博士論文

# Study for the development of vaccines against cryptosporidiosis of bovine.

（ウシのクリプトスポリジウム症ワクチン開発
に関する基礎研究）

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# 目次

# 日本語要旨

*Cryptosporidium parvum* (*C. parvum*) は家畜、伴侶動物、ヒトに感染し下痢などの症状を引き起こす原虫で、下痢をおこす病原体としては一般的なものである。本研究の目的は、DNA ワクチンや組換えウイルスベクターの手法を用いてウシのクリプトスポリジウム症ワクチンを開発することにある。

第 1 章（chapter 1）では、ウイルスベクターを用いたワクチンの可能性について検討するため、組換えの技術やウイルス自体の性状についてすでに多くの知見がえられているワクシニアウイルスをベクターとして *C. parvum* sporozoites の免疫原性抗原である p23 を発現する組換え体を作製した。この組換えウイルスで BALB/c マウスを免疫したところ、p23 抗原を認識する抗体の産生が確認できた。C57BL マウスにおいては、抗体産生は見られなかったものの p23 抗原に対する遅延型過敏反応が検出された。免疫反応の違いはマウスの系統によると考えられる。

第 2 章（chapter 2）ではウシヘルペスウイルス 1 型（BHV-1）をベクターとして p23 を発現させるための基礎研究を行った。ヘルペスウイルスを用いる利点としては、 BHV-1 やオーエスキー病ウイルス（PrV）など一部のウイルスにおいて弱毒生ワクチンが既に使用されており、これらの株がベクター候補に

なりうるとがあげられる。　またヘルペスウイルスのゲノムには複数の外来遺伝子挿入可能可能部位がある点からも利便性の高いベクターであるといえる。加えて BHV-1 はワクシニアウイルスと異なってヒトに感染しないことから安全性も高い。

しかし BHV-1 のベクター利用にあたって以下の 2 点が大きな問題となる。すなわち、適切な外来遺伝子挿入部位の検討が不可欠である点と、BHV-1 は宿主域が狭くウシ以外の動物にほとんど感染しないことから実験動物を用いた感染実験が困難な点である。

本研究では、BHV-1　US4 遺伝子領域を p23 遺伝子挿入部位とすることを計画したが、US4 遺伝子領域の改変は近傍の US3 遺伝子の発現を抑制する可能性がある。BHV-1 と近縁のヒト単純ヘルペス 1 型(HSV-1)において US3 遺伝子欠損株が感染細胞にアポトーシスをおこすことが知られており、このような性質を持つウイルスはベクターに適さない。 Chapter 2.1 において US３遺伝子を欠損する BHV-1 組換え体を作製しＢＨＶ-1　US3 遺伝子産物の性状を解析した。US3 遺伝子は HSV-1 の US3 protein kinase の相同タンパクであるとされている。HSV-1 においてこのタンパクは感染細胞のアポトーシスを阻害することが知られているが、本研究で作製した US3 欠損 BHV-1 はアポトーシス抑制に関して HSV-1 で報告されているような効果を示さなかった。これらの結果は、BHV-1 組換え体作

成時、US３遺伝子もしくは近傍の US4 遺伝子が外来遺伝子挿入部位として使用可能であることを示唆している。

続いて、実験動物を用いた感染実験を可能にするため、Chapter 2.2 では PRV の糖タンパク（gB および gC）を発現する組換え BHV-1 を作製した。PRV はマウスなど実験動物を含む多くの種類の動物に感染する。いっぽう、BHV-1 の宿主域は狭くマウス体内では全く増殖しない。chapter 2.3 では作製したこの組換え体が効率よくマウスの細胞に感染しうることを確認し、本来 BHV-1 が感染しにくい小型実験動物をもちいた BHV-1 感染実験が可能であることを示した。

以上の知見を踏まえ、p23 を発現する BHV-1 組換え体の作製において遺伝子挿入部位は US4 遺伝子領域、親株には PrV gB および gC を発現している株を用いることしたが、その前段階として BHV-1 と同じアルファヘルペスウイルス亜科に属するウイルスの中で、組換えが技術的に容易な PRV をベクターとして p23 の発現に成功した（chapter 2.3）。この結果を踏まえ、Chapter 2.4 にて p23 抗原を発現する BHV-1 組換え体の作製に着手した。P23 遺伝子は BHV-1 ゲノム中の US4 遺伝子領域に、緑色蛍光タンパクをコードするマーカー遺伝子とともに挿入した。BHV-1 の組換え頻度は極めて低いが、蛍光を発するプラークを単離することで、組換え体は容易にクローニングできた。近縁のウイルスである PRV では、US4 遺伝子領域への外来遺伝子挿入によって US3 遺伝子の発現が抑制されること

が報告されているが、chapter 2.1 で示したようにBHV-1 ではUS3 遺伝子の欠損による悪影響は少ないと思われる。この組換え体をウサギに接種したところp23を認識する抗体の産生が確認された。また、この抗体は *C. parvum* の細胞への感染を抑制した。これらの結果から、本組換えウイルスのワクチンとしての可能性が明らかになった。

以上の結果から、クリプトスポリジウム症ワクチン開発におけるヘルペスウイルスベクターの可能性が示されたが、ヘルペスウイルスは宿主免疫を抑制することも知られている。Chapter 2.5 では家畜に感染するヘルペスウイルスによる免疫抑制について新たな知見を得るため、PRV をマウスリンパ球とともに培養した。この条件ではマウスリンパ球の増殖は抑制され、この効果は紫外線照射によって不活化されたウイルスでも同様の効果をもつことが明らかになった。これらの結果は、PRV によるリンパ球増殖抑制はウイルス遺伝子の発現によるものではなくウイルス粒子を構成する成分によるものであると考えられた。

第３章（Chapter 3）ではイムノグロブリン G1（gG1）の Fc 領域をアジュバントとして利用する事を試みた。Chapter3.1 では、Fc 領域を含む膜タンパクをコードする融合遺伝子を作成し、RK13 細胞を用いて、この遺伝子産物を細胞表面に発現する細胞株を樹立した。この細胞をマクロファージとともに培養したところ、通常細胞に比べて激しく傷害された。このことはマクロファージ上の

Fc レセプターが細胞表面の融合遺伝子産物を認識することを示している。また この細胞をマウスに接種したところ通常細胞を接種するより効率よく抗 RK13 細胞抗体を誘導できた。このことから Fc 領域を含む融合タンパクがアジュバント効果をもつことが明らかになった。

Chapter 3.2 では p23 を発現するプラスミド(pCX-p23)と p23 と Fc 領域の融合タンパクを発現するプラスミド(pCX-p23fc)を作製し、DNA ワクチンとしてマウスに接種した。融合タンパクをコードする pCX-p23fc プラスミドを接種したマウスでは、pCX-p23　接種マウス群に比べて高いレベルのインターフェロンγ産生がみられた。このことから、p23 をもちいたクリプトスポリジウム症ワクチンにおいても Fc 領域がアジュバントとして有効であることが示唆された。

本研究では、いくつかのワクチン候補を作製しその性状を分析した。本稿に示したように組換えヘルペスウイルスや抗体分子 Fc 領域と *C. parvum* 抗原の融合タンパクによる免疫はクリプトスポリジウム症ワクチンとして効果が期待できる。

## General introduction

Aim of this study is development of vaccine for cattle against *Cryptosporidium parvum* infection using a DNA vaccines and recombinant virus vectors. Here, the author reviews *C. parvum*, immunological activity associated with milk and recombinant viral vector.

1. Cryptosporidum and cryptosporidiosis.

*Cryptosporidium parvum* is a recognized protozoan parasite of livestock, companion animals and human (Tzipori, 1983; Fayer et al., 1990). It is now one of the most commonly found enteropathogens causing diarrheal illness in human worldwide. It is likely that cryptosporidiosis was previously included in the 25 to 35% of diarrheal illness with unknown etiology (Current and Garcia, 1991). Recently, outbreaks of cryptosporidiosis have been reported, and contamination of watersheds by cattle operations has been suggested as a source of these outbreaks (Smith and Rose, 1990). Controlling *C. parvum* infections in cattle is important not only to reduce the economic impact, but also to resolve public health concerns. In this review

chapter, the author focuses on life cycle, clinical sign, economic impact and immunology of cryptosporidiosis.

## 1.1 Life cycle of *C. parvum* infection.

The organisms have wide host range and isolates from mammals can transmitted to both homologous and heterogonous host species. The transmission is considered to be by oocysts. During the patent period, a large number of oocysts are passed in feces and contaminate the environment (Angus, 1983). Transmission may occur directly or via contaminated water and feed. The thick-wall oocyst can survive for several months in cool and moist condition (Radostitis et al., 1994). The life cycle of *C. parvum* consists of six major developing event, excystation, merogony, gametogony, fertilization, oocyst wall formation and sporozoite formation. When a oocyst is ingested, it releases infectious sporozoites (excystation) in the intestine. In many cases of cattle, the infection occurs in lower part of the small intestine and same times in the cecum and colon (Radostitis et al., 1994). The free sporozoite in lumen of the intestine attaches to microvillous region of the villous epithelial cell and becomes embedded at the base of the microvilli,

and start asexual proliferation (merogony). The parasites differentiate into the intracellular trophozoite and then schizont, which produces eight merozoites. The parasitophorous envelope of the trophozoites and schizonts are derived from the host microvilli (Pearson and Logan, 1983). Thus, the parasites exist intracellular but extracytoplasmic. A merozoite infects to host cell and repeats asexual proliferation, or differentiates into either a micro- or macrogametocyte (gametogony). After the fertilization, it becomes a zygote, which is called oocyst. During the intracellular stage, organisms are within a vacuole, which is confined to the microvillous region of the villous epithelial cells. After the formation of wall and sporozoites, the sporulated oocyst can exist in the host intestine before the excretion to the feces. The prepatent periods range 2 to 7 days in cattle (Radostitis et al., 1994).

## 1.2 Clinical sign of *C. parvum* infection on human and cattle.

*C. parvum* is an intestinal protzoan parasite that causes enteric infection and diarrhea in human and animals (Tzipori, 1983: Fayer et al 1990). In the case of adult human and cattle, in usual, severe clinical symptoms are not observed. However, in children, old patients and immunocompromised

patients, severe diarrhea caused by *C. parvum* may be chronic and life threatening (Current et al., 1983). In the case of animals, the infection of *C. parvum* in young calves results in a severe diarrhea and death.

## 1.3 Economic impact of *C. parvum* infection.

*Escherichia coli*, rotaviruses, and *C. parvum* are the three major microorganisms are frequently incriminated as causative agents in diarrheas among neonatal food animals, and humans. (Current and Garcia, 1991; Holland, 1990). These agents are a major cause of economic loss to the producer because of costs associated with therapy, reduced performance, and high morbidity and mortality rates. Moreover, diarrheic animals infected with *C. parvum* may act as a source to healthy animals and humans of oocyst of *C. parvum* (Holland, 1990).

## 1.4 Immunology of *C. parvum* infection.

Cryptosporidiosis occurs in immunocompetent persons, and as opportunistic infections in immunocompromised individuals. Recent work supports the view that *C. parvum* infection can be prevented or interrupted

by antibody that binds to sporozoites and merozoites, which are the life cycle stages present in the intestinal lumen (Heyworth, 1992). Although the mechanism of cellar immunity to prevent *C. parvum* infection was not yet known so well, the importance of IFN-γ to prevent *C. parvum* infection was suggested (Hayward et al., 2000; Pollok et al., 2001).

2. Immunological activity associated with milk.

It was suggested that neonatal calves were exposed to *C. parvum* within hours of birth and the vaccination after few days from the birth was too late (Harps and Goff, 1998). Therefore, a vaccine for dams that could induce the production of colostrums for protecting calves from cryptosporidiosis would be a great advantage. In this review capture, the author focuses on immunological activity associated with milk.

The ruminant mammary gland is responsible for providing protective immunity to neonates and for defending itself from invading pathogens concluding *C. parvum* (Kehrli Jr. and Harp, 2001; Fayer et al., 1989). The ruminant mammary gland is also unique in that lymphocyte trafficking, which is essential to adaptive immunity, is shared with the peripheral

immune system rather than the common mucosal immune system (Kehrli Jr. and Harp, 2001). Milk contains a multitude of components providing immune protection to the suckling offspring. Maternal immunity can be transferred to the infant via colostrums containing antibodies (Kelleher and Lonnerdal, 2001). In this way, protection is provided passively against the pathogens to which the mother has been exposed or vaccinated.

It is reported that the major part of immunogloblin in colostrums of ruminant animals is IgA produced by B cells located in an udder and IgG1 derived from maternal blood (Lascelles, 1979). Therefore, in this study, the author focued on IgG in the serum of small experiment animals rather than IgA.

## 3. Recombinant virus vector

### 3.1 Vaccinia virus vectors.

During past century, vaccination with the vaccinia virus, a member of poxviridae, has protected many people worldwide from smallpox. Vaccinia virus is widely studied as a viral vector for expression of foreign genes (Moss, 1991; Yilma, 1994). Among a large number of genes of the vaccinia virus,

several genes are nonessential ones. Some strains lacking or deleted these genes were reported as reduced virulent strain (Lee et al., 1992; Bloom et al., 1991). These genes have been used as insertion sites for foreign genes (Panicali et al., 1983; Smith et al., 1983). Several vaccinia virus recombinants expressing protozoan antigen were also reported as candidates of vacinies against deceases caused by parasites (Nishikawa et al., 2000; Nishikawa et al., 2001; Takasima et al., 1999). Vaccinia virus is known as a heat-stable virus and can be delivered without cold chain. Therefore, recombinants vaccinia viruses are suitable as vaccines for developing countries. However, vaccinia virus can infect many different species of mammalian containing human, and infection of the vaccinia virus remains a major health problem in immunocompromised individuals. Therefore, reduced pathogenecity of this virus or other virus vectors, which do not infect to human, are more suitable to use in the field.

### 3.2 Herpesvirus vectors.

Like the vaccinia virus, herpesviruses also have large genome, in which there are several nonessential genes. The recombinant herpesviruses lacking

gnens, which involved in the virulence, are used as safe and effective vaccines (Mcfarland et al. 1987. Mcfarland and Hill 1987, Kit et al., 1985; Kit et al., 1986; Kit et al.1987; Kit et al., 1990). By replacing nonessential genes with foreign genes, recombinant virus expressing foreign genes can be constructed. Theses recombinant herpesviruses were reported as candidates for polyvalent vaccine (Dormitzer et al., 1992; Nishikawa et al. 2000; Otsuka et al., 1996; Shin et al, 1984; Shiraki et al. 1999; Yokoyama et al. 1996).

Bovine herpesvirus-1 (BHV-1) causes diseases in cattle, such as infectious bovine rhinotracheitis, encephalitis and so on. However, it has a narrow host range and infection in animals other than bovine is very rare. And attenuated live vaccine was developed and widely used (Kit et al., 1985; Kit et al., 1986; Kit et al., 1990). The attenuated bovine herpesvirus-1 has a great possibility as a vector candidate for a safe vaccine development.

Objective

In this thesis, the author focused on the establishment of vaccine candidates against cryptosporidiosis on cattle. In capture 1, the author cloned a gene coding immunogenic antigen of *C. parvum* and constructed the

recombinant vaccinia virus expressing the antigen. Moreover, the immune reaction against the antigen expressed by the recombinant vaccinia virus was examined using mouse model.

In capture 2, the author constructed some recombinant BHV-1 recombinants and investigated the property of the recombinants. In addition, using one of the recombinants as vector, the author constructed recombinant BHV-1 expressing the immunogenic antigen of *C. parvum*. The immunogenic property of the recombinant BHV-1 was examined using rabbit model.

In capture 3, the author modified the gene, coding the antigen of *C. parvum,* to produce a modified antigen, which have higher immunogenisity. And then, the author also modified a culture cell line to produce a virus virion with higher immunogenisity.

# Chapter 1:

## Immunogenic property of a recombinant Vaccinia virus expressing p23 of *Cryptosporidium parvum*

## 1. ABSTRACT

To develop a vaccine against cryptosporidiosis in cattle, the author cloned a gene coding an immunodominant surface protein p23 of *Cryptosporidium parvum* (*C.parvum*) sporozoites and constructed a recombinant vaccina virus expressing p23. 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. The immunization of C57BL mice with the recombinant induced the delayed type hyper sensitivity but antibody production against p23 was not detected.

## 2. INTRODUCTION

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). 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 has 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, p23 is considered to be an important vaccine candidate for dams to produce colostrums that can protect neonatal calves from cryptosporidiosis.

In this study, the author 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.

## 3. MATERIALS AND METHODS

Parasite and its gene coding p23: *C.parvum* isolate (Mito strain) (Abe et al. 2002) 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 oligonucleotide primers, 5'-ACGGATCCAAAAATGGGTTGTT-3' and 5'-ACGGATCCTAATTTAGGCATCA-3' were designed using the published sequence of the p23 gene of *C. parvum* (Perryman et al., 1996). To amplify the p23 gene, the template DNA was extracted from the oocysts prepared as described above, and PCR was performed as described previously (Xuan et al., 1999). Amplified DNA was separated by 1.5% agarose gel electrophoresis. A DNA fragment was recovered from the gel and inserted into the *Bam* HI site of a pUC19 vector. The resulting plasmid was designated as pUC/p23, and the nucleotide sequence of the p23 gene was confirmed as described previously (Xuan et al., 1999).

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: To obtain a recombinant p23 protein as an antigen for producing anti-p23 mouse serum, a p23 expressing vector for an *Escherichia coli* expression system was constructed as follows. The plasmid pUC/p23 was digested with *Bam* HI, and the fragment containing the complete p23 gene was inserted into the *Bam* HI site of the bacterial expression vector pGEMEX-2 (Promega, USA). *E. coli* JM 109 (DE3) was transformed with the resulting plasmid, and p23 protein was expressed in the transformed *E. coli*. One-hundred μg of the lysate of *E. coli* expressing the recombinant fusion protein was injected intraperitoneally into a mouse (BALB/c, 8 wk old) with Freund's complete adjuvant. On days 14 and 28 PI, the same antigen was injected with Freund's incomplete adjuvant. The sera from immunized mice were collected 10 days after the last immunization, and the sera were used as anti-p23 protein antibody to detect p23 protein expressed by BHV-1 recombinants.

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 $3\times10^6$ 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, USA) in frame. The p23 expressed in *E.coli* DH5α 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 $Na_2CO_3$. 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℃ 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℃ 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).

## 4. 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 expression and to determine the molecular weight of the p23, Western blotting analysis was performed. As shown in Fig. 1-1, 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 (Fig1-2). However, DTH reaction against p23 was

not detectable (Fig. 1-3). 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 1-1, 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. 1-3). 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 with mouse serum harvested from the same mouse pre- and post immunization by Western blotting analysis. As shown Fig.1-4, 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. 1-5, 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.

## 5. DISCUSSION

In this study, the author focused on vaccinia virus, which can induce humoral, and cell mediated immunity (Andrew et al., 1984) 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 than the parental virus did (Fig.1-5). 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.1-2).

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, the author demonstrated that immunization with vv/p23 induce greater level of IgG1 production than IgG2a in Balb/c mice (Fig1-3). 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)-γ 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.

## 6. LEGENDS FOR FIGURE

Fig. 1-1. 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. 1-2 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. 1-3 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. 1-4 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. 23 kDa and 35 kDa bands were indicated by black and gray arrows, respectively.

Fig. 1-5 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 indicates the O.D. value of IgG1 and IgG2a in each mouse, respectively.

Table1-1. The end-point titer of IgG1 and IgG2a against p23 in Balb/c mice immunized with vv/p23

| | IgG1 | IgG2a |
|---|---|---|
| Mouse 1 | 4.0 | N |
| Mouse 2 | 3.8 | N |
| Mouse 3 | 3.3 | N |
| Mouse 4 | 3.1 | N |
| Mouse 5 | 3.3 | 3.3 |

The titer of IgG1 and IgG2a are shown as log.
N: under detectable.

Fig. 1-1

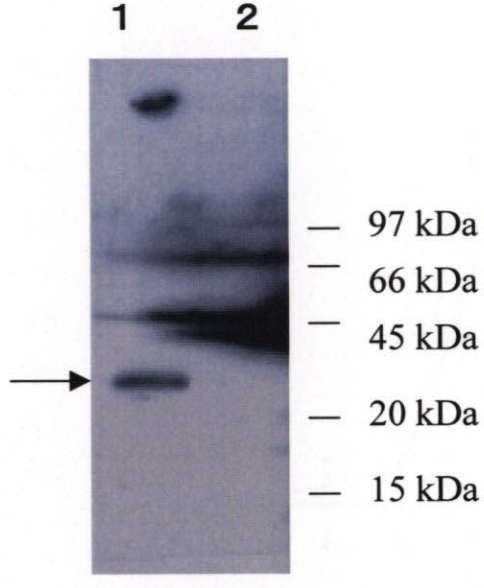


Fig. 1-2

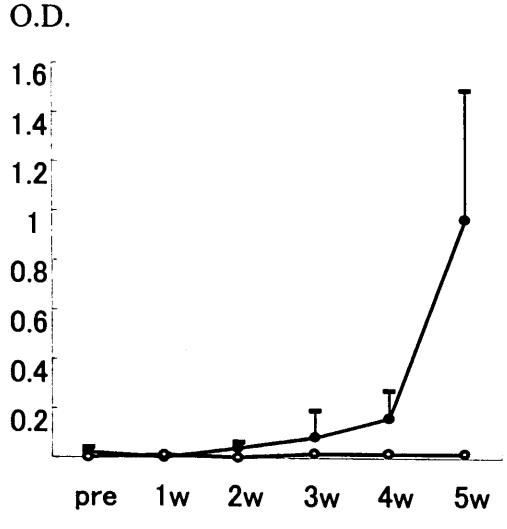


Fig.1-3

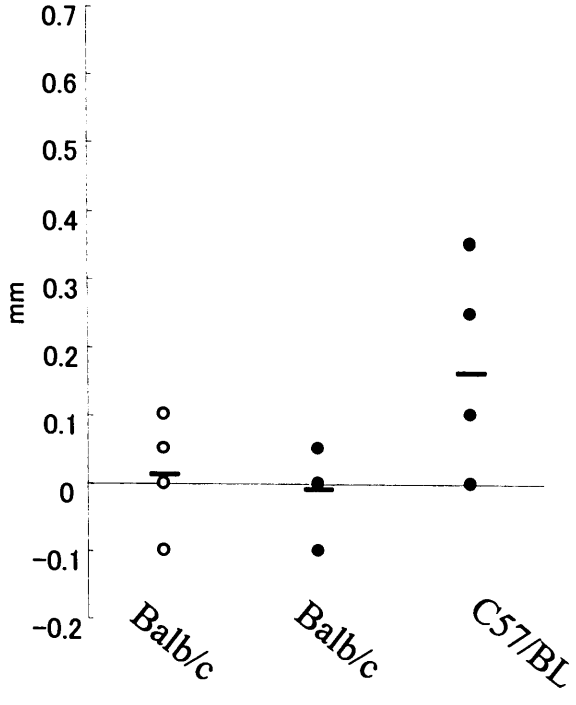


Fig.1- 4

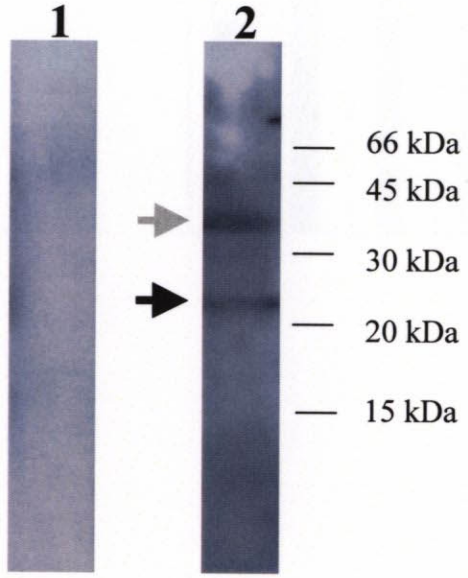


Fig. 1-5

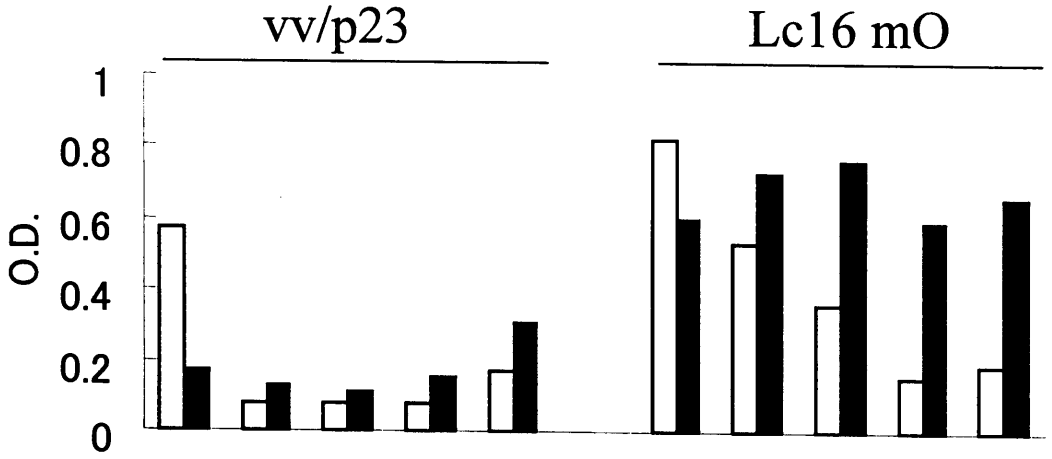
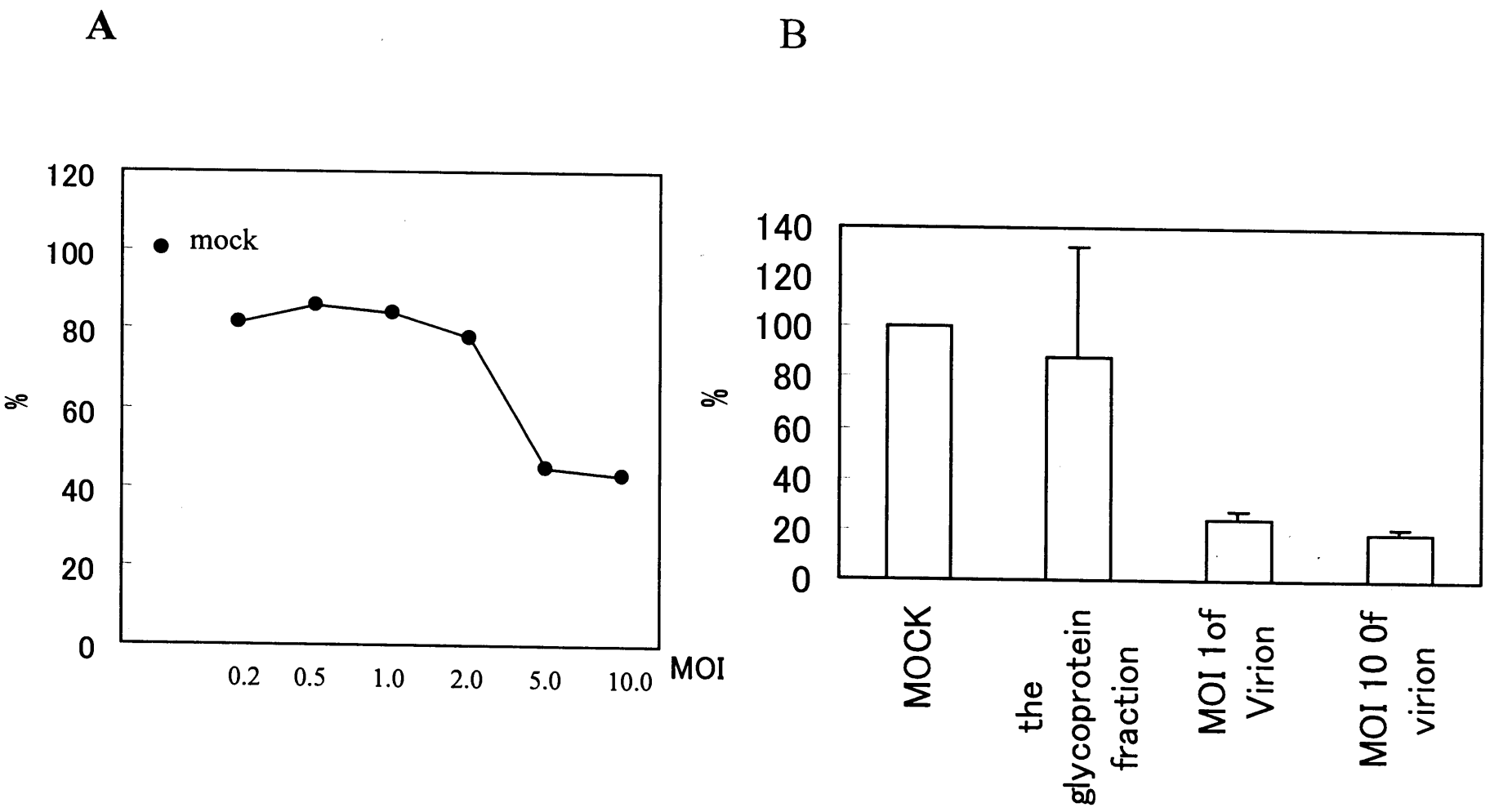


Fig. 2.5-3


A
120
100
80
60
40
20
0
%
mock
0.2
0.5
1.0
2.0
5.0
10.0
MOI
B
140
120
100
80
60
40
20
0
%
MOCK
the glycoprotein fraction
MOI 1of Virion
MOI 10 0f virion

Chapter 2:

# Development of herpesvirus vectors and the construction of a recombinant herpesvirus expressing p23 of *Cryptosporidium parvum*

## 2.1 The characterization of BHV-1 mutants of the US3 gene.

## 2.2 Bovine Herpesvirus-1 (BHV-1) Recombinant Expressing Pseudorabies Virus (PrV) Glycoproteins B and C Induce Type 1 Predominant Immune Response in BALB/c mice.

## 2.3 Construction of the recombinant pseudorabies viruses expressing *Cryptosporidium parvum* an immunodominant surface protein

## 2.4 Recombinant Bovine Herpesvirus-1 (BHV-1) Expressing p23 Protein of *Cryptosporidium parvum* Induces Neutralizing

Antibodies in Rabbits.

## 2.5 Suppression of the Proliferation of Mouse Splenocytes by Pseudorabies virus.

## 2.1 The characterization of BHV-1 mutants of the US3 gene.

### 1. ABSTRACT

The product of the US3 gene of bovine herpes virus type 1 (BHV-1), which is homologous to the herpes simplex virus type 1 (HSV-1) US3 protein kinase (PK) gene. Recent reports indicating that the US3 gene of HSV-1 is involved in the blockage of apoptosis in virus infected cells. As to the apoptosis in BHV-1 infected cells, the author found following: (1) no apoptosis was observed in cells infected with wild type BHV-1 and the US3 mutants (2) the apoptosis induced by the osmotic shock of sorbitol treatment was blocked when cells were infected by the wild type BHV-1 (3) the US3 mutants of BHV-1 blocked the apoptosis of sorbitol treated cell, but the suppressive effect was delayed relative to that of wild type BHV-1 (4) the other BHV-1 mutants, with the intact US3 gene but with some other non-essential gene (genes) deleted behaved similar way to the US3 mutant. It is concluded that the US3 gene of BHV-1 is not directly involved in the blockage of apoptosis in infected cells.

