EFFICIENT INTRANASAL IMMUNIZATION OF NEWBORN MICE WITH RECOMBINANT ADENOVIRUS EXPRESSING ROTAVIRUS PROTEIN VP4 AGAINST ORAL ROTAVIRUS INFECTION

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Summary. – Efficacy of passive protection of newborn mice against rotavirus infection by the rotavirus VP4 protein expressed by an adenoviral vector in mice was studied. The VP4 gene was inserted into the E1 region of adenoviral vector pJM17. Recombinant adenovirus Ad5/VP4 was grown in 293 cells. Intramuscular (i.m.), oral or intranasal (i.n.) immunization of newborn mice with Ad5/VP4 resulted in appearance of VP4-specific antibodies. Specific IgG antibodies were detected in the serum and intestine specimens of i.m. vaccinated mice. Oral immunization elicited serum IgG antibodies and intestinal IgG and IgA antibodies. Compared with i.m. and oral applications, i.n. immunization led to higher levels of serum IgG and intestinal IgG and IgA antibodies. Pups were challenged twice with simian rotavirus SA11 strain orally at the days 7 and 8 after birth. Pups born to i.n. immunized dams achieved 100% protection from rotavirus-induced diarrhea after both challenges. The protection of pups born to orally immunized dams was 80%, while only 30% of pups born to i.m. immunized dams were protected after both challenges. I.n. immunization was most efficient in inducing rotavirus VP4-specific serum, intestinal and milk IgG or IgA in mice that protected newborn mice completely.

Key words: rotavirus; antibodies; adenoviral vector; diarrhea; immunization; mice; protection; VP4 protein

Introduction

Rotaviruses are the major cause of severe diarrhea in infants and young children worldwide and can also cause acute viral gastroenteritis in young mammalian and avian species (Burns *et al*., 1996). VP4 is an 88 K non-glycosylated protein involved in the attachment and penetration of the virus into the host cell (Mason *et al*., 1980; Enouf *et al*., 2003; Arias *et al.*, 2002). In the infected cells, VP4 is synthesized in the cytoplasm, lacking signal sequence and posttranslational modifications (Estes *et al*., 1996). VP4 or its subfragment elicits efficient immune response that protects animals actively or passively (Mackow *et al*., 1990; Ijaz *et al*., 1998; Gil *et al*., 2000). Recombinant adenoviruses expressing rotavirus VP7 protein induce in newborn mice specific systemic antibodies and protected them passively from homologous infection (Both *et al.*, 1993). The immunogenicity of VP4 produced by a recombinant adenovirus had not been reported earlier. Based on these results, we constructed a recombinant adenovirus that expressed a complete rotavirus VP4 protein and determined its passive protection efficacy in newborn mice.

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Abbreviations: Ad5 = adenovirus type 5, referred to the recombinant adenoviral vector pJM17 in the manuscript; Ad5/VP4 = recombinant adenovirus expressing rotavirus VP4; DAB = diaminobenzidene; ICGEB = International Center for Genetic Engineering and Biotechnology; IF = immunofluorescence; i.m. = intramuscularly; i.n. = intranasally; OPD = *o*-phenylenediamine; p.i. = post infection; p.imm. = post immunization; RT = room temperature

Materials and Methods

Virus and cells. Simian rotavirus strain SA11 (obtained from International Center for Genetic Engineering and Biotechnology (ICGEB), Italy) was grown in fetal monkey kidney MA104 cells. These cells were grown in the Modified Eagle's Medium (MEM) supplemented with 10% of fetal bovine serum. The recombinant adenovirus was grown in human 293 cells in the Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

Construction of the recombinant adenovirus. Complete VP4 gene obtained by RT-PCR was inserted into the E1 region of the adenovirus type 5-based vector pJM17 by homologous recombination. Recombinant adenovirus Ad5/VP4 was obtained using a method described previously (Sun *et al.*, 2001). In this way the recombinant adenovirus Ad5/VP4 was obtained. Adenovirus type 5 DNA containing only a part of the E1 gene (Ad5) was used as control. After growing in 293 cells and purification by CsCl density gradient centrifugation, both viruses were dialyzed and stored at -80°C. VP4 expression in MA104 cells infected with either recombinant virus was assayed by immunofluorescence (IF) test and Western blot analysis.

