Immune Milk Suppresses Herpes Simplex Virus Type 1

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(Received June 1, 2015)

Summary Immune milk has been developed as a substitute for colostrum and contains a high concentration of IgG antibodies specific to the immunized pathogens. Meanwhile, bovine herpesvirus type-1 (BHV-1) naturally infects cattle worldwide, and its antibody is found in milk. Moreover, BHV-1 glycoprotein K, the major antigen, exhibits substantial homology with human herpes virus simplex 1 (HSV-1) glycoprotein K. On the basis of this evidence, we hypothesized BHV-1 antibody exists in immune milk and suppresses HSV-1 activity. This study investigated whether immune milk IgG recognizes HSV-1 and suppresses HSV-1 activity. IgG in immune milk was purified by affinity Protein A columns, and HSV-1–reactive IgG in immune milk IgG was detected and quantified by ELISA. The efficacy of the IgG against HSV-1 was analyzed using a reduction assay based on the cytopathic effect due to HSV-1 in the presence of macrophages. We detected a high concentration of HSV-1–reactive IgG in immune milk. Furthermore, IgG suppressed HSV-1 pathogenicity in the presence of macrophages. These results indicate immune milk has protective activity against HSV-1 by opsonic activity owing to its high concentration of HSV-1–reactive IgG, which is likely the BHV-1 antibody. HSV-1 is currently a refractory infection with a worldwide distribution. Primary infection occurs via the oral cavity, but there is no effective precaution at this time. However, the present results suggest that taking oral immune milk may be an effective measure to prevent primary HSV-1 infection in the oral cavity.

Key Words immune milk, human herpes simplex virus type-1, bovine herpesvirus type-1, glycoprotein K, cytopathic effect

Colostrum contains large quantities of bioactive substances (1) and plays several important roles in the health maintenance of newborns (2–4). In particular, the higher concentration of immunoglobulin (Ig) in colostrum than in mature milk supports neonatal immunity (3, 5–7). In addition, to support neonatal health, colostrum is a physiologically functional food (6). However, the commercial use of colostrum is limited owing to difficulties in harvesting and scaling up production.

Bovine “hyperimmunized milk,” commonly termed “immune milk,” is a substitute for colostrum originally produced by Stolle Milk Biologics International (Cincinnati, OH) in 1957. Immune milk is obtained from dairy cattle immunized with the antigens of 26 different bacteria and viruses affecting human digestive and respiratory organs, and is characterized by high levels of IgG antibody (Ab) with activity specific to the immunized pathogens (8, 9). Several clinical studies have evaluated its efficacy in the prevention and treatment of various human infections after oral ingestion (5, 9–13).

Meanwhile, cattle worldwide are infected by bovine herpesvirus type 1 (BHV-1), which causes such conditions as rhinotracheitis, conjunctivitis, and encephalitis (14–17). BHV-1 Ab is also detected in the milk of BHV-1–infected bovines (18–20). Importantly, BHV-1 shares homology with herpes simplex type-1 (HSV-1) with respect to major antigens recognized by the infected host’s immune system (21).

Therefore, we hypothesized that BHV-1 Ab exists in immune milk and suppresses HSV-1 activity. Accordingly, this study investigated whether immune milk IgG recognizes HSV-1 and suppresses HSV-1 pathogenicity.

Materials and Methods

Milk and HSV-1. The immune milk used was Stolle Milk Gold® (Kanematsu Wellness Co., Ltd., Tokyo, Japan), which contains 6.4 mg/g immunoglobulin (http://www.s-milk.com/). Skim milk (Kanematsu Wellness), which is whole milk with all milk fat removed, was used as a comparative control.

HSV-1 (HSV-1 HF strain) was supplied by Dr. Morita.
Purified HSV-1 pools ($4 \times 10^7$ PFU/mL) were prepared by infecting Vero cells at a multiplicity of infection of 0.01. Forty-eight hours post-infection, the supernatants were harvested for cell-free viruses and centrifuged at 1,750 g for 10 min at 4˚C. The virus titers in the supernatants were measured, and the supernatants were subsequently stored at -80˚C. HSV-1 solution ($1 \times 10^{10}$ PFU/mL) was prepared and inactivated by 1 J UV radiation (UV crosslinker Spectrolinker XL-1500, Tomy Seiki, Tokyo, Japan) before use.

