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Construction of the recombinant pseudorabies viruses expressing *Cryptosporidium parvum* an immunodominant surface protein, p23

Yasuhiro Takashima[1], Xuenan Xuan[2], Hideyuki Nagasawa[2], Yasunobu Matsumoto[1], Ikuo Igarashi[2], Kozo Fujisaki[2], Takeshi Mikami[2] and Haruki Otsuka[1].

[1]*Department of Global Agricultural Science, Graduate School of Agriculture and Life Science, The University of Tokyo, Japan.*

[2]National Research Center for Protozoan Diseases, Obihiro University, Japan

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Corresponding author: Haruki Otsuka, PhD.
Department of Global Agricultural Science, Graduate School of Agriculture and Life Science, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
Tel: +81-3-3812-2111, Fax:+81-3-5802-9519, e-mail: aotsuka@ mail.ecc.u-tokyo.ac.jp

ABSTRACT

To develop a vaccine against cryptosporidiosis in animals, we constructed recombinant pseudorabies virus (PrV), a member of the Herpesviridae Alphaherpesvirus subfamily, expressing an immunodominant surface protein p23 of *Cryptosporidium parvum* sporozoites. Because of the wide host range of PrV, it has the possibility as the vector to delivery the foreign genes to several species of animals containing experiment animal. In the recombinant constructed in this study, the p23 gene under the control of CAG promoter was integrated into the thymidine kinase (TK) gene of PRV. Antibody against p23 recognized p23 expressed in CPK cells infected with the recombinant, as the approximate 23 kDa specific band in Western blotting analysis. This study showed the possibility of a PrV recombinant as a vaccine against cryptosporidiosis in animals.

INTRODUCTION

Cryptosporidium parvum is an intestinal protozoan parasite that causes enteric infection and diarrhea in human and animals (Tzipori, 1983: Fayer et al 1990). In the case of animals, the infection of *C. parvum* in young calves results in a severe economic concern. Although there is currently no vaccination or specific therapeutic regiments for control cryptosporidiosis, it was reported that receiving colostrum from hyperimmunized cows provide partial protection against

cryptosporidiosis with neonatal calves (Fayer et al. 1989). Furthermore, 23-kDa glycoprotein of *C. parvum* (p23) was identified as a protein with neutralization-sensitive epitopes (Perryman, L.E et al.1996). And it was suggested that immunoglobulin A (IgA) directed to neutralization-sensitive epitope of p23 have utility in passive immunization against *C. parvum* infection on mice (Enriquez, F.J. et al. 1998). Therefore the p23 peptide is considered to be an important candidate for development of an effective vaccine against cryptosporidiosis.

Pseudorabies virus (PrV), a member of the Herpesviridae Alphaherpesvirus subfamily, which has plural number of non-vital genes in which foreign genes can be interrupted. Although PrV causes Aujeszky's disease, a serious illness of almost all spices of mammalians, safe and effective vaccine strains, which lack thymidine kinase (TK) gene, had been established (Mcfarland et al. 1987, Mcfarland and Hill 1987, Kit et al.1987). These attenuated vaccine strains have the possibility as useful vector for expression of foreign genes. And PrV vector system is useful as the experimental model because it can infect to almost all species of mammalian including experimental animals (Takashima et al. 2000). In this study we have constructed recombinant PRV expressing p23 gene of *C. parvum*, to develop the herpes virus vector vaccine against cryptosporidiosis, as the candidates for the useful vaccine model that can infect to experimental animals.

MATERIALS and METHODS

Virus and cells: CPK cells were cultured in Eagle's MEM (autoclavable, Nissui) containing 7.5 % of fetal bovine serum and 60 μl/ml kanamycin. The TK deficient cell line, MDBK Bu100 cells (Otsuka and Xuan, 1996) were cultured in the same medium with 100 μg/ml of 5-buromo-2'-deoxyuridine PrV Indiana strain (IND) and its recombinant were prepared in CPK cells.

Parasites and p23 gene*: C. parvum* isolate (Mito strain) used for all experiments was obtained as described previously (Xuan et al. 1999). The cloning of p23 gene of *C. parvum* (Mito strain) was performed and cloned into *Bam*HI site as described at another paper (Takashima et al. 2001). The gene cording p23 used for all experiments in this study was obtained from the resulting plasmid by digestion with *Bam*HI.

