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IMMUNOGENIC PROPERTY OF A RECOMBINANT VACCINIA VIRUS EXPRESSING P23 OF *CRYPTOSPORIDIUM PARVUM*

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ABSTRACT

To develop a vaccine against cryptosporidiosis in cattle, we constructed a recombinant vaccina virus expressing an immunodominant surface protein p23 of *Cryptosporidium parvum* (*C. parvum*) sporozoites. Antibody against p23 recognized the p23 expressed in RK13 cells infected with the recombinant vaccinia virus as an approximately 23 kDa specific band in Western blotting analysis. The immunization of Balb/c mice with the recombinant induced the production of immunoglobulin G1 (IgG1). However, the level of immunoglobulin G2a (IgG2a) production was very low. These results indicate that the p23 expressed by the recombinant vacinia virus induced predominantly a Th2 response in Balb/c mice. In the case of C57BL mice, delayed type hyper sensitivity was observed. However, the induction of antibody production against p23 was not detected.

INTRODUCTION

Cryptosporidium parvum (*C. parvum*) is an intestinal protozoan parasite that causes enteric infection and diarrhea in human and animals (Tzypori, 1983). In the case of animals, the infection of *C. parvum* is in young calves can be a severe economic concern. Despite of the importance of the control of *C. parvum,* there are currently no vaccination or specific therapeutic regimens.

Although it was reported that an experimental oral vaccination could protect young cattle from cryptosporidiosis, a field test of the vaccine resulted in failure (Harps and Goff. 1998). It has been suggested that the young calves were exposed to *C. parvum* in the initial days of life in the field and the vaccination few days after the birth was already too late. The report indicates the importance of providing neonatal calves with immunity against *C. parvum* as early as possible. Therefore a passive immunization via colostrums has a great possibility as a method to control the infection of *C. parvum* to neonatal calves. It was reported that receiving colostrums from hyperimmunized cows provide a partial protection against cryptosporidiosis in neonatal calves (Fayer, et al., 1989).

It was been reported that 23 kDa glycoprotein of *C. parvum* was identified as an antigen with neutralization sensitive epitopes of sporozoite stage (Perryman et al., 1996). It was also suggested that IgA

directed p23 have an utility in passive immunization against *C. parvum* infection in mice (Enriquez and Riggs, 1998). Therefore, vaccination of dams with p23 is considered to be an important vaccine candidate to produce colostrums that can protect neonatal calves from cryptosporidiosis.

Vaccinia virus is a member of Poxviridae, which is known as a widely studied viral vector for expression of foreign genes. (Moss, 1991; Yilma, 1994). Several vaccinia virus recombinants expressing protozoan antigen were reported as candidates of vacinies (Nishikawa et al., 2000; Nishikawa et al., 2001; Takasima et al, 1999).

In this study, we constructed a vaccinia virus recombinant expressing p23 of *C. parvum* as a vaccine candidate for dams to produce colostrums containing antibodies and investigated the property of the recombinant virus.

MATERIALS AND METHODS

Parasite and its gene coding p23: *C. parvum* isolate (Mito strain) used for all experiments was obtained from National Institute of Animal Health, Japan and oocysts were prepared and the DNA was extracted as described previously (Xuan et al., 1999) . The gene coding p23 was amplified by PCR as described previously, using the oligonucleotide primers, 5'-ACGGATCCAAAAATGGGTTGTT-3' and 5'-ACGGATCCTAATTTAGGCATCA-3'. The amplified fragment was inserted into Bam HI site of pUC19. The p23 gene used in all experiments was obtained from the resulting plasmid, designated as pUC/p23.

Viruses and cells: Vaccinia virus LC16mO (mO) strain and its recombinant were propagated in RK13 or 143TK- cells in Eagle's minimum essential medium (EMEM) supplemented with 7.5% fetal calf serum.

Anti-p23 serum: The production of anti-p23 was described previously (Takashima et al, in press). Briefly, Balb/c mouse was immunized with one hundred μg of p23 protein expressed by *E. coli* expressing system with Freund's complete adjubant. On day 14 and 28 post immunization, the same antigen was injected with Freund's incomplete adjubant. The serum of immunized mice were collected 10 days after the last immunization and used as anti-p23 serum.