## 2. INTRODUCTION

The US3 genes of alphaherpesviruses was found to be homologous to the protein kinase (PK) gene family of eukaryotes and retrovirus by DNA sequencing analysis (McGeoch and Davison, 1986). The US3 gene of HSV-1 code for a protein kinase with an apparent molecular weight of 68kDa and transfer phosphate from ATP to the seryl or threonyl residues of basic peptides (Purves et al., 1987). Similarly the US3 gene of HSV-2 codes for a 66 kDa PK (Daikoku et al., 1993). On the other hand the US3 gene equivalent of pseudorabies virus (PRV) also codes for a PK with an apparent molecular weight of 38 kDa (Stevely et al., 1985 ; Zhang et al., 1990). Despite the possibility it may contribute to the phosphorylation of the major virion phosphoprotein, the role of the US3 PK in virus infection is unknown so well. Since the deletion mutants of US3 PK can grow in tissue culture cells, it is not required for lytic growth of virus in cell culture.

It has been reported earlier that a mutant of HSV-1 lacking both copies of the major regulatory gene ($\alpha$4) induced DNA degradation characteristic of apotosis in infected cells, whereas the wild-type virus protected cells from apotosis induced by thermal shock (Leopardi and Roizman, 1996). A viral

mutant carrying the wild type α4 gene but from which the US3 gene had been deleted showed that it induced fragmentation of cellular DNA, suggesting that the protein kinase encoded by the US3 gene as the principal viral product required to block apotosis (Leopardi et al., 1997).

A homologous sequence of the US3 PK gene has been identified in the US region of BHV-1, which belongs to the alphaherpesvirus subfamily and is an etiological agent of some of the economically important cattle diseases (Leung-Tack et al., 1994). In this study the constructed the deletion mutants and studied the effects of the deletion on apoptosis in infected cells.

## 3. MATERIALS and METHODS

Cells and viruses: Madin-Darby bovine kidney (MDBK) cells and its thimidine kinase (TK) negative derivative, MDBK (tk$^-$) cell (Otsuka and Xuan, 1996) are cultured in Eagle's minimum essential medium (EMEM) supplemented with 7.5% fetal calf serum (FCS) and 60 μg/ml Kanamycin. A TK deletion mutant of BHV-1 (Los Angeles), IBRV(NG)dltk, (Kit et al., 1986) was used throughout the experiments as a parental virus for construction of the PK mutants and for identification and purification of the PK gene product. The BHV-1 mutants, IBRV(NG)dltk, IBRV(NG)dltk-dlgIII and BHV-1/TF7-1 were described earlier (Otsuka and Xuan, 1996).

Cloning and expression of the BHV-1 US3 gene in *E. coli*: The plasmid pLAH-K was constructed by inserting into the *Hind* III site of plasmid pUC19 the *Hind* III K fragment of BHV-1 genomic DNA. The expression plasmid pGEMEX/BPK was constructed by inserting into the *Eco* RI site of the expression vector pGEMEX2 (Promega, USA) the 1407 bp NcoI-SmaI fragment of pLAH-K, which contained the open reading frame (ORF) of the BHV-1 US3 gene. *E. coli* JM109 (DE3) was transformed with pGEMEX/BPK and the US3 gene product was expressed as the fusion protein of

bacteriophage T7 gene 10 product as follows. A clone of the transformed *E. coli* JM109 (DE3) was inoculated in 200 ml L-broth and incubated at 37°C with shaking until the OD reached to 0.6 and then 200 μl 1 M isopropyl-1-thio-β-D -galactopyranoside was added. After 4 hr incubation at 37°C, *E. coli* was harvested, suspended in a mixture of 9 ml TNE buffer (100 mM NaCl, 50 mM Tris–HCl pH 7.5, 2 mM EDTA) and 1 ml lysozyme solution (1 mg/ml). The suspension was sonicated, incubated at 30°C for 1 hr and centrifuged at 15000 rpm for 5 min. The precipitates were re-suspended in 1 ml TNE buffer, sonicated and centrifuged at 15000 rpm for 5 min. The precipitates were suspended in 3 ml PBS.

Immunization: A volume of 1 ml of the *E. coli* extracts was mixed with an equal volume of Freund's complete adjuvant and inoculated intraperitoneally to four mice (ddy). Mice were immunized with the *E. coli* extracts with Freund's incomplete adjuvant twice more at 10 days intervals. Sera were harvested 10 days after the final immunization.

Construction of the US3PK mutants of BHV-1: The insertion mutant, BHV-1/TF9-1L1, which has an insertion mutation at the US3 gene, was

constructed by homologous recombination between transfer vector plasmid and the parental BHV-1, IBRV(NG)dltk, as described earlier(Otsuka and Xuan, 1996). The transfer vector plasmid, pLAH-K/PTK, was constructed by inserting into the *Mlu*I site of pLAH-K the thymidine kinase (TK) gene of PRV. The parental virus has a TK negative phenotype while recombinants virus had a TK positive phenotype and selected in HAT medium in MDBK($tk^-$) cells. BHV-1/TF9-1L1 was obtained after repeated plaque isolation in MDBK($tk^-$) cells in HAT medium. The insertion-deletion mutant, BHV-1/TF9-1dlPK, was obtained by homologous recombination as described above by using another transfer vector, pLAHKdlPK/PTK, in which the *Nco*I-*Mlu*I fragment of pLAH-K was replaced with the PRV TK gene.

Sorbitol treatment and induction of apoptosis: MDBK cells were infected with BHV-1, BHV-1/TF9-1 at a moi of 10 or mock infected. After various time intervals, infected cells were treated with sorbitol and DNA fragmentation was examined as described below. Appproximately $1.5\times10^6$ infected or mock infected MDBK cells were incubated in the medium containing 0.5 M sorbitol

for 1 h at 37°C and then maintained in the sorbitol-free medium for 1 h. Detached cells were removed with the medium and attached cells were suspended with 0.02% trypsin containing 0.02% EDTA and combined with the detached cells. Cells were pelleted at 2000×*g* for 5 min and suspended with 70 μl of PBS and lysed by adding 280 μl TE buffer (10 mM of Tris–HCl [pH 7.4], 10 mM of EDTA) containing 0.6% sodium lauryl sulfate. The cell lysate was gently mixed with 87.5 μl of 5 M NaCl and incubated at 4°C overnight. The mixture was centrifuged at 14000×*g* for 30 min to remove the chromatin. The supernatant was treated with 0.1 mg RNaseA at 37°C for 1 hr and then with 0.05 mg of proteinase K at 50°C for 1 hr. The mixture was treated with water-saturated phenol and DNA was precipitated with ethanol. DNA samples were dissolved with distilled water and analyzed for a nucleosomal DNA ladder by electrophoresis on a 1.5% agarose gel to detect the fragmentation of DNA.

## 4. RESULTS

US3PK mutants of BHV-1: To investigate the function of the US3 gene product on the blockage of apoptosis in virus infected cells, two mutants, BHV-1/TF9-1L1 and BHV-1/TF9-1dlPK, were constructed as described in the methods. Restriction maps of the PK region of these mutants are shown in Fig. 2.1-1. In BHV-1/TF9-1L1 the US3 PK gene is interrupted by the insertion of the PRV TK gene at the *Mlu* I site. There is a stop codon in the PRV TK gene in the same frame with the initiation codon of the PK gene. The nucleotide sequence of the mutated gene predicts that the size of the polypeptide from the initiation codon to the *Mlu* I site of the PK gene is about 20 kDa and from the *Mlu* I site to the stop codon in the PRV TK gene is about 11 kDa. Therefore it is expected that this mutant synthesize a polypeptide of 31 kDa in which 20 kDa is derived from the N-terminus of the BHV-1 US3 PK. Indeed, a polypeptide of about 33 kDa was detected by the anti-BHV-1 PK antibody in Western blot analysis of the BHV-1/TF9-1L1 infected cells (Fig. 2.1-2).

The insertion-deletion mutant, BHV-1/TF9-1dlPK, contains a deletion at the 5' side of the PK gene in such a way that a part of the promoter and the

initiation codon of the PK gene is lost and it is expected that no US3 PK related polypeptide is synthesized by this mutant. As shown in Fig. 2.1-2, anti-BHV-1 US3 PK antibody did not detect any protein in BHV-1/TF9-1dlPK infected cells.

To investigate the effect of the mutation in the US3 gene, the growth of the mutants was compared with that of wild type virus. The mutants grew well in MDBK cells as shown in Fig. 2.1-3 the growth rates were slightly lower than that of the parental virus. And the mutants exhibited unaltered plaque mophology.

Suppression of fragmentation of DNA by BHV-1 infection and effect of mutation in the US3 PK gene: It was reported that Hep-2 cells treated with the osmotic shock of a high concentration of sorbitol underwent apoptosis and infection of HSV-1 suppressed the apoptosis (Koyama and Miwa, 1997). The author examined the effect of BHV-1 infection on the apoptosis in sorbitol-treated MDBK cells. When MDBK cells were treated with sorbitol at various concentrations, fragmentation of chromosomal DNA, a typical sign of

apoptosis, was observed within 1 hr after sorbitol treatment at concentration of more than 0.5 M (Fig. 2.1-4). Infection of MDBK cells by BHV-1, BHV-1/TF9-1L1 or BHV-1/TF9-1dlPK, without sorbitol treatment, did not cause major breakdown of the chromosomal DNA in any stage of viral growth (data not shown). MDBK cells were infected with wild type BHV-1, BHV-1/TF9-1L1 or BHV-1/TF9-1dlPK at the moi of 10. After various time intervals, infected cells were treated with sorbitol and fragmentation of DNA was examined. Results are shown in Fig. 2.1-5. The cells treated with sorbitol immediately after the infection with wild type BHV-1 showed a marked fragmentation of chromosomal DNA similar to that of sorbitol treated un-infected cells. However, hardly any fragmentation was observed, when cells were treated with sorbitol at 3 or 6 h after the infection by BHV-1. However, the suppressive effects of the US3 mutants, BHV-1/TF9-1L1 and BHV-1/TF9-1dl PK, were observed later than that of wild type BHV-1. When the US3 mutant infected cells were treated with sorbitol at 3 h, the fragment ladder was still visible but at 6 h hardly any fragmentation was observed. Similarly, the other mutants of BHV-1 [IBRV(NG)dltk, IBRV(NG)dltk-dlgIII and BHV-1/TF7-1], with the intact US3 gene but with the other gene (genes)

deleted or added, behaved similar way to US3 mutants (Fig. 2.1-6A). When MDBK cells were infected with these mutants, the fragmentation by sorbitol was partially blocked at 3 h and completely blocked at 6 h. To examine the possibility that delayed apoptosis inhibitory effects of these mutants may be related to the reduced growth rate, the growth rates of these mutants which have intact US3 gene were measured. As shown in Fig. 2.1-6B, all the mutants demonstrated reduced growth rate when compared that of wild type virus.

## 5. DISCUSSION

At this time, the function of the US3 PK in replication of BHV-1 is not known so well. However, the PK$^-$ mutants, BHV-1/TF9-1L1 and BHV-1/TF9-1delPK, grew well in MDBK cells suggesting that the US3 PK is not essential for the growth at least in vitro in some cell lines.

Leopardi et al. (1997) reported that cells infected with a HSV-1 mutant carrying a wild type $\alpha$4 gene but from which the US3 gene had been deleted underwent apoptosis whereas recombinant virus in which the deleted sequence s of the US3 gene had been restored did not cause the cellular DNA to fragment. It would appear, therefore, that the PK encoded by the US3 gene of HSV-1 is the viral product required to block apoptosis.

However, the US3 gene of BHV-1 seems to have no functions in apoptosis of infected cells. Wild type as well as the PK$^-$ mutants of BHV-1 did not cause apoptosis in infected cells and wild type BHV-1 blocked the apoptosis induced in MDBK cells by osmotic shock. The US3 mutants (BHV-1/TF9-1L1 and BHV-1/TF9-1dlPK) and other mutants with the intact US3 gene [IBRV(NG)dltk, IBRV(NG)dltk-dlgIII and BHV-1/TF7-1] suppressed the apoptosis induced by sorbitol treatment but the suppressive effect were

observed later than that of wild type BHV-1. IBRV(NG)dltk has the inactivated TK gene, IBRV(NG)dltk-dlgIII has the inactivated TK gene with the gC gene replaced with pseudorabies virus (PRV) gC gene and BHV-1/TF7-1 has the inactivated TK and gC genes and the PRV gC, gD, gE and gI genes were integrated into the BHV-1 genome (Otsuka and Xuan, 1996). The common characteristics of these US3 and other mutants were their slower growth rates than the wild type virus (Fig. 2.1-3 and Fig. 2.1-6B). Therefore it would appear that the apoptosis induced by osmotic shock was suppressed by BHV-1 infection but the US3 PK, gC or TK genes of BHV-1 was not involved directly in the blockage of apoptosis. The deletion of these non-essential genes caused the reduction of the growth rates of virus, which may cause delayed expression of the apoptosis suppressing effect.

## 6. LEGENDS of FIGURE

Fig. 2.1-1. Structure of wild-type BHV-1, BHV-1/TF9-1 L1 and BHV-1/TF9-1delPK. The restriction map of the *Hind*III K fragment of BHV-1 is shown below a schematic diagram of prototype orientation of the genome. The same fragment of BHV-1/TF9-1L1 and BHV-1/TF9-1delPK are shown below. The location and direction of transcription of the genes are represented by open box and solid arrows, inserted PRV TK genes are represented by stippled arrows.

Fig. 2.1-2. Western blot analysis the Us3 gene mutants of BHV-1. infected MDBK cell lysates (lane 1), TF9-1 L1 infected MDBK cells lysates (lane 2), TF9-1 del PK infected MDBK cell lysates (lane3) and mock infected MDBK cells lysates (lane 4).

Fig. 2.1-3. Comparison of the growth of wild-type BHV-1, BHV-1/TF9-1L1 and BHV-1/TF9-1delPK. MDBK cells were infected with wild-type BHV-1 (●), TF9-1 L1 (■), TF9-1 del PK (▲) at a m.o.i of 0.01 and at indicated time virus was titrated on MDBK cell.

Fig. 2.1-4. DNA fragmentation in MDBK cells after sorbitol treatment. MDBK cells were incubated in medium containing 0.5 M sorbitol for 1 h. The cells were incubated in medium without sorbitol. At the indicated intervals, fragmented DNA was extracted, precipitated and analyzed in a 1.5% of agarose gel.

Fig. 2.1-5. Effect of BHV-1 infection on DNA fragmentation in sorbitol treated MDBK cells. MDBK cells were infected with wild-type BHV-1, TF9-1L1 and TF9-1delPK. At the indicated times after infection, cells were treated with 0.5 M sorbitol for 1 h, followed by incubation in mediume without sorbitol for 1 h. The fragmented DNA was detected in the same way as in Fig. 2.1-4. As controls, DNAs were extracted from non-infected MDBK cells with or without sorbitol treatment and analyzed.

Fig. 2.1-6. (A) effect of BHV-1 mutants infection. MDBK cells were infected with wild-type BHV-1, IBRV(NG)dltk, IBRV(NG)dltk-dlgIII and TF7-1. At the indicated times after infection, the cells were induced

apoptosis and DNA fragmentation was detected in the same way as in Fig.2.1-4. (B) comparison of the growth of wild-type BHV-1, BHV-1/TF7-1, IBRV(NG)dltk-gIII and IBRV(NG)dltk. MDBK cells were infected with wild-type BHV-1 (●), BHV-1/TF7-1 (■), IBRV(NG)dltk-gIII (▲) and IBRV(NG)dltk (◆). And then growth rates were determined as described in Fig. 2.1-3.

## 2.2 Bovine Herpesvirus-1 (BHV-1) Recombinant Expressing Pseudorabies Virus (PrV) Glycoproteins B and C Induce Type 1 Predominant Immune Response in BALB/c mice.

### 1. ABSTRACT

Bovine herpesvirus 1 (BHV-1) attached poorly and penetrated into a mouse cell line, BALB 3T3/A31, but a recombinant BHV-1/TF7-6, which expresses pseudorabies virus (PRV) gB and gC genes, did attach and penetrated into cells more efficiently. In this study the gene green fluorescent protein (GFP) has been integrated into genome of BHV-1/TF7-6 and its parental line of BHV-1. When the mouse mesenteries were incubated *in vitro* and infected with BHV-1/TF7-6/GFP, strong fluorescence was observed while BHV-1/GFP infection hardly demonstrated fluorescence, suggesting that BHV-1 recombinant expressing PRV gB and gC can infect mouse tissue cells more efficiently than the parental BHV-1 does. When BALB/c mice were inoculated with purified BHV-1/TF7-6 or its parental BHV-1, the former induced lower level of anti-BHV-1 immunogloblin G (IgG) than the latter did. When

sub-classes of anti-BHV-1 IgG were analyzed, it was found that mice immunized with BHV-1/TF7-6 or the parental BHV-1 demonstrated the same level of IgG2a. Since anti-BHV-1 IgG1 level was lower in mice inoculated with BHV-1/TF7-6, the IgG2a:IgG1 ratio was higher in BHV-1/TF7-6 inoculated mice than in the parental BHV-1 inoculated ones. These results indicate that BHV-1/TF7-6 induces type 1 predominant immune to BALB/c mice.

## 2. INTRODUCTION

Pseudorabies virus (PRV), a member of the Alphaherpesvieinae, is the etiological agent of the Aujeszky's disease (AD) which is often fatal in new born pigs, causes respiratory disorders in older pigs and reproductive failures (Baskerville et al., 1973; Igresias and Harkness, 1988; Igresias et al., 1992). In other animals such as cattle, sheep, dogs and cats, PRV causes a severe neurological, and often fatal disease. PRV can infect experimentally small laboratory animals. Bovine herpesvirus-1 (BHV-1) infects not only cattle but also pigs (Derbyshire and Caplan, 1976; Nelson et al., 1972), goat (Mohanty et al., 1972), minks, ferrets (Porter et al., 1975), and rabbits (Lupton et al., 1980) but infection in animals other than cattle is rare. BHV-1 does not grow in mice at all (Gibbs and Rweyemamu, 1977). Therefore it would appear that BHV-1 has narrower host range than PRV. The wide host range of PRV correlates with wide host range *in vitro*. In tissue culture PRV can grow in many cell lines derived from pigs, cattle, hamster, rats, rabbits and mice while BHV-1 can grow well in bovine derived cells but poorly in hamster cells and not at all in mouse cells.

Previously, it was reported that a BHV-1 recombinant which expresses PRV gB and gC exhibited remarkable competence in virus attachment and penetration into non-bovine cells (Nakamich and Otsuka, 2000). If the recombinant, BHV-1/TF7-6, can enter into the cells and express genes on its genome in cells of animals other than cattle, BHV-1/TF7-6 may be used as a vector for recombinant vaccines not only for cattle but also for other animals. In this study, the author investigated the infectivity and immunogenic properties of BHV-1/TF7-6 in mice, which are refractory to BHV-1 infection.

## 3. MATERIALS and METHODS

Viruses and cells: IBRV(NG)dltk, is a thymidine kinase (TK) deletion mutant of BHV-1 (LA) (Kit et al., 1986). BHV-1/TF7-6was a recombinant derived from IBRV(NG)dltk containing the PRV gB and gC genes (Otsuka and Xuan, 1996). Viruses were propagated in bovine kidney derived MDBK cells. MDBK cells and hamster lung derived HmLu-1 cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with 7.5% fetal calf serum (FCS) and 60 μg/ml Kanamycin. BALB/c 3T3, A31-1-1 cells (A31 cells) were cultured in Dulbecco's modified EMEM (DMEM) supplemented with 10 % FCS and 60 μg/ml Kanamycin.

Purification of virus: MDBK cells were infected with IBRV(NG)dltk or BHV-1/TF7-6, at a multiplicity of infection (moi) of 0.1 and incubated at 37 ℃ for 72 hr. The medium was collected from the infected cultures and centrifuged twice at 2,000 rpm for 5 min at room temperature to remove cell debris. The supernatant was then centrifuged at 100,000×g for 120 min at 4 ℃ to pellet the virus. The virus was re-suspended in phosphate-buffered

saline (PBS), gradient in TNE (50 mM Tris HCl, pH7.5; 130 mM NaCl, 1 mM EDTA) and centrifuged at 100,000×g for 120 min at 4 ℃. The gradient was fractionated into 12 fractions. The purified virus fraction was centrifuged at 100,000×g for 120 min at 4 ℃. The precipitates were re-suspended in EMEM containing 1 % FCS and stored at –80 ℃ as purified virus.

Immunization: Five BALB/c mice were immunized intraperitoneally with $10^6$ plaque formation unit (PFU) of purified BHV-1/TF7-6 or IBRV(NG)dltk. After immunization serum samples were collected once a week.

ELISA for detection of the antibody against BHV-1: Purified IBRV(NG)dltk was diluted in carbonate buffer to $1\times10^6$ PFU/ml and 100 μl aliquots were added to each well of 96-well ELISA plate (Corning, USA). The plate was incubated at 4 ℃ for overnight, washed 3 times with PBS-Triton100. After the fixation with 3 % skim milk in PBS, mouse serum samples were diluted 1:200 and, and 100 μl of the diluted serum sample was added to a well and incubated at 37 ℃ for 1hr. The wells were washed 3 times with PBS-TritonX100, added with 100 μl of 1:3000 diluted horseradish

peroxidase-conjugated goat anti mouse IgG, IgG1 or IgG2a (ZYMED, USA) and incubated at 37℃ for 1hr. After washing 3 times with PBS-TritonX100, 100 μl of 0.04% o-phenylendiamine and 0.003% $H_2O_2$ in pH5.0 phosphate-citrate buffer 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.

Delayed type hypersensitivity (DTH) assay: Six weeks after the immunization, the test antigen suspended in 30 μl was injected intradermally into the left footpad of a mouse. Purified IBRV(NG)dltk was UV inactivated and used as the test antigen. Each mouse was inoculated with $1 \times 10^6$ PFU equivalent of UV inactivated IBRV(NG)dltk. 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).

Construction of recombinant BHV-1 expressing green fluorescent protein (GFP): The *Pst* I fragment of BHV-1 (LA) containing tk gene and flanking

sequence was cloned into *Pst* I site of the plasmid vector, pUC19 and resulting plasmid was designated as pLATK. The *Hind* III-*Sal* I fragment of PRV containing the promoter of immediate early (IE) gene was inserted into *Bgl* II site of the plasmid, pLATK and resulting plasmid was designated as pLATK/pie. The *Xba* I-*Hind* III fragment of pCX-eGFP (provided from Dr. Miyazaki, Osaka Univ.) containing the gene coding for enhanced GFP and poly A signal was inserted into the *Xba* I site of the plasmid, pLATK/pie. The resulting plasmid was designated as pLATK/GFP. The *Hind* III K fragment of BHV-1 genome was inserted into the *Hind* III site of pUC19, and *Eco* RI-*Xba* I fragment of the resulting plasmid was deleted to construct pLAH-KdelMCS. pUC19 was digested with *Hind* III and treated with *Klenow* fragment and re-ligated to eliminate *Hind* III site in the multi-cloning site (MCS) and the *Pvu* II fragment containing the MCS without *Hind* III site (MCSdlH) was obtained from this plasmid. The *Xho* I fragment of pLAH-KdelMCS was replaced with above mentioned MCSdlH. The resulting plasmid was designated as pA3/MCSdelH. The *Pst* I-*Kpn* I fragment of the PRV genome was cloned at *Pst* I-*Kpn* I site of pUC19 and the fragment containing the tk gene was obtained by digesting this plasmid with

*Kpn* I and *Hind* III. After treating this fragment with *Klenow* fragment, it was inserted at *Kpn* I and *Eco* RI sites of pA3/MCSdelH to construct pLAH-K/pTK. The *Hind* III-*Bam* HI fragment containing the promoter of the PRV IE gene was obtained from the plasmid pIE (Yamada and Shimizu, 1993), treated with Klenow fragment and inserted at the *Xba* I site of pLAH-K/pTK. The resulting plasmid was designated as pLAH-K/pTK/pIE. The *Hind* III-*Xba* I fragment of pCX-eGFP, containing enhanced GFP cDNA and poly A site was inserted into *Xba* I site of the plasmid, pLAH-K/pTK/pIE. The resulting plasmid was designated as p25/GFP. The recombinant BHV-1/GFP was constructed by homologous recombination between pLATK/GFP and BHV-1 LA strain. BHV-1/TF7-6/TK-GFP was constructed by homologous recombination between p25/GFP and BHV-1/TF7-6.

Detection of virus entry to tissue of adult mouse mesenteries: Mesenteries of 8 weeks old female BALB/c mouse was harvested and cut into approximate 5 mm×5mm pieces. The piece of mesenteries was incubated for 9 hr at 37℃ with $2\times10^7$ PFU of IBRV/TK-GFP or BHV-1/TF7-6/TK-GFP in RPMI1640 (GIBCO BRL, USA) medium containing 10 % FCS. After the incubation, the

pieces of mesenteries were picked up and incubated again in fresh RPMI 1640 medium containing 10 % FCS for 6 hr. And then the pieces were observed under a fluorescent microscope to detect specific fluorescence of GFP.

Statistical analysis: The data of different group were analyzed for statistical significance by the student *t* test.

## 4. RESULTS

Construction of BHV-1 recombinant expressing GFP as a marker of virus entry: GFP expressing recombinants of BHV-1, which formed fluorescent plaques in permissive MDBK cells, were constructed to investigate the entry of BHV-1 recombinants into semi- or non-permissive cells. BHV-1/GFP was constructed by integrating the GFP gene under the control of PRV IE promoter at the site of the thymidine kinase gene of BHV-1 (LA). BHV-1/TF7-6/TK-GFP was constructed by inserting both the GFP gene under the control of PRV IE promoter and the tk gene of PRV at the *Xho* I sites of the gG gene of BHV-1/TF7-6. The schematic diagrams of the genome structure of these recombinants are shown in Fig. 2.3-1. Virions of these recombinant BHV-1 are not fluorescent but infected cells became fluorescent after virions enter the cells and GFP is expressed. BHV-1/GFP was tk negative while BHV-1/TF7-6/TK-GFP was tk positive and gG negative. Since the tk and gG are not contained in the BHV-1 virion (Keil et al., 1996), the deletion of these genes are not involved in the early events of the BHV-1 infection such as attachment, penetration and expression of the IE genes.

Therefore the author used these recombinants to investigate the entry of BHV-1 and BHV-1/TF7-6 into cells of tissue.

Entry of BHV-1 recombinants into semi-permissive and non-permissive cells: BHV-1 grows poorly in HmLu-1 cells and not at all in A31 cells although PRV can grow in both cell lines very well. Therefore HmLu-1 cells are semi-permissive and A31 cells are non-permissive for BHV-1 and both cells are permissive for PRV (18). As shown in Fig. 2.3-2, BHV-1/TF7-6/TK-GFP showed much greater level of specific fluorescence than BHV-1/GFP both in A31 and in HmLu-1 cells. However, plaque formation of BHV-1/TF7-6/TK-GFP was not observed in A31 cells. These results confirmed the earlier data obtained by measuring BHV-1 DNA by quantitative competitive PCR that PRV gB and gC on the BHV-1 recombinant increased the level of viral DNA entry into non-permissive A31 cells but they did not make BHV-1 to multiply (Nakamich and Otsuka, 2000).

Entry of BHV-1 recombinant into cells of mouse mesenteries: To determine whether the BHV-1 recombinants expressing gB and gC of PRV, can infect

mouse tissue cells, following experiments were carried out. The mesenteries were harvested from mouse and cut into small pieces. The pieces were incubated with BHV-1/GFP or BHV-1/TF7-6/TK-GFP and then the expression of GFP was followed as a marker of virus entry into the mesentery cells. As shown in Fig. 2.3-3, GFP is hardly expressed in the mesentery cells infected with BHV-1/GFP but strong fluorescence of GFP was observed in mesentery cells infected with BHV-1/TF7-6/TK-GFP. Therefore it can be cocluded that PRV gB and gC on the BHV-1 recombinant increase the ability of entry into not only tissue culture cell line but also mouse mesentery cells.

Induction of IgG in mice immunized with IBRV(NG)dltk and BHV-1/TF7-6: When BHV-1 is injected into intraperitoneal cavity of a mouse, virus contacts with mesenteries immediately. BHV-1/TF7-6 virion may attach and be taken up by cells of the mesenteries but BHV-1 virion may stay in the peritoneal cavity without being taken up by the mesenteries. It is of interest to know whether the immunogenic properties of BHV-1/TF7-6 and BHV-1 are different when inoculated intraperitoneally into mice. BALB/c mice were

immunized with $10^6$ PFU of purified virus, BHV-1/TF7-6 or IBRV(NG)dltk intraperitoneally. Both were tk negative and gG positive. BHV-1/TF7-6 derived from IBRV(NG)dtk expressed gB and gC of PRV. Sera were collected from each mice every week and anti BHV-1 IgG was measured. As shown in Fig. 2.3-4, immunization with BHV-1/TF7-6 induced lower level of IgG against BHV-1 virion than with IBRV(NG)dtk. Especially, at one week and three week post immunization significant difference was observed ($p<0.01$).

Induction of DTH response in immunized mice: For the evaluation of cell mediated immunity, the DTH responses were measured. Six weeks post immunization, the UV inactivated IBRV(NG)dltk was injected as a test antigen in the left footpad of a mouse. As a control, the same volume of PBS was injected into the right footpad. After 2days from the injection, positive DTH reactions were observed as the swelling of left footpad, in both groups immunized with BHV/TF7-6 or IBRV(NG)dltk (Fig. 2.3-5). The average of swelling value of BHV/TF7-6 immunized group was slightly higher than that of IBRV(NG)dltk group ($0.29 \pm 0.19$mm and $0.20 \pm 0.15$mm, respectively). But statistical difference was not observed ($p>0.05$).

IgG1 and IgG2a production in immunized mice: Antigens on BHV-1 virion specific serum antibody isotype was measured as an *in vivo* indicator of type 1 and type 2 immune response. At four weeks post immunization, BV-1/TF7-6 immunized mice produced lower level of IgG1 comparing that induced in IBRV(NG)dltk immunized mice ($p<0.05$). However, the statistical difference of the induced IgG2a level was not observed between two grops of mice immunized with BHV-1/TF7-6 or IBRV(NG)dltk (Fig. 2.3-6). And BHV-1/TF7-6 immunized mice have a significantly higher IgG2a:IgG1 ratio than that of IBRV(NG) immunized mice (Fig. 2.3-6B). These results indicate that immunization with BHV-1/TF7-6 induce type 1 predominant immune response than IBRV(NG)dltk control.