IF test was carried out as follows. Twelve hrs post infection (p.i.), the cells were washed twice with PBS and fixed with 2% formaldehyde at room temperature (RT) for 30 mins, then with 0.1% Triton X-100 at RT for 10 mins. After blocking with 2% BSA, a guinea pig antiserum to SA11 VP4, produced using a recombinant baculovirus (Sun *et al.,* 1997) and diluted to 1:500, was added to cells for incubation at 37°C for 30 mins. A non-immune guinea pig serum was used as control. FITC-conjugated pig anti-guinea pig IgG (Sigma) was then added for incubation at 37°C for 2 hrs. The cells were washed between each step.

Western blot analysis of VP4 protein expressed from the recombinant adenovirus was performed as follows. Sixteen hrs p.i. the 293 cells were harvested, washed with ice-cold PBS pH 7.4 and incubated in 5% NP40 in 50 mmol/l Tris-HCl pH 8.0 and 0.1 mol/l NaCl for 20 mins on ice. The cells were sonicated for 3 mins and then centrifuged at 14,000 x g for 10 mins at 4°C. The supernatant and pellet were subjected to electrophoresis in discontinuous 10% polyacrylamide gel under reducing conditions (PAGE). The resolved viral proteins were electroblotted to a nitrocellulose membrane (Gelman). The blot was incubated at 37°C for 40 mins in 10 mmol/l Tris-HCl pH 7.5 with 150 mmol/l NaCl and 3% bovine serumalbumin (BSA) and then with a guinea pig antiserum to a baculovirus-expressed SA11 VP4 (Sun *et al.,* 1997) for 2 hrs at RT. The bound antibody was detected by incubation with a rabbit anti-guinea pig IgG peroxidase conjugate (Sigma). After washing, development of the blot was performed with DAB (Sigma).

Immunization and challenge. Before immunization, 4 week-old female Balb/C mice were bled and found to be negative for pre-existing rotavirus antibodies by ELISA. Six groups of mice (6 mice per group) were immunized i.n., i.m. and orally with Ad5/VP4 and Ad5, respectively with a dose of 2.8 x 10^8 PFU (day 0) and boosted twice with the same inoculum on days 28 and 42 post immunization (p.imm.). At the weeks 2, 4, 6, 7 and 8 p.im. (first), blood and intestine specimens were collected and assayed for rotavirus antibodies. Naive mice were mated. Three days later, the female mice divided into 6 groups (2 mice per group) were immunized with 5.6 x 10^8 PFU of Ad5/VP4 or Ad5 i.n., i.m. and orally. Another control consisted of mock-infected mice. On the days 7 and 8 after birth, pups were challenged with 10^6 PFU of rotavirus SA11 strain. At the end of the assessment period, blood and intestine specimens were collected from dams to assay the antibodies. The pups were sacrificed and the milk plugs were removed from the pups' stomach for the antibody assay. Two pups from dams immunized i.n. with Ad5 were foster-nursed by the dams immunized i.n. with Ad5/VP4 and other 2 pups from dams immunized i.n. with Ad5/VP4 were foster-nursed by the dams immunized with Ad5 i.n. after birth. They were marked to observe diarrhea incidence.

Preparation of samples for ELISA of VP4 antibodies. Immediately after collection, stool specimens (approx. 2 mg) were suspended in ice-cold PBS (10 ml), incubated for 30 mins at 4°C and centrifuged at 8,000 x g for 10 mins at 4°C. A hundred µl of the supernatant (final stool concentration 0.2 mg/ml) was assayed immediately for VP4-specific IgA antibodies by ELISA. (Xiang *et al.,* 1999). The mice were sacrificed and the contents of the small intestine was flushed with 5 ml of PBS and centrifuged at 1,500 x g for 30 mins at 4°C. The supernatant was diluted 1:4 with PBS made in aliquots (200 µl each) and diluted three fold in PBS (starting dilution of 1:4) to detect VP4 specific IgG and IgA. Milk plugs removed from the sucking mice were mixed with PBS according to the plugs' weight (100 µl/0.05 g). After centrifugation, the supernatant was diluted 3-fold with PBS and subjected to ELISA of VP4-specific antibodies.