Construction of a recombinant vaccinia virus: The gene coding p23 was inserted into the *Bam* HI site of the vaccinia virus transfer vector, pAK8 (Yasuda et al., 1990). The transfer vector contains thymidin kinase (tk) gene and flanking region of vaccinia virus, and the early-late promoter of vaccinia virus p7.5 kDa polypeptide was inserted into the vaccinia virus tk gene derived sequence. The *Bam* HI site was located at downstream of the promoter. Therefore in the resulting plasmid, the p23 gene is under the control of the early-late promoter. The plasmid was designated as pAK/p23. A recombinant vaccinia virus was constructed by homologous recombination between parental virus and pAK/p23 as described previously (Xuan et al, 1999). The recombinant virus expressing p23 was checked by indirect immuno-fluorescence assay as described previously (Xuan et al, 1997). A clone expressing p23 was designated as vv/p23 and used in following experiments.

Western blotting analysis: RK13 cells were infected with vaccinia virus mO strain or vv/p23 at a multiplicity of infection (moi) of 5 and incubated at 37□ for 24h. The infected cells were then harvested, lysed and cellular proteins were separated by SDS-polyacrylamide gel electrophoresis. The lysate of oocyst was also separated by SDS-polyacrylamide gel electrophoresis. The protein bands were transferred to a transfer membrane (Millipore, USA). And the p23 band on the transfer membrane was detected by anti-p23

serum or mouse sera collected from mice immunized with the recombinant vaccinia virus, as the primary antibodies. As the secondary antibody, rabbit anti mouse IgG antibody (ZYMED, USA) was used.

Immunization: Seven or eight weeks old Balb/c female mice and C57BL mice were inoculated intraperitoneally with 3×10^6 pfu vaccinia virus mO strain or vv/p23.

Recombinant p23 peptide: The gene cording p23 was inserted into the *Sma* I site of the bacterial expression vector pGEX5×2 (Amersham Pharmacia Biotech, USA) in frame. The p23 expressed in *E.coli* DH5□ transformed with the resulting plasmid, as a fusion protein with glutathion S-transferase was purified according to the manufacture's instruction. The p23 protein expressed by *E.coli* was used as an antigen for ELISA and DTH assay.

ELISA to detect antibodies against p23: Purified p23 protein was diluted in carbonate buffer (74 mM $NaHCO_3$, 26 mM Na_2HCO_3. pH 9.6) to 10 μg/ml and the 100 μl aliquots were added into each well of 96-well ELISA plate (Corning). The plate was incubated at 4^0C over night and washed with PBS containing 0.1% Triton X100 (PBS-TritonX100). After the fixation with 3 % skim milk in PBS, mouse serum samples were diluted 1:100 and, and 100 μl of the diluted serum sample was added to a well and incubated at 37^0C for 1hr. The wells were washed 3 times with PBS-TritonX100, added with horseradish peroxidase-conjugated goat anti mouse IgG, IgG1 or IgG2a antibody (ZYMED, USA), and incubated at 37□ for 1hr. After washing3 times with PBS-TritonX100, 100 μl of 0.04% o-phenylendiamine and 0.003% H_2O_2 in pH5.0 phosphate-citrate buffer (52 mM citric acid, 103 mM Na_2HPO_4) was added, and incubated at room temperature for 30 min. The reaction was stopped by adding 20 μl of 6N H_2SO_4, and the absorption at 490 nm was determined. For end-point assay, after making 5-fold serial dilutions of serum samples, 100 μl of diluted serum samples were added to each well of the plate. O.D. value was measured as described above. The mean of O.D. of 1:100 diluted pre-immunized serum samples was calculated and added six standard divisions as the cut-off point. The dilution rate of immunized serum samples, at which O.D. is same to the cut-off point, was determined as end-point titer.

