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Characterization of P15 Antigen of *Cryptosporidium parvum* Expressed by a Recombinant Vaccinia Virus

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ABSTRACT

The gene encoding P15 of *Cryptosporidium parvum* (*C. parvum*) sporozoites was amplified from Mito strain isolated from calf in Japan, and inserted into the thymidine kinase gene of vaccinia virus LC16mO strain under the control of the early-late promoter for the vaccinia virus 7.5-kilodalton (kDa) polypeptide. The P15 expressed by recombinant vaccinia virus in mammalian cells (RK13) had apparent molecular masses of 18-22 kDa. The vaccinia virus-expressed P15 was glycosylated and transported to the surface of infected cells. Mice inoculated with the recombinant vaccinia virus produced high titers of antibodies against sporozoites or merozoites of *C. parvum*. Our next step will be to examine the ability of the recombinant P15 as a potential subunit vaccine to control cryptosporidiosis in animals.

INTRODUCTION

Cryptosporidium parvum (*C. parvum*) is an intracellular coccidian parasite that infects intestinal epithelium and causes diarrhea in humans and animals. Cryptosporidiosis is characterized by self-limited infection in immunocompetent

hosts, but persistent infection in immunodeficient hosts. There are currently no vaccination or specific therapeutic regimens for control of cryptosporidiosis. Control efforts are hampered further by the fact that oocysts are infective at the time of passage in feces and are not inactivated by commonly used disinfectants. However, recently several studies have shown that administration of polyclonal antibodies against whole *C. parvum* organisms, can confer passive immunity against cryptosporidiosis in mice (Arrowood et al. 1989; Riggs et al. 1989; 1997). Furthermore, Tilley et al. (1991) have shown that a monoclonal antibody against a single immunodominant protein P15 of *C. parvum* sporozoite can neutralize the parasites. Therefore, the P15 is considered to be an important candidate for development of an effective subunit vaccine against cryptosporidiosis in animals and humans. The gene encoding P15 of *C. parvum* sporozoites was cloned and sequenced by Jenkins et al. (1993). However, the product of the P15 gene have not yet been characterized in detail.

In this study we established a high-level production system for P15 of *C. parvum* using a vaccinia virus expression system, and demonstrated that the recombinant P15 was similar to the native P15 in both structure and antigenicity. Our results indicated that the recombinant P15 antigen could be used to immunize cows for production of hyperimmune colostrum that may confer effective passive immunity to humans and animals against cryptosporidiosis.

MATERIALS AND METHODS

Parasites:

C. parvum isolate (Mito strain) used for all experiments was obtained from National Institute of Animal Health, Japan. Calf feces containing oocysts were stored in an equal volume of 2.5% potassium dischromate solution at 4°C.

Viruses and cells:

Vaccinia virus LC16mO (mO) strain and its recombinants were propagated in RK13 or 143TK$^-$ cells in Eagle's minimum essential medium (EMEM) supplemented with 8% heat-inactivated fetal calf serum.

Isolation of oocysts:

A modified ether extraction procedure was used to separate oocysts from calf feces (Riggs et al. 1987). The fecal-potassium dichromate slurry was washed five times in 10 volumes of phosphate-buffered saline (PBS; 1,400×g, 10 min) and suspended in 5 volumes of 1% sodium bicarbonate solution and a quantity of ether equal to 1.2 times the original fecal pellet volume. The resulting mixture was vigorously shaken for 30 sec. Following centrifugation at 1,400×g for 10

min, the ether-extracted fecal layer was removed, and the oocyst pellet was suspended in PBS and sieved with a filter (pore size, 45 μm). Sieved oocysts were washed 5 times in PBS.

Extraction of parasite DNA:

A hundred million oocysts were lysed in 0.1 M Tris-HCl (pH 8.0), containing 1% SDS, 0.1 M NaCl and 10 mM EDTA, and then treated with proteinase K (100 μg/ml) for 2 hr at 55°C. The parasites DNA was extracted with phenol/chloroform and precipitated by ethanol. The DNA pellet was resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and stored at 4°C until use.

Cloning of the P15 gene:

Two oligonucleotide primers (5'-ACGGATCCAAACATGGGTAAC-3' and 5'-ACGGATCCCCTGTTTTAGTTAA-3') were designed by the published sequence of the P15 gene of *C. parvum* (Jenkins et al. 1993). PCR was performed in 50 μl of a mixture containing 1 μg of template DNA, 50 pmol of each primer, 200 μM of dNTPs, 1.25 U of Taq Gold DNA polymerase in 1× Buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin). PCR was performed by 10 min at 95°C to activate the Taq Gold DNA polymerase, and then the reaction was repeated for 40 cycles under the following conditions: 1 min of denaturation at 94°C, 1 min of annealing at 50°C, and 2 min of extension at 72°C. The amplified DNA was loaded on to 1.5% agarose gel eletrophoresis. A DNA band of the expected size (479 bp) was recovered from the gel and inserted into the *Bam*HI site of pUC19 vector. The resulting plasmid was designated as pUCP15.