## 5. DISCUSSION

Previously it was reported that BHV-1 recombinants expressing PRV gB and gC had marked increase in attachment and penetration abilities to semi-permissive and non-permissive cells (Nakamich and Otsuka, 2000). The increase of virus titer was observed in semi-permissive cells but not in non-permissive cells. In this study the marker GFP gene was integrated into the genome of BHV-1/TF7-6 and BHV-1 to construct BHV-1/TF7-6/TK-GFP and BHV-1/GFP, respectively. As expected from the previous reports, stronger fluorescence of GFP was observed in semi-permissive HmLu-1 or non-permissive A31 cells, infected with BHV-1/TF7-6/TK-GFP than by BHV-1/GFP. The strongerfluorescence by BHV-1/TF7-6/TK-GFP infection was observed not only in the established cell lines but also in the mesentery cells fresly obtained from mouse peritoneal cavity. The results suggest that PRV gB and gC on BHV-1 virion increase the competence of attachment and penetration into mouse mesenteries cells and the expression of foreign genes integrated into its genome.

When the immunogenic properties of BHV-1 and BHV-1/TF7-6 was

investigated in mice, results suggested that BHV-1/TF7-6 induced more of the type 1 immune response than parental BHV-1 did. The reason of the type 1 immune responses has not been understood so well.

It is suggested that the BHV-1/TF7-6 could enter the mesentery cells and probably other abdominal types cells more efficiently than the parental BHV-1 did. On the other hand, BHV-1 was likely taken by phagocytic cells. Difference in types of immune response induced by these recombinants might be due to cell types where the virus antigens were expressed.

In this study, it was confirmed that BHV-1/TF7-6/TK-GFP could enter the mouse mesentery cells and expressed GFP efficiently. It suggests that BHV-1/TF7-6 can be used as a virus vector to delivery some antigens into small experimental laboratory animals which are refractory to BHV-1 infection.

## 6. LEGENDS of FIGURE

Fig. 2.2-1. Schematic diagram of GFP expressing recombinants, BHV-1/GFP and BHV-1/TF7-6TK-GFP. (A) BHV-1. Open boxes indicate repeat region. Solid bars indicate unique long (UL) and unique short (US) regions. (B) BHV-1/GFP. GFP gene is inserted into the tk gene of BHV-1. (C) BHV-1/TF7-6/TK-GFP. PRV gB gene is inserted into the tk gene of BHV-1. PRV gC gene is replaced with BV-1 gC gene. PRV tk gene and GFP gene are inserted into the BHV-1 gG gene.

Fig. 2.2-2. Expression of GFP by BHV-1/GFP and BHV-1/TF7-6/TK-GFP in semi-permissive HmLu cells and non-permissive A31 cells. The cells were infected with BHV-1/GFP or BHV-1/TF7-6/TK-GFP and observed at 24 hr post infection. (A) HmLu cells infected with BHV-1/TF7-6/TK-GFP. (B) HmLu cells infected with BHV-1/GFP. (C) A31 cells infected with BHV-1/TF7-6/TK-GFP. (D) A31 cells infected with BHV-1/GFP.

Fig. 2.2-3. Expression of GFP by BHV-1/GFP and BHV-1/TF7-6/TK-GFP in

mesenteries of mouse. (A)(C) BHV-1/GFP infected mesenteries. (B)(D) BHV-1/TF7-6/TK-GFP infected mesenteries. The observations with differential microscope (A)(B) and fluorescent microscope (C)(D).

Fig. 2.2-4. Induced anti BHV-1 IgG in mice immunized with IBRV(NG)dltk or BHV-1/TF7-6 was detected by ELISA. Solid circles and open circles indicate O.D. of serum samples from IBRV(NG)dltk or BHV-1/TF7-6/TK-GFP immunized mice, respectively.: * indicate $p<0.01$.

Fig. 2.2-5. DTH reaction against BHV-1 virion. Open circles indicate net swelling of each mouse. Abbreviations, del TK and TF7-6 indicate IBRV(NG)dltk immunized mice and BHV-1/TF7-6/TK-GFP immunized mice, respectively.

Fig. 2.2-6. IgG1 and IgG2a produced in immunized mice. (A) Produced IgG1 and IgG2a against BHV-1 virion in each mouse. O.D. was measured using HRP conjugated anti mouse IgG1 antibody and an HRP conjugated anti mouse IgG2a antibody as secondly antibodies. (B) IgG2a:IgG1 ratio in

immunized mice. After making the 2-fold serial dilutions of serum samples, 100 μl of diluted samples were added to each wells of the plate. And O.D. was measured as described in MATERIALS and METHODS. And then the mean of O.D. of pre-immunized serum samples was calculated and added six standard deviations as a cut off point. The highest dilution number, at which O.D. is larger than the cut-off point, was determined as the end-point titer. Open circles indicate the ratio IgG2a:IgG1 endpoint-titer. Abbreviations, del TK and TF7-6 indicate IBRV(NG)dltk immunized mice and BHV-1/TF7-6/TK-GFP immunized mice, respectively.

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

### 1. ABSTRACT

To develop a vaccine against cryptosporidiosis in animals, the author constructed recombinant pseudorabies virus (PrV) , a member of the Herpesviridae Alphaherpesvrus subfamily expressing an immunodominant surface protein p23 of *Cryptosporidium parvum* sporozoites. 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 23kDa specific band in Western blotting analysis. This study showed the possibility of recombinant herpesvirus as a vaccine against cryptosporidiosis in animals.

## 2. INTRODUCTION

Pseudorabies virus (PrV), a member of the Herpesviridae Alphaherpesvrus 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 thymidin 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. 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.

## 3. MATERIALS and METHODS

Viruess and cells: CPK cells were cultured in Eagle's minimum essential medium (EMEM) ontaining 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 descrived previously (Xuan et al. 1999). The p23 gene of *C.parvum* (Mito strain) was obtained from pUC/p23 by the 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.2.2-1).

Infectious DNA of PrV: CPK cells cultured were infected with PRV (IND) at moi of 5.0 After incubating at 37℃ 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 X100, 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 2000rpm for 10 min. And then the proteinase K was added to the supernatant transferred into a new cloven to a final concentration of 200 □g/ml and incubated at 37 ℃ 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

and co-transfected with 1.5 $\mu$ g of pUC/pTK/CAG/p23 digested with *Kpn* I and 1 $\mu$ g of infectious virul DNA using Lipofectamine PLUS™ Reagent (GIBCO BRL, USA). After the transferred culture was incubated at 37℃ 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 (EMEM containing 7.5 % FCS and 50 $\mu$ g of 5-iodo-2-deoxyuridine). After the culture was incubated at 37℃ 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 analysys: SDS-polyacrylamid gel electrophoresis and Western blotting analysis were carried out as described previously (Takashima et al.1999) using mouse anti-serum against p23.

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 $\mu$ l of PBS previously. As the control 3 mice were inoculated with 200 $\mu$ l of PBS.

## 4. RESULTS

Expression of p23 by the recombinant PrV: PrV expressing p23 peptide of *C.parvum* was constructed by the homologous recombination with the plasmid, pUC/pTK/CAG/p23 (Fig. 2.2-1) and infectious DNA of PrV. Among the progeny virus, recombinants were enriched by TK selection (Otsuka and Xuan, 1996). The obtained recombinant PRV was designated as IND/p23. CPK cells infected with IND/p23 were analyzed by Western blotting analysis using anti-p23 mouse serum. As shown in Fig.2.2-2, 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.

Mortality of mice infected with the recombinant PrV: 4 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. 3 of 5 mice inoculated with $10^2$ or $10^3$ pfu of the recombinant survived during all period of this experiment.

## 5. DISCUSSION

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 disease (Kit,S. et al. 1987). However, comparing with the TK deleted vaccine strain, IND/p23 was high virulence. 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.2.2-1. 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). These report and results indicate that, although ICP0 of BHV-1 and UL24 gene of PRV are nonessential, these genes should not be deleted.

The recombinant constructed in this study is virulent and not suitable for

a vaccine. However, p23 expressed by the recombinant PRV has similar molecular weight to that of authentic one. The p23 peptide expressed in IND/p23 infected CPK cells seemed to be glycosilated as same as authentic p23 expressed in parasite. It suggests the possibility of nonvirulent recombinant herpesvirus as a vector to express p23 and to develop a vaccine for cryptosporidiosis.

LEGENDS of FIGURE

Fig. 2.3-1: 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.

Fig.2.3-2: Western blot analysis of CPK cells infected with recombinant PrV. CPK cells infected with IND/p23 and PrV (IND) were lysated and separated by SDS-PAGE (lane1 and lane2, respectively) following immunoblotting using anti-p23 mouse serum as the primary antibody.

## 2.4 Recombinant Bovine Herpesvirus-1 (BHV-1) Expressing p23 Protein of *Cryptosporidium parvum* Induces Neutralizing Antibodies in Rabbits.

### 1. ABSTRACT

In order to develop a vaccine against cryptosporidiosis in cattle, the author constructed a recombinant bovine herpesvirus-1 (BHV-1) expressing an immunodominant surface protein p23 of *Cryptosporidium parvum* (*C. parvum*) sporozoites. In the recombinant virus, the p23 gene under the control of a CAG promoter and a gene coding for an enhanced green fluorescent protein were integrated into the gG gene of BHV-1. Despite a low frequency of homologous recombination, cloning of the recombinants was easy because of the specific fluorescence of the plaques formed by recombinants. These plaques were among the plaques of the non-fluorescent parental virus. All clones selected for fluorescence also contained the p23 gene. In MDBK cells infected with the recombinant BHV-1, antibody against the p23 protein recognized the p23 protein as an approximately 23kDa

specific band in Western blotting analysis. Rabbits immunized with the recombinant produced IgG against the p23 protein. It was also demonstrated that the sera of immunized rabbits reduced infection of *C. parvum* sporozoites into HCT-8 cells. The serum of an immunized rabbit reduced infection compared to the normal rabbit serum control. These results indicate that the recombinant BHV-1 induces neutralizing antibodies in rabbits.

## 2. INTRODUCTION

A 23kDa glycoprotein p23 of *C. parvum* was identified as an antigen with neutralization-sensitive epitopes. Monoclonal antibodies, which significantly reduced infection with *C. parvum* in mice, reacted with a 23-kDa glycoprotein of *C. parvum* (Perryman et al., 1996), and the gene encoding the protein was cloned (Perryman et al., 1999). Immunoglobulin A (IgA)-secreting hybridomas were developed using Peyer's patch lymphocytes from mice inoculated with *C. parvum.* The hybridomas produced monoclonal antibodies that reacted with the p23 protein and reduced the number of intestinal parasites in mice (Enriquez and Riggs, 1998). Prophylactic and therapeutic efficacies of the monoclonal antibody against the p23 protein were also reported (Riggs et al., 1997; Riggs et al., 2002). A study revealed that immunization with a recombinant p23 protein induced immune bovine colostrums that protected calves against cryptosporidiosis (Perryman et al., 1999). Therefore, p23 protein is considered to be an important candidate for the development of an effective vaccine against cryptosporidiosis.

Bovine herpesvirus-1 (BHV-1), a member of the herpesviridae alphaherpesvirus subfamily, is a major pathogen of cattle. This virus causes a variety of diseases, including respiratory infection, conjunctivitis, encephalitis, genital infections, abortions, and fetal multi-systemic infection of neonates (Gibbs et al., 1977; Yates et al., 1980). However, safe and effective live vaccine strains have been established (Kit et al., 1985; Kit et al., 1986; Kit et al., 1990). These attenuated vaccine strains are useful as vectors for the expression of foreign proteins. Recombinants of BHV-1 expressing immunogenic foreign proteins have been reported as vaccines for other infectious diseases (Otsuka and Xuan, 1996; Taylor et al., 1998; Kweon et al., 1999; Ikeda et al., 2000).

In this study, a BHV-1 recombinant expressing the p23 protein of *C. parvum* was constructed as a candidate for a vaccine for cryptosporidiosis.

## 3. MATERIALS and METHODS

Virus and cells: MDBK cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with 7.5% fetal calf serum (FCS) and 60□g/ml of kanamycin. Wild type BHV-1 LA strain and recombinant BHV-1TF7-6 derived from BHV-1 (LA) containing the pseudorabies virus (PrV) glycoprotein B (gB) and glycoprotein C (gC) genes (Otsuka and Xuan, 1996) were propagated and titrated in MDBK cells as described previously (Kit et al., 1992). HCT-8 cells were cultured in RPMI-1640 medium supplemented with 10% FCS, 20 mM HEPES, and 4 mM L-Glutamine.

Parasites : *Cryptosporidium parvum* isolate (HNJ-1 strain) was used for all experiments (Abe et al., 2002). The parasite stock was maintained as described in capture 1. The p23 gene was obtained from pUC/p23 as described in capture 1.

Construction of the transfer vector plasmid for BHV-1 recombination: A BHV-1 recombinant harboring the p23 gene was constructed by the

homologous recombination between the viral genome and the transfer vector plasmid. The transfer vector plasmid was constructed as follows. An approximately 45 kbp cosmid pAxCAwt (TaKaRa, Japan) containing a CAG promoter and a rabbit β-globin poly A signal (Kanegae et al., 1995) was digested with *Sal* I and re-ligated to construct a small size plasmid of approximately 5 kbp containing the CAG promoter and the rabbit β-globin poly A signal. The resulting plasmid was designated as pCAG. The open reading frame of the p23 gene obtained by digesting pUC/p23 with *Bam* HI was inserted into the pCAG plasmid at the *Swa* I site which is located between the CAG promoter and the β-globin poly A signal site. The resulting plasmid was designated as pCAG/p23. The *Not* I fragment of the plasmid pGreen LANTERN™-1 (GIBCO.BRL., USA) contains the open reading frame of green fluorescence protein (GFP), and this was inserted into the pCAG at the *Cla* I site, which is located between the CAG promoter and the β-globin poly A signal site. The resulting plasmid was designated as pCAG/GFP. The expression unit of p23 and GFP was obtained from these plasmids pCAG/p23 and pCAG/GFP as follows. By digesting these plasmids with *Sal* I and *Xho* I, *Sal* I-*Xho* I fragments

containing the CAG promoter, GFP or p23 gene and rabbit β-globin poly A signal (Figs. 2.4-1C, D) were obtained. The obtained fragments were used to construct the transfer vectors as follows.

The plasmid pLAH-K (Otsuka and Xuan, 1996) was constructed by the insertion of the *Hind* III -K fragment (Fig. 2.4-1B) of the BHV-1 (LA) genomic DNA (Fig. 2.4-1A) into the *Hind* III site of the plasmid pUC19. As shown in Fig.2.4-1A and Fig. 2.4-1B, the *Hind* III -K fragment spans the entire unique short (US) region in which the genes coding gG, gD, gI, and gE are present (Leung-Tack et al., 1994). The pLAH-K was digested with *Xho* I to remove the 397 bp *Xho* I fragment in the gG gene, and the *Sal* I-*Xho* I fragment of the plasmid pCAG/GFP containing the GFP expression unit was inserted at this site (Fig. 2.4-1C). The resulting plasmid was designated as pLAH-K/GFP. The *Sal* I-*Xho* I fragment of the plasmid pCAG/p23 containing the p23 expression unit was then inserted into the *Xho* I site of the plasmid pLAH-K/GFP to construct pLAH-K/GFP-p23 (Fig. 2.4-1D). The plasmid pLAH-K/GFP and pLAH-K/GFP-p23 were used as transfer vectors to construct BHV-1 recombinants.

Construction of the recombinant BHV-1: To linearize the plasmids, the plasmids pLAH-K/GFP and pLAH-K/GFP-p23 were digested with *Hind* III. After purification by ethanol precipitation, these linearized plasmids were used as transfer vector plasmids. MDBK cells cultured in a 6-well plate were transfected with 2 μg of these transfer vector plasmids pLAH-K/GFP or pLAH-K/GFP-p23 using Superfect™ Transfer Reagent (QIAGEN, Germany). Transfected cells were then infected with approximately 1,000 plaque formation units (pfu) of the parental virus BHV-1 TF7-6 (Otsuka and Xuan, 1996). The parental viral genomes and the transfer vector plasmid pLAH-K/GFP or pLAH-K/GFP-p23 co-existed in the cells, 48 hr after inoculation, the infected cells and medium were harvested and frozen and thawed to allow the virus in the cells to be released into the medium. The sample was centrifuged at 3,000 rpm for 5 min, and the supernatant was stocked at –80℃ as the progeny virus pool. MDBK cell monolayers were infected with diluted progeny virus pool and incubated in culture medium containing 0.5% of methylcellulose. After incubation for 2 days, many plaques were observed. Among the plaques, fluorescent plaques were picked up as candidates for recombinant virus clones expressing the

marker gene GFP. After recloning by limiting dilution, the virus was used as a recombinant virus clone.

Detection of p23 protein expressed *in vitro* by recombinant BHV-1: MDBK cells were infected with recombinant BHV-1 at a multiplicity of infection (moi) of 10 and incubated at 37℃ until a cytopathic effect was observed in all cells. The infected cells and mock-infected cells were then harvested. These cells or oocysts of *C. parvum* prepared as described above were suspended in PBS, and an equal volume of extract buffer (0.02% blomophenolblue, 20% glycerol, 4.0% SDS, 125 mM Tris-HCl, pH 6.8, 1.4 M 2-mercaptoethanol) was added, and this was then heated in boiling water for 5 min. These samples were subjected to electrophoresis in SDS-polyacrylamide gel. The protein bands were transferred to a transfer membrane (Immobilon, Millipore, USA). The p23 protein was detected by Western blot using anti-p23 mouse serum as the primary antibody. The specific bands were visualized as described previously (Takasima et al., 1999).

Infection of rabbits with recombinant BHV-1: Five New Zealand White female rabbits weighing 2.0-2.5 kg were purchased from Saitama Experimental Animals Supply Company (Japan). The rabbits were divided into 2 groups. The first group of 3 rabbits were inoculated with $1\times10^6$ pfu of BHV-1/TF7-6/GFP-p23 intraperitoneally, and the second group of 2 rabbits were inoculated with $1\times10^6$ PFU of BHV-1/TF7-6/GFP. After 4 wk and 7 wk from the first inoculation, all rabbits of both groups were inoculated with $1 \times 10^6$ pfu of BHV-1/TF7-6/GFP-p23 intraperitoneally. Rabbits were examined once a day for clinical signs, and rectal temperatures were measured for 7 days after the first inoculation. Nasal swabs were collected every day for 7 days after the first injection. The nasal swabs were inoculated in MDBK cell cultures to determine whether virus was being shed. Blood samples were collected once a wk.

The p23 protein for ELISA antigen: The *Bam* HI fragment of pUC/p23 containing the p23 gene was blunt ended by a *Klenow* fragment and inserted into the *Sma* I site of the bacterial expression vector pGEX5×2 (Amersham Pharmacia Biotech, Sweden). The p23 protein expressed in *E.*

*coli* DH5 $\alpha$ as a fusion protein with glutathione S-transferase was purified according to the manufacturer's instructions.

ELISA for detection of anti-p23 protein antibody produced in rabbits: Purified p23 protein was diluted in carbonate buffer (74 mM $NaHCO_3$, 26 mM $Na_2CO_3$, pH 9.6) to 5 μg/ml, and 100 μl aliquots of the diluted polypeptide were added to each well of a 96-well ELISA plate (Corning, USA). The plate was incubated at 4℃ overnight and washed 3 times with PBS containing 0.1% Triton X100 (PBS-Triton X-100). Rabbit serum samples were diluted 1:100, and 100 μl of the diluted serum sample was added to a well and incubated at 37℃ for 1 hr. The wells were washed 3 times with PBS-Triton X-100. Horseradish peroxidase-conjugated goat anti rabbit IgG Fc antibody was added, and this was incubated at 37℃ for 1 hr. After washing with PBS-Triton X-100, 100 μl of 0.04% o-phenylenediamine and 0.003% $H_2O_2$ in pH 5.0 phosphate-citrate buffer (52 mM citric acid, 103 mM $Na_2HPO_4$) was added, and this was incubated at room temperature for 30 min. The reaction was stopped by adding 20 μl of 6 N $H_2SO_4$, and the absorbance at 490 nm was determined.

Virus neutralization assay: The virus neutralizing activity of the antiserum from immunized rabbits was tested in a 50% plaque reduction assay as described previously (Ikeda et al., 2000). Briefly, BHV-1 (LA) was incubated for 1 hr at 37℃ with heat-inactivated rabbit serum and 5% normal rabbit serum as a source of complement. The infectious titer was then measured using a plaque titration assay. The end point was determined by the serum dilution required for 50% plaque reduction.

Fluorescent antibody assay: The oocysts of *C. parvum* were purified by sucrose gradient purification and treated with 0.01 M HCl at 37°C for 1 hr and then washed twice in PBS. Oocysts were incubated at 37°C for 1 hr in RPMI-1640 medium containing 1% Gall (Wako Chemicals, Japan) to excyst oocysts and then washed twice in PBS. The excystation mixture, which contains free sporozoites and intact and empty oocysts, was placed on slides, air dried, and then fixed in acetone. The slides were incubated at 37°C with rabbit serum (1:100) for 1 hr. Antibody-bound sporozoite cells were revealed with Alexa Fluor 488-labeled goat anti-rabbit IgG antibody

(1:200) (Molecular Probes, USA). Slides were washed in PBS and analyzed using a fluorescence microscope.

Parasite neutralization assay: Cover well (19 mm L×6 mm W×2.0 mm D ×4 well (GRACE Bio-Labs, USA) was put on micro slide glasses coated with 0.01% poly-L-lysine solution (Sigma, USA). HCT-8 cells were seeded into the wells at 2 × $10^5$ cells/well and cultured in 0.2 ml medium. The oocysts were pre-treated with 0.5% NaClO to insure excystation, and suspended at 5 × $10^5$ /ml with media containing 10% normal- or immunized rabbit serum, and 0.2 ml of this suspension was added to HCT-8 cell culture after removing the medium. After 24 hr, the infected cells were washed by PBS and fixed with 10% formaldehyde for 1 hr, washed with PBS, frozen, and thawed. To detect the parasite in the cells, the cells were then reacted with anti-recombinant p23 chicken yolk antibody (Ghen Co., Japan) as a primary antibody and FITC-conjugated anti chicken IgY (Jakson, USA) as a secondary antibody. After the washing and fixation, some empty oocysts and sporozoites, which did not invaded into cells were observed. However, using the primary antibody, trophozoite

and type I meront were discriminated from these oocysts and sporozoites (Fig. 2.4-6). The number of trophozoites and type I meronts in 8 $mm^2$ mono-layer of cells was counted under a fluorescent microscope. The percentage of inhibition is expressed as ({1-[The number of infected parasite treated with immunized serum]/[The number of infected parasite treated with normal control serum]}$\times$100).

## 4. RESULTS

Construction of the recombinant BHV-1 expressing p23 and GFP: BHV-1/TF7-6 is a recombinant BHV-1 harboring PrV gB and gC genes (Otsuka and Xuan, 1996). The open reading frames of PrV gC and gB genes was inserted downstream of the promoter of BHV-1 gC and into the BHV-1 thymidine kinase (TK) gene, respectively. To integrate the p23 gene and the GFP gene into the gG gene of BHV-1/TF7-6, the transfer plasmid pLAH-K/GFP-p23 (Fig. 2.4-1D) was constructed. The recombinant virus BHV-1/TF7-6/GFP-p23 was constructed by the homologous recombination between the BHV-1/TF7-6 genome and the transfer plasmid pLAH-K/GFP-p23 in MDBK cells. Although the frequency of fluorescent plaques was very low (<0.01%), it was easy to find them under the fluorescent microscope because of the bright fluorescence of the plaques formed by recombinant viruses (Fig. 2.4-2). As a control, the BHV/TF7-6/GFP, which does not harbor the p23 gene, but has the GFP gene, was also constructed by homologous recombination between the transfer plasmid pLAH-K/GFP (Fig. 2.4-1C) and BHV-1/TF7-6. The

frequency of the fluorescent plaques among the progeny viruses after the homologous recombination experiment was about the same in constructing BHV-1/TF7-6/GFP-p23 or BHV-1/TF7-6/GFP (<0.01%).

Expression of p23 protein by BHV-1 recombinant BHV-1/TF7-6/GFP-p23: To detect p23 protein in MDBK cells infected with BHV-1/TF7-6/GFP-p23 and to determine the molecular weight of the p23 protein, Western blot analysis was performed. A specific band of 23-kDa, which was the same size as that of the authentic p23 polypeptide expressed by *C. parvum,* was detected in BHV-1/TF7-6/GFP-p23- infected MDBK cells (Fig. 2.4-3). The anti-p23 mouse serum did not react with any polypeptide in MDBK cells infected with BHV-1/TF7-6 or BHV-1/TF7-6/GFP. The results indicate that the p23 protein expressed in MDBK cells infected with BHV-1/TF7-6/GFP-P23 was glycosylated in the same way as authentic p23 protein.

Pathogenic and immunogenic properties of the recombinant BHV-1 in rabbits: Three rabbits were inoculated intraperitoneally with $1 \times 10^6$ pfu

of BHV-1/TF7-6/GFP-p23 and 2 rabbits were inoculated with $1 \times 10^6$ pfu of BHV-1/TF7-6/GFP. The rectal temperature of each rabbit was measured for 7 days after the first inoculation, but no change of body temperature was observed (data not shown). There were no clinical symptoms, and no virus was isolated from the nasal swabs of any rabbit during the experiment (data not shown).

All 5 rabbits were inoculated with BHV-1/TF7-6/GFP-p23 at 4 and 7 wk after the first inoculation. No clinical symptoms were observed in any of the rabbits after the second and third inoculations. Serum samples collected from these rabbits were subjected to ELISA tests to measure antibodies against p23 protein (Fig. 2.4-4). A virus neutralization test was also carried out to confirm the infection of BHV-1 recombinants in the rabbits. In the case of rabbits 1 and 2, significant increases in virus neutralizing antibody titer against BHV-1 was observed within 2 wk of the first inoculation (Table 2.4-1). During the same period, increasing IgG titer against p23 protein was also observed (Fig. 2.4-4). In rabbit 3, virus neutralizing antibody titer against BHV-1 was lower than in rabbit 1 and 2, but IgG titers against the p23 polypeptide increased to a similar level as

rabbit 1 (Fig. 2.4-4, Table 2.4-1). After the second inoculation of BHV-1/TF7-6/GFP-p23, a booster effect on the anti-p23 antibody titer was seen in rabbits 1, 2 and 3; an especially high titer was observed in rabbit 2. The third inoculation of BHV-1/TF7-6/GFP-p23 generated another booster effect (Fig. 2.4-4).

No antibody against p23 protein was detected in the control rabbits (4 and 5), which were inoculated with BHV-1/TF7-6/GFP at first and then with BHV-1/TF7-6/GFP-p23 at 4 and 7 wk later. However, neutralizing antibody titer against BHV-1 was increased (Table 2.4-1).

Reactivity of immunized rabbit sera against *C. parvum*: The reactivity of immunized rabbit serum against *C. parvum* was investigated by fluorescent antibody assay. Sporozoites were reacted with normal rabbit serum or the serum of rabbit 2, which was harvested 8 wk after the first immunization. The sporozoites reacted with the serum of the immunized rabbit showed specific fluorescence (Fig. 2.4-5). The result indicates that antibody induced by BHV-1/TF7-6/GFP-p23 can recognize an authentic p23 protein expressed by a parasite. To investigate whether the immunized

rabbit sera neutralize parasites, a parasite neutralizing assay was carried out using serum harvested 8 wk after the first immunization of the rabbits (1, 2, and 3). Each serum was investigated in 2 different wells, and the tests were repeated 3 times. All sera of the immunized rabbits inhibited the infection of *C. parvum* into HCT-8 cells (Table 2.4-2).

## 5. DISCUSSION

In the present study, the recombinant BHV-1/TF7-6/GFP-p23 which expresses the *C. parvum* p23 gene was constructed using BHV-1/TF7-6 as a vector. As shown in chapter 2.3 and previous reports, BHV-1/TF7-6 expresses PrV gB and gC (Otsuka and Xuan, 1996) and can attach and penetrate non- or semi-permissive cells much more efficiently than wild type BHV-1 (Nakamichi and Otsuka, 2000). Although BHV-1 cannot efficiently infect small experiment animals, the recombinant BHV-1/TF7-6 is a vector that can infect experimental animal models more efficiently than wild type BHV-1.

The alphaherpesvirus US4 gene encodes glycoprotein G (gG), which is conserved in most viruses of the alphaherpesvirus subfamily. In the case of pseudorabies virus (PRC), it was reported that US3 protein is reduced by insertion of foreign genes at the gG locus (Demmin et al., 2002). However, as shown in chapter 2.1., BHV-1 mutant lacking US3 can proliferate at lease *in vitro*, and had no effect on the suppression of apoptosis. Therefore, the author chose gG locus as a site to which p23 gene was inserted.

Previous experiments were carried out using homologous recombination to construct BHV-1 recombinants by transforming cells with transfer plasmid and then infecting the cells with parental BHV-1 virion (Otsuka and Xuan, 1996). This was because the infectious titer of BHV-1 DNA was so low that the usual method of co-transfecting infectious viral DNA and transfer plasmid to cells did not work. Infecting with virion instead of infectious viral DNA made the frequency of recombinants among the progeny virus pool very low, and the recombinants had to be enriched for a selective marker, usually TK. To simplify the construction of BHV-1 recombinants, we made use of GFP of the jellyfish *Aequorea victoria*. GFP is a 27-kDa monomer protein that is naturally fluorescent (Foster et al., 1998). Because of its lack of toxicity and small size, the protein is useful as a marker (Chalfie et al., 1994). It is also reported that the GFP gene integrated into the genomes of herpesvirus or other viruses has no effect on the properties of the parental virus (Jones and Mettenleiter, 1997; Page et al., 1997; Foster et al., 1998; Mahalingam et al., 1998; Agungpriyono et al., 2000).

BHV-1/TF7-6/GFP-p23 was constructed by inserting the *C. parvum*

p23 gene and the GFP gene in tandem at the site of the gG gene of BHV-BHV-1/TF7-6. The frequency of fluorescent homologous recombinants in the progeny pool was less than 0.01%, but it was easy to find them among the non-fluorescent parental viruses. Therefore, there was no need to enrich the recombinants in selective media, and pure clones of recombinants were obtained after several plaque purifications. All clones selected for fluorescence also contained the p23 genes (data not shown). The growth behavior of the recombinants BHV-1/TF7-6/GFP and BHV-1/TF7-6/GFP-p23 did not appear to be affected by GFP (data not shown).

