

2.3. Animal studies

Female guinea pigs at the indicated weeks after birth (Hartley, Japan SLC, Inc.) were inoculated intraperitonealy (i.p.) with 10^6 IUs of GPCMV and euthanized 3-weeks later. Blood specimens were drawn directly from the heart, and organ specimens, including liver, spleen, kidney, lung, and salivary gland, were harvested. Dams at 1-week of gestation (Japan SLC, Inc.) were inoculated i.p. with 10^{10} transducing units (TUs) of rAd viruses, infected subcutaneously with 10^6 IUs of GPCMV (SG-P5) 3-weeks after the inoculation, and euthanized 3-weeks later. Blood specimens were drawn from the dams and their fetuses. Salivary glands were also obtained from the dams. The placentas and fetuses were weighed and organs were harvested from the fetuses. All animal procedures were approved by the Animal Care and Use Committee of the National Institute of Infectious Diseases (NIID), and were conducted according to the 'Guidelines for Animal Experiments Performed at the NIID'.

2.4. Immunological assays

Transfection was performed by using a commercial reagent (Fugene6, Roche). GPCMV-infected and -transfected cells were fixed with acetone for 5 min and expression of antigens were examined by an immunofluorescence assay as described previously [19].

Anti-gB antibody levels in the dams and fetuses were measured by ELISA using the cytoplasmic fraction (0.8 μg of protein/well) of 293T cells transfected with a gB construct. Absorbance values obtained using sera diluted at 1:200 had a good correlation with titers determined as a maximum dilution (in a range of 1:200–3200) that gives the threshold absorbance (data not shown). Avidity indices of anti-gB IgG were determined by a 10-min treatment with 4 M urea.

Neutralizing activities in sera were measured as follows. A total of 1×10^4 IUs of GPCMV-RFP(4A) in 50 μl of medium was mixed with 50 μl of serially diluted serum specimens, and incubated at 37 °C for 1 h. The reaction mixtures were then diluted and inoculated into GPL cell cultures. RFP-positive foci were counted 3 days after infection.

2.5. Immunohistochemistry

All organs obtained from sacrificed animals were fixed in 10% buffered formalin. Formalin-fixed specimens were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE), as described previously [8]. Immunohistochemical analysis was performed using the monoclonal antibody g-1, which detects a GPCMV early antigen, or the rabbit polyclonal antibody against immediate-early proteins 1 and 2, which was generated by the immunization of rabbits with a GST-IE1/2 fusion protein, as primary antibodies. For the second- and third-phase immunostaining reagents, a biotinylated $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulin (DAKO) or of goat anti-rabbit immunoglobulin (DAKO) and peroxidase-conjugated streptavidin (DAKO) were used. DAB was used as a chromogen and the slides were counterstained with hematoxylin.

2.6. Quantification of viral DNA

DNA samples were prepared from the placentas and fetal organs, and viral DNAs in the samples were detected by real-time PCR assays for GPCMV GP83 and β-actin genes as described previously [16].

2.7. Statistical analysis

Mann–Whitney *U* test was used to analyze statistical differences in the weight of animals, fetuses, and placentas, and in the number of viral foci in placentas. Chi-square test was also used to analyze differences in the rates of CMV-positive placentas and fetal organs.