DNA sequenc analysis:

Nucleotide sequencing was performed on both strands with double-stranded plasmid template pUCP15, using the Taq DNA polymerase cycle sequencing method supplied by Applied Biosystems (ABI, USA) and then analyzed with a model 377A ABI autosequencer.

Production of anti-P15 antibody in mice:

The P15 gene was recovered from pUCP15 after digestion with *Bam*HI, and ligated into the *Bam*HI site of bacterial expression vector pGEMEX-2, and then the P15 was expressed as a fusion protein of the bacteriophage T7 gene 10 protein in *E. coli* JM109 (DE3) according to the manufacture's instructions (Promega, USA). One hundred μg of the recombinant fusion protein was injected into a mouse (BALB/c, 8 weeks old) intraperitoneally in Freund's complete adjuvant.

The same antigen in Freund's incomplete adjuvant was injected intraperitoneally into the mouse on days 14 and 28. Sera from immunized mice were collected 10 days after the last immunization.

Construction of transfer vector:

The P15 gene was recovered from pUCP15 after digestion with *Bam*HI, and ligated into the *Bam*HI site of vaccinia virus transfer vector pAK8 (Yasuda et al., 1990). The structure of recombinant plasmid pAKP15 was checked by restriction enzyme analysis.

Transfection and selection of recombinant vaccinia virus:

RK13 cells infected with vaccinia virus (mO strain) were transfected with the recombinant transfer vector pAKP15 by using Lipofectin reagent. Thymidine kinase-negative (TK^-) virus was isolated by plaque assay on $143TK^-$ cells in the presence of 5-bromo-2'-deoxyuridine at a concentration of 100 μg/ml. TK^- plaque was picked and after three cycles of purification, a recombinant vaccinia virus VV/P15 was obtained.

Immunofluorecence test:

Indirect immunofluorecence test was done as described previously (Xuan et al. 1997).

Western blot analysis:

SDS-polyacrylamid gel electrophoresis and Western blot analysis were carried out as described in our previous paper (Xuan et al. 1997).

RESULTS

Cloning of the P15 gene:

The gene encoding P15 of *C. parvum* was amplified from Mito strain isolated in Japan. As shown in Fig. 1, the expected 479 bp fragment was amplified only from *C. parvum* DNA, but not from negative control DNA. The PCR product was inserted into pUC19, and then subjected to DNA sequencing. The sequence of PCR product was completely identical to the published DNA sequence of the P15 gene of *C. parvum* ksu-1 strain isolated from calf in USA (Jenkins et al. 1993). The predicted amino acid sequence appeared to contain a signal sequence (1-17 aa), transmembrane region (26-47 aa), and two N-linked glycosylation sites (25-27 aa and 148-150 aa).

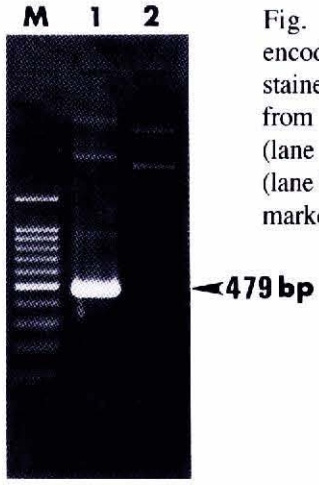

Fig. 1. Amplification of the gene encoding P15 of *C. parvum*. Ethidium stained agarose gel of PCR products from *C. parvum* Mito strain DNA (lane 1) and negative control DNA (lane 2). Lane M, 100 bp DNA ladder marker.

Expression of P15 in E. cili by pGEMEX2 vector:

The P15 gene was ligated into the bacterial expression vector pGEMEX-2, and then P15 was expressed as a fusion protein of the bacteriophage T7 gene 10 protein in *E. coli*. As shown in Fig. 2, the molecular weights of gene 10 and gene 10-P15 fusion protein were estimated as 35 kDa and 50 kDa as expected, respectively. Mice immunized with gene 10-P15 fusion protein induced specific antibodies against *C. parvum* sporozoite (data not shown).

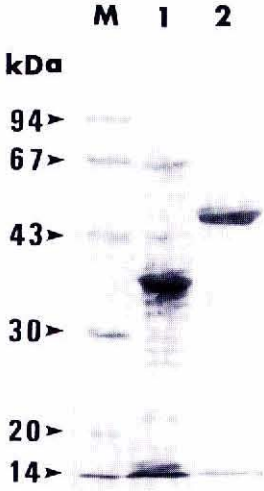

Fig. 2. SDS-PAGE analysis of recombinant P15 expressed in *E. coli*. The protein expressed by pGEMEX-2 vector without insert (lane 1) or pGEM/P15 (lane 2) was detected by CBB staining.