MDBK cells infected with BHV-1/TF7-6/GFP-p23 expressed a p23 protein with the same molecular weight as that of the authentic p23 protein expressed by parasite (Fig. 2.4-3). This suggests that the p23 protein expressed by the recombinant BHV-1 was glycosylated in the same manner as the authentic one. BHV-1/TF7-6/GFP-p23 and the control BHV-1/TF7-6/GFP were not pathogenic in rabbits, and no virus was isolated from the nasal swab of infected rabbits. This suggests that the replication of the recombinants occurred in limited tissue. It was reported

that TK defective BHV-1 produced acute infection in cattle but did not reactivate from latency (Gilliam et al., 1993). The non-pathogenesis of these recombinants may be because of the defect in the TK gene.

Anti-p23 IgG was induced in all rabbits inoculated with BHV-1/TF7-6/GFP-p23. The sharp rise of anti-p23 IgG titers after the second and third inoculations with BHV-1/TF7-6/GFP-p23 suggests that there were booster effects after the second and third inoculations. Anti-p23 IgG from immunized rabbits react with parasites (Fig. 2.4-5) and inhibit their infectivity in HCT-8 cells (Table 2.4-2). Comparing a control in media which did not contain any serum, normal rabbit serum inhibited the infectivity slightly (data not shown). However, as shown in Table 2.4-2, immunized rabbits sera inhibited it more effectively. No anti-p23 IgG was induced in rabbits that were immunized with BHV-1/TF7-6/GFP at the first day and then inoculated with BHV-1/TF7-6/GFP-p23 at wk 4 and 7 (Fig. 2.4-4, open symbols). Since virus neutralizing antibody against BHV-1 was induced by the first inoculation of BHV-1/1TF7-6/GFP (Table 2.4-1), this probably limited the replication of BHV-1/TF7-6/GFP-p23 inoculated later and prevented the induction of anti-p23 IgG. These results

suggest that the first inoculation with a BHV-1 vector vaccine must be done before the animals obtain immunity against BHV-1.

Cryptosporidiosis in young calves is a severe economic concern, but currently no vaccine is available to control the disease. It was reported that colostrum from immunized dams protects calves against cryptosporidiosis (Perryman et al., 1999). It was also suggested that it is important to control the exposure to *C. parvum* within hours of birth to prevent cryptosporidiosis of young calves (Harps and Goff, 1998). Therefore, a vaccine for dams that could induce the production of colostrum for protecting calves from cryptosporidiosis would be a great advantage. In the present study, it was demonstrated that BHV-1/TF7-6/GFP-p23 induced anti-p23 IgG and virus neutralizing antibody in rabbits. This suggests the possibility of the recombinant BHV-1/TF7-6/GFP-p23 or a similar recombinant without the PrV gB and gC gene as a divalent vaccine to protect against BHV-1 infection and to protect calves from cryptosporidiosis.

## 6. LEGENDS FOR FIGURE

Fig. 2.4-1. The genome of BHV-1. (A) Solid bars indicate unique long (UL) and unique short (US) regions. Open boxes indicate repeat regions. The structure of *Hind* III K fragment of BHV-1 genome. (B) Solid arrows indicate genes of BHV-1 coded on the fragment. Construction of the plasmid pLAH-K/GFP and Construction of the plasmid pLAH-K/GFP-p23 (C, and D).

Fig. 2.4-2. Plaques formed by recombinant BHV-1 on a monolayer of MDBK cells. Cells were infected with a mixture of BHV-1/TF7-6 and BHV-1/TF7-6/GFP-p23 (approximate 100:1). The plaques were observed using a differential microscope (A) or a fluorescent microscope (B). The arrow indicates a plaque of BHV-1/TF7-6/GFP-p23.

Fig. 2.4-3. Western blot analysis of MDBK cells infected with recombinant BHV-1 and authentic parasite. Oocysts of *C. parvum* (lane 1), mock-infected MDBK cells (lane 2) and MDBK cells infected with BHV-1/TF7-6/GFP and BHV-1/TF7-6/GFP-p23 (lanes 3 and 4, respectively) were lysated and

separated by SDS-PAGE following immunoblotting using anti-p23 mouse serum as the primary antibody.

Fig. 2.4-4. Detection of antibody against p23 protein in rabbits by ELISA. Rabbits 1, 2 and 3 were inoculated with BHV-1/TF7-6/GFP-p23 on the first day and at 4 wk and 6 wk after the first inoculation (●, ■, and ▲ respectively), and rabbits 4 and 5 were inoculated with BHV-1/TF7-6/GFP at the first day and inoculated with BHV-1/TF7-6/GFP-p23 4 wk and 7 wk after the first inoculation (○ and □, respectively). As the antigen, p23 protein expressed by a GST gene fusion system was used.

Fig.2.4-5. Sporozoites were incubated at 37°C with serum harvested from rabbit 2 (A) or normal rabbit serum (B). Antibodies bound to sporozoite cells were revealed with Alexa Fluor 488-labeled goat anti-rabbit IgG antibody.

Fig.2.4-6. Parasites in HCT-8 cells. The oocysts were pre-treated with 0.5% NaClO, suspended at $5 \times 10^5$/ml with media and added to HCT-8 cell culture. The infected cells were reacted with anti-recombinant p23 chicken yolk

antibody as a primary antibody and FITC-conjugated anti chicken IgY. The reacted cells were observed using a fluorescent microscope (A) or a differential microscope (B). Trophozoites and type I meronts are indicated by large arrows. Sporozoites, which did not invade into cells, were indicated by small arrows.

## 2.5 Suppression of the Proliferation of Mouse Splenocytes by Pseudorabies virus.

### 1. ABSTRACT

Pseudorabies virus (PRV) infection in resistant swine causes immunosuppression which sometimes results in secondary infection by other virus or bacteria. However the mechanism of the immunosuppression is not yet well understood. In this study, the effect of PRV on the immune system was examined in the mouse model. Splenocytes or lymphocytes prepared from the spleen of BALB/c mice were incubated *in vitro* with mitogen, and the ability of cells to proliferation was measured. When the cells were incubated with PRV, the ability of cells toproliferate was inhibited, although PRV did not multiply in the lymphocytes. UV-inactivated PRV also suppressd the proliferation of mouse splenocyte. This result suggests that the structural component of PRV virion might cause the immunosuppression.

## 2. INTRODUCTION

Previously recombinant herpesviruses were reported as candidates for polyvalent vaccine (Dormitzer et al., 1992; Nishikawa et al. 2000; Otsuka et al., 1996; Shin et al, 1984; Shiraki et al. 1999; Yokoyama et al. 1996). On the other hand, it has also been suggested that some of the ailphaherpesviruses are immunosuppressive (Braun and Reiser, 1986; Hammer et al., 1982). The attachment of Bovine herpesvirus-1 (BHV-1), one of the ailphaherpesviruses, induces apoptosis to bovine peripheral blood mononuclear cells, and the phenomenon was suggested as one of the mechanism of immunosuppression by BHV-1 (Hanon et al., 1998).

In the case of swine, higher incidents of pneumonia caused by bacteria or mycoplasma were observed in swie herds infected with PRV (Chinsakchai and Molitor, 1994). Swine experimentally co-infected with PRV and several bacteria or mycoplasma suffered more severe case of pneumonia (Fuentes and Pijoan, 1987; Sakano et al., 1993; Shibata et al., 1997), suggestiong that PRV is also immunosuppressive. However the mechanism of the immunosuppression caused by PRV is not well known. In this study, the

author examined the effects of PRV on the immune system in the mouse model. The mouse model has been used to study immunity against PRV infection (Bianchi et al., 1998) and to assess the protective effect of DNA vaccine against PRV challenge (Haagmans et al, 1999; Ho et al., 1998).

## 3. MATERIALS and METHODS

Viruses and cells: Porcine kidny (CPK) grown in Eagle's minimum essential medium (EMEM) supplemented with 7.5% fetal calf serum (FCS) and 60 μg/ml Kanamycin were infected with PRV Indiana strain and incubated at 37 ℃ for 48 hr. The supernatant of the infected cell culture was harvested, centrifuged at 3,000 rpm to remove cell debris and stored at -80 ℃ as a virus stock.

Separation of virion and supernatant: The virus stock was centrifuged at 100,000×g for 120 min on 80 % (w/v) sucrose cushion and the pellet was re-suspended with fresh medium and stored at -80 ℃ as purified virion. The supernatant was filtered through a 0.45 mm filter (MILLIPOTR, USA) and stored at -80 ℃ as a glycoprotein fraction containing large quantities of glycoproteins, which were not integrated into virions, fragmented envelope and defective virus.

Splenocytes and lymphocytes: Splenocytes were harvested from the

homogenized spleens of 8 weeks old female BALB/c mice and suspended with RMMI 1640 medium containing 10 % FCS at the concentration of $2\times10^6$ cells/ml. To obtain lymphocytes, the monocytes containing in the splenocytes suspension were allowed to adhere to tissue culture dishes by incubation in a tissue culture dish for 2hr and non-adhering cells were harvested. After repeating this procedure for three times, the lymphocytes preparation containing less than 1 % of monocytes when the cell morphology was assessed after Giemsa's staining.

Splenocytes and lymphocytes proliferation assay: Splenocytes were suspended at $2\times10^6$ cells/ml in RPMI1640 medium supplemented with 10 % FCS, 14 mM HEPES and $5\times10^{-5}$ M 2-mercaptoethanol, containing 10 μg/ml of Concanavalin A (con A) or lipopolysaccharide (LPS). And 100 μl of the lymphocytes suspension added to each well of 96-well cell culture plate and added with 100 μl of the supplemented RPMI1640 medium containing PRV or not. After the incubation at 37 ℃ for 42 hr, the cells were labeled with 1 μCi of [$^3$H] tymidine for 6 hr. The lymphocytes were suspended at $1\times10^6$ cells/ml in supplemented RPMI1640 medium containing PRV. After

incubation at 37 ℃ for 3 hr, the lymphocytes were washed with phosphate-buffered saline (PBS) to remove viruses in the medium and suspended at $1 \times 10^6$ cells/ml in the supplemented RPMI1640 containing 10 μg/ml of Con A, and then 100 μl of the suspension was added to a well of 96-well cell culture plate. The thymidine incorporation was measured as described above.

PRV replication in lymphocytes: The enriched lymphocytes were suspended at $1 \times 10^6$ cells/ml with supplemented RPMI1640 medium with or without 10 μg/ml of Con A, and distributed into 3.5 cm dishes at 1ml/dish. The lymphocytes cultures were then infected with PRV at multiplicity of infection (moi) of 1. After incubation of various times at 37 ℃, the infected cells were harvested, frozen and thaw and the virus titers were evaluated by plaque titration on CPK cells. As a control, PRV was also incubated in the medium without any cells and virus titers were determined.

## 4. RESULTS

Suppression of the proliferation of splenocytes by PRV: As shown in Fig. 2.5-1A and 2.5-1B, PRV infected mouse splenocytes showed lower level of proliferation comparing with that of mock infected control. The suppression of the proliferation was observed in the splenocytes stimulated with both Con A and LPS. In other experiments, splenocytes were activated with Con A or LPS for 42 hr prior to the PRV infection. In this condition, inhibitory effects of PRV were not observed (data not shown). The splenocytes preparation in the experiments contained lymphocytes and macrophage (monocytes). To determine whether the suppressive effects of PRV on mouse lymphocytes was due to the direct effects on lymphocytes or is caused via the effects on monocytes, the same experiment was carried out using enriched lymphocytes preparation, which contained less than 1 % of monocytes. As shown in Fig. 2.5-1C, PRV reduced the proliferation of lymphocytes stimulated with Con A. Lymphocytes without any stimulation, incorporated 3H-thymidine at low level but PRV infection did not affect this low level incorporation (data not shown). These results suggest that PRV

infection to lymphocytes caused the inhibition of lymphocytes activation by the mitogen and this inhibition is not the secondary effect of PRV infection to monocytes.

PRV replication in mouse lymphocytes: To determine whether PRV can replicates in mouse lymphocytes, and PRV infection induces necrosis or apoptosis on the lymphocytes, PRV was incubated in lymphocytes cell culture or RPMI medium without any cells in the presence or absence of Con A stimulation. As shown in Fig. 2.5-2, PRV titer in infected lymphocytes with or without Con A decreased with the incubation time and this decrease was almost the same in the absence of lymphocytes. This decrease of infectious titer appears to be due to the instability of PRV at 37 ℃. To detect the necrosis and apoptosis, PRV infected lymphocytes were stained with trypan blue and in Situ Cell Death Detection Kit (Boehringer Mannheim, Germany), respectively. The numbers of stained cells were not increased by PRV infection (data not shown), suggesting that neither necrosis nor apoptosis was induced by PRV.

Suppression of the proliferation of lymphocytes by PRV virion: Since PRV did not replicate in lymphocytes, and cell death by PRV infection was not observed, it appeared that early stage of PRV infection induce the suppression of the lymphocytes proliferation. To determine whether the expression of virus genes is necessary to inhibit the proliferation of lymphocytes, the effect of UV inactivated PRV on mouse lymphocytes was investigated. As shown Fig. 2.5-3A, although the effect was smaller than that of live PRV, it was clearly seen that UV exposed PRV also reduced the proliferation of lymphocytes. The result suggests that inhibition is not due to only infectious PRV but also to one of the viral components present in the viral stock. In the next experiment, virus stock was separated into virus virion and supernatant as described MATERIALS and METHODS. As shown in Fig. 2.5-3B, virus virion reduced the proliferation but supernatant fraction was not inhibitory at all. These results indicate that the presence of virion is necessary to inhibit the proliferation of lymphocytes.

## 5. DISCUSSION

In this study the author found that PRV infection of mouse splenocytes results in a reduction of the mitogenic activation by Con A and LPS. Con A is one of the T cell mitogens and LPS is known to induce cytokine release of macrophage. In the assay system, therefore, lymphocytes in splenocytes were activated by Con A directly, while LPS activates macrophage and cytokines released by activated macrophage induce proliferation of lymphocytes. It has been reported that PRV infection has suppressive effect on the function of alveolar macrophages of swine (Fuentes and Pijoan, 1986; Igresias et al., 1989; Narita et al., 1993). As shown in Fig.2.5-1, lymphocytes, in this system, PRV reduced the proliferation of lymphocytes stimulated by Con A. The result suggested that the inhibition is not the secondary effect of PRV infection to monocytes but a direct effect on lymphocytes.

It has been reported that PRV suppressed the proliferation of lymphocytes and multiplies in the swine lymphocytes (Chinsakchai and Reiser, 1986). In this study, as shown in Fig. 2.5-3, UV-inactivated virus was also immunosuppressive. It suggests the inhibition is due to the early stage of

infection, prior to the exression of viral genes.

When PRV infects permissive cells, it attached to the cell surface via the interaction between glycoproteins of the viral envelope and the cell surface receptors and then the nucleocapsids penetrate into the cells without the envelope. This period is called the eclipse period because the infectious virus titer drops sharply and it does not rise until the progeny viruses emerges. As shown in Fig. 2.5-2, there is no eclipse period in PRV infected lymphocytes. It suggests that PRV does not even penetrate into lymphocytes, and attachment of PRV to lymphocytes suppresses the proliferation of lymphocytes.

## 6. LEGENDS FOR FIGURE

Fig. 2.5-1. Effect of PRV on proliferation of mouse splenocytes (A,B) and lymphocytes (C). (A, B) Splenocytes were infected with PRV at moi of 1, and cultured in supplemented RPMI1640 medium containing 10 μg of Con A (A) or LPS (B). After 48 hr, incorporation of $^{3}$H-thymidine was measured, and CPM data was standardized as % of mock-infected control of each experiment. (C) Lymphocytes were incubated with PRV at moi of 1 for 3 hr and washed. And then the lymphocytes were cultured in supplemented RPMI1640 medium containing 10 μg of Con A. The CPM data was measured and standardized as described above.

Fig. 2.5-2. The replication of PRV on mouse lymphocytes. Lymphocytes was suspended in supplemented RPMI1640 medium with or without 10 μg of Con A. Each culture was infected with PRV at moi of 1. After incubated for appropriate hours, the virus titer was evaluated. As the negative control, virus titer incubated in the medium without any cells were also measured. Open symbols and solid symbols indicate virus titers of lymphocytes

suspension or medium without any cells, respectively. Circles and squares indicate virus titers of cultures with or without Con A, respectively.

Fig. 2.5-3. Effect of UV inactivated PRV and purified virions on proliferation of mouse lymphocytes. (A) After incubated with UV inactivated PRV for 3 hr at appropriate moi (moi before inactivation), the lymphocyte were washed and incubated in supplemented RPMI1640 medium with or without 10 μg of Con A, and incorporation of $^{3}$H-thymidine was measured at 48hr post infection. (B) After incubated with the purified virion or the glycoprotein fraction for 48 hr, the incorporation of $^{3}$H-thymidine was measured. The irus virion and the glycoprotein fraction, containing glycoproteins were prepared as described in MATERIALS and METHODS.

Table 2.4-1. Virus neutralizing activity of antisera from immunized rabbits appropriate diluted (ca. 100 pfu per well) of BHV-1 (LA) were incubated for 1 hr at 37 ℃ with the sera of immunized rabbits and complement as described in materials and methods. The end point was determined by the serum dilution required for 50 % plaque reduction.

| | Pre | 2 wk PI | 6 wk PI |
|---|---|---|---|
| Rabbit 1 | <50× | 800× | >1600× |
| Rabbit2 | <50× | >1600× | >1600× |
| Rabbit 3 | <50× | 50× | 200× |
| Rabbit 4 | <50× | 50× | 100× |
| Rabbit 5 | <50× | 200× | 1600× |

Table 2.4-2. Parasite neutralizing activity of antisera from immunized rabbits. $5 \times 10^5$/ml of oosysts were suspended with the sera of immunized rabbits or normal rabbit serum. And the oosysts were added onto the culture of HCT-8 cells. After 24 hr, the number of trophozoite and type I meronts were counted. The experiment was repeated 3 times. Each experiment was carried out using 2 different wells.

| | The number of infected parasites | | | | | | Parentage of inhibition |
|---|---|---|---|---|---|---|---|
| | Experiment 1 | | Experiment 2 | | Experiment 3 | | |
| Normal serum | 105 | 96 | 144 | 116 | 96 | 91 | — |
| Rabbit 1 | 78 | 68 | 78 | 85 | 64 | 66 | 31.8 % |
| Rabbit 2 | 85 | 70 | 76 | 65 | 70 | 61 | 32.9 % |
| Rabbit 3 | 60 | 64 | 65 | 70 | 54 | 52 | 43.3 % |

Fig. 2.1-1

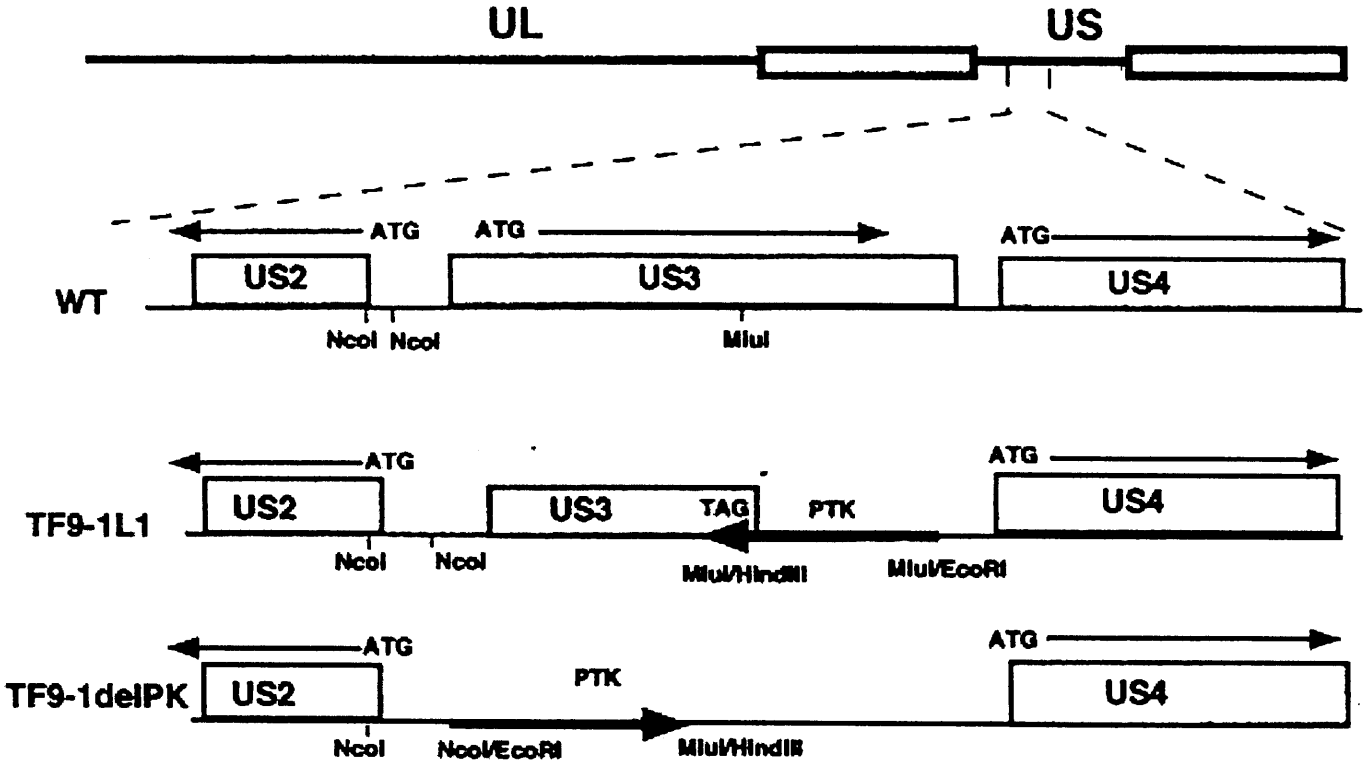

UL
US
ATG
ATG
ATG
WT
US2
US3
US4
NcoI NcoI
MluI
ATG
ATG
TF9-1L1
US2
US3
TAG
PTK
US4
NcoI
NcoI
MluI/HindIII
MluI/EcoRI
ATG
ATG
TF9-1delPK
US2
PTK
US4
NcoI
NcoI/EcoRI
MluI/HindIII

Fig. 2.1-2

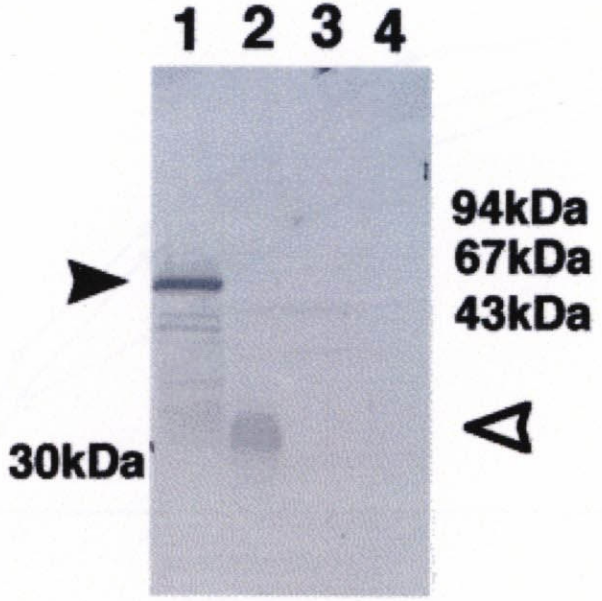


Fig. 2.1-3

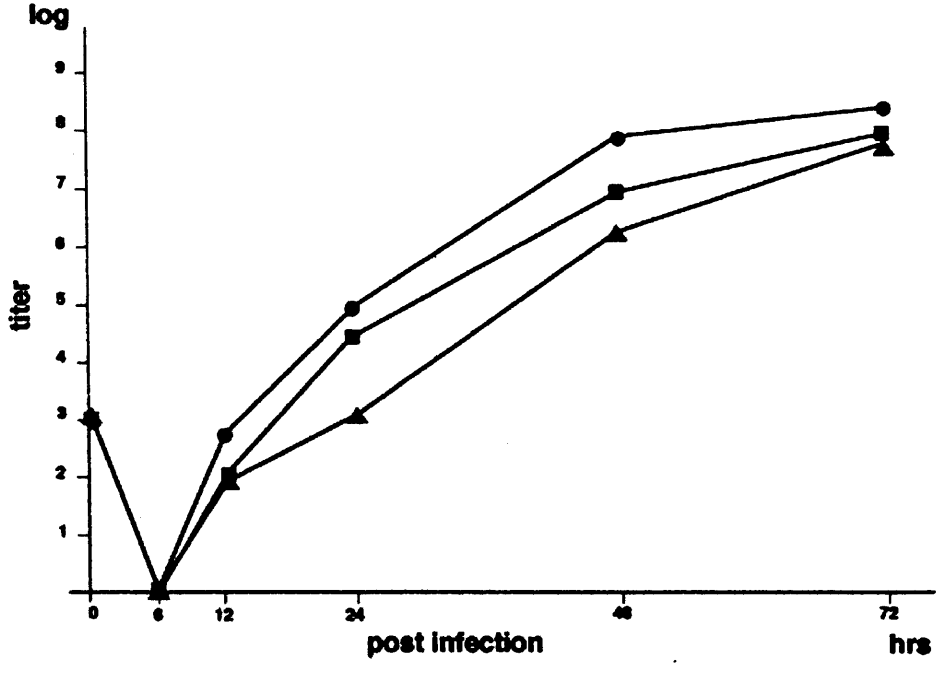


Fig. 2.1-4

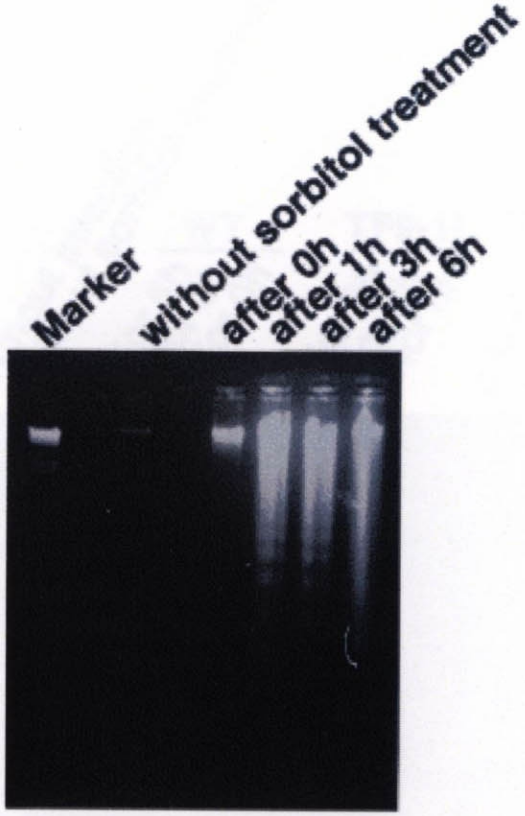

Marker
without sorbitol treatment
after 0h
after 1h
after 3h
after 6h

Fig. 2.1-5

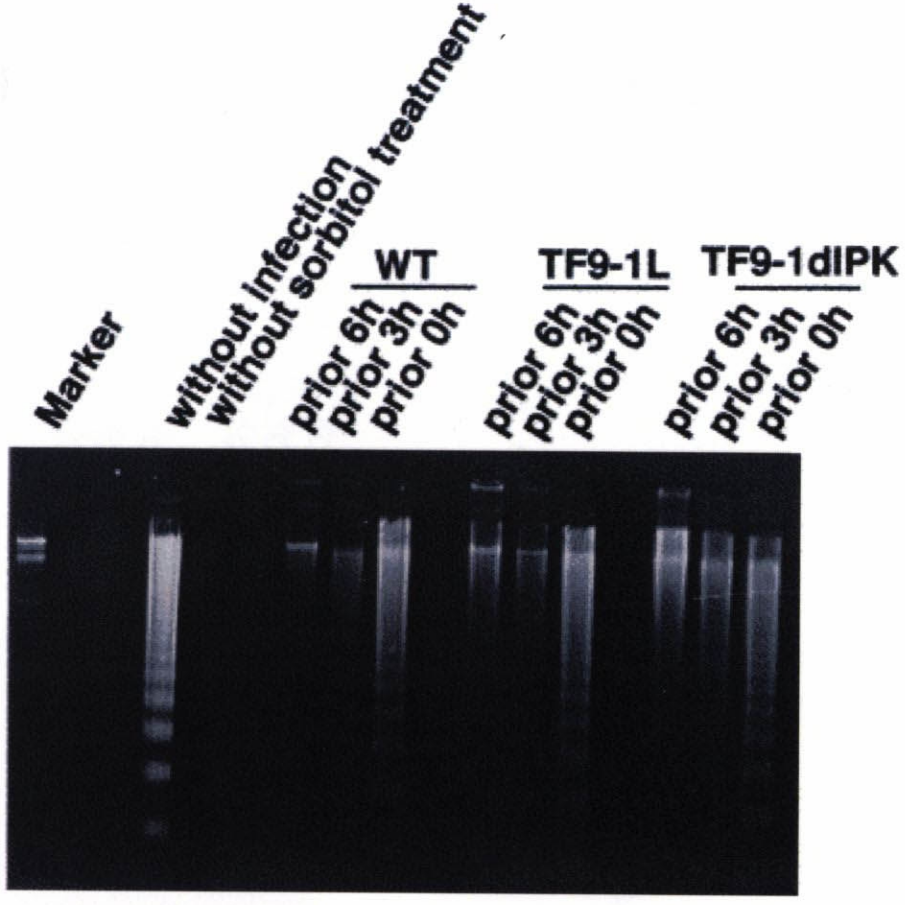
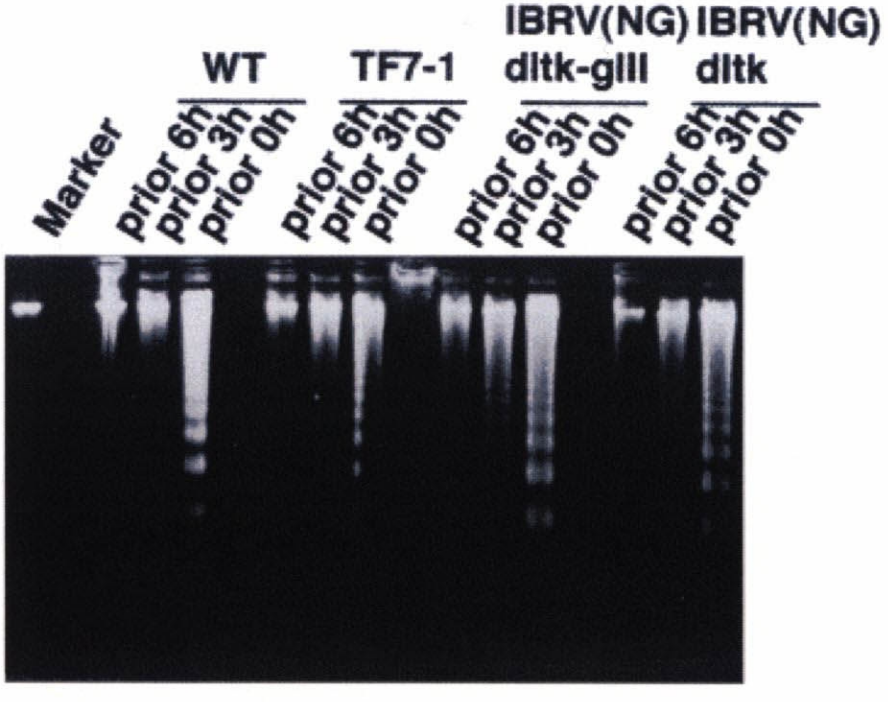

