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DNA vaccine coding a p23 protein of *Cryptosporidium parvum* fused with Fc portion of immunogloblin G induces higher level of interferon-γ expression in mice

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ABSTRACT

To develop DNA vaccines against cryptosporidiosis, a plasmid coding an immunodominant protein of *Cryptosporidium parvum* sporozoite, p23 (pCX-p23) and another plasmid coding a fusion protein containing whole the p23 and the Fc portion of mouse immunogloblin G1 (pCX-p23Fc). Vaccination of BALB/c mice with the plasmid, pCX-p23 and pCX-p23Fc induced the production of antibodies against p23. Although both of splenocytes of mice immunized with the plasmids pCX-p23 and pCX-p23Fc expressed interleukin-4 and interferon-γ, after the in vitro stimulation by p23 antigen, the interferon-γ expression level of pCX-p23Fc immunized mice was much higher than that of pCX-p23 immunized mice. These results suggest a possibility of the plasmid pCX-p23Fc as a DNA vaccine candidate against cryptosporidiosis.

INTRODUCTION

It was reported that the immunization of mice with DNA coding a *Cryptosporidium parvum* sporozoite antigen induced an antigen-specific T cell proliferation both in spleen and mesenteric lymph node (Sagodira et al., 1999). It was also known that a recombinant protein derived from *C. parvum* expressed by eucaryotic system is a better antigen as a vaccine than that expressed by a prokaryotic system (Iochman et al., 1999). These studies suggested that a DNA vaccine may be a feasible candidate for controlling cryptosporidiosis. Although, the weak antigenicity for a DNA vaccine is a major obstacle for its practical use in the field, it was reported that a DNA vaccine coding a hepatitis B virus antigen fused with Fc portion of immunogloblin G (IgG) had enhanced vaccine potency, comparing a plasmid simply coding a hepatitis B virus antigen (You et al., 2001). In this study, a plasmid DNA coding the p23 kDa glycoprotein of *C. parvum*, p23, fused with Fc portion of mouse IgG1, and its feasibility as a DNA vaccine was tested.

MATERIALS AND METHODS

Mice and cells:

Seven weeks female BALB/c mice were used. CPK cells were cultured in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% fetal bovine serum and 60 mg/ml of kanamycin.

Constraction of the fusion gene:

To generate a 5'-*Bam* HI and 3'-*Kpn* I terminal restriction enzyme site, the p23 gene was amplified from the plasmid pUC/p23 (Takashima et al., 2003), using the primers, 5'-GGTACCAAAAATGGGTTGTTCATC-3' and 5'-GGTACCGGCATCAGCTGGCTTGTC-3'. The amplified fragment was cloned into a pT7Blue vector (Novagen, USA) as described in Fig. 1A. The resulting plasmid was designated as prp23. The gene coding Fc portion of mouse IgG1 was amplified from BALB/c mouse spleen cDNA, using the primer 5'-GGATCCTTATTTACCAGGAGAGTGGGAG-3' and 5'-GGTACCGTGCCCAGGGATTGTGGTTGTA-3', and cloned into a pT7Blue vector a pT7Blue vector as described in Fig. 1A. The resulting plasmid was designated as pIgG. Digesting the plasmid pIgG with *Kpn* I, the gene coding Fc portion was obtained and the gene was inserted into the *Kpn* I site of the plasmid prp23. The fusion gene was obtained from the resulting plasmid, designated as p23Fc, digesting with *Bam* HI.

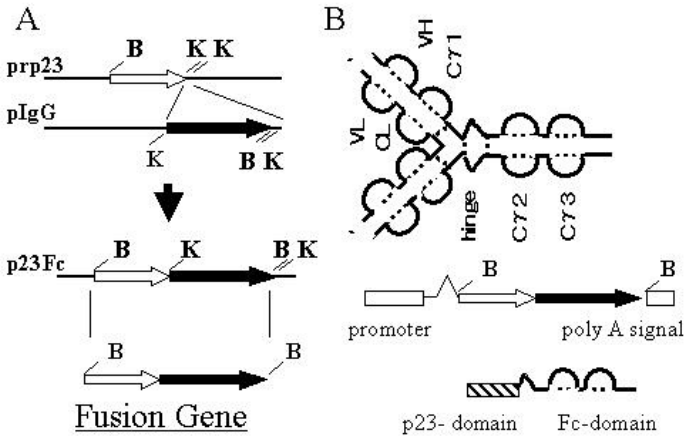

Fig. 1. Schema of the construction of the fusion protein. (A) Construction of the p23 and IgG Fc fusion gene. The capital letters, 'B' and 'K' indicate *Bam* HI site and *Kpn* I recognized sites, respectively. Open and solid arrows indicate p23 gene and Fc gene, respectively. (B) Schema of the fusion gene and protein. Upper panel is the schema of IgG. Middle panel is the schema of the fusion gene expression vector, pCX-p23Fc. Open and solid arrows indicate p23 gene and Fc gene, respectively. Lower panel is the schema of the fusion protein. The striped box indicates the p23-derived domain.