ELISA to detect antibodies against virus antigens: RK13 cells infected with vaccinia virus mO strain or vv/p23 at mi of 10 was sonicated and soluble fraction was diluted in carbonate buffer to 50 μg/ml. The 100 μl of aliquots of soluble fraction were added to each well. Following procedures were carried out as described above.

DTH assay: After 40 days post immunization, 40 μl of antigen solution (500 μg/ml) was injected into the left footpad of immunized mouse. As a control, the same volume of PBS was injected into the right footpad. After 48 hr from the injection, swelling of each footpad was measured. Net swelling was calculated as described as previously (Takasima et al 1999).

RESULTS:

Construction of the recombinant vaccinia virus: The coding region of p23 gene under the control of the early-late promoter of the vaccinia virus 7.5 kDa polypeptide, was inserted into the tk gene of vaccinia virus mO strain by homologous recombination. At the stationary phase of virus proliferation, the resulting recombinant virus, vv/p23, reached a titer at least ten-fold less than that of parental virus (data not shown). To detect the p23 expreson and to determine the molecular weight of the p23, Western blotting analysis was performed. As shown in Fig. 1, an approximately 23 kDa specific band was detected in RK13 cells infected

with vv/p23. The molecular weight of p23 expressed by recombinant virus was the same size as that of the authentic p23 expressed by *C. parvum* was detected (data not shown). The anti-p23 serum did not react with any protein in RK cells infected with parental mO strain.

Immunogenic properties of p23 expressed by recombinant vaccinia virus: Five Balb/c mice were immunized with vv/23 or mO strain. After the immunization, the increasing of IgG titer against p23 was observed in mice immunized with vv/p23 (Fig2). However, DTH reaction against p23 was not detectable (Fig. 3). These results suggested that immunization with vv/p23 induce type 2 immune reaction on Balb/c mice. To confirm this, the titers of IgG1 and IgG2a were measured as an indicator of immuno reaction type. As shown in Table 1, production of only IgG1 but IgG2a was not detected except one mouse. In C57BL mice, the production of antibody against p23 was not observed. However, DTH reaction against p23 was observed (Fig. 3). These results suggested that immunization with vv/p23 induce type 1 immune reaction on C57BL mice.

To investigate the reactivity against parasite of induced antibody in Balb/c mice, lysate of oocyst was reacted reacted with mouse serum harvested from the same mouse pre- and post immunization by Western blotting analysis. As shown Fig.4, approximately 23 and 35 kDa specific bands were reacted with the immunized serum but not with pre-immunized serum. The anti-serum against p23, which was produced by immunizing *E.coli* expressed p23 protein, was also reacted to not only 23 kDa protein but also 35 kDa protein (Data not shown). It is not clear whether the 35 kDa protein band is related with p23 or not. However, these results suggested that the band was not the artifact of using vaccinia virus vector.

Immune reaction against virus antigens: The immune reaction against vaccina virus antigen was investigated in immunized Balb/c mice. IgG1 and IgG2a antibodies against virus antigens in sera harvested from immunized mice were detected by ELISA assay. As shown in Fig. 5, in all mice immunized with vv/p23 or mO strain, both of IgG1 and IgG2a against virus antigens were detected. Parental virus induced antibodies more effectively than vv/p23 did.

Table 1 - 1 . The end-point titer of IgG1 and IgG2a against p23 in Balb/C mice immunized with vv/p23

Mouse No.	IgG1	IgG2a
1	4.0	N
2	3.8	N
3	3.3	N
4	3.1	N
5	3.3	3.3

The titer of IgG1 and IgG2a are shown as $\log_{10}$.
N: under detectable.