Marker
without infection
without sorbitol treatment
WT
prior 6h
prior 3h
prior 0h
TF9-1L
prior 6h
prior 3h
prior 0h
TF9-1dIPK
prior 6h
prior 3h
prior 0h

Fig. 2.1-6

A


IBRV(NG) IBRV(NG)
WT
TF7-1
dltk-gIII
dltk
Marker
prior 6h
prior 3h
prior 0h
prior 6h
prior 3h
prior 0h
prior 6h
prior 3h
prior 0h
prior 6h
prior 3h
prior 0h

B

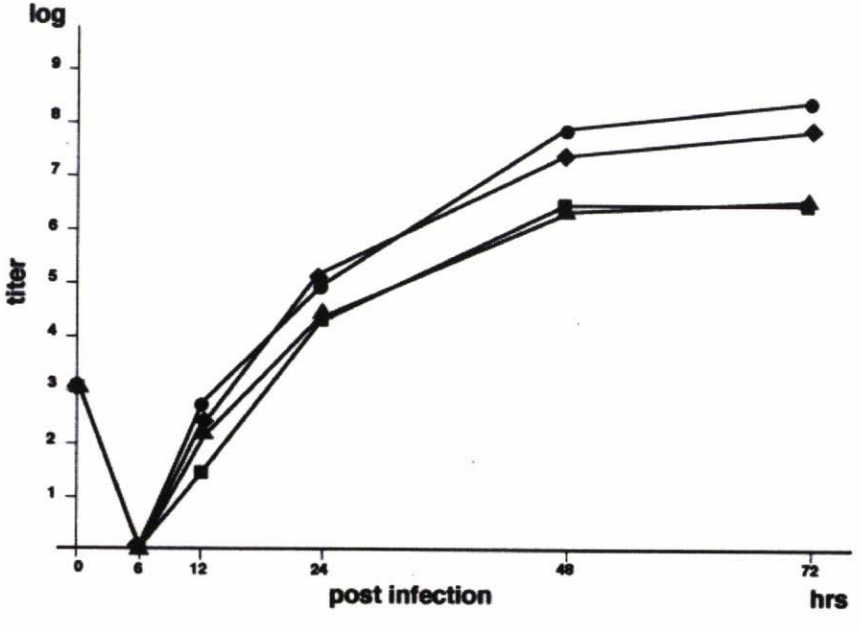

log
titer
9
8
7
6
5
4
3
2
1
0
6
12
24
48
72
post infection
hrs

Fig. 2.3-1

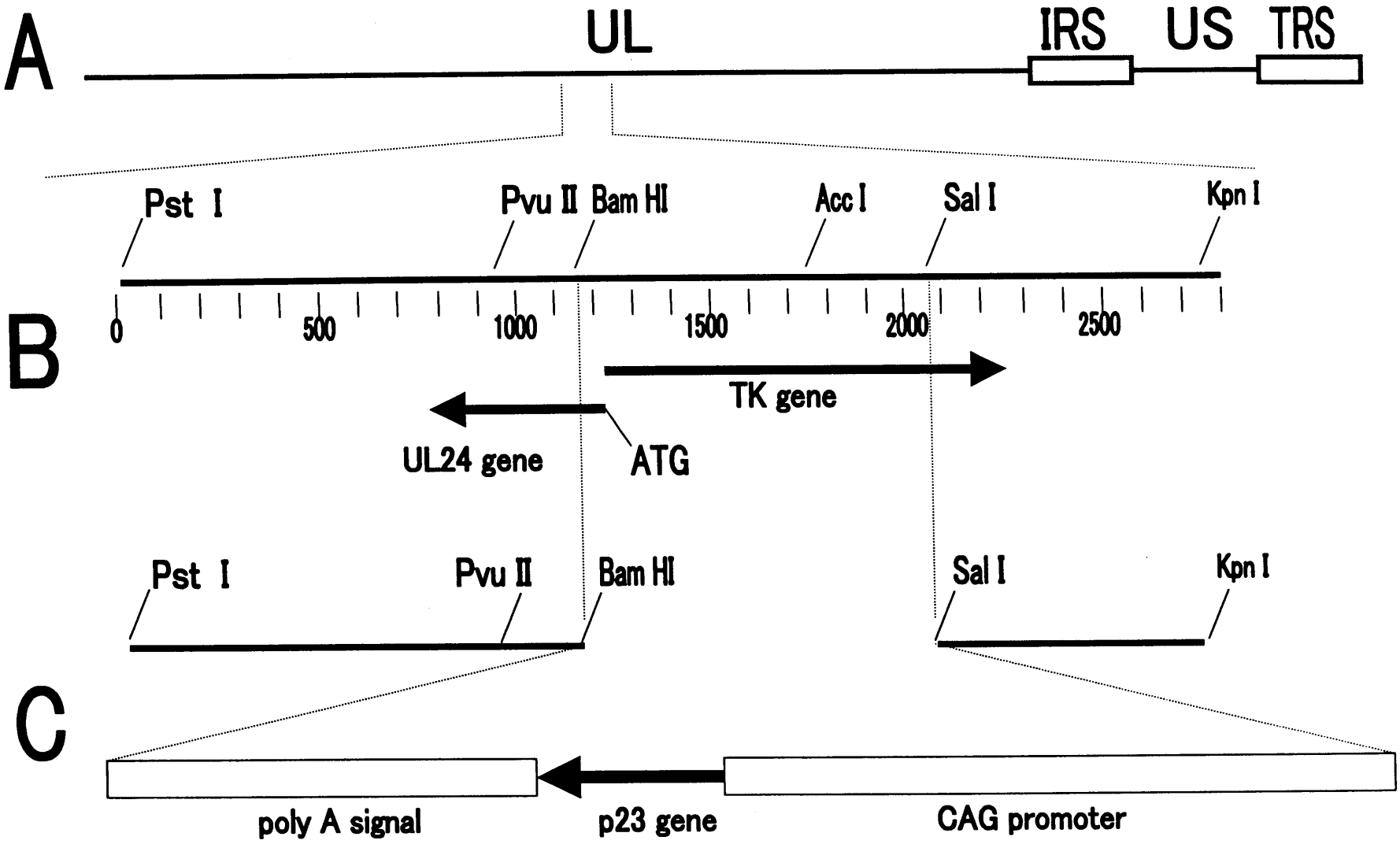

A
UL
IRS
US
TRS
B
Pst I
Pvu II
Bam HI
Acc I
Sal I
Kpn I
0
500
1000
1500
2000
2500
TK gene
UL24 gene
ATG
Pst I
Pvu II
Bam HI
Sal I
Kpn I
C
poly A signal
p23 gene
CAG promoter

Fig. 2.3.-2

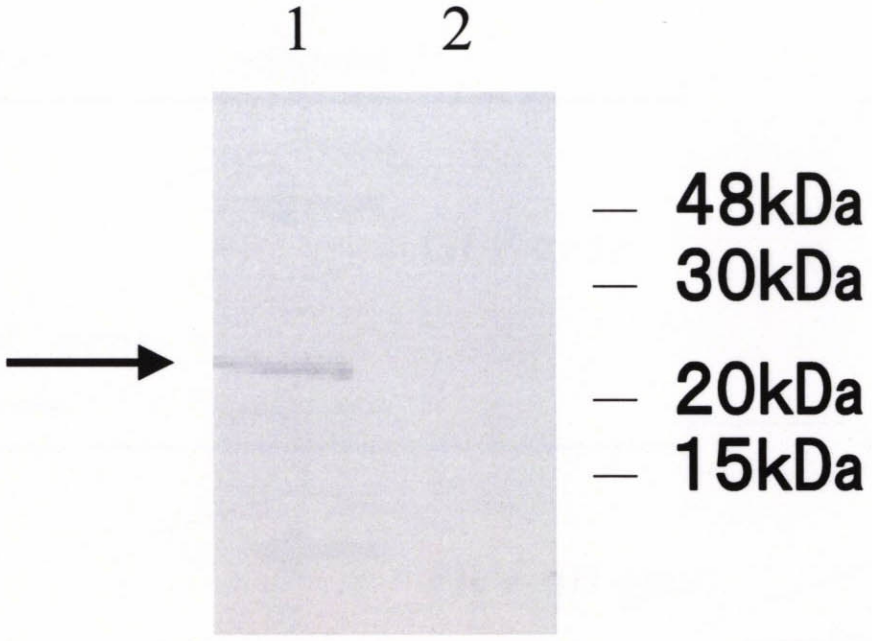


Fig. 2.2-1

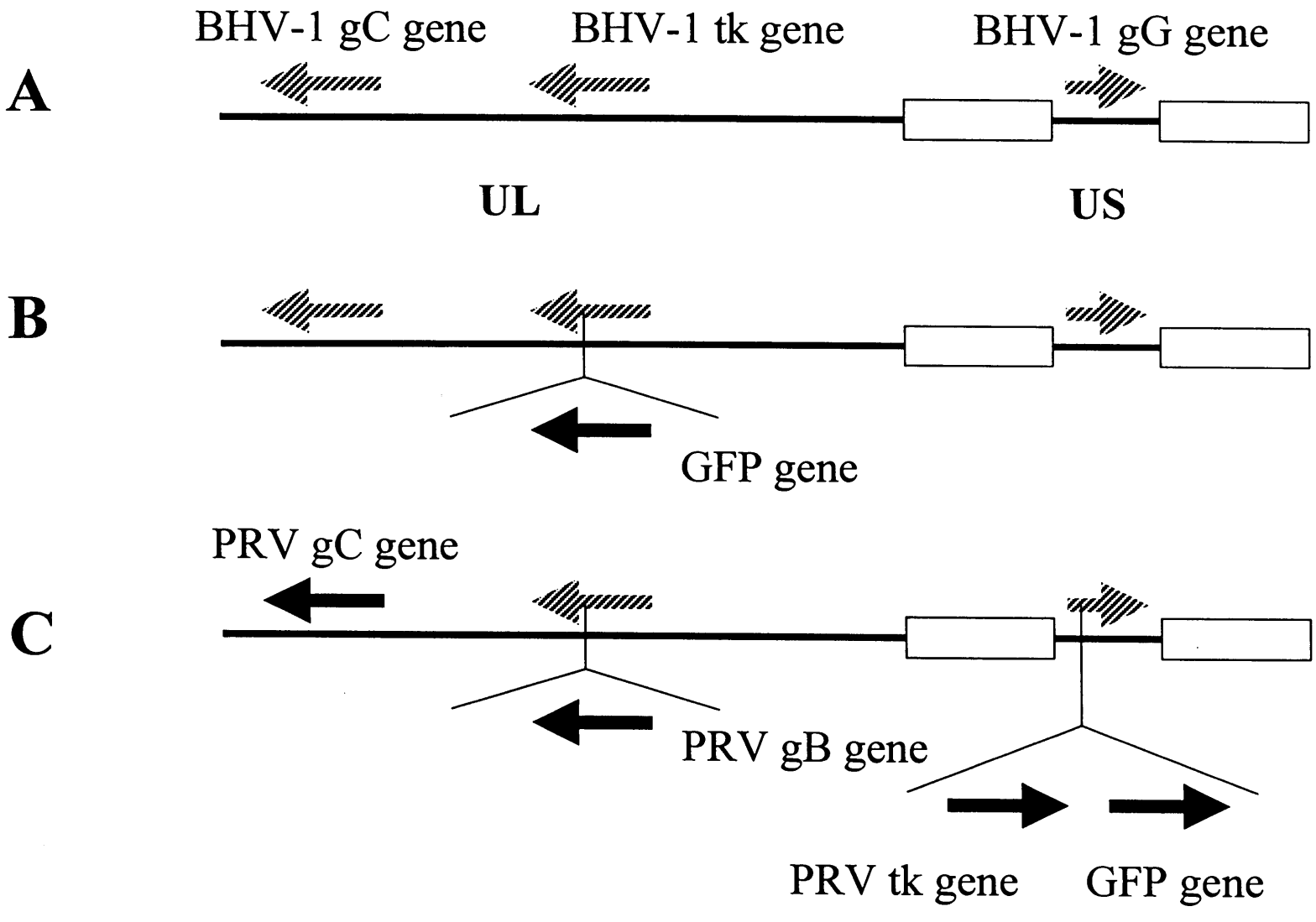

A
BHV-1 gC gene
BHV-1 tk gene
BHV-1 gG gene
UL
US
B
GFP gene
C
PRV gC gene
PRV gB gene
PRV tk gene
GFP gene

Fig. 2.2-2

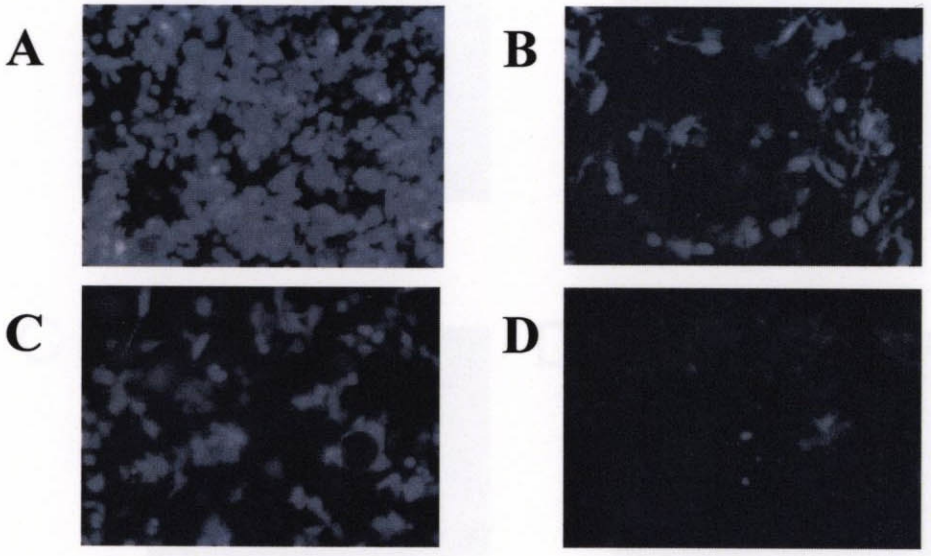


Fig.2.2-3

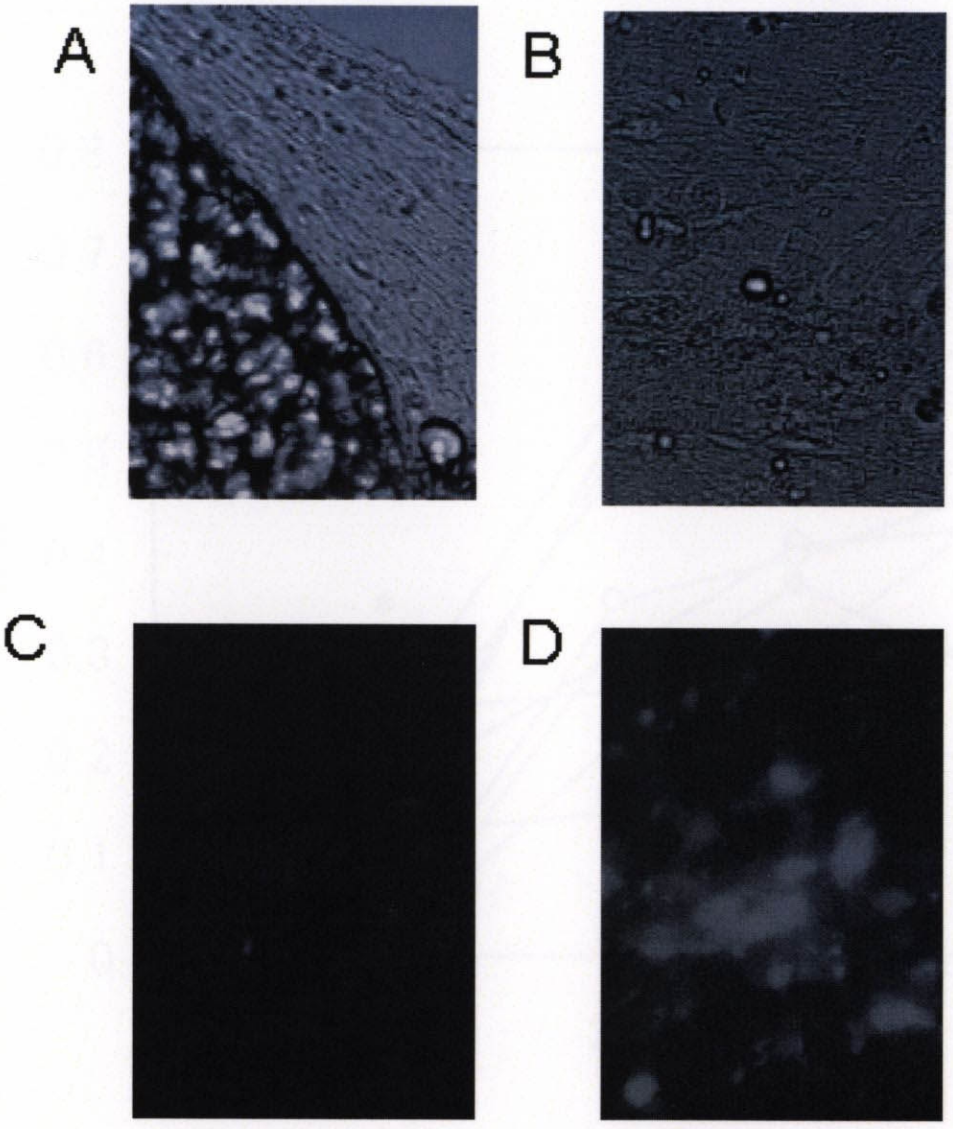


Fig. 2.2-4

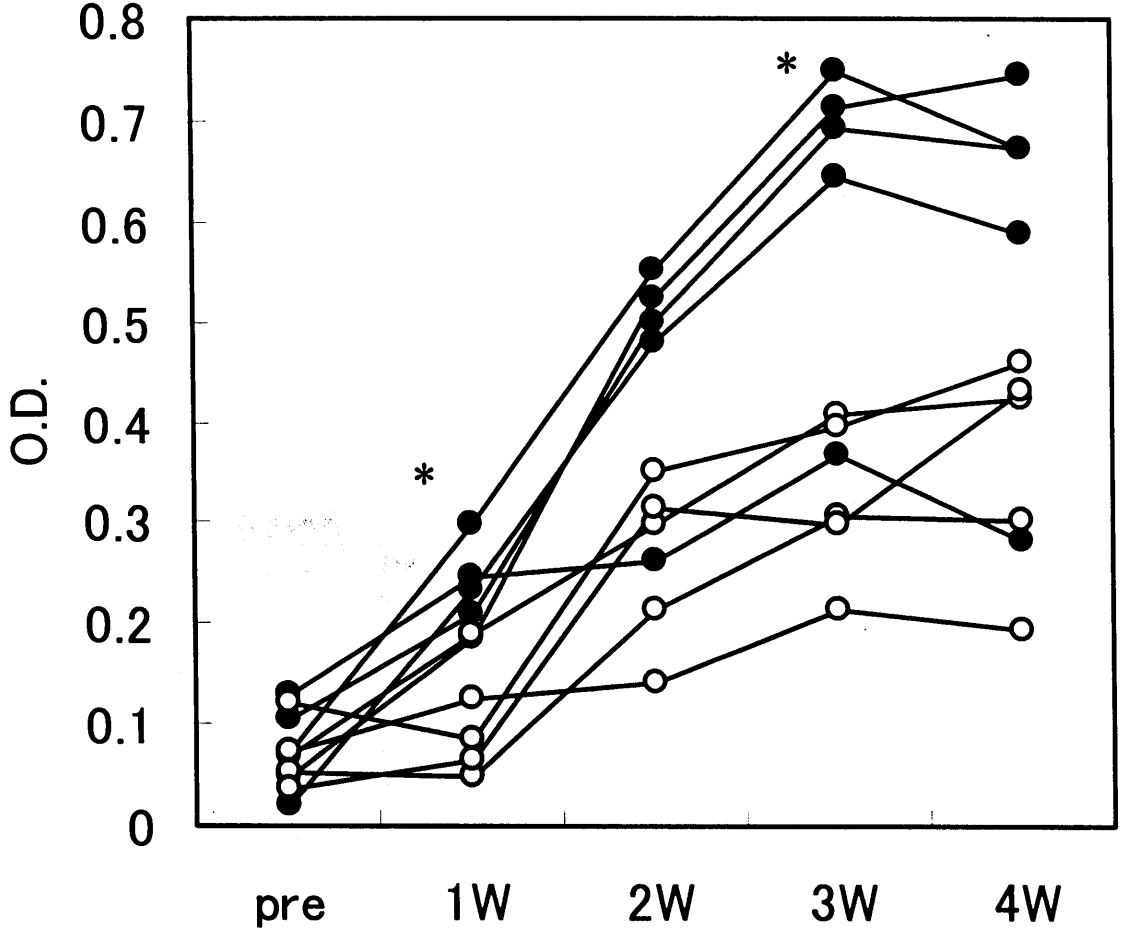


Fig. 2.2-5

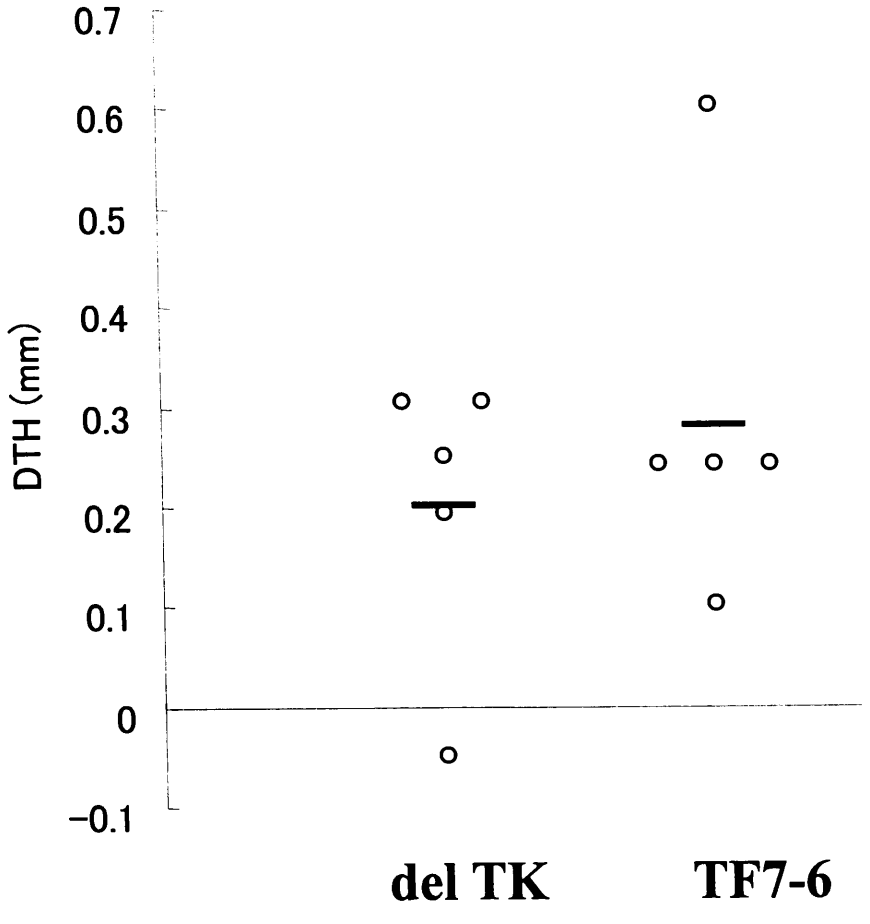


Fig. 2.2-6

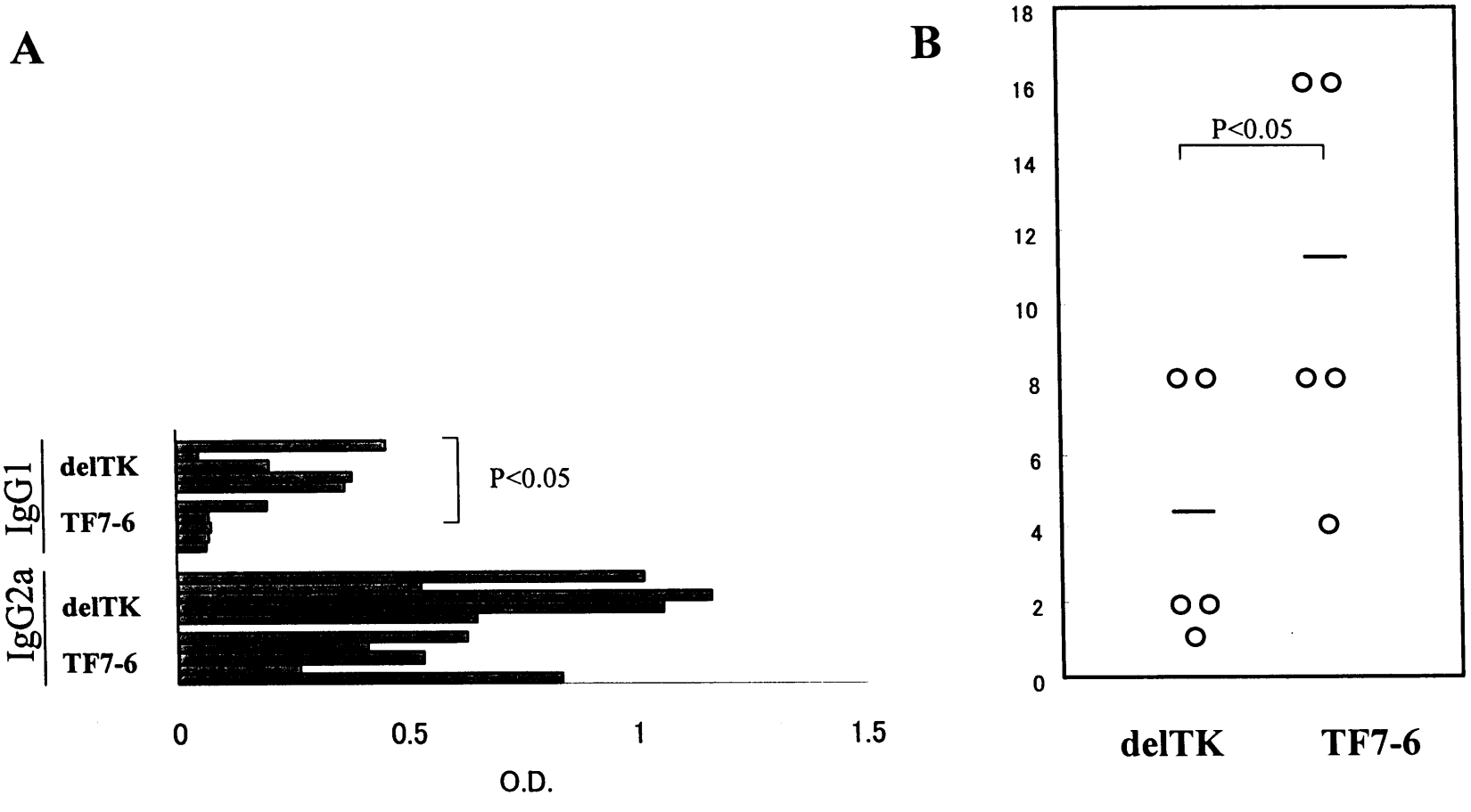

A
IgG1
delTK
TF7-6
IgG2a
delTK
TF7-6
P<0.05
0
0.5
1
1.5
O.D.
B
18
16
14
12
10
8
6
4
2
0
P<0.05
delTK
TF7-6

Fig. 2.4-1

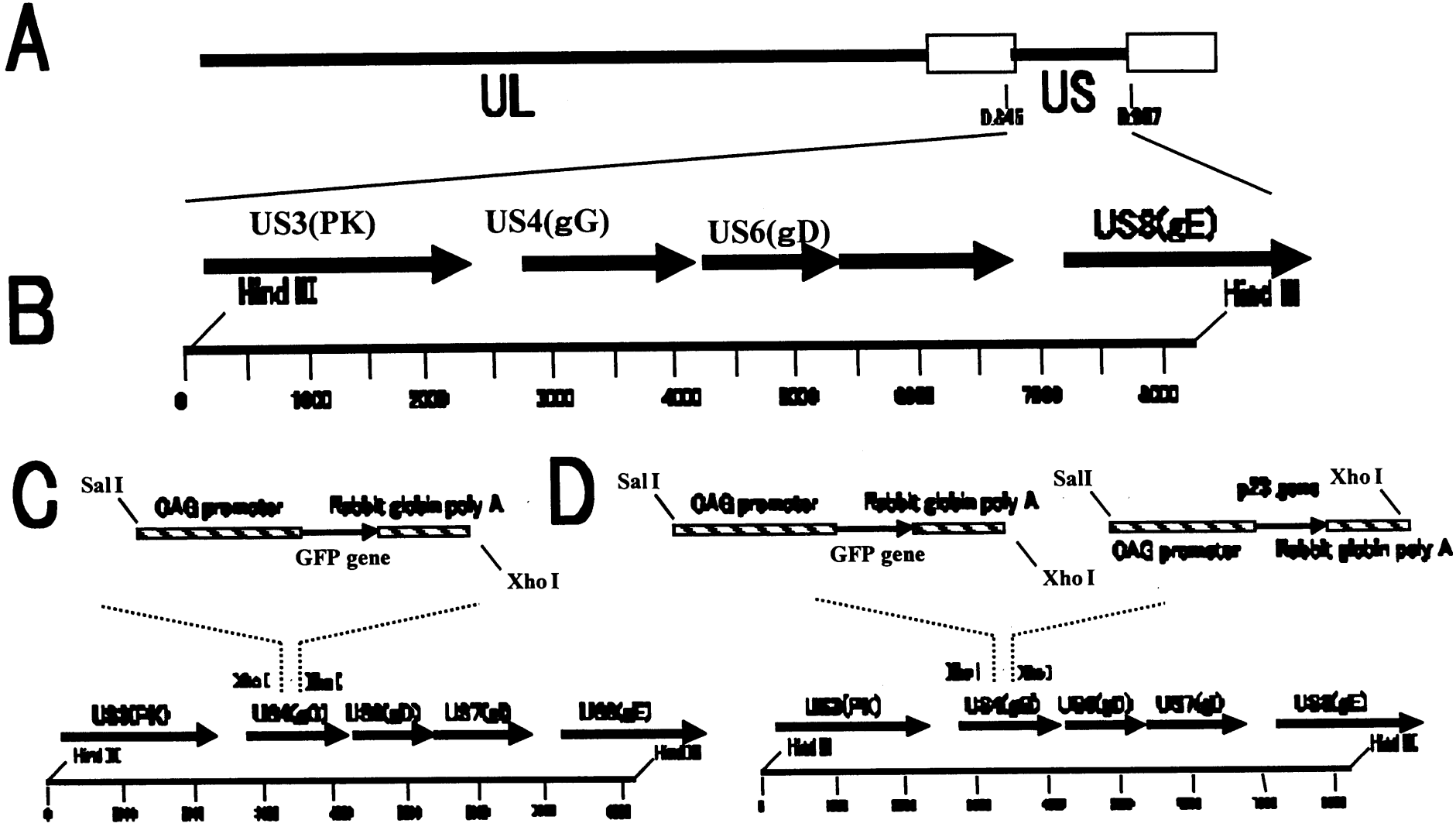

A
UL
US
B
US3(PK)
US4(gG)
US6(gD)
US8(gE)
Hind III
C
Sal I
CAG promoter
Rabbit globin poly A
GFP gene
Xho I
D
Sal I
CAG promoter
Rabbit globin poly A
GFP gene
Xho I
Sal I
p23 gene
Xho I
CAG promoter
Rabbit globin poly A