**Affinity protein A-column.** IgG was purified from milk using affinity protein A-columns (GE Healthcare, Uppsala, Sweden). Milk was diluted 1 : 5 in phosphate-buffered saline (PBS) before being loaded into the columns. Protein A-bound IgG was eluted with 0.1 M glycine-HCl buffer (pH 2.7) into tubes containing 0.1 M Tris-HCl (pH 9.0) for neutralization. Fractions were pooled and dialyzed against PBS. IgG was measured by ELISA.

**ELISA.** ELISA plates (Corning, Corning, NY) were coated overnight at 4˚C with inactivated HSV-1 ($1 \times 10^7$ PFU·100 μL⁻¹·well⁻¹). HSV-1–reactive IgG in milk IgG or rabbit anti-HSV 1 polyclonal Ab (Chemicon International, Temecula, CA) were detected with horseradish peroxidase-labeled protein A (Thermo Fisher Scientific, Rockford, IL) supplemented with TMB (3,3',5,5'-tetramethylbenzidine). ELISA plates were read at a wavelength of 450 nm by a microplate reader (Spectrafluor Plus, Tecan, Osaka, Japan) according to the manufacturer’s instructions. Respective absorbance was measured in triplicate.

**Cytopathic effect reduction assay.** A CPE reduction assay was used to evaluate the inhibitory effect of immune milk IgG on HSV-1 transmitted to Vero cells. B6 and BALB/c mice splenic macrophages ($5 \times 10^5$) and HSV-1 (8 PFU/mL) were incubated with or without 100 μg/mL immune milk IgG at 37˚C for 2 h. Then, 100 μL supernatant was incubated with Vero cells for 1 h at 37˚C. After the supernatant was removed, the cells were cultured with Eagle minimal essential medium containing 1% fetal bovine serum for 6 d. The CPE was observed under an inverted microscope (CK40, Olympus, Tokyo, Japan). The assay was performed a total of seven times in two species of mouse.

**Statistical analyses.** All data are expressed as mean ± SE. Statistical significance was determined by Student’s t-test using Microsoft Excel 2003 (Microsoft, Redmond, WA). The level of significance was set at $p<0.05$.

**Results**

**Detection of HSV-1–reactive IgG in immune milk IgG**

IgG was isolated from 1 g milk by protein A column.
was calculated as follows: \( \frac{0.23}{100} \times \frac{0.01}{0.05} = 0.40 \). The concentration of HSV-1-reactive IgG in immune milk was calculated as follows: \( \frac{6.4 \, \text{mg/g}}{125 \, \text{µg/mL}} = 0.05 \). Therefore, the concentration of HSV-1-reactive IgG in immune milk was calculated as follows: 6.4 mg/g \( \times 2 \, \text{µg/mL} \times 125 \, \text{µg/mL} = 102 \, \text{µg/mL} \). This is as high as the concentration of each Ab against its respective immunized pathogen in immune milk (9). Therefore, HSV-1 Ab would be overproduced against latent BHV-1 owing to the influence of the hyperimmunity-inducing procedure. Moreover, the high amount of HSV-1-reactive IgG in immune milk had opsonic activity against HSV-1.

As a result of oral ingestion, the pathogen-specific antibodies in immune milk reduce several pathogens in the human gut (9, 12). Anti-rotavirus-specific Ab included in immune milk is reported to improve rotavirus-induced enteritis (12). In addition, the specific antibodies of five oral cavity pathogens (Haemophilus influenzae, Streptococcus sanguis, Streptococcus salivarius, Streptococcus mitis, and Streptococcus mutans) are found in immune milk. Therefore, immune milk is also expected to reduce pathogen levels in the oral cavity (10–12). For instance, Filler et al. (10) report that rinsing the oral cavity with immune milk protects against colonization of, and dental caries due to, S. mutans owing to the specific Ab, and that this efficacy is maintained for up to 2 wk; this is likely to be the case for HSV-1.

HSV-1 is a refractory infection and has a global prevalence of approximately 90% (23). Primary HSV-1 infection is an unapparent asymptomatic infection of the mouth and lips (24), but there is currently no effective preventive measure. However, the present results suggest that the amount of HSV-1-reactive Ab in immune milk prevents primary HSV-1 infection in the oral cavity. Moreover, we forecast the clinical effect of immune milk to suppress the recurrence of HSV-1. We already have a clinical trial study in view.

**Acknowledgments**

We would like to thank Editage (www.editage.jp) for English language editing.
REFERENCES

1) Biswas P, Vecchi A, Mantegani P, Mantelli B, Fortis C, Lazzarin A. 2007. Immunomodulatory effects of bovine colostrum in human peripheral blood mononuclear cells. New Microbiol 30: 447–454.