Construction of the plasmid*:*

The *Pst*I- *Kpn*I fragment of PrV genome containing thymidine kinase (TK) gene and flanking region was replaced to *Pst* I- *Kpn* I fragment of pUC19. And then *Bam* HI- *Sal* I fragment of the resulting plasmid, containing start codon of TK gene and promoter region of UL24 gene, was

replaced to *Bam* HI- *Sal* I fragment of pAxCAwt (Takara, Japan) containing CAG promoter and rabbit β-globin poly A signal (Kanegae et al.1995). And the p23 gene was inserted into *Swa* I site of the plasmid. The resulting plasmid was designated as pUC/pTK/CAG/p23 and used as the transfer vector (Fig.1).

Infectious DNA of PrV: CPK cells cultured were infected with PRV (IND) at m.o.i. of 5.0 After incubating at 37 ^{o}C for 16 h, infected cell were scraped into the media and centrifuged at 2000 rpm for 10 min and the infected cells were resuspended with 1.5 ml of lysis buffer (0.25 % triton X-100, 10 mM Tris-HCl [pH 7.3], 10 mM EDTA) and homogenized. After added 60 µl of 5 M NaCl and incubated on ice for 10 min, the homogenized cells were centrifuged at 2000 rpm for 10 min. And then the proteinase K was added to the supernatant transferred into a new cloven to a final concentration of 200 µg/ml and incubated at 37 ^{o}C for 1h. The mixture was treated with water-saturated phenol and DNA was precipitated with ethanol.

Construction of recombinant PrV: CPK cells were inoculated in a 6-well plate (Corning) and co-transfected with 1.5µg of pUC/pTK/CAG/p23 digested with *Kpn* I and 1 µg of infectious virus DNA using Lipofectamine PLUS^{TM} Reagent (GIBCO BRL). After the transferred culture was incubated at 37 ^{o}C for 72 h, the progeny virus was harvested. To enrich TK-negative recombinant viruses among the progeny viruses, MDBK Bu100 cells (Otsuka and Xuan, 1996) were infected with harvested virus. And infected cells were cultured in selecting medium (Eagle's MEM containing 7.5 % FCS and 50 µg of 5-iodo-2-deoxyuridine). After the culture was incubated at 37 ^{o}C for 24 h, the reproduced virus was harvested. And then monolayer of CPK cells was infected with the harvested virus and the plaques were picked up. The obtained recombinant virus was designated as IND/p23.

Western blotting analysis: SDS-polyacrylamide gel electrophoresis and Western blotting analysis were carried out as described previously (Takashima et al.1999) using mouse anti-serum against p23 (Takashima et al. 2001).

Inoculation to mice:

Each 5 Balb/c female 7-10 weeks old mice were fallen into 3 groups and inoculated intraperitoneally with 10^2, 10^3 or 10^4 pfu of IND/p23 in 200 µl of PBS previously. As the control 3 mice were inoculated with 200 µl of PBS.