Fig. 2.4-2

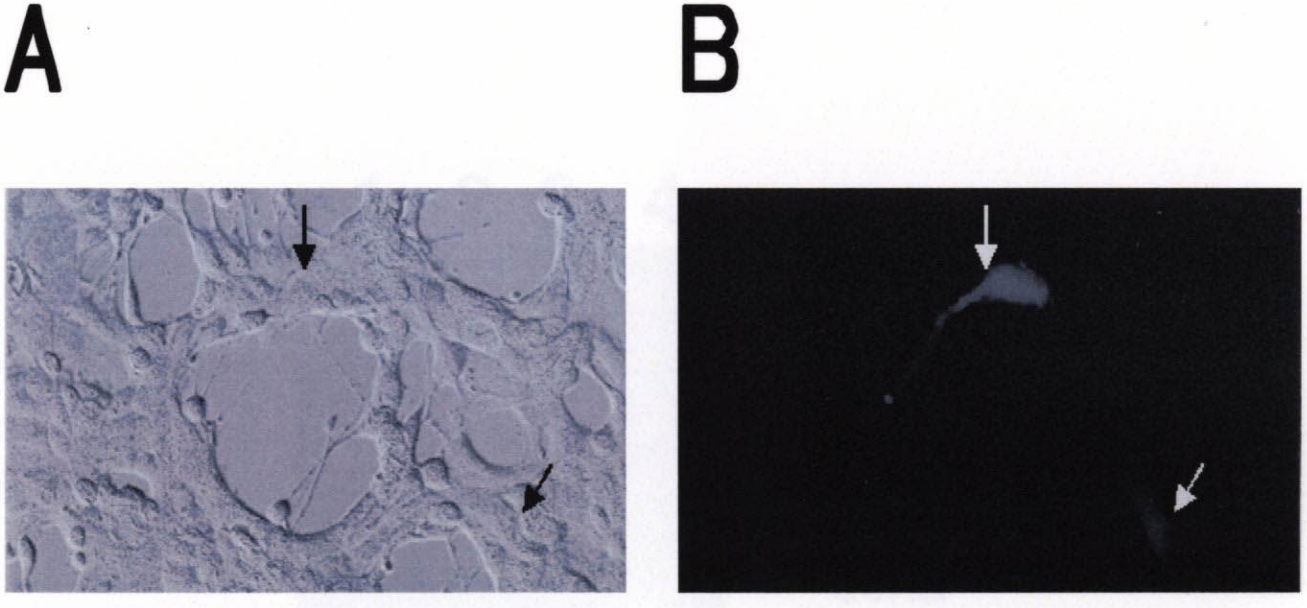


Fig. 2.4-3

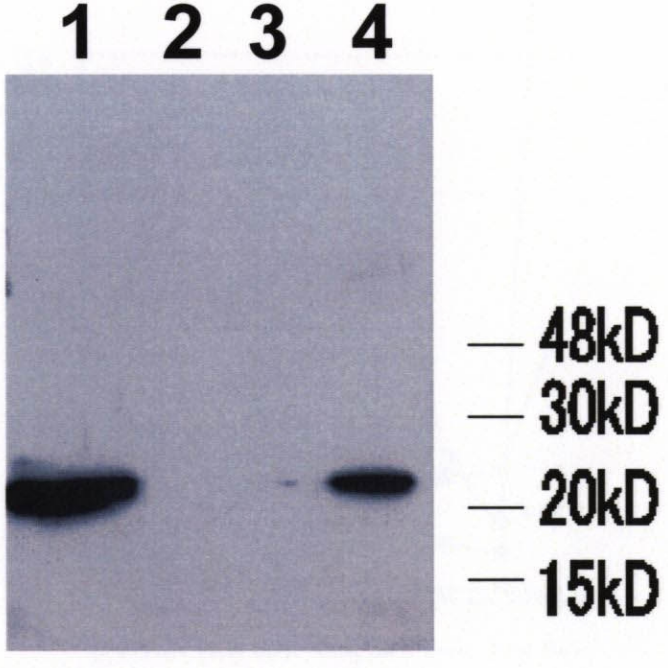


Fig. 2.4-4

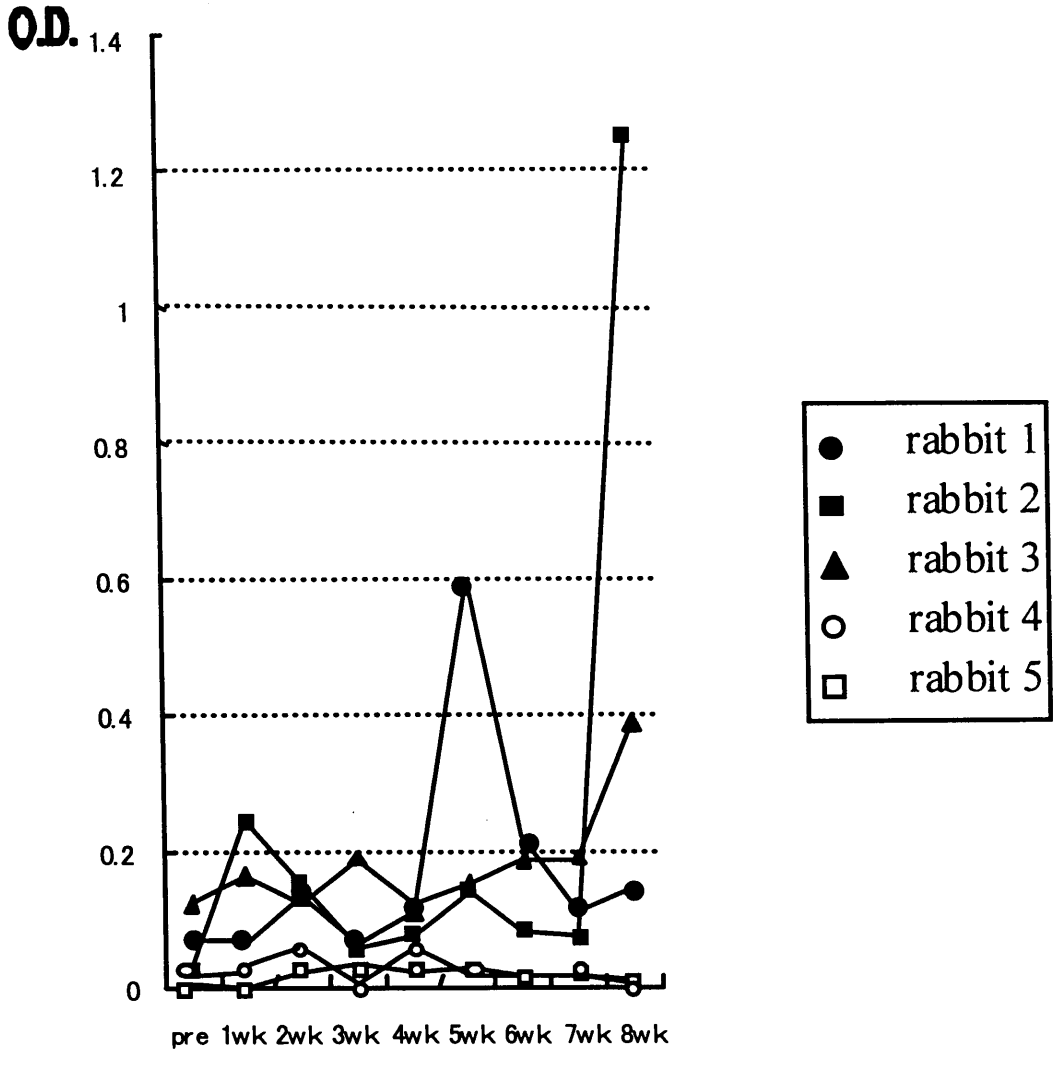

O.D.
1.4
1.2
1
0.8
0.6
0.4
0.2
0
pre 1wk 2wk 3wk 4wk 5wk 6wk 7wk 8wk
rabbit 1
rabbit 2
rabbit 3
rabbit 4
rabbit 5

Fig. 2.4-5

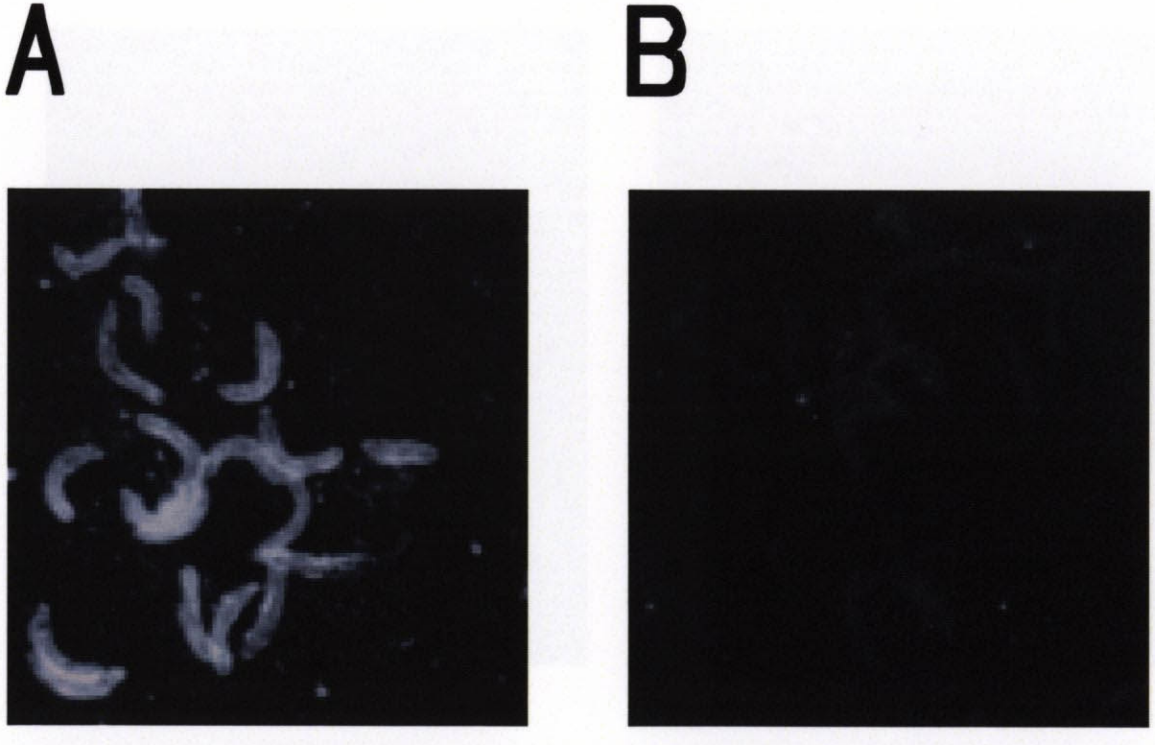


Fig. 2.4-6

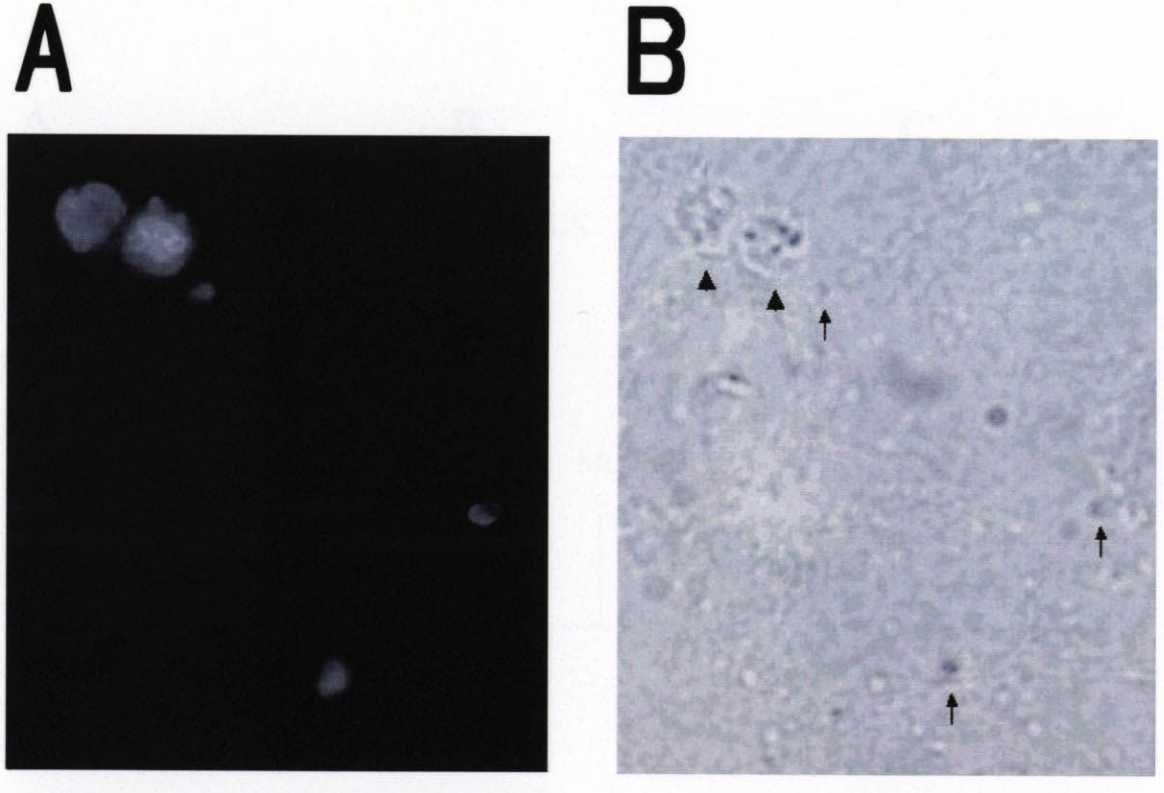


Fig. 2.5-1

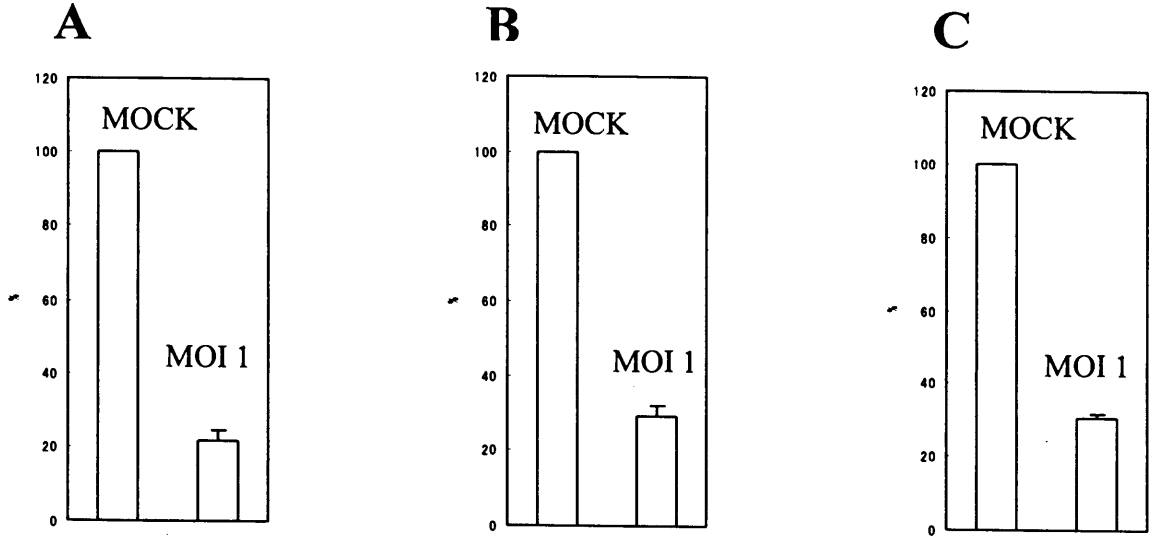
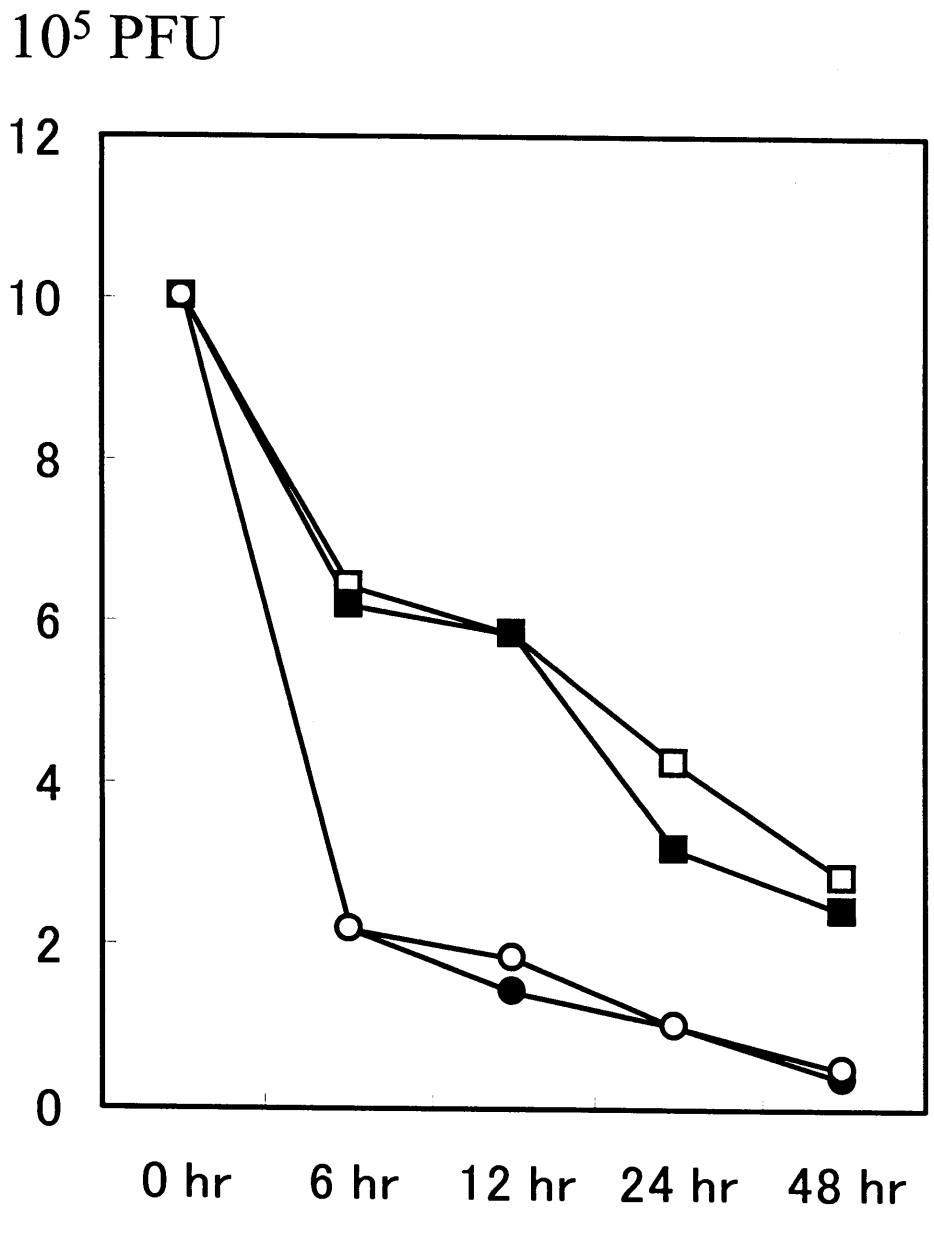


Fig. 2.5-2



# Chapter 3: Modification of antigens by adding Fc region of antibody molecule.

## 3.1 Construction of the cell line expressing Fc region of mouse IgG and adjuvant effect of the Fc region.

## 3.2 DNA vaccine, coding p23 of *Cryptosporidium parvum* fused with the Fc portion of mouse immunoglobulin G, efficiently induces antigen specific interferon-γ production.

## 3.1 Construction of the cell line expressing Fc region of mouse IgG and adjuvant effect of the Fc region.

### 1. ABSTRACT

The author established RK13 cell expressing a chimeric antibody-like molecule, containing Fc region of mouse IgG1. The chimeric protein was a type II membrane protein in such an orientation that Fc portion projected away from the cell surface. The cell line expressing the chimeric protein was designated as RK/Fc. When RK13 or RK/Fc cells were cultured with mouse macrophages *in vitro*, RK/Fc cells were damaged much more than RK/13 cells, indicating that the Fc region can be recognized by Fc receptors of macrophages. The activation of the complement pathway by the molecule was not detected. The immunization of BALB/c mice with RK/Fc cells induced much greater level of anti-RK cell antibody, comparing the immunization with parental RK13 cells. These data suggested that Fc region of the chimeric protein was recognized by Fc receptor (FcR) on macrophage , and that such a chimeric protein enhance the uptake by antigen presenting

cells via FcRs.

## 2. INTRODUCTION

Opsonization of foreign antigens with immunogloblin G (IgG) enhances their removal from the host by macrophage (Allen and Aderem, 1996; Huber et al., 2001; Miller et al., 1996; Raghavan and Bjorkman, 1996). Antibody-dependent cell mediated cell toxicity (ADCC) is one of the mechanism of the enhancement (Durden et al., 1994; Hulett and Hogarth, 1994; Raghavan and Bjorkman, 1996). However, the enhancement of removal is due to not only ADCC but also by acquired immune reactions. Antigen presenting cells (APCs) expressed receptors for the Fc portion of immunogloblin (FcRs), which mediate internalization of antigen-IgG complexes and promote efficient MHC class II-restricted antigen presentation (Ravetch, 1994; Regnault et al., 1999; Sallust and Lanzavecchia, 1994). In addition, it was reported that FcR-targeted antigenic uptake led to both MHC class I- and class II-restricted responses (Rafiq et al., 2002). Previously several trials were carried out to enhance a vaccine potential, using the interaction of FcR and IgG treated antigens (Bot et al., 2001; Gosselin et al., 1992; Liu et al., 1996).

Recently, the cells expressing human IgG Fc portion was constructed (Stabila et al., 1998). The author also genetically modified cells to express mouse IgG Fc portion. In this study, the author not only constructed genetically modified cells, but also investigated the immunogenic property of the cells. The chimeric protein on the established cell was a type II membrane protein in such an orientation that Fc portion projected away from the cell surface (Fig. 3.1-1).

## 3. MATERIALS and METHODS

Cells: CPK, RK13 or established cell lines from RK 13 cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with 7.5 % fetal calf serum (FCS) and 60 μg/ml of kanamycin. Peritoneal exudative cells were harvested from female BALB/c mice, purchased from Japan Clea Co. (Tokyo, Japan), suspended at $10^6$ cells/ml in RPMI 1640 medium supplemented 10 % FCS and incubated in a culture dish 37℃ for 1hr. After the incubation, the non-adherent cells were removed, and adherent cells were gentry washed with warmed medium. The adherent cells were harvested and used as peritoneal excudative macrophages.

Plasmid vectors: The gene coding Fc portion of immunogloblin G1 (IgG1) containing hinge, CH2 and CH3 domain was amplified from BALB/c mouse spleen cDNA. The oligonucleotide primers were designated to generate 5'-*Kpn* I and 3'-*Bam* I terminal restriction enzyme site (5'-GGTACCGTGCCCAGGGATTGTGGTTGTA-3' and 5'-GGATCCTTATTTACCAGGAGAGTGGGAG-3', respectively). To clone the

gene coding mouse transferrine receptor (mTR) trans membrane domain, the oligonucleotide primers were designated to generate 5'-*Bam* HI and 3'-*Kpn* I terminal restriction enzyme site (5'-GGATCCGCCACCATGATGGATCAAGCCAGATCA-3' and 5'-GGTACCCTCCTCTTTTTGTTCTACACG-3', respectively). The amplified genes coding mTR and IgG1 Fc domain were ligated in flame, to generate the gene coding sequence for the chimeric molecule. The gene coding the chimeric molecule (mTR-Fc) was replaced with *Eco* RI fragment of the mammarian expressing vector pCX-eGFP, which cods a enhanced green fluorescent protein (eGFP) gene (provided by Dr. Miyazaki, Osaka Univ.) and resulting plasmid was designated as pCX-mTRfc. To construct a vector coding a control chimeric molecule containing Fc domain, which is not transferred into cell membrane, the plasmid, pCX-p23fc was constructed as following. The p23 gene was further amplified from pUC/p23 using a pair of oligonucleotide primers, 5'-GGATCCAAAAATGGGTTGTTCATC-3' and 5'-GGTACCGGCATCAGCTGGCTTGTC-3', which were designed to delete the stop codon and to generate a 5'-*Bam* HI and 3'-*Kpn* I terminal restriction enzyme site. The amplified fragment was cloned into a pT7Blue vector

(Novagen, USA) with the same direction of the lacZ gene as the vector. The resulting plasmid was designated as prp23. The p23 gene and the Fc portion gene were ligated in frame as follows. The fragment containing the Fc portion gene was obtained by digesting pIgG with *Kpn* I, as described in chapter 3.1. The plasmid prp23 was digested with *Kpn* I, and the Fc portion gene was ligated at the *Kpn* I site of prp23 in such a way that the p23 gene and the Fc portion gene were in proper orientation. The resulting plasmid was designated as p23fc. The chimera p23fc gene was obtained from the plasmid p23fc by digestion with *Bam*HI and replaced with the *Eco* RI fragment of the expression vector pCX-eGFP, and the resulting plasmid was designated as pCX-p23fc.

Establishment of cell lines: RK13 cells cultured in six-well cell culture plate were transfected with 3 μg of pCX-mTRfc and 0.15 μg of the pRVSVneo (provided from Dr. Adachi, Kyoto Univ.), or 3 μg of pCX-eGFP and 0.15 μg of the pRVSVneo, using lipofectamin plus reagent (Gibco BRL, Gaithersburg, MD) according to manufacture's instruction. Stable cell lines were selected by G418 (Gibco BRL, Gaithersburg, MD) at a concentration of 400 μg/ml.

Detection of the expression of eGFP and mTR-Fc: To select eGFP expressed in cloned cell lines, the cells were observed by fluorescence microscope, and a clone with the strongest fluorescence was designated as RK/GFP. To determine the relative mTR-Fc expression, ELISA was used. RK13 cells, established cells or RK cells transfected with plasmid vectors were added to each well of 96-well cell culture plate at the concentration of $2 \times 10^4$ cells/well and incubated for 6h to allow the cells to adherent to the bottom of each well. The wells were washed with PBS and 100 μl of 3 % skim milk was added to each well. After the inoculation for 40 min at room temperature, the cells were reacted with 1:1000 diluted horseradish peroxidase-conjugated rabbit anti mouse IgG antibody (ZYMED, San Francisco, CA). Substrate $\sigma$phenilendiamin was added and the plate was read after 30 min at $OD_{490}$. Selected clones expressing mTR-Fc were designated as RK/Fc.

Western blotting assay: RK13, RK/Fc or RK13 cells transfected with plasmid vectors were lysed in a lysis buffer (0.01% blomophenolblue, 10% glycerol, 2.0% SDS, 62.5 mM Tris-HCl, pH 6.8) with or without 1.4 M

2-mercaptoethanol. These samples were subjected to electrophoresis in SDS-polyacrylamid gel. The protein bands were transferred to the transfer membrane and visualized by the reaction with peroxidase-conjugated rabbit anti mouse IgG antibody (ZYMED).

Cell lysis by complements: 100 μl of supplemented EMEM and 50 μl of rabbit serum as a source of complement was added onto $1.5 \times 10^5$ RK/GFP or RK/Fc cells cultured in 24-well cell culture plate. After adding of 20 μl of PBS, 10 μl of mouse anti-RK13 cell serum (Maeda et al., 2002), or 20 μl of anti-mouse IgG (SIGMA, St.Luis, MO), the cells were inoculated at 37℃ for 1 hr, and the cells were stained with trypan blue. The number of stained or non-stained cells were counted as that of died or live cells.

Cell lysis by macrophages: Splenocytes of BALB/c mice were cultured in RPMI 1640 medium containing 10 μg/ml concanavalin A at 37℃for 48 hr, and the supernatant was harvested. The peritoneal excudative macrophages prepared as described above were cultured in the supernatant for 24 hr at 37℃. The macrophages were washed with PBS and suspended in RPMI 1640

medium at the concentration of $1 \times 10^6$ cells/ml. The 200 μl of suspended macrophages were added to the confluent monolayer of RK13 or RK/Fc cells in 96-well cell culture and incubated for 16 hr at 37℃. After removing the supernatant, adherent cells were stained with crystal violet and observed by microscope. To determine the relative lysis level, the release of lactose dehydrogenase (LDH) from damaged RK13 or RK/Fc cells were measured using Cyto Tox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madisson, WI). $5 \times 10^3$ RK13 or RK/Fc cells and $3 \times 10^5$ macrophages, which was pre-cultured for 24 hr as described above, were cultured with 100 μl of RPMI 1640 medium for 7 hr at 37℃. After that, the released LDH into medium was measured and cell lysis level was determined according to manufacture's instruction.

Immunization: 8 weeks old female BALB/c mice were immunized with $10^5$ UV-exposed RK13 or RK/Fc cells suspended in 20 μl of PBS from right footpad. Blood samples were collected two and four weeks later from the immunization.

Detection of anti-RK13 cell antibodies: RK13 cells were cultured in 96-well cell culture were washed with PBS and 100 μl of 3 % skim milk was added to each well. After the inoculation for 40 min at room temperature, 1:100 diluted serum samples were added and incubated for 40 min. After washing with PBS, 1:3000 diluted horseradish peroxidase-conjugated rabbit anti mouse IgG, IgG1 or IgG2a antibody (ZYMED, San Francisco, CA) were added. After the incubation for 40 min, substrate *o*-phenilendiamin was added and the plate was read after 30 min at $OD_{490}$.