Construction of plasmids for DNA vaccines:

The p23 gene and the fusion gene were obtained by the digestion with *Bam* HI of pUC/p23 and p23Fc, respectively. And these genes were replaced to *Eco* RI fragment of the expression vector pCX-eGFP (kindly provided from Dr. Miyazaki, Osaka University). The resulting plasmids were designated as pCX-p23 and pCX-p23Fc, respectively. The empty control plasmid, pCX was constructed by removing the *Eco* RI fragment coding eGFP from the plasmid pCX-eGFP.

Western blot analysis:

The CPK cells were transfected with pCX-p23 or pCX-p23Fc. After 24 hrs incubation, the transformed cells were lysed, subjected to electrophoresis and transferred a transfer membrane (Millipore, USA), as described previously (Takashima et al., 1999). To detect Fc portion, the membrane was reacted with horseradish peroxidase-conjugated rabbit anti-mouse IgG, IgG1 or IgG2a antibody (ZYMED, USA). To detect the p23 or p23 derived domain of the fusion protein, the membrane was reacted with mouse anti-p23 serum and horseradish peroxidase-conjugated rabbit anti-mouse IgG2a antibody. It had been confirmed that

the mouse anti-p23 serum contains IgG2a against p23 (data not shown). The band was visualized using ECL™ western blotting detection reagent (Amersham Pharmacia biotech, U.K.).

Immunization and detection of the production of antibodies against p23:

The purified plasmids, pCX, pCX-p23 and pCX-p23Fc were diluted in 25% sucrose solution at 1 mg/ml concentration. Mice were anaesthetized by 75 mg/kg sodium pentobarbital and each tibial anterior muscle was injected with 50 µl of diluted plasmid (100 µg/mouse). Four and eight wks after the first immunization, the second and third inoculation were carried out as described above. Four wks after the first inoculation, serum samples were harvested and the productions of antibodies against p23 were detected by ELISA as described previously (Takashima et al., 2003).

Detection of the cytokine expression:

Four wks after the third immunization, mice were sacrificed and splenocytes were harvested. The splenocytes harvested from mice in each group were pooled and cultured for 48 hrs in RPMI-1640 medium in the presence or absence of 30 µg/ml p23 protein expressed by *E. coli* system (Takashima et al., 2003). The secreted interleukin-4 (IL-4) and interferon-γ (IFN-γ) were detected by sandwich ELISA kits to detect IL-4 and IFN-γ (Pharmingen, USA).

RESULTS AND DISCUSSION

Expression of p23 protein fused to the Fc:The gene coding p23 protein fused with Fc portion (Fig. 1A) of mouse IgG1 was constructed as shown in Fig. 1B. CPK cells were transfected with the plasmids coding the p23 protein or the fusion protein with p23 and Fc, pCX-p23 or pCX-p23Fc, respectively. By the Western blot analysis using horseradish peroxidase-conjugated rabbit antibodies against mouse IgG1, the specific band of approximately 50 kDa, which was the expected molecular weight of the fusion protein, was detected in CPK cells transfected with pCX-p23Fc (Fig. 2) but not with pCX-p23 (data not shown), resulting that CPK cells transfected with pCX-p23Fc expressed a fusion containing Fc portion derived domain. When the proteins were visualized using anti-p23 mouse serum and horseradish peroxidase-conjugated goat anti mouse IgG2a as a first- and second-antibodies, a 50 kDa specific band was detected in the extract of pCX-p23Fc transfected cells (Fig. 2). However, when the membrane was reacted with only the horseradish peroxidase-conjugated goat anti mouse IgG2a, any specific band was not detected. The result indicates that the protein expressed by the plasmid vector, pCX-p23Fc contains p23-derived domain. In the extract of the pCX-p23 transformed cells, when the anti-p23 serum and horseradish peroxidase-conjugated goat anti mouse IgG2a as a first- and second-antibodies, approximate 23 kDa specific band was detected (data not shown). These results indicate that pCX-p23 and pCX-p23Fc express approximate 23 kDa p23 protein and 50 kDa fusion protein containing p23 and Fc domain, respectively.

Induction of antibodies production following DNA immunization:

BALB/c mice were immunized with pCX-p23, pCX-p23Fc and negative control empty vector pCX. After the immunization, both pCX-p23 and pCX-p23Fc immunized mice produced antibodies against p23 protein (Fig. 3). The result indicates that the fusion protein expressed by pCX-p23Fc conserve immunogenic properties of p23 antigen. A statistically significant difference in the level of produced antibodies was not observed between pCX-p23 and pCX-p23Fc immunized mice.