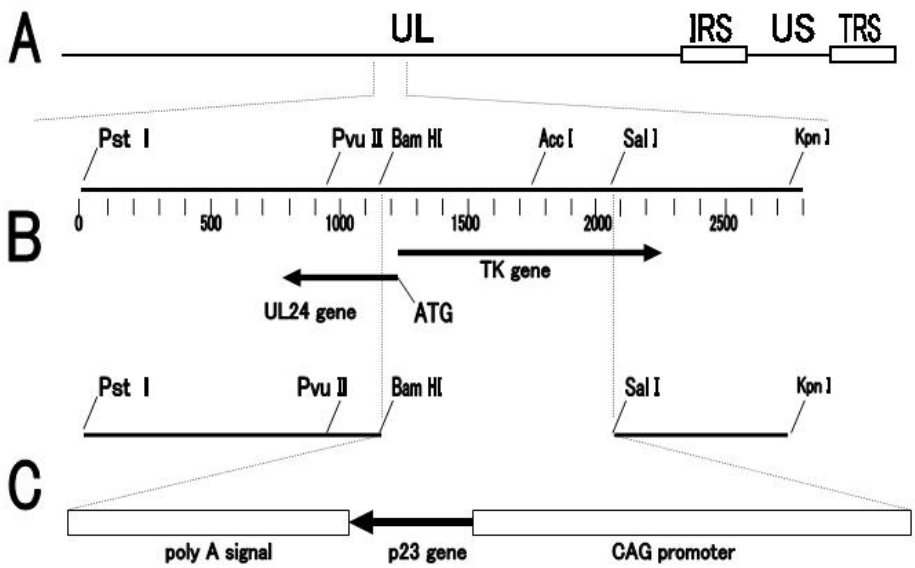

Fig.1: Construction of the transfer plasmid vector. (A) The genome of PrV. Solid bars indicate unique long (UL) and unique short (US) region. Open boxes indicate repeat regions. (B) *Pst*I-*Kpn*I fragment of PrV genome. Solid arrows indicate the open reading flames of PrV genes, TK gene and UL24 gene. (C) Construction of the transfer plasmid vector, pUC/pTK/CAG/p23. The solid arrow indicates eGFP gene.

RESULTS and DISCUSSION

Expression of p23 by the recombinant PrV:

To construct the recombinant PrV expressing p23 peptide of *C. parvum*, CPK cells were transfected with the plasmid, pUC/pTK/CAG/p23 (Fig. 1) and infectious DNA of PrV. After incubated at 37 ^{o}C for 72 h, the progeny virus was harvested. And then to enrich TK-negative recombinant viruses among the progeny viruses, MDBK Bu100 cells (Otsuka and Xuan, 1996) were infected with harvested virus and cultured in selecting medium as described in above. After several times of plaque purification, the obtained recombinant virus was designated as IND/p23.

CPK cells infected with IND/p23 were analyzed by Western blotting analysis using anti-p23 mouse serum. The result of the Western blotting analysis is shown in Fig. 2. The

approximate 23 kDa specific band, same size to that of the authentic p23 peptide expressed by *C. parvum*, was detected in CPK cells infected with recombinant PRV, IND/p23. The anti-p23 mouse sera did not react against any peptide in CPK cells infected with parental virus. The results suggested indicate that p23 peptide expressed in IND/p23 infected CPK cells is glycosilated as same as authentic p23 expressed in the parasite.

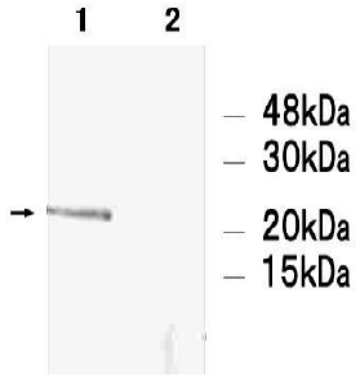

Fig.2: Western blot analysis of CPK cells infected with recombinant PrV. CPK cells infected with IND/p23 and PrV (IND) were lysed and separated by SDS-PAGE (lane 1 and lane 2, respectively) following immunoblotting using anti-p23 mouse serum as the primary antibody.

Mortality of mice infected with the recombinant PrV:

Four days post inoculation, all mice infected with 10^4 pfu of IND/p23, 2 of 5 mice infected with 10^3 pfu of the recombinant and 2 of 5 mice infected with 10^2 pfu of the recombinant died. Three of 5 mice inoculated with 10^2 or 10^3 pfu of the recombinant survived during all period of this experiment.

It has reported that the deletion of TK gene of PrV results in decreasing of virulence. And TK deleted PrV had been constructed and used as a live vaccine against Aujeszky's disease (Kit, S. et al. 1987). However, comparing with the TK deleted vaccine strain, IND/p23 was high virulence (data not shown). In the case of the recombinant, which has constructed in this study, not only TK gene but also UL24 gene are inactivated as shown in Fig.1. In the case of bovine herpesvirus-1 (BHV-1) lacking ICP0 gene, it was suggested that the cloning of a recombinant lacking ICP0

gene was very difficult because of the contamination of the parental virus providing ICP0 to the ICP0 lacking recombinant (Koppel, R. et al.1996). At the procedure of cloning of IND/p23, as like the case of BHV-1 lacking ICP0, parental virus might be a helper virus to provide US24 gene product to the recombinant. There is possibility of the undetectable level of high virulent parental virus, PrV (IND). It is necessary to confirm the effect of deletion of UL24 gene to virulence of the recombinant PrV to develop an effective and safe vaccine against cryptosporidiosis.

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