## 4. RESULTS

Expression of the chimeric molecule, mTR-Fc: The expression of a chimeric molecule, mTR-Fc, which contains trans membrane domain of mTR and Fc domain of mouse IgG1, in the RK13 cells transiently transfected with pCX-mTRfc was detected as an approximately 44 kDa specific band (Fig. 3.2-2A) by Western blotting analysis. At the non-reduced condition, a broad band was appeared around 66 and 97 kDa molecular markers (Fig. 3.2-2B). The results suggested that mTR-Fc conformed dimmer as like IgG heavy chain. Cell-surfaced mTR-Fc was detected by ELISA. As shown in Fig. 3.3-3, RK13 cells transfected with pCX-mTRfc exhibits high signal compared with RK13 cells transformed an empty vector, pCX or a control vector, pCX-p23fc. The vector pCX-p23 codes a chimeric molecule containing Fc domain, which is accumulated in cytoplasm and not transferred into cell membrane (data not shown). These results indicate that the chimeric molecule mTR-Fc existed on cell surface. Similar results were obtained in the RK/Fc cell, which is the stable cell line established using pCX-mTRFc (data not shown). The results confirmed the existence of mTR-Fc on the surface of RK/Fc cells.

To determine whether the mTR-Fc on the surface of RK/Fc cells can activate complement classic pathway, several clones of RK/Fc cells, which express several levels of mTR-Fc (Fig. 3.1-4A), and RK/GFP cells was treated with rabbit serum. As a control, RK/GFP cells were also treated in the presence of mouse anti-RK13 cells serum. As shown in Fig. 3.1-4B, only the cells treated in the presence of mouse anti-RK13 cells serum were lysed. And a positive correlation was not observed between the mTR-Fc expression level and cell lysis level. These results indicated that mTR-Fc did not activate complement classical pathway. In the following experiments, RK/Fc clone which express highest level of mTR-Fc was used.

Recognition of mTR-Fc by macrophage: RK13 and RK/Fc cells were incubated with mouse peritoneal exudative macrophages. As shown Fig. 3.1-5A, when the RK13 cells were opsonized by mouse anti-RK13 cells serum, the damage of RK13 cells were observed morphologically. Without any opsonization, on RK13 cells, any damage was not observed (Fig.3.1-5A). However, in the absence of anti-RK13 cells antibodies, the damage of RK/Fc cells was observed morphologically (Fig.3.1-5A). In addition, release of an

intracellular enzyme, LDH, from RK/Fc cells were also detected. These data indicate that RK/Fc cells were attacked by macrophages, suggesting mTR-Fc on RK/Fc cells was recognized by Fc receptor of macrophage.

Adjuvant effect of the fusion protein on the established RK/Fc cells: To determine whether Fc domain on the established cell line, RK/Fc, has adjuvant effects, RK13 cells or RK/Fc cells were injected into mice footpad and the level of produced anti-RK13 cell antibodies was measured. As shown Fig. 3.1-6, in the mice immunized with RK/Fc, higher level of antibodies production was detected just after two weeks from the immunization, whereas, at that time, only the very low level of antibodies were detected in mice immunized with RK13 cells. The result suggests that Fc domain on RK/Fc cells has the adjuvant effects. As shown in Fig. 3.1-7, RK/Fc induce not only higher level of IgG1 but also IgG2a (Fig. 3.1-7).

## 5. DISCUSSION

In this study, the author construct a gene coding a chimeric protein containing Fc portion of mouse IgG, mTR-Fc, and establish the cell line, RK/Fc, expressing mTR-Fc on their surface. As shown in Fig.3.1-6, RK/Fc cells induced higher level of IgG, suggesting that the chimeric protein mTR-Fc has an adjuvant potential. It was reported that mice vaccinated with IgG-opsonized antigens made high levels of IgG1 isotype and that when antigen is targeted to Fc receptors on macrophages, the T cell response was biased toward a Th2-like response (Anderson and Mosser, 2002). However, as shown in Fig. 3.1-7, RK/Fc induced higher level of IgG2a. The result indicates that the fusion protein, mTR-Fc, does not bias T cell response toward Th2-like response.

These results show the possibility of chimeric proteins containing Fc portion as a vaccine adjuvant to induce both of production of antibodies and cell mediated immune reactions.

## 6. LEGENDS of FIGURE

Fig. 3.1-1: Design of chimeric molecule. (A) Cell surface IgG. Solid boxes indicate type I transmembrane anchor domain of C-terminal. (B) Reverse cell surface chimeric molecule. Open boxes indicate type II transmembrane anchor domain of N-terminal.

Fig. 3.1-2: Western blotting analysis of RK13 cells transiently transfected with pCX-mTRfc (lane 1, 3) or pCX (lane 2, 4) in non-reduced (lane 1,2) or reduced (lane 3, 4) condition. The protein bands were visualized by the reaction with peroxidase-conjugated rabbit anti mouse IgG antibody.

Fig. 3.1-3: Cell surface Fc domain detected by ELISA. Solid bar, striped bar and open bar indicate results of RK13 cells transfected with pCX, pCX-p23fc or pCX-mTRfc, respectively.

Fig. 3.1-4: Cell lysis by complement. (A) Cell surface Fc domain was detected by ELISA. Solid bar and open bars were O.D. values of RK/GFP and four clones of RK/FC. (B) Correlation between Fc domain expression level and cell

lysis. The horizontal axis indicates O.D. value of ELISA to detect cell surface Fc domain. The vertical axis indicates cell mortality. Solid and open symbols indicate results of RK/GFP and RK/Fc, respectively. Square and circles indicates results in the presence or absence of opsonization by mouse anti-RK13 cell serum.

Fig. 3.1-5: Identification of Fc domain of cell surface by macrophage. (A) RK13 (left panel) or RK/Fc cells (right panel) cultured with macrophage for 16 hr. As a control, RK13 cells opsonized with mouse anti-RK13 cell serum was also cultured with macrophages (lower panel).
(B) LDH release from RK13 or RK/Fc cells (solid or open bar, respectively) cultured with macrophage for 7 hr.

Fig. 3.1-6: Production of IgG against RK13 cell. The serum samples were harvested from mice immunized with RK/Fc or RK13 cells, two weeks after the immunization, and antibodies in the serum samples were detected by ELISA. Black dots indicate the O.D. value of individual samples.

Fig. 3.1-7: Production of IgG1 and IgG2a against RK13 cell. The serum samples were harvested from mice immunized with RK/Fc or RK13 cells, four weeks after the immunization, and antibodies in the serum samples were detected by ELISA. Black dots indicate the O.D. value of individual samples.

## 3.2. DNA vaccine, coding p23 of *Cryptosporidium parvum* fused with the Fc portion of mouse immunoglobulin G, efficiently induces antigen specific interferon-γ production

### 1. ABSTRACT

To develop a DNA vaccine against cryptosporidiosis, the author constructed a plasmid coding an immunodominant surface protein p23 of *Cryptosporidium parvum* (*C.parvum*) sporozoites (pCX-p23) and another plasmid coding a fusion protein containing whole p23 and the Fc portion of mouse immunoglobulin G1 (IgG1) (pCX-p23fc). 3T3, A31-1-1 cells transformed with pCX-p23fc expressed a fusion protein containing the IgG Fc portion and p23 derived domain. Vaccination of BALB/c mice with pCX-p23 and pCX-p23fc induced the production of antibodies against p23. Lymphocytes harvested from the mice immunized with pCX-p23 and pCX-p23fc expressed interleukin-4 (IL-4) and interferon-γ (IFN-γ) after in vivo stimulation by p23 antigen. The IFN-γ level expressed by the lymphocytes of pCX-p23fc immunized mice was much higher than that of

pCX-p23 immunized mice. The plasmid pCX-p23fc has a possibility as a vaccine against cryptosporidiosis because it can induce antigen specific IFN-γ production, which is important in recovering from a *C. parvum* infection. .

## 2. INTRODUCTION

It was reported that the immunization of mice with DNA encoding a *Cryptosporidium parvum* (*C. parvum*) sporozoite antigen induced an antigen-specific T lymphocyte proliferative response in both spleen and mesenteric lymph nodes (Sagodira et al., 1999). It is also known that a recombinant protein derived from *C. parvum* synthesized by a eukaryotic system is a better antigen as an immunogen than that synthesized by a prokaryotic system (Iochman et al., 1999). These studies suggest that a DNA vaccine may be a feasible candidate for controlling cryptosporidiosis. However, the weak antigenicity of a DNA vaccine is a major obstacle for its practical use in the field.

Previously, a complex of a hepatitis B virus surface antigen (HBsAg) and the anti-HBsAg antibody was used as a vaccine, and it was found that the complex induced a stronger immune response than the antigen alone (Wen et al., 1999). It was also reported that a hepatitis B virus *e* antigen fused with the Fc portion of immunoglobulin G (IgG) had an enhanced vaccine potency (You et al., 2001). This is probably due to antigens being more efficiently

taken up by antigen presenting cells (APC) through the Fc receptors on these cells.

In this study, the author constructed a plasmid coding the 23 kDa glycoprotein p23 of *C. parvum* fused to the Fc portion of immunogloblin G1 (IgG1) and tested its feasibility as a DNA vaccine in mice.

## 3. MATERIALS and METHODS

Mice and Cells: Seven weeks old female BALB/c mice were used. Porcine kidney derived CPK cells and mouse BALB/c 3T3, A31-1-1 cells (A31 cells) were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10 % fetal calf serum (FCS) and 60 μg/ml of kanamycin.

Parasite and cloning of p23 gene: A *C. parvum* isolate (HNJ-1 strain) was used (Abe et al., 2002). The parasite stock was maintained and the template DNA for cloning the p23 gene was obtained as described in chapter 1.
The p23 gene was further amplified to generate a 5'-*Bam* HI and 3'-*Kpn* I terminal restriction enzyme site and the fragment was cloned into a pT7Blue vector (Novagen, USA) with the same direction of the lacZ gene as the vector as described in chapter 3.1. The resulting plasmid was designated as prp23.

Preparation of the gene coding the Fc region of IgG: The gene coding for the Fc portion of immunogloblin G1 (IgG1) containing hinge, CH2 and CH3

domains was amplified from BALB/c mouse spleen cDNA as described in chapter 3.1.

Construction of the p23fc fusion gene: The p23 gene and the Fc portion gene were ligated in frame as described in chapter 3.1. The schema of the construction of the p23fc fusion gene was shown (Fig. 3.2-1A). The resulting plasmid was designated as p23fc.

The expression vectors: The plasmid vector expressing the chimera p23fc gene, pCX-p23fc was constructed as described in chapter 3.1. The gene coding normal p23 was obtained from pUC/p23 by the digestion with *Bam* HI and replaced with the *Eco* RI fragment of the expression vector pCX-eGFP. The resulting plasmid was designated as pCX-p23. The empty control plasmid pCX was constructed by removing the *Eco* RI fragment containing the eGFP gene from the plasmid pCX-eGFP.

Direct fluorescent assay: A31 cells were transformed with pCX-p23 or pCX-p23fc using Superfect™ Transfer Reagent (QIAGEN, Germany). After a

48 hr incubation, the cells were fixed with chilled acetone and reacted with fluorescein-conjugated goat anti-mouse IgG and IgM (Jackson ImmunoResearch Laboratories, Inc., USA). After incubation at 37℃ for 1 hr, the cells were observed using a fluorescent microscope.

Mouse anti-p23 serum: 8 weeks old female mouse was immunized intraperitoneally with $3 \times 10^5$ plaque formation units (PFU) of recombinant vaccinia virus expressing p23, vv/p23. Four weeks after immunization, mouse serum was harvested and used as a mouse anti-p23 serum. The construction and properties of the recombinant vaccinia virus expressing p23 were described in chapter1. When BALB/c mouse was immunized with $3 \times 10^5$ PFU of vv/p23, both of IgG1 and IgG2a against p23 were induced (data not shown).

Western blotting assay: The A31 cells or CPK cells were transformed with pCX-p23 or pCX-p23fc as described above. After 24 hr incubation, the transformed cells were lysed in a lysis buffer (0.01% blomophenolblue, 10% glycerol, 2.0% SDS, 62.5 mM Tris-HCl, pH 6.8, 1.4 M 2-mercaptoethanol).

These samples were subjected to electrophoresis in a SDS-polyacrylamide gel. To detect the fusion protein containing the Fc portion, the bands were transferred to a transfer membrane, Immobilon (Millipore, USA). The membrane was reacted with horseradish peroxidase-conjugated rabbit anti mouse IgG, IgG1 or IgG2a antibody (ZYMED, USA), and the band of fusion protein was visualized using ECL™ western blotting detections reagent (Amersham Pharmacia biotech, U.K.). To detect p23 or the fusion protein containing the p23 derived domain, the transferred protein bands were reacted with mouse anti-p23 serum and horseradish peroxidase-conjugated rabbit anti mouse IgG2a antibody (ZYMED, USA). The band which reacted with mouse anti-p23 serum was visualized as described above.

DNA preparation and immunization: The plasmids pCX, pCX-p23 and pCX-p23fc were purified by a Qiagen column (Qiagen, Germany) and diluted in a 25% sucrose solution at 1mg/ml and stored at –20℃. Mice were anaesthetized by 75 mg/kg sodium pentobarbital. Each tibial anterior muscle was injected with 50 μl of diluted plasmid (100 μl/mouse). 4 weeks and 8 weeks after the first inoculation, a second and third inoculation was carried out as described above. Twelve weeks after the first inoculation, serum

samples were harvested.

Recombinant p23 peptide: The recombinant p23 protein was propagated as described in chapter 1. The p23 protein expressed by *E.coli* was used as an antigen for ELISA and for *in vivo* stimulation of mouse splenocytes.

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 100 μl aliquots were added into each well of a 96-well ELISA plate. The plate was incubated at 4℃ over night and washed with PBS containing 0.1% Triton X100 (PBS-TritonX100). After blocking with 3 % skim milk in PBS, mouse serum samples were diluted 1:100, and 100 μl of the diluted serum samples was added to each well and incubated at 37℃ for 1hr. The wells were washed 3 times with PBS-TritonX100, and horseradish peroxidase-conjugated goat anti mouse IgG +A +M antibody (ZYMED, USA) was added, and this was incubated at 37℃ for 1hr. After washing3 times with PBS-TritonX100, 100 μl of 0.04% o-phenylenediamine and 0.003% $H_2O_2$ in pH5.0 phosphate-citrate buffer (52 mM citric acid, 103 mM

$Na_2HPO_4$) was added, and this was 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.

Detection of the cytokine expression: Twelve weeks after the first inoculation, mice were sacrificed, and splenocytes were harvested. The splenocytes harvested from mice in each group were pooled and cultured for 48 hr in RPMI1640 medium in the presence or absence of 30μg/ml p23 peptide. The secreted interleukin-4 (IL-4) and interferon-γ (IFN-γ) in the supernatant was detected by sandwich ELISA as follows. Capture antibodies, Anti-Mouse IL-4 and Anti-Mouse IFN-γ (Pharmingen, USA), were diluted in carbonate buffer 1:250 and 1:2000, respectively. 100μl aliquots of capture antibodies were added to each well of a 96-well ELISA plate (Corning). The plate was incubated at 4℃ overnight and washed with PBS-TritonX100. After fixation with PBS containing 10% fetal bovine serum, 100 μl of the supernatant of the splenocytes was added to each well and incubated at room temperature for 2hr. The wells were washed with PBS-TritonX100, added with detection antibody, Biotilated anti-Mouse IL-4

or IFN-γ (Pharmingen, USA), which had been diluted 1:250 and pretreated with Avidin-horseradish peroxidase conjugate (Pharmingen, USA), and incubated at room temperature for 1hr. After washing with PBS-TritonX100, 100 μl of 0.04% o-phenylenediamine and 0.003% $H_2O_2$ in pH5.0 phosphate-citrate buffer was added, and this was 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.

## 4. RESULTS

Expression of p23 protein fused to the Fc region of IgG:

The gene coding the Fc fragment of mouse IgG1 was ligated in frame to the 3' terminal of the p23 gene. The gene coding the fusion protein (p23fc) (Fig. 3.2-1B) was inserted into an expression vector, and the resulting plasmid was designated as pCX-p23fc. A31 cells were transformed with pCX-p23fc or the control plasmid pCX-p23, which contained the normal p23 gene. Western blotting was performed using rabbit antibody against mouse IgG with A31 cell extracts transformed with pCX-p23fc or pCX-p23. A specific band of approximately 50kDa, which was the expected molecular weight of p23fc, was detected in A31 cells transformed with pCX-p23fc but not with pCX-p23 (Fig.3.2-2). When the proteins were visualized using anti-p23 serum and horseradish peroxidase-conjugated goat anti mouse IgG2a as a first- and second-antibody, a 23 kDa band was detected in pCX-p23 transformed cell extracts. In pCX-p23 transformed cell extracts, a 50 kDa band of the fusion protein was detected as shown in Fig. 3.2-3. The results indicate that the fusion protein p23fc contained the p23 derived domain as

well as the Fc region of mouse IgG.

The fusion protein expressed by pCX-p23fc transformed cells was detected by direct fluorescent assay after fixation but not detected without fixation (data not shown), suggesting that p23fc was not transferred onto the cell surface.

Induction of antibody production following DNA immunization: BALB/c mice were immunized with pCX-p23, pCX-p23fc, and pCX as a negative control. Twelve weeks after the first immunization, serum samples from immunized mice were assayed by ELISA to detect antibodies against p23. Both pCX-p23 and pCX-p23fc induced antibody production against p23 (Fig. 3.2-4). A statistically significant difference in the antibody levels was not observed between pCX-p23 or pCX-p23fc immunized mice.

Induction of the expression of IL-4 and IFN-γ: Splenocytes of mice immunized with pCX, pCX-p23 or pCX-p23fc were harvested, cultured, and the level of IL-4 and IFN-γ in splenocytes culture medium were compared by sandwich ELISA. As shown in Fig. 3.2-5, very low O.D. values were

observed in all samples, indicating that neither IL-4 nor IFN-γ production was induced by the administration of pCX-p23 or pCX-p23fc. However, when lymphocytes were stimulated with p23 antigen *in vitro*, lymphocytes of mice immunized with pCX-p23 and pCX-p23fc produced IFN-γ (Fig. 3.2-5). The IFN-γ level expressed by the lymphocytes of pCX-p23fc immunized mice was much higher than that of pCX-p23 immunized mice. Compared with the splenocytes of mice immunized with negative control plasmid pCX, those of mice immunized with pCX-p23 or pCX-p23fc produced a slightly higher level of IL-4. A statistically significant difference in IL-4 productions levels was not observed between the lymphocyte samples of pCX-p23 or pCX-p23fc immunized mice.

## 5. DISCUSSION

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 vector pCX-p23fc which expressed the fusion protein of p23 with the Fc portion of mouse IgG1. The plasmid vector pCX-p23 expressing normal p23 was also constructed as a control. The inoculation of pCX-p23fc or pCX-p23 into Balb/c mice induced about the same level of anti-p23 IgG. The antigen specific IL-4 production was stimulated by pCX-p23fc better than by pCX-p23, but only by a small margin. On the other hand, production of IFN-γ was far more efficiently induced by inoculation with pCX-p23fc than with pCX-p23 (Fig. 3.2-4).

It has been reported that neonatal C57BL/6 mice are able to clear the *C. parvum* within 3 weeks after infection, whereas C57BL/6 IFN-γ knock out mice, depending on age, die rapidly (Lacroix et al., 2001). It is also known 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 data indicate the importance of IFN-γ in protection against infection and shedding of *C. parvum.* The fusion protein p23fc constructed in this study induced antigen specific IFN-γ expression more efficiently and therefore is more suitable as a vaccine against cryptosporidiosis than normal p23.

It is known that antigen presenting cells (APCs) express receptors for the Fc region of IgG (FcRγs) and that FcRγs mediate endocytosis of antigen-IgG complex, MHC-restricted antigen presentation, and activation of APCs (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 may be because p23 with the Fc region can activate APCs and be taken up by APCs more efficiently than p23 without the Fc region.

## 6. LEGENDS of FIGURE

Fig. 3.2-1 (A) Schema of the construction of the plasmid p23fc. Gray and black bars indicate the gene coding the p23 derived domain and the Fc portion, respectively. (B) Construction of IgG and the gene coding the fusion protein. Upper panel is the construction of IgG. Lower panel is the construction of the genes coding the p23 derived domain and the Fc portion of IgG. Gray Box and black arrow indicate the p23 derived domain.

Fig. 3.2-2 Expression of a fusion protein containing the IgG Fc portion by the plasmid pCX-p23fc. A31 cells transformed with pCX-p23 (left lane) and pCX-p23fc (right lane) were analyzed by Western blotting analysis using horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody.

Fig. 3.2-3 Detection of the fusion protein by anti-p23 serum. CPK cells transformed with pCX-p23fc were analyzed by Western blotting analysis using horseradish peroxidase-conjugated rabbit anti-mouse IgG1 or IgG2a, with or without prior treatment with anti-p23 serum.

Fig. 3.3-4 Production of antibodies against p23 in Balb/c mice immunized with DNA vaccines. Antibodies against p23 in sera of mice immunized with pCX, pCX-p23fc or pCX-p23 were detected by ELISA. Solid, striped and open bars indicate O.D. values of sera harvested from mice immunized with pCX, pCX-p23fc and pCX-p23, respectively.

Fig. 3.2-5 Detection of the expression of IFN-γ and IL-4. Splenocytes harvested from mice immunized with pCX, pCX-p23fc and pCX-p23 were cultured in the absence (left columns indicated by 'w/o') or presence (right columns indicated by 'p23') of *in vivo* stimulation by adding p23 antigen. Solid, striped and open bars indicate O.D. values of splenocytes harvested from mice immunized with pCX, pCX-p23fc and pCX-p23, respectively.

Fig. 3.1-1

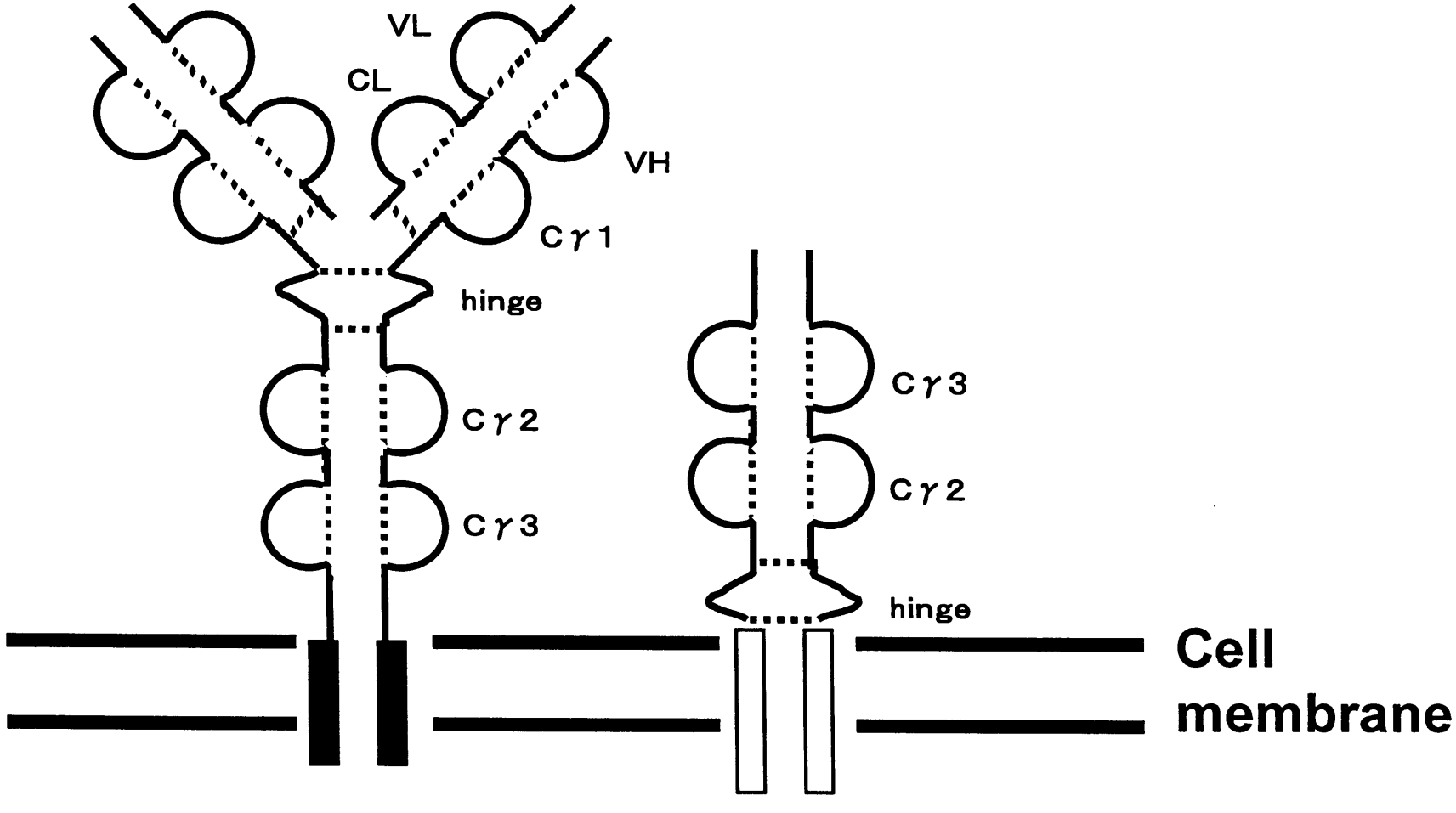


Fig. 3.1-2

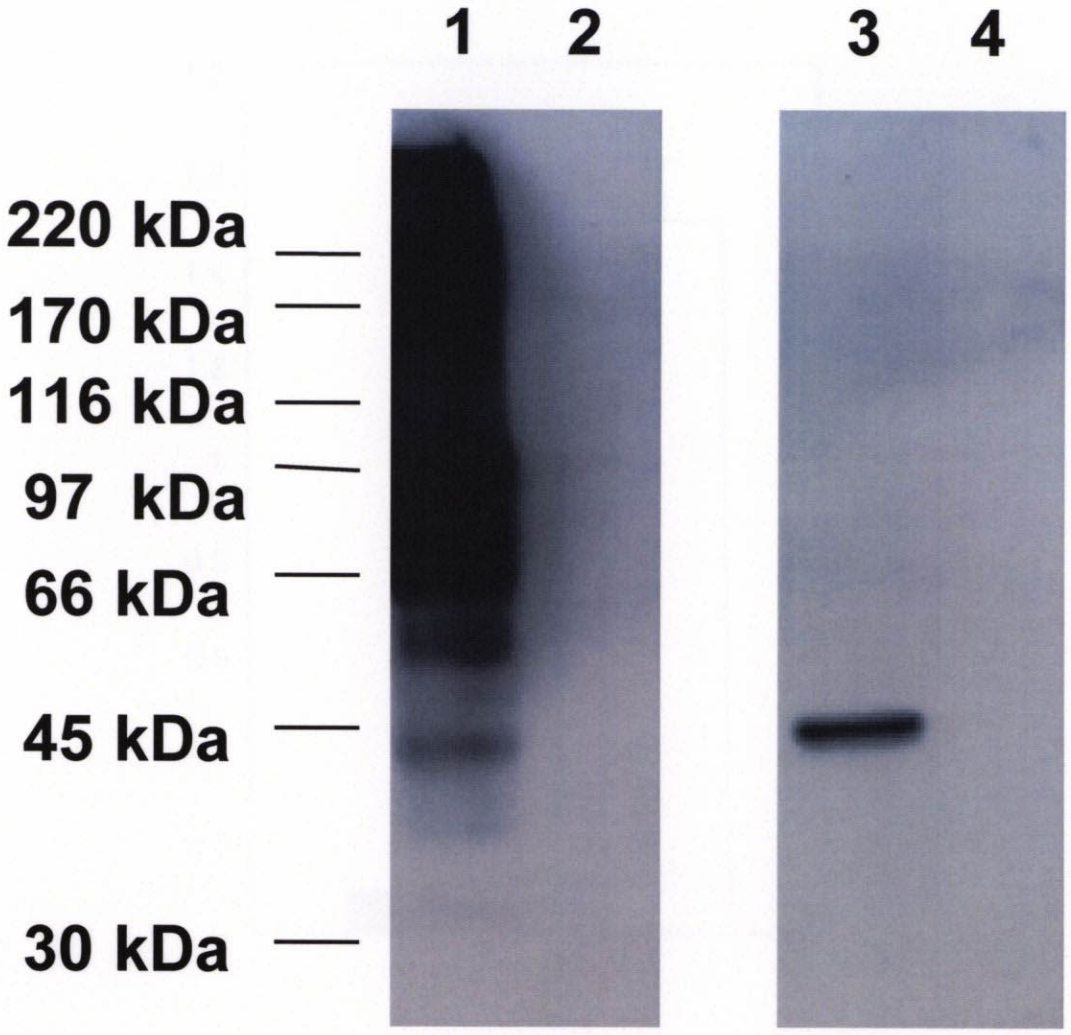


Fig. 3.1-3

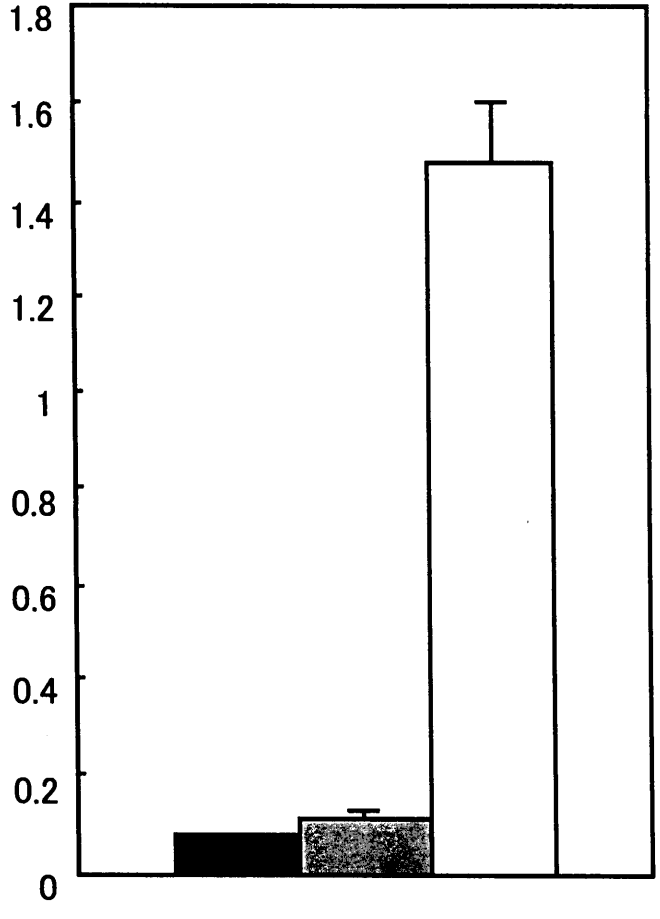


Fig. 3.1-4

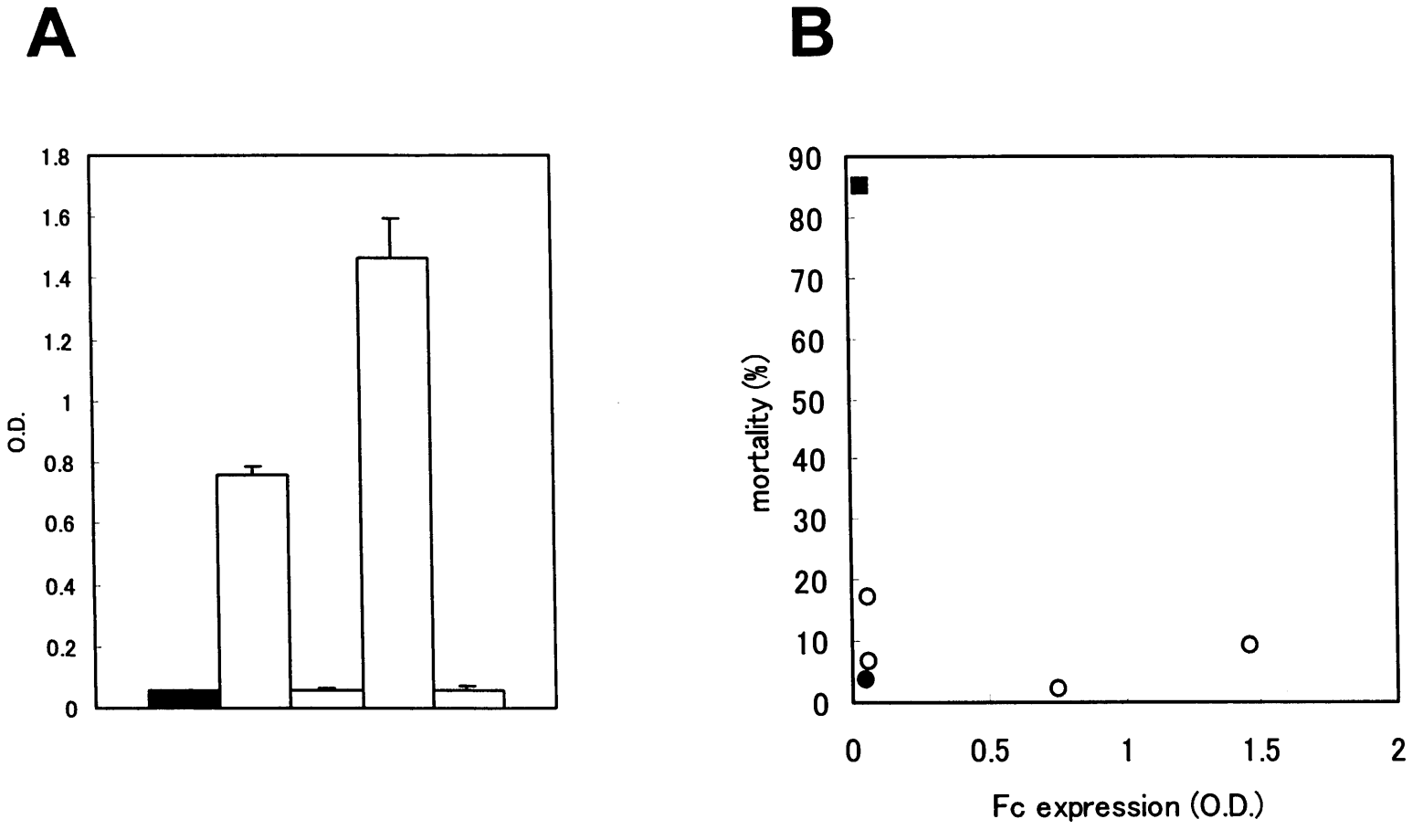

A
B
O.D.
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
mortality (%)
90
80
70
60
50
40
30
20
10
0
0
0.5
1
1.5
2
Fc expression (O.D.)