ELISA of VP4 antibodies. Simian rotavirus type 5 SA11 strain VP4 expressed by *E. coli* was employed as antigen. It was dissolved in 8 mol/l urea and purified by ion-exchange chromatography. Briefly, fast flow DEAE-Sepharose (Pharmacia) was balanced with 8 mol/l urea in PBS and VP4 was eluted with a solution containing 8 mol/l urea and 0.6 mol/l NaCl in PBS using the AKTA-purifier Box-900. After identification by Western blot analysis, VP4 was diluted in 50 mmol/l carbonate bicarbonate buffer pH 9.6 to a concentration of 157 µg/ml and used to coat polyvinyl 96-well microtiter plates (Titertek). The plates were incubated overnight at 4°C, blocked with 3% BSA in PBS for 40 mins at 37°C, and washed three times with 0.05% Tween-20 (Sigma) in PBS in a Thermo Microplate Washer. Sera diluted three fold serially in PBS (starting dilution of 1:4) were added to the plates and incubated at 37°C for 2 hrs. After washing the plates four times, a horseradish peroxidase-conjugated anti-mouse IgG or IgA (Sigma) was added. The plates were incubated at 37°C for 1 hr, washed and *o*-phenylenediamine (OPD) in H_2O_2 was added for 15 mins in the dark. The reaction was stopped with 2 M H_2SO_4 and A_{490} was read. The analysis was run in duplicate and the average A_{490} value of blank wells was subtracted from that of the test wells. A sample was considered positive for $A_{490} \geq 2.1$-fold of negative (mock-infected) control samples at the same dilution and ≥ 0.2. Except for the antibody titers in fecal specimens, IgG and IgA antibody titers in serum, intestine and milk plug specimens were calculated as geometric means. For this purpose, individual highest titers were employed. For fecal specimens, the IgA antibody titer of each group was calculated using the mean A_{490}.