DISCUSSION:

In this study, we focused on vaccinia virus, which can induce humoral, and cell mediated immunity (Andrew et al., 1989) as a live virus vector to develop a vaccine for cryptosporidiosis. The recombinant vaccinia virus, vv/p23 is tk negative in phenotype. It is known that tk negative viruses have a reduced pathogenesity *in vivo* (Buller et al 1985). But the deletion of tk gene of vaccinia virus mO strain does not have an effect on the growth rate *in vitro* (Nishikawa et al., 2000). However, the growth rate of vv/p23 was approximately 10 fold less than that of parental mO strain. This might be due to the effect of the produced p23 protein. The immunization of mice with vv/p23 induced lower level of antibodies against virus antigens

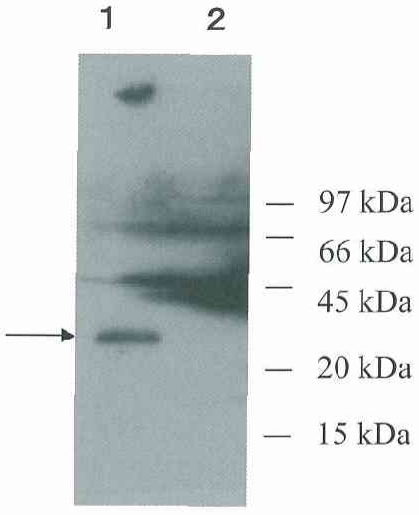

Fig. 1 Western blotting analysis to detect the expression of p23 by the recombinant vaccinia virus, vv/p23. RK13 cells infected with vv/p23 (lane 1) or parental mO strain (lane 2) were reacted with anti-p23 mouse serum.

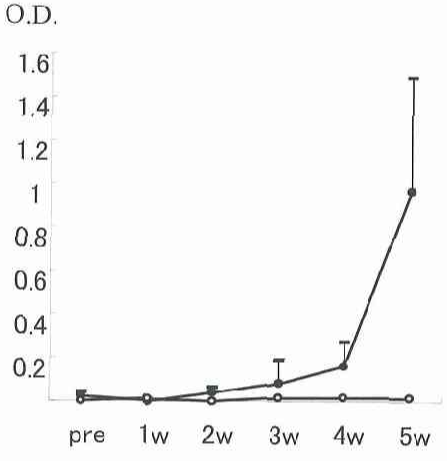

Fig. 2 The specific antibody response of mice immunized with vv/p23 (solid circles) or parental mO strain (open circles). Antibodies in serum harvested once a week were detected by ELISA. The data is shown as O.D. value.

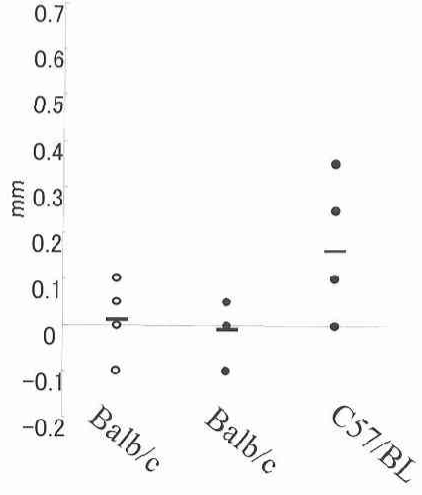

Fig. 3 DTH reaction of mice immunized with vv/p23 (solid circles) or parental mO strain (open circles). The circle indicates net swelling of each mouse. The bar indicates average of the net swellings of each group.

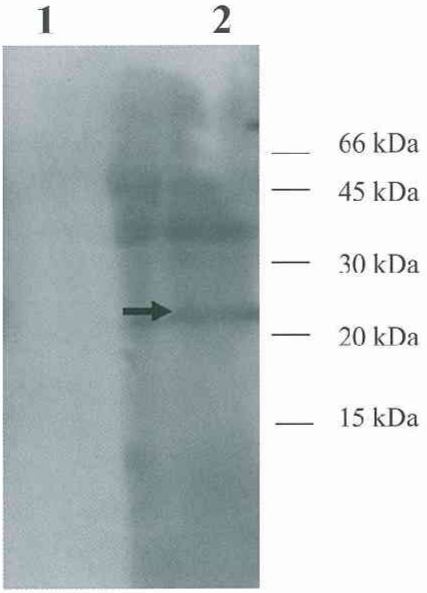

Fig. 4 Reactivity of mouse serum immunized with vv/p23. The mouse serum, harvested pre-immunization (lane 1) or post-immunization (lane 2), was reacted with *C. parvum* oocysts antigen by Western blotting analysis.