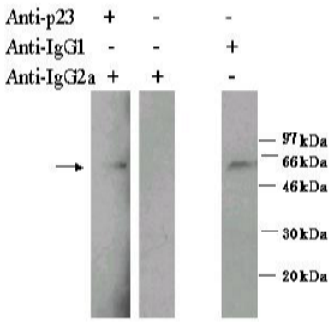

Fig. 2. Detection of the fusion protein. Cells transfected with pCX-p23Fc were analyzed by Western blot analysis using horseradish peroxidase-conjugated rabbit anti-mouse IgG1 or IgG2a, with or without prior reaction with anti-p23 serum.

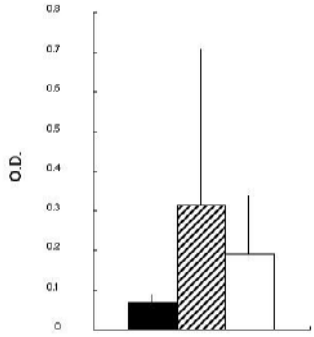

Fig. 3. Productions of antibodies against p23 in BALB/c mice immunized with DNA. Solid, striped and open bars indicate O.D. values of ELISA for sera harvested from mice immunized with pCX, pCX-p23 or pCX-p23Fc, respectively.

Induction of the expression of IL-4 and IFN-γ:

Splenocytes of mice immunized with pCX, pCX-p23 and pCX-p23Fc were harvested and cultures in the presence or absence of re-stimulation with purified p23 protein. As shown in Fig. 4., in the presence of re-stimulation, splenocytes of pCX-p23 or pCX-p23Fc immunized mice expressed both of IL-4 and IFN-γ. The IFN-γ. expression level of pCX-p23Fc immunized mice was much higher than that with pCX-p23 (Fig. 4).

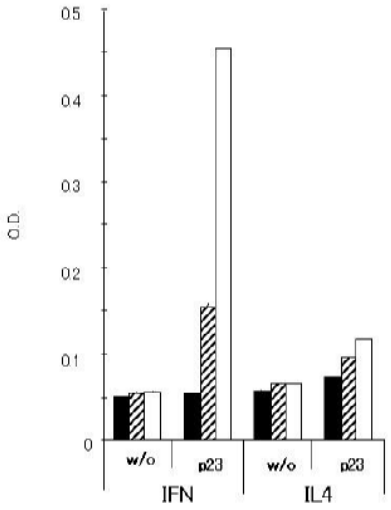

Fig. 4. Detection of the expression of IFN- γ and IL-4. Splenocytes harvested from mice immunized with DNA were cultured in the absence (w/o) or presence (p23) of in vitro stimulation by adding p23 protein. Solid, striped and open bars indicate O.D. values of sandwich ELISA for the sera of mice immunized with pCX, pCX-p23 or pCX-p23Fc, respectively.

Glycoprotein p23 of *C. parvum* had been identified as an immunogenic antigen with neutralization-sensitive epitopes (Perryman et al., 1996). In this study, we constructed the plasmid, pCX-p23Fc, which expresses the fusion protein containing p23-derived and Fc-derived domains. As shown in Fig. 4., immunization with the pCX-p23Fc induced much higher level of IFN-γ expression than the plasmid expressing p23, pCX-p23. It was reported that neonatal C57BL/6 mice are able to clear the *C. parvum* within 3 wks after infection, whereas C57BL/6 IFN-γ knock out mice, depending on age, die rapidly

(Lacroix et al., 2001). It was also reported that IFN-γ directly induces enterocyte resistance against *C. parvum* infection (Pollok et al., 2001). In humans, it was reported that IFN-γ in the jejunum was associated with the absence of oocyst shedding (White et al., 2000). These reports indicate the importance of IFN-γ in protection against infection and shedding of *C. parvum*. Therefore, pCX-p23Fc which induced much higher level of IFN-γ expression has a possibility of a effective vaccine. It is known that antigen presenting cells express receptors for the Fc of IgG (FcgR) and that FcgR mediate endocytosis of antigen-IgG complex, MHC-restricted antigen presentation, and activations of antigen presenting cells (Albert et al., 1998; Carbone and Bevan, 1990; Huang et al., 1994; Kovacsovics-Bankowski and Rock, 1995; Ravetch, 1994; Regnault et al., 1999; Sigal et al., 1999). Therefore effective production of IFN-γ by pCX-p23Fc might be because of p23 with the Fc can activate antigen presenting cells and be taken up by antigen presenting cells more efficiently than p23 without Fc.

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