Expression of P15 in mammalian cells by recombinant vaccinia virus:

The P15 gene was inserted into the TK gene of vaccinia virus mO strain under the control of the early-late promoter for the vaccinia virus 7.5-kDa polypeptide. To determine whether the P15 was expressed in RK13 cells by

recombinant vaccinia virus, the VV/P15-infected cells were examined by indirect immunofluorescence analysis using anti-P15 serum (Fig. 3). Specific fluorescence was observed in VV/P15-infected cells, but not in VV/WT- infected cells. Also, the result shows that the recombinant P15 was likely expressed on the cell surface.

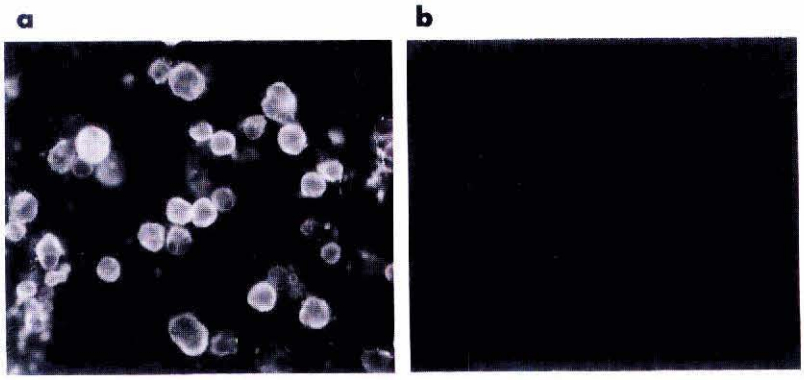

Fig. 3. Immunofluorescent observation of recombinant P15 expressed in mammalian cells using anti-P15 serum. a, VV/P15-infected RK cells; b, VV/WT-infected RK13 cells.

Fig. 4 shows the result of Western blot analysis for P15 expressed by recombinant vaccinia virus VV/P15. The P15 expressed in RK13 cells by recombinant vaccinia virus had apparent molecular masses of 18-22 kDa. Mice inoculated with the recombinant vaccinia virus VV/P15 produced high titers of antibodies against *C. parvum* sporozoite (data not shown).

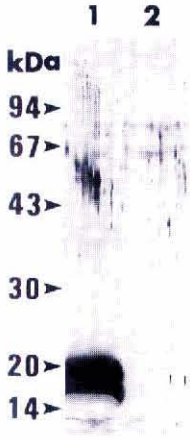

Fig. 4. Western blot analysis of recombinant P15 expressed in mammalian cells using anti-P15 serum. Lane 1, VV/P15-infected cell extract; lane 2, VV/WT-infected cell extract.

DISCUSSION

In host-parasite interaction, the surface proteins of both host and parasite

cells play an important role and the surface antigens of the parasites are therefore logical targets for subunit vaccine development. Several surface antigens, such as P15 and P23 of *C. parvum* sporozoites or merozoites have been reported previously (Jenkins et al. 1993; Perryman et al. 1996). Since the monoclonal antibodies against these antigens can neutralize the *C. parvum* sporozoites, the antigens were proposed as a candidates for development of recombinant vaccine to control the cryptosporidiosis (Tilley et al. 1991; Enriquez et al. 1998). In the present study, we constructed a recombinant vaccinia virus which expresses the P15 of *C. parvum* and demonstrated that the recombinant P15 was similar to the native P15 in structure and antigenicity.

The gene encoding P15 of *C. parvum* was cloned from Mido strain isolated in Japan. The nucleotide sequence of the P15 gene of Mido strain was completely identical to that of kus-1 strain. This result indicates that the P15 gene was perfectly conserved between USA isolate (kus-1) and Japan isolate (Mito). The P15 produced in mammalian cells by recombinant vaccina virus had apparent molecular masses of 18-22 kDa, which was larger than the native P15 (15 kDa). This size difference may be due to the difference in post-translational modifications, such as difference in glycosylation level between parasite and mammalian cells. In addition, immunofluorescence analyses showed that the recombinant P15 was likely transported to and anchored in the plasma membrane of VV/P15-infected cells.

Vaccinia virus has been widely used as a live vector to express foreign genes mainly from other viruses. In general, immunization of laboratory animals or natural host animals with these recombinant vaccinia viruses induced neutralizing antibodies and protected the animals from challenge with corresponding viruses. Recently, vaccinia virus vector has been also used as a live vector to express foreign genes from parasites, and demonstrated that the animals immunized with recombinant vaccina viruses induced protective immunity against parasite infections (Honda et al. 1998; Miyahira et al. 1998). In the present study, mice inoculated with the recombinant vaccinia virus expressing P15 of *C. parvum* produced high titers of antibodies against sporozoite of *C. parvum*. Our next step will be immunization trails with cows to check the potency of the hyperimmune colostrum to confer effective passive immunity of humans and animals against cryptosporidiosis.

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