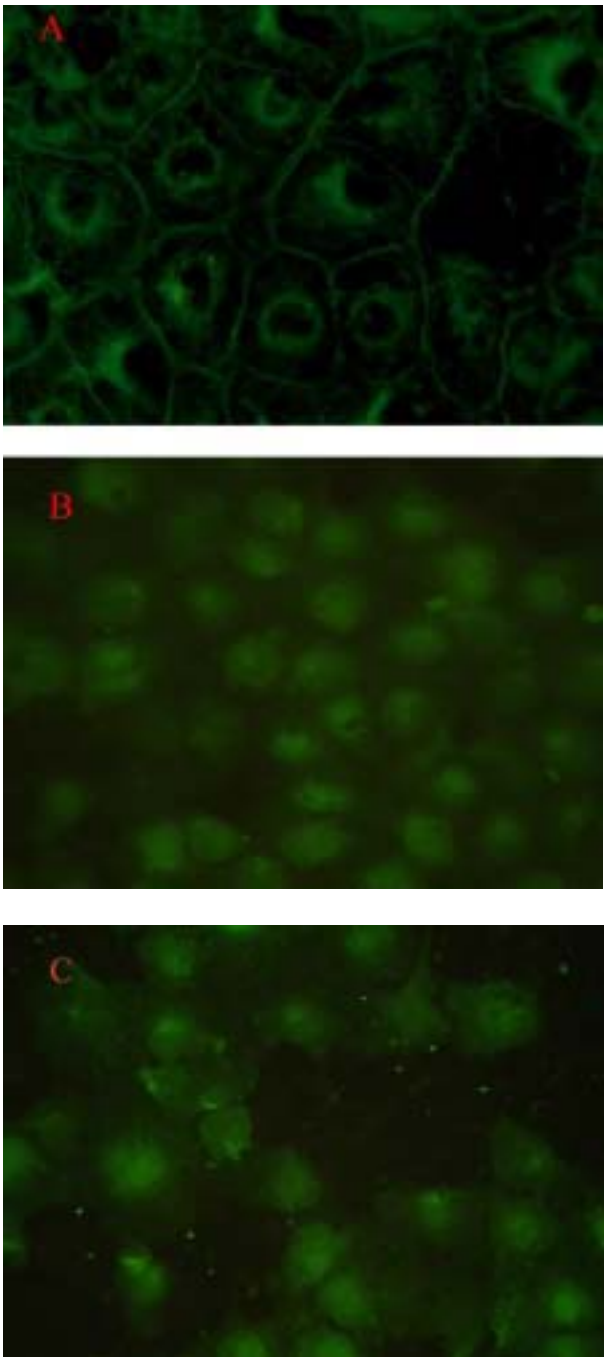

Fig. 1

VP4 protein expression in MA104 cells

IF test. Infection with Ad5/VP4 (A). Infection with Ad5, negative control (B). Infection with Ad5/VP4, detection with non-immune guinea pig serum. (C). Amplification 500x.

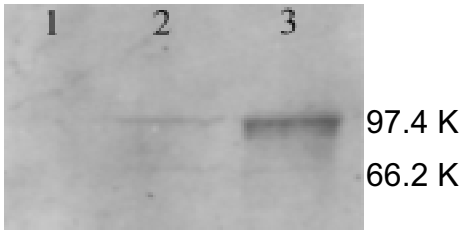

Fig. 2

VP4 protein expression in 293 cells

Western blot analysis. Infection with Ad5, negative control (lane 1). Infection with Ad5/VP4, the cell lysate supernatant (lane 2). Infection with Ad5/VP4, the cell lysate pellet (lane 3).

Statistical analysis. Data were analyzed using the SPSS 11.5 software. Significance of differences in ELISA A_{490} values of antibodies between groups of mice was determined by independent sample *t*-test. A difference was considered significant for $P \leq 0.05$.

Results

Expression of VP4 in Ad5/VP4-infected cells

To examine the expression of VP4 protein, IF test and Western blot analysis were performed. Specific fluorescence was observed in the cytoplasm and plasma membrane of about 90% of Ad5/VP4-infected cells but not in the Ad5-infected cells or Ad5/VP4-infected cells stained with a non-immune guinea pig serum (Fig. 1). Western blot analysis confirmed the expression of VP4 protein in Ad5/VP4-infected cells (Fig. 2).

Systemic and intestinal antibody responses in immunized mice

In general, immunization of newborn mice with Ad5/VP4 by all three routes elicited VP4 specific serum and intestinal antibodies. Specific serum IgA antibodies could not be detected. The Ad5/VP4-immunized mice had significantly much higher serum and intestinal antibody titers than the Ad5-immunized mice for all three immunization routes. Of these, the i.n. route was most efficient in eliciting serum and intestinal immunity. I.n. and oral but not i.m. immunization with Ad5/VP4 induced fecal and intestinal IgA antibodies (Fig. 3, Tables 1 and 2). In the i.n. immunized group, the highest titer of serum IgG antibodies was at the week 7 p.im., reaching about 15,000 (Fig. 4). The highest titers of intestinal antibodies were detected at the week 8 p.im. These antibodies were predominantly of IgG (the titer of 74.8) and less of IgA isotype (the titer of 36) (Table 1). In the i.m. and oral groups, the