Fig. 3.1-5

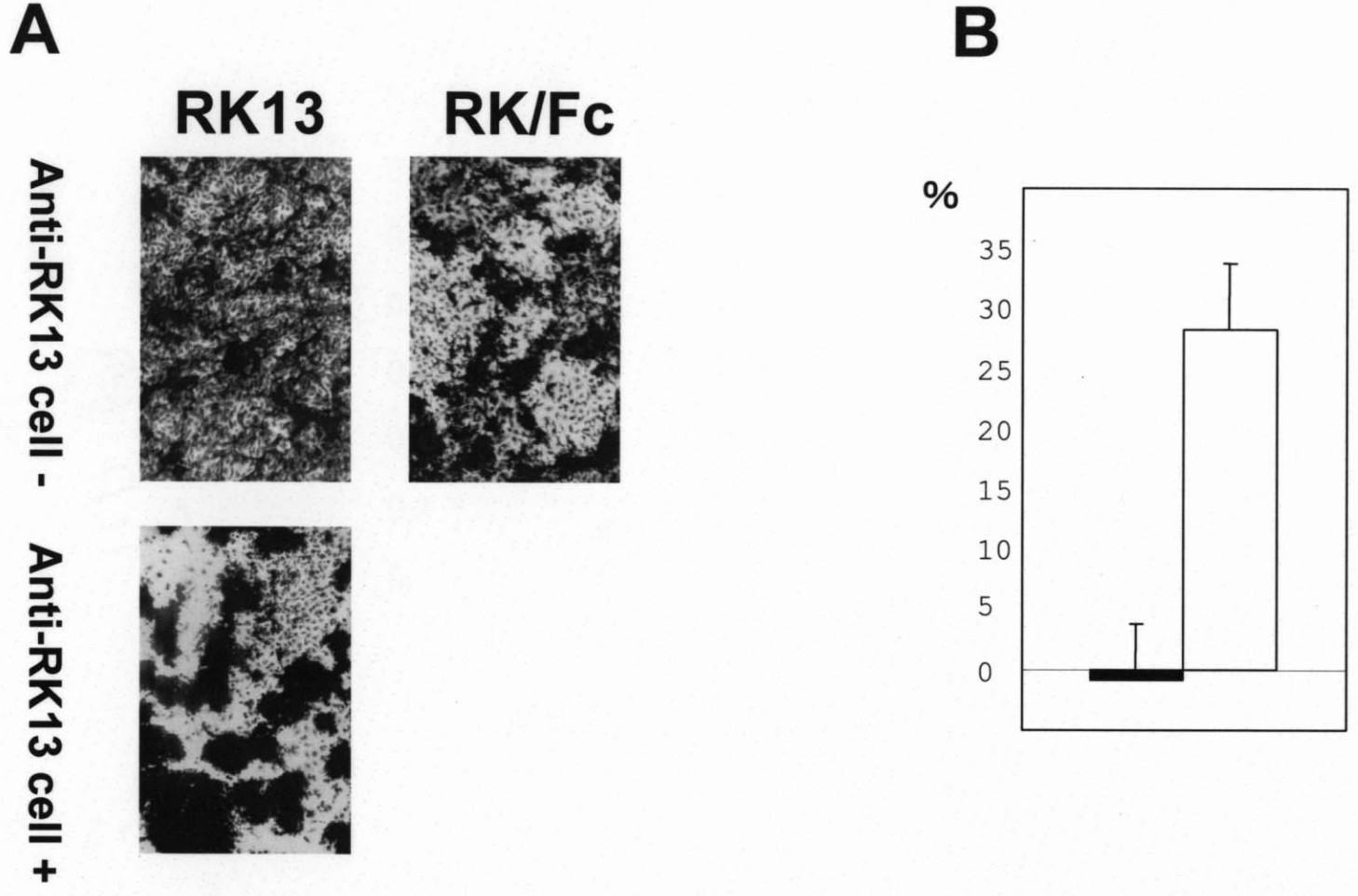


Fig. 3.1-6

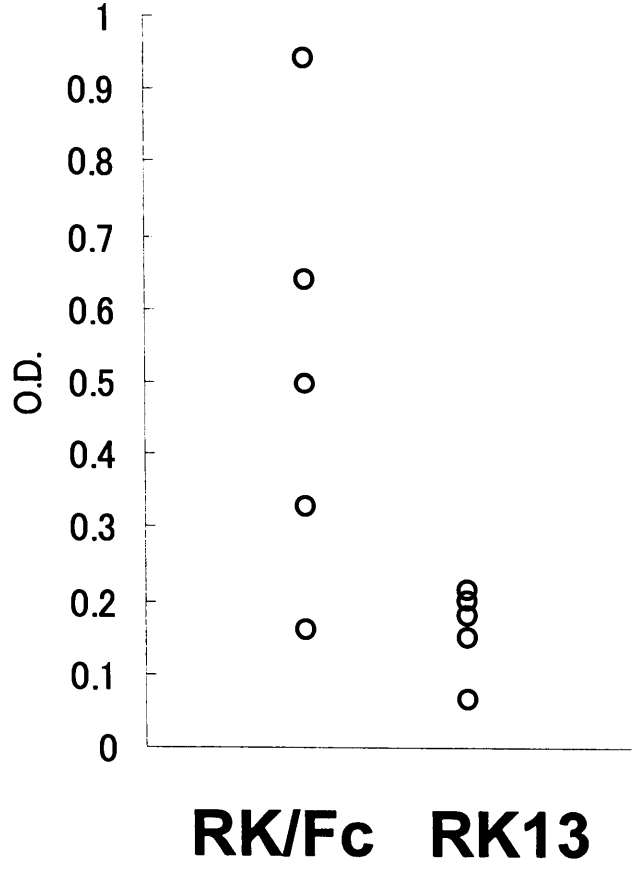


Fig. 3.1-7

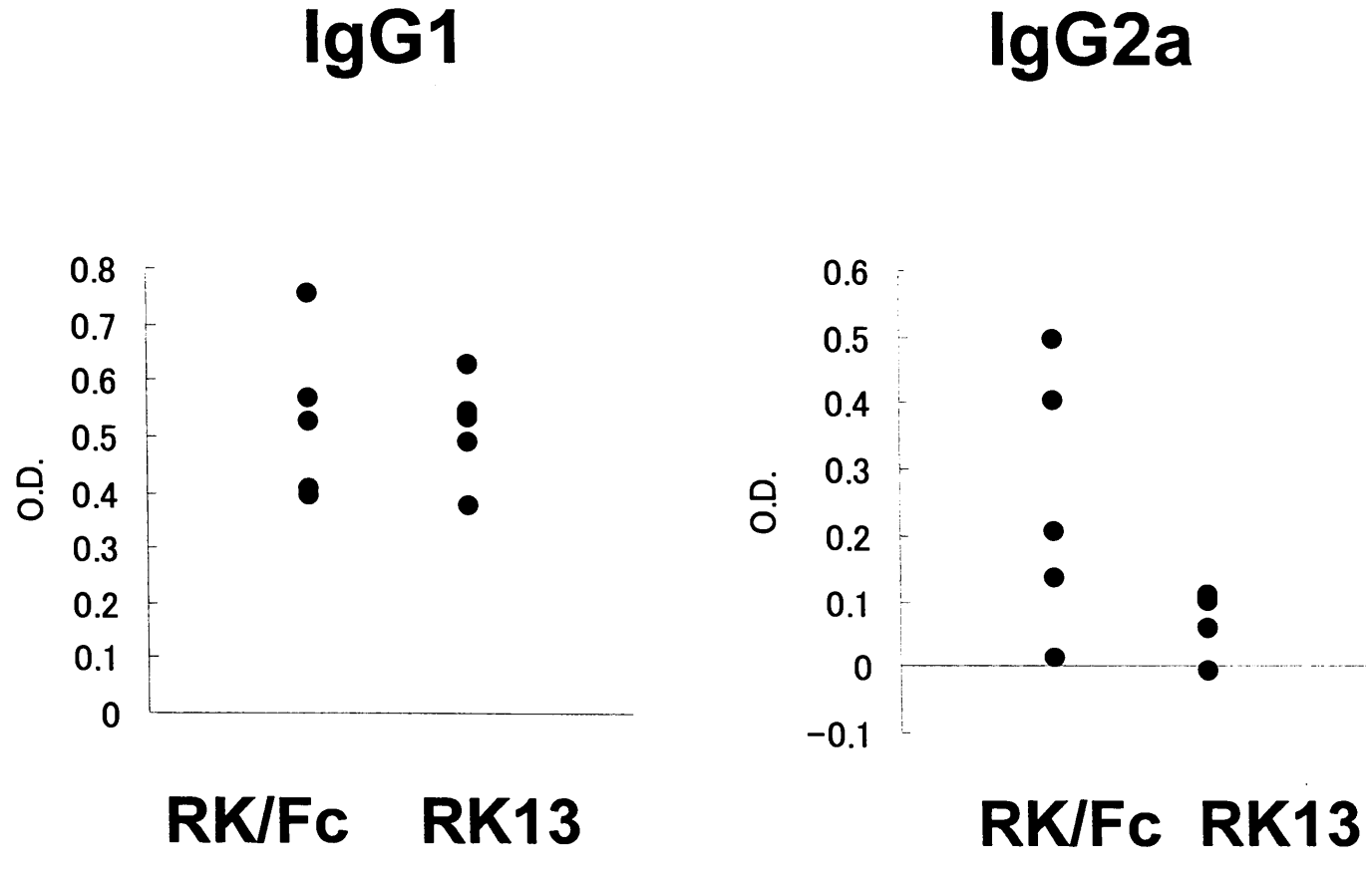
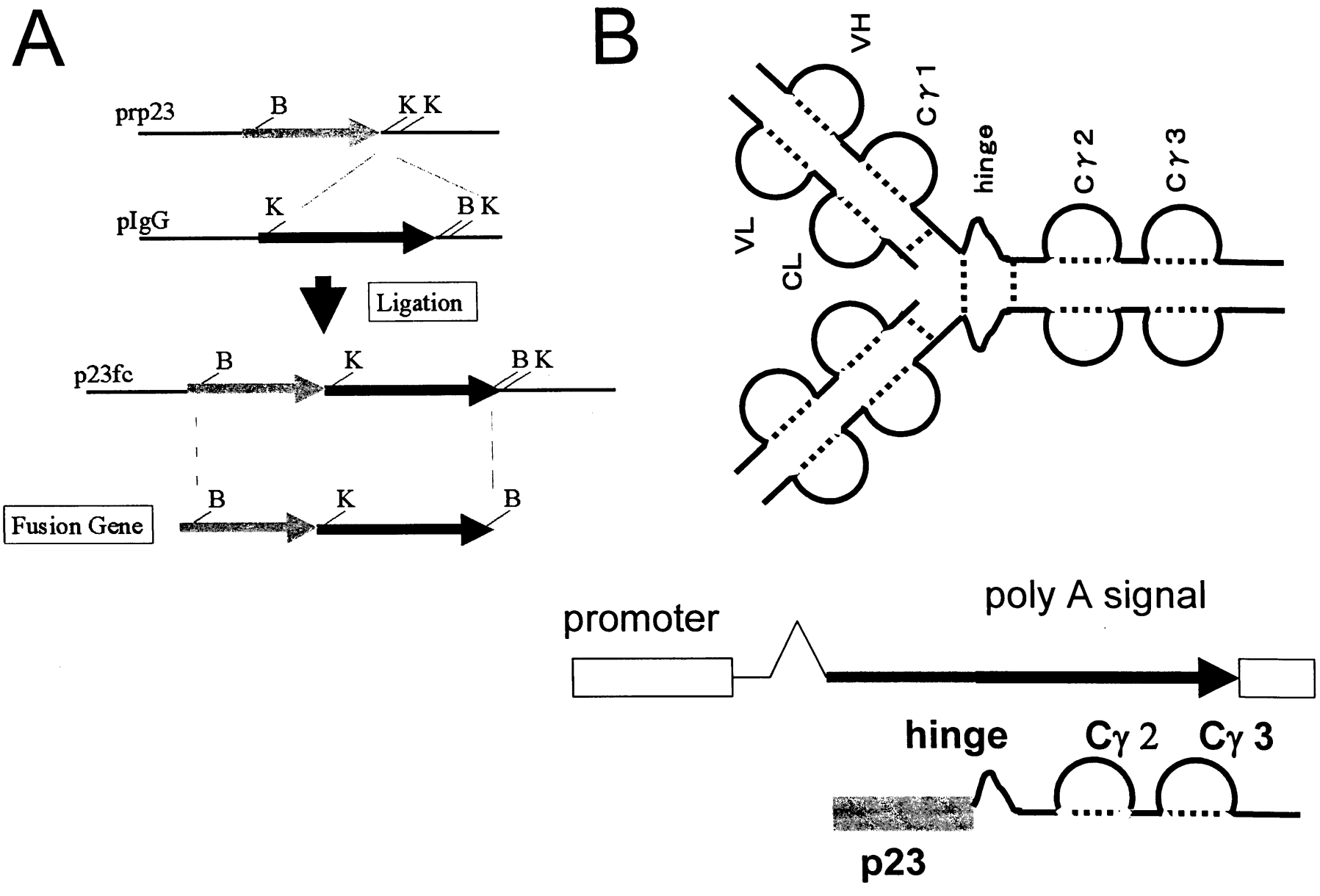


Fig. 3.2-1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prp23
B
K K
pIgG
K
B K
Ligation
p23fc
B
K
B K
Fusion Gene
B
K
B
B
VH
Cγ1
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Cγ2
Cγ3
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CL
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poly A signal
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Cγ 2
Cγ 3
p23

Fig. 3.2-2

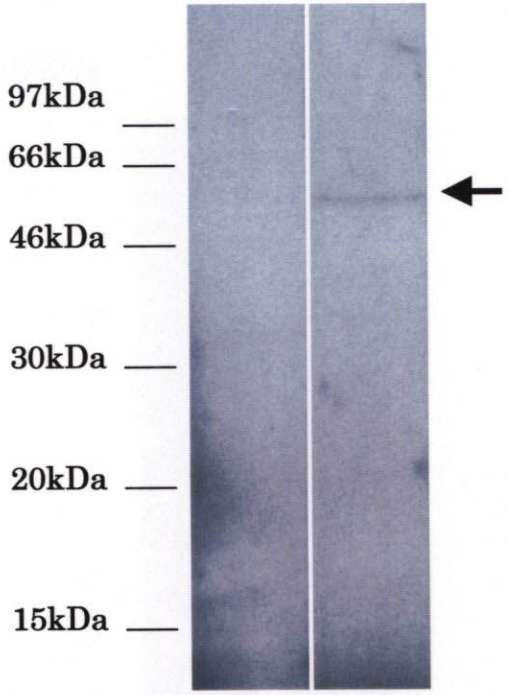


Fig. 3.2-3

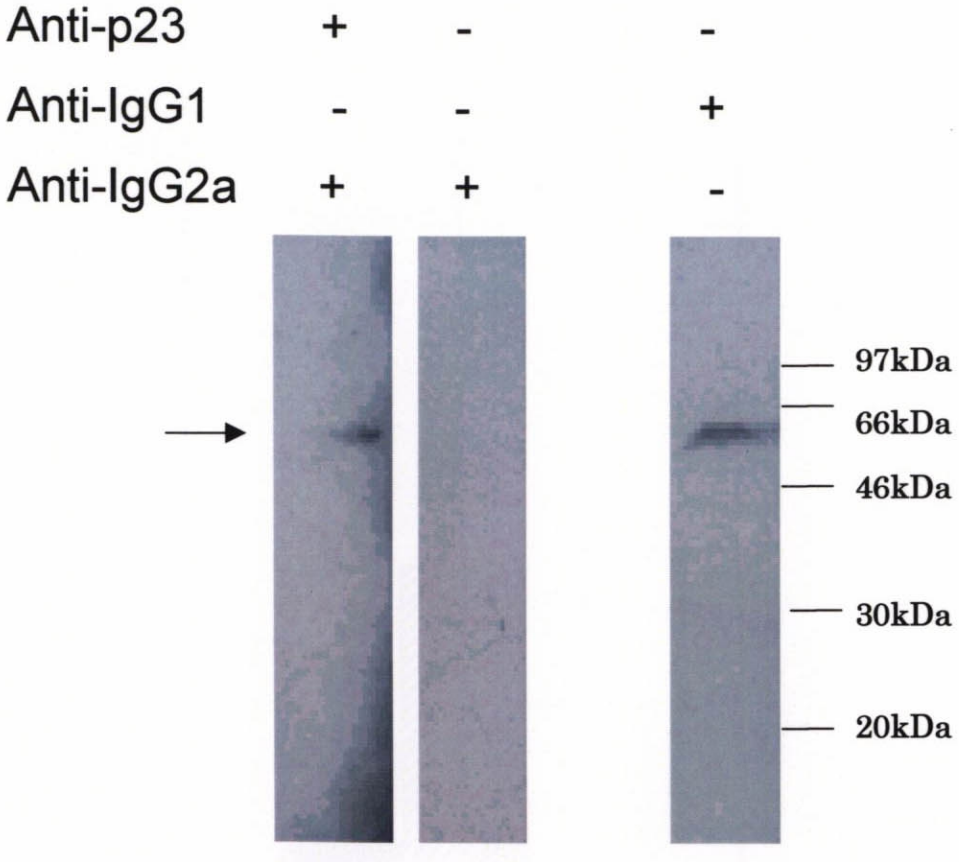


Fig. 3.2-4

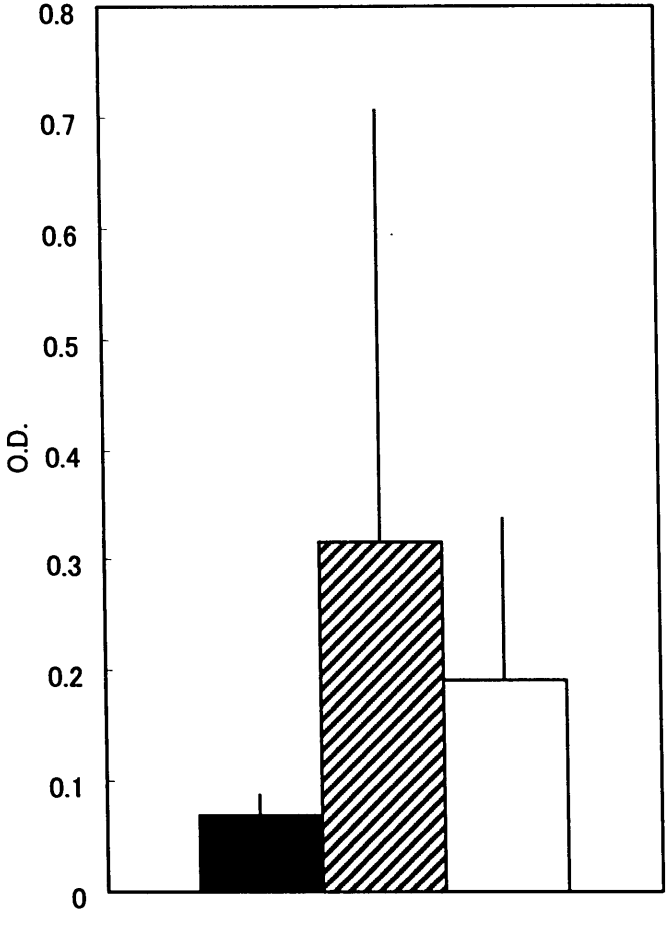
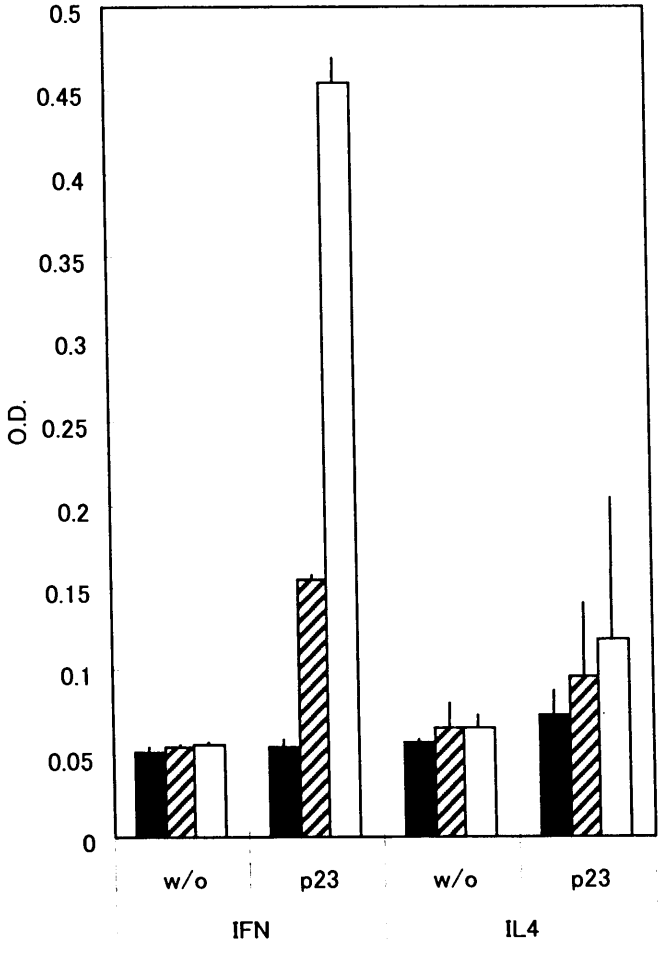


Fig. 3.2-5



## General summary

*Cryptosporidium parvum* (*C. parvum*) is a recognized protozoan parasite of livestock, companion animals and human (Tzipori, 1983; Fayer et al., 1990). It is now one of the most commonly found enteropathogens causing diarrheal illness in humans worldwide. The aim of this study is development of vaccine for cattle against *C. parvum* infection using a DNA vaccines and recombinant virus vectors.

In chapter 1, the author constructed the recombinant vaccinia virus expressing an immunodominant surface protein p23 of *C. parvum* sporozoites and constructed a recombinant vaccina virus expressing p23. By the immunization of the recombinant vaccinia virus, in BALB/c mice, the production of antibodies against p23 was induced. In the case of C57BL mice, the production of antibodies was not observed. However, the delayed type hyper sensitivity reaction against p23 was detected. The difference of immune reaction seemed to be dependent on the strain of mice.

In chapter 2, the author constructed a bovine herpesvirus-1 (BHV-1)

recombinant expressing p23, and investigated the its properties as a vaccine candidate. In addition, to improve herpesvirus vectors for animal, the author investigated some properties of BHV-1 and pseudorabies virus (PRV).

In chapter 2.1, the author constructed BHV-1 recombinants lacking US3 gene. The product of the US3 gene of BHV-1is homologous to the herpes simplex virus type 1 (HSV-1) US3 protein kinase (PK) gene. Recent reports indicating that the US3 gene of HSV-1 is involved in the blockage of apoptosis in virus infected cells. However, in the case of BHV-1, the product of US3 gene had no effect on the blockage of apoptosis induced by the osmotic shock. These results suggest that US3 gene and genes, of which expression are affected by US3 gene product, are the candidates for the foreign gene insertion sites.

In the chapter 2.2, to develop a vaccine against cryptosporidiosis in animals, the author constructed herpesvirus recombinant expressing p23. Among some herpesviruses, the construction of PRV recombinant is technically easy than other members of herpesviruses. Therefore, as the first step, the author constructed a PRV recombinant expressing p23. The recombinant expressed the approximate 23kDa protein. This study showed

the possibility of recombinant herpesvirus as a vaccine against cryptosporidiosis in animals.

PRV can infect experimentally small laboratory animals. In contrast, BHV-1 has narrow host range; it does not grow in mice at all. In the chapter 2.3, the author constructed BHV-1 recombinants expressing PRV glycoproteins B and C (gB and gC), and investigate the infectivity of the recombinants on mice. The recombinant BHV-1 expressing the PRV gB and gC infected mouse tissue cells more efficiently than the parental BHV-1 does. The results indicate the possibility of the recombinant BHV-1 as a experimental tool, which can use for mouse model.

In chapter 2.4, the author constructed BHV-1 recombinant expressing p23 as a vaccine candidate against cryptosporidiosis in cattle. The p23 gene was inserted in glycoprotein G (gG) gene locus of BHV-1 genome, with a marker gene coding green fluorescent protein. Despite a low frequency of homologous recombination, cloning of the recombinants was easy because of the specific fluorescence of the plaques formed by recombinants. In the case of PRV, a member of alphaherpesvirus, it was reported that US3 protein is reduced by insertion of foreign genes at the gG locus. However, as shown in

the chapter 2.1, the BHV-1 gene, of which expression is affected by US3 product, can be an foreign gene insertion site. Therefore p23 gene was inserted into gG gene locus. The recombinant BHV-1 induces antibodies against p23, inhibiting *C. parvum* infection. The recombinant BHV-1 has a great possibility as a vaccine against cryptosporidiosis for cattle.

As shown in the chapter 2, herpesvirus recombinants expressing foreign genes have a possibility as a vaccine. However, at the same time, herpesvirus infection cause the immune suppression. In the chapter 2.5, the effect of PRV on the immune system was examined in the mouse model. When lymphocytes were incubated with PRV, the ability of cells to proliferate was inhibited, although PRV did not multiply in the lymphocytes. UV-inactivated PRV also suppressd the proliferation of mouse splenocyte. This result suggests that the structural component of PRV virion might cause the immunosuppression.

In the chapter 3, the author cloned mouse cDNA coding Fc region of immunogloblin G1, and investigated the adjuvant effect of its product. In the chapter 3.1, the author established RK13 cell expressing a chimeric antibody-like molecule, containing Fc region of mouse IgG1 in such an

orientation that Fc portion projected away from the cell surface. The cell line was damaged by mouse macrophages much more than parental RK/13 cells, indicating that the Fc region can be recognized by Fc receptors of macrophages. The immunization of BALB/c mice with RK/Fc cells induced much greater level of anti-RK cell antibody, comparing the immunization with parental RK13 cells. These data suggested that Fc region of the chimeric protein have adjuvant effects.

In the chapter 3.2, the author constructed a plasmid coding an immunodominant surface protein p23 (pCX-p23) another plasmid coding a fusion protein containing whole p23 and the Fc portion of mouse IgG1 (pCX-p23fc). Vaccination of BALB/c mice with these plasmids induced the production of antibodies against p23. The IFN-γ level expressed by the lymphocytes of pCX-p23fc immunized mice was much higher than that of pCX-p23 immunized mice. The results suggested that Fc portion can be used as an adjuvant for vaccines against cryptosporidiosis.

In this study, the author constructed several vaccine candidates against cryptosporidiosis, and investigated their effect as a vaccine. As shown in this thesis, herpesvirus recombinant expressing *C. parvum* antigen(s) or

modified antigen with Fc portion has a great possibility as a vaccine.

関連論文リスト

1．発表済

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2．印刷中

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