2) Kelly GS. 2003. Bovine colostrums: a review of clinical uses. Altern Med Rev 8: 378–394.

3) Wheeler TT, Hodgkinson AJ, Prosser CG, Davis SR. 2007. Immune components of colostrum and milk—a historical perspective. J Mammary Gland Biol Neoplasia 12: 237–247.

4) Ballard O, Morrow AL. 2013. Human milk composition: nutrients and bioactive factors. Pediatr Clin North Am 60: 49–74.

5) Lilius EM, Marnila P. 2001. The role of colostral antibodies in prevention of microbial infections. Curr Opin Infect Dis 14: 295–300.

6) Gapper LW, Copestake DE, Otter DE, Indyk HE. 2007. Analysis of bovine immunoglobulin G in milk, colostrum and dietary supplements: a review. Anal Bioanal Chem 389: 93–109.

7) Hurley WL, Theil PK. 2011. Perspectives on immunoglobulins in colostrum and milk. Nutrients 3: 442–474.

8) Golay A, Ferrara JM, Felber JP, Schneider H. 1990. Cholesterol-lowering effect of skim milk from immunized cows in hypercholesterolemic patients. Am J Clin Nutr 52: 1014–1019.

9) Ishida A, Yoshikai Y, Muroasaki S, Kubo C, Hidaka Y, Nomoto K. 1992. Consumption of milk from cows immunized with intestinal bacteria influences age-related changes in immune competence in mice. J Nutr 122: 1875–1883.

10) Filler SJ, Gregory RL, Michalek SM, Katz J, McGhee JR. 1991. Effect of immune bovine milk on Streptococcus mutans in human dental plaque. Arch Oral Biol 36: 41–47.

11) Loimaranta V, Nuutila J, Marnila P, Tenovuo J, Korhonen H, Lilius EM. 1999. Colostral proteins from cows immunised with Streptococcus mutans/S. sobrinus support the phagocytosis and killing of mutans streptococci by human leucocytes. J Med Microbiol 48: 917–926.

12) Korhonen H, Marnila P, Gill HS. 2000. Bovine milk antibodies for health. Br J Nutr 84(Suppl 1): S135–146.

13) Huang XH, Chen L, Gao W, Zhang W, Chen SJ, Xu LB, Zhang SQ. 2008. Specific IgG activity of bovine immune milk against diarrhoea bacteria and its protective effects on pathogen-infected intestinal damages. Vaccine 26: 5973–5980.

14) Straub OC. 1991. BHV1 infections: relevance and spread in Europe. Comp Immunol Microbiol Infect Dis 14: 175–186.

15) Hutchinson L, Johnson DC. 1995. Herpes simplex virus glycoprotein K promotes egress of virus particles. J Virol 69: 5401–5413.

16) Turin L, Russo S, Poli G. 1999. BHV-1: new molecular approaches to control a common and widespread infection. Mol Med 5: 261–284.

17) Nandi S, Kumar M, Manohar M, Chauhan RS. 2009. Bovine herpes virus infections in cattle. Anim Health Res Rev 10: 85–98.

18) Frankena K, Franken P, Vandevoorde J, Kocks G, Kramps JA. 1997. Probability of detecting antibodies to bovine herpesvirus 1 in bulk milk after the introduction of a positive animal on to a negative farm. Vet Rec 140: 90–92.

19) Van Wuijckhuise L, Bosch J, Franken P, Frankena K, Elbers AR. 1998. Epidemiological characteristics of bovine herpesvirus 1 infections determined by bulk milk testing of all Dutch dairy herds. Vet Rec 142: 181–184.

20) Nylin B, Stroger U, Rønsholt L. 2000. A retrospective evaluation of a bovine herpesvirus-1 (BHV-1) Ab ELISA on bulk-tank milk samples for classification of the BHV-1 status of Danish dairy herds. Prev Vet Med 47: 91–105.

21) Khadr A, Tikoo SK, Babik LA, van Drunen Littel-van den Hurk S. 1996. Sequence and expression of a bovine herpesvirus-1 gene homologous to the glycoprotein K-encoding gene of herpes simplex virus-1. Gene 168: 189–193.

22) Davison AJ. 2010. Herpesvirus systematics. Vet Microbiol 143: 52–69.

23) Nicoll MP, Proença JT, Elstathiou S. 2012. The molecular basis of herpes simplex virus latency. FEMS Microbiol Rev 36: 684–705.

24) Cohrs RJ, Gilden DH. 2001. Human herpesvirus latency. Brain Pathol 11: 465–474.