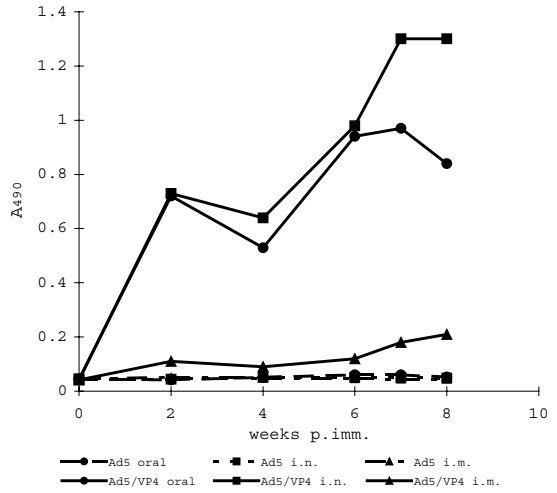

Fig. 3

IgA antibodies in the stool of mice following i.n., i.m. and oral immunization with Ad5/VP4 and Ad5

Ordinate: A_{490} values determined by ELISA.

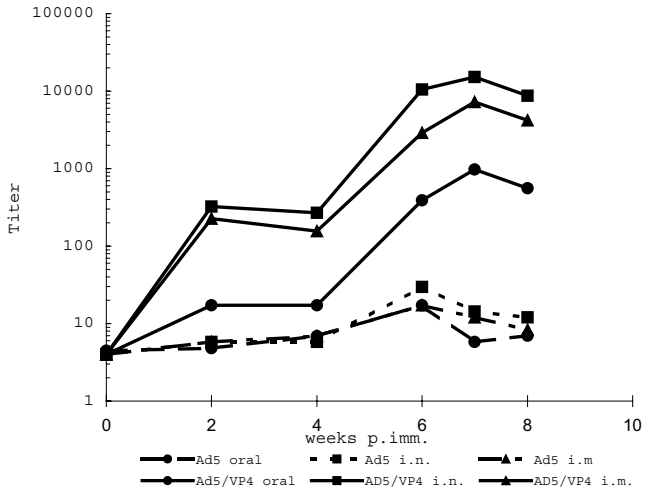

Fig. 4

Serum antibody titers in mice following immunization with Ad5/VP4 or Ad5 by i.n., i.m. and oral routes

Antibody titers determined by ELISA.

Table 1. Titers of intestinal rotavirus antibodies in mice at the 8th week p.imm. with Ad5/VP4 or Ad5

Route of immunization	Inoculated virus	No. of mice	Antibody titer	
			IgG	IgA
i.n.	Ad5	6	<4	<12
	Ad5/VP4	6	74.8	36
i.m.	Ad5	6	<4	<4
	Ad5/VP4	6	51.9	<4
Oral	Ad5	6	<4	<12
	Ad5/VP4	6	46	29.5

For details see Materials and Methods.

highest titers of serum IgG antibodies were observed at the week 7 p.im., reaching 7282 and 972, respectively (Fig. 4).

Protection of neonates against rotavirus infection transferred from dams immunized with Ad5/VP4

After Ad5/VP4 immunization, VP4-specific antibodies were detected in the serum, and stool of the dams and in the milk plug specimens from the pups (Table 2). The protective efficacy of the VP4 protein delivered by the recombinant adenovirus was studied using a protocol similar to that described earlier (Both *et al.,* 1993). Pups were delivered at various times. At days 7 to 8 after birth, the pups were challenged orally with the virus.

Table 2. Titers of rotavirus antibodies and diarrheal incidence for individual litters of mice after the 2nd challenge

Group	No. of mice in litter	Day p.imm.	No. of surviving mice/total mice	Serum IgG	Milk IgA[a]	Milk IgG[a]
Ad5 i.n.	10	42~45	0/10	<4	<4	<4
Ad5 i.m.	11	43~46	0/11	<12	<4	<4
Ad5 orally	9	43~46	0/9	<4	<4	<4
Ad5/VP4 i.n.	11	42~45	11/11	1572	48	340
Ad5/VP4 i.m.	10	42~47	3/10	906	<4	44
Ad5/VP4 orally	10	43~46	8/10	162	28	18

For details see Materials and Methods.
[a]Titers in pups' stomach milk plugs.