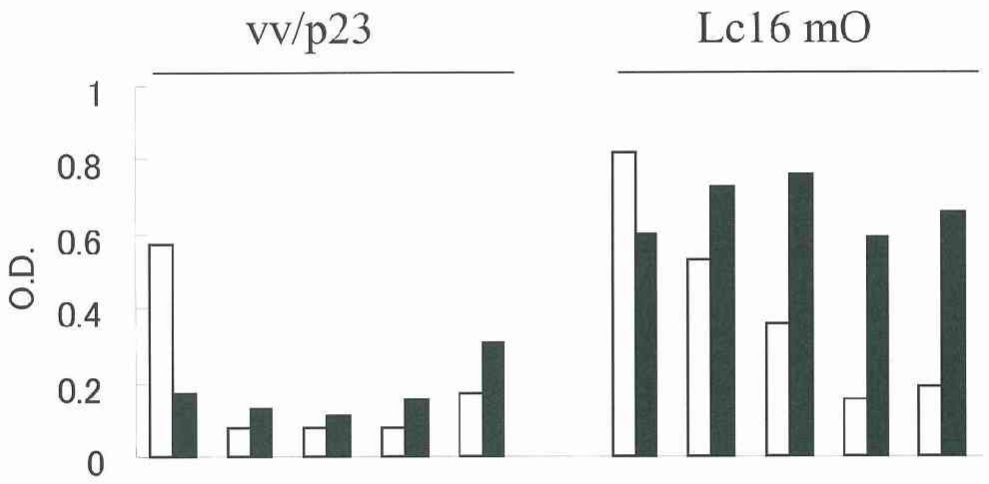

Fig. 5 The antibodies against vaccinia virus antigens, in Balb/c mice immunized with vv/p23 or parental mO strain, was detected by ELISA. The open bar and solid bar indicate the O.D. value of IgG1 and IgG2a in each mouse, respectively.

than the parental virus did (Fig.5). It suggests that the growth rate of vv/p23 is also lower *in vivo* than that of parental virus. However, despite of the low growth rate of vv/p23, we could demonstrate that immunization with vv/p23, without any booster, induced a significant increasing of antibody against p23 in Balb/c mice (Fig.2).

It is reported that the major part of immunoglobulin in colostrums of ruminant animals is IgG1, which is derived from blood (Lascelles, 1979). In this study, we demonstrated that immunization with vv/p23 induce greater level of IgG1 production than IgG2a in Balb/c mice (Fig3). These results indicate a possibility of vv/p23 as a vaccine for dams to produce colostrums containing high dose antibodies against p23. It is known that Th1 cells, which produce interleukin (IL)-2, interferon (IFN)-□ induce the secretion of IgG2a and that Th2 cells, which produce IL-4 and IL-5 induce that of IgG1 (Stevens et al., 1988). The results indicate that the p23 expressed by vv/p23 activate Th2 cells and induce type 2 immune reaction, resulting in the production of IgG1 in Balb/c mice. However, Th2 predominant immune reaction was observed only Balb/c mice but not in C57BL mice. These results indicate that the bias of induced immune reaction type was due to strains of mice.

To develop a vaccine against cryptosporidiosis for dams to protect neonatal animals, it remains to be investigated whether a Th2 predominant immune reaction in bovine can be induced by the immunization with vv/p23.

References

Andrew M.E., Coupar, B.E., Boyle, D.B. 1989. Humoral and cell-mediated immune responses to recombinant vaccinia virus in mice. *Immunol. Cell. Biol.* 67: 331-337.

Buller, R.M. Smith, G.L., Gremer, K., Notkins, A.L., Moss, B. 1985. Decreased virulence of vaccinia virus expression vectors is associated with thymidine kinase-negative phenotype. *Nature* 317:813-815.

Enriquez, F.J. Riggs, M.W. 1998. Role of immunoglobulin A monoclonal antibodies against p23 in controlling murin Cryptosporidium parvum infection. *Infect. Immunol.* 66: 4469-4473.