A complete protection was achieved in pups born to dams immunized with Ad5/VP4 i.n. and challenged twice (Table 2). Eight pups born to orally immunized dams were protected and two of them had diarrhea after the second challenge. I.m immunization led to partial protection of the pups; three of ten pups were protected after the second challenge. In contrast, all pups born to dams immunized with Ad5 had diarrhea after the virus challenges. Thus, the i.n. route was most efficient both in antibody induction and passive protection.

The two pups born to the dams immunized i.n. with Ad5 that were foster-nursed by dams immunized i.n. with Ad5/VP4 did not suffer from diarrhea after the challenge. In contrast, the two pups born to the dams immunized i.n. with Ad5/VP4 that were foster-nursed by the dams immunized i.n. with Ad5 developed diarrhea after the challenge.

Discussion

We evaluated the protecting efficacy of a recombinant adenovirus expressing complete rotavirus VP4 protein. According to our results the VP4 protein protected efficiently newborn mice against rotavirus-induced diarrhea. Previous studies have indicated that this protection does not depend on serum neutralizing antibodies (Choi *et al.,* 1998, 1999; Ward *et al.,* 1992) but is based on local intestinal antibodies (Gil *et al*., 2000; Feng *et al.,* 2002; Herrmann *et al.*, 1999). In our study, we detected in immunized mice both serum and intestinal rotavirus VP4-specific antibodies with titers correlating well with the protection efficacy. It has been assumed that IgA antibodies in maternal milk protect mainly against enteric infections (Van *et al*., 2003). As in the intestine and milk the IgG antibody titers were higher than the IgA antibody titers in conformity with data reported earlier (Gil *et al.*, 2000) and correlated with the serum IgG antibody titers, we deduce that IgG antibodies may neutralize the rotavirus infection in the stomach and intestines locally.

The demonstration of viral antigen in the Ad5/VP4-infected cells indicated the attachment of the VP4 protein to the cell plasma membrane after expression from the recombinant adenovirus. It has been speculated that a rotavirus VP7 protein modified for expression at the cell surface could have a better immunogenicity (Andrew *et al.*, 1990) and the VP4 protein, having been transported to the plasma membrane during rotavirus infection or transfection with plasmid DNA may have advantage over other rotavirus antigens in the immunogenicity (Nejmeddine *et al.,* 2000).

The route of immunization may influence the immunodominant neutralization responses elicited to rotavirus infection (Herrmann *et al.,* 1999; Giammarioli *et al.,* 1996). In our study, i.n., i.m. and oral routes of immunization with Ad5/VP4 affected both immunoreaction and protection. The immunization effects corresponded to the antibody titers. I.n. inoculation had the best immunization effect probably due to the induction of mucosal immunity (Xiang *et al.,* 1999). Oral inoculation induced lower antibody titers compared to i.n. application due to possible defense mediated by gastric acid and pepsin secretion (Chen *et al.,* 1993; Bass *et al.*, 1992). In our study, the mice boosted twice could have elicited adenovirus-specific antibodies in the serum and gut that rejected more immunization (Russell *et al*., 2000).

Since the rotavirus SA11 strain used in our study was a cell-cultured one, its two doses were applied to get a more virulent challenge. We exchanged pups born to immunized dams and naive dams right after the birth as described by Gil *et al.* (2000).

It has been reported that whereas the rotavirus VP7 protein inserted in adenovirus or vaccinia vectors did not protect adult mice from challenge, a gene gun injection of VP7 DNA vaccine did protect adult mice from challenge (Chen *et al.,* 1997). To obtain a satisfactory answer to the question if rotavirus VP4 expressed by a recombinant adenovirus can protect adult mice more work has to be done.

Vaccination with attenuated virus, protein subunits or virus like particles (VLPs) formed from protein subunits can protect animals partially or totally against homologous or heterologous rotavirus infection (Ijaz *et al*., 1991; Crawford *et al*., 1999; Kim *et al*., 2002; Corner *et al*., 1993). To design more effective and safe vaccines against infections with rotaviruses of different serotypes it is necessary to understand better the mechanisms of rotavirus infection and immunity against it.

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