Fayer, R., C. Andrews, B. L. Unger, and B. Blagburn. 1989 Efficacy of hyperimmune bovine colostrum for prophylaxis of cryptosporidiosis in neonatal calves. *J. Parasitol.* 75:393-397.

Harps, J. A., and J. P. Goff. 1998. Strategies for the control of *Cryptosporidium parvum* infection in calves. *J. Dairy Sci.* 81:289-294.

Lascelles, A.K. 1979. The immune system on the ruminant mammary gland and its role in the control of mastitis. *J. Dairy Sci.* 62:154-167.

Moss, B. 1991. Vaccinia virus: A tool for research and vaccine development. *Science* 252:1662-1667.

Nishikawa, Y., Kousaka, Y., Fukumoto, S., Xuan, X., Nagasawa, H., Igarashi, I., Fujisaki, K., Otsuka, H. and Mikami, T. 2000. Delivery of Neospora caninum surface protein, NcSR (Nc-p43), to mouse using recombinant vaccinia virus. *Parasitol. Res.* 86:934-939.

Nishikawa, Y., Xuan, X., Nagasawa, H., Igarashi, I., Fujisaki, K., Otsuka, H. and Mikami, T. 2001. Prevention of vertical transmission of Neospora caninum in BALB/c mice by recombinant vaccinia virus carrying NcSRS2 gene. *Vaccine.* 19:1710-1716.

Perryman, L. E.,, D. P. Jasmer, M. W. Riggs, S. G. Bohnet, T. C. McGuire, and M. J. Arrowood. 1996. A cloned gene of *Cryptosporidium parvum* encodes neutralization-sensitive epitopes. *Mol. Biochem.*

Parasitol. 80:137-147.

Stevens, T.L., Bossie, A., Sanders, V.M., Fernandez-Botran, R., Coffman, R.L., Bossie, A., Mosmann, T.R. and Vitetta, E.S. 1988. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature* 1334: 252-258.

Takasima, Y., X. Xuan, Y. Matsumoto, M Onuma, and H. Otsuka. 1999. Antibodies produced by mice immunized with recombinant vaccinia viruses expressing two different types of a major Theileria sergenti surface antigen (p32) react with the native surface antigen. *Vet. Parasitol.* 84:65-73.

Takashima, Y., Xuan, X., Kimata, I., Iseki, M., Kodama, Y., Nagane, N., Nagasawa, H., Matsumoto, Y., Mikami, T and Otsuka, H. Recombinant bovine herpesvirus-1 (BHV-1) expressing p23 protein of Cryptosporidium parvum induces neutralizing antibodies in rabbits. *J. Parasitol.* (In prss)

Tzipori, S. Cryptosporidiosis in animals and humans. 1983. *Microbiol. Rev.* 47: 84-96.

Xuan, X., Kojima, A., Murata, T., Mikami. T. and Otsuka, H. 1997. Analysis of canaine herpesvirus gB, gC and gD expressed by a recombinant vaccinia virus. *Arch. Viol.* 142:1003-1010.

Xuan, X., Zhang, S. Kamio, T., Tsushima, Y., Kamada, T., Nishikawa, Y., Otsuka, H., Karanis, P., Igarashi, I., Nagasawa, H., Fujisaki, K. and Mikami, T. 1999. Characterization of P15 antigen *Cryptosporidium parvum* expressed by a recombinant vaccinia virus. *J. Protozool. Res.* 9: 32-40.

Yasuda, A., Kimura-Kuroda, J., Ogimoto, M., Sata, T., Takamura, c., Kurata, T., Kojima, A. and Yasui, K. 1990. Induction protective immunity in animals vaccinated with recombinant vaccinia virus that express pre M abd E glycoproteins Japanese enthephalitis virus. *J. Viol.* 64:2788-2797.

Yilma, T. 1994. Genetically engineered vaccines for animal viral diseases. J. *Am. Vet. Med. Assoc.* 204: 1606-1615.