Acta virologica **57**: 397 – 404, 2013 doi:10.4149/av_2013_04_397

Mice immunogenicity after vaccination by DNA vaccines containing individual genes of a new type of reovirus

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Received March 14, 2013; accepted September 24, 2013

Summary. – In this study, we investigated humoral and cellular immune responses in mice to DNA vaccines containing individual S or M genes of a new type of reovirus (nRV) isolate from a severe acute respiratory syndrome (SARS) patient in Beijing, China. Mice were immunized intramuscularly (i.m.) with 100 μg of S1, S2, S3, S4, M1, M2, and M3 DNA vaccine each 4 times in 2-week intervals and assayed for humoral IgG, IgG1, IgG2, and IgG2b antibodies by ELISA and for cellular immune response, particularly IFN-γ induction by ELISpot assay. Moreover, CD4+ and CD8+ T cell levels in peripheral blood mononuclear cells were assayed by flow cytometry. We found that all DNA vaccines induced IgG antibodies, predominantly of the IgG2a class and S3 DNA vaccine was the strongest inducer. M2 and S3 DNA vaccines elicited Th1- and Th2-based immune responses, respectively, while S1 and M3 DNA vaccines induced a mixed Th1/Th2 response. M1, S2, and S4 DNA vaccines were poorly immunogenic. To our knowledge, this is the first report characterizing mammalian reovirus DNA vaccines applied to a mouse model.

Keywords: reovirus; DNA vaccine; SARS; mouse; immunogenicity

Introduction

Mammalian reoviruses (respiratory enteric orphan viruses), members of the family *Reoviridae*, are non-enveloped, double-stranded (ds) RNA viruses with a genome composed of 10 genes. Although orthoreoviruses have been identified as the causative agents of diseases in animals, infections in humans are generally benign, resulting in rare cases of mild upper respiratory tract illness or enteritis in infants or children.

In reovirions, the dsRNA is surrounded by a double capsid comprised of an internal core that contains the viral transcriptase and an outer capsid. The outer capsid contains 3 polypeptides, μ1, σl, and σ3, which are encoded by genes of M2, S1, and S4, respectively (Weiner *et al*., 1978). The σl polypeptide is the viral hemagglutinin and has been linked to host cell attachment, type-specific neutralization, cytotoxic T cell recognition, tissue tropism, and pathogenesis of reovirus-mediated diseases (Finberg *et al*., 1979, 1982; Lee *et al*., 1981; Weiner *et al*., 1977). Viral nonstructural proteins μNS (encoded by M3) and σNS (encoded by S3), as well as core protein μ2 (encoded by M1) play key roles in forming viral inclusions and recruiting other viral proteins and RNA to these structures for replication and assembly (Becker *et al*., 2001, 2003; Broering *et al*., 2004; Mbisa *et al*., 2000; Parker *et al*., 2002). Tyler *et al*. identified the viral S1 and M2 genes as the major determinants of differences in the capacities of reovirus type 1 Lang (T1L) and type 3 Dearing (T3D) to induce apoptosis of L cells (Tyler *et al*., 1996). The segmented genome of the virus allows for the generation of intertypic reassortants, which have been exploited to assign biological functions to individual genes and their protein products.

A new type of reovirus was isolated from the first case of severe acute respiratory syndrome (SARS) in Beijing,

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Abbreviations: ELISpot = enzyme-linked immunosorbent spot assay; IFN-γ = interferon gamma; i.m. = intramuscularly; R4 = new type of reovirus; SARS = severe acute respiratory syndrome; SFC = spot-forming cells

China (He *et al.*, 2005, 2006; Song *et al.*, 2008). Based on electron microscopy and DNA sequence analysis, this virus, designated R4, represents a new type of reovirus that is most closely related to the mammalian orthoreovirus 2 isolate. Owing to the potential advantages of DNA vaccines (Chen *et al.*, 2003; Gurunathan *et al.*, 2000; Hung *et al.*, 2007; Osorio *et al.*, 1999; Rogers *et al.*, 1999; Saldarriaga *et al.*, 2006), in this study, we constructed DNA vaccines containing individual S or M genes of nRV and investigated their immunogenicity for mice. The results showed that (*i*) all of them induced specific humoral antibodies predominatly of IgG2a class, (*ii*) S3 DNA vaccine was the strongest inducer, (*iii*) M2 and S3 DNA vaccines elicited Th1- and Th2-based immune responses, respectively, while S1 and M3 DNA vaccines induced a mixed Th1/Th2 response, and (*iv*) M1, S2, and S4 DNA vaccines were poorly immunogenic. We hope that these results will be helpful in efforts to control possible reovirus-associated epidemics.

Table 1. Primers used to amplify individual reovirus genes by PCR

Gene Primer
S1 Forward 5′-AAGAATTCATGTCTGAGCTGATTCAGCTTA-3′ Reverse 5′-AAGAGCTCTCAGCCTAAGCATGGATACA-3′
S2 Forward 5′-AACTCGAGTCGCTGGTCAGTTATGGCTC-3′ Reverse 5′-GGTCTAGAGGATCCCCTCACTCCAAGACG-3′
S3 Forward 5′-AAAAGCTTCCTGTTGTCGTCACTATGGCTT-3′ Reverse 5′-GGTCTAGAAGCTCACTCACCCATTACACG-3′
S4 Forward 5′-AACTCGAGATGGAGGTGTGCTTACCCAATGG-3′ Reverse 5′-GGGATATCTTAGCCAAGAATCATCGGATCGC-3′
M1 Forward 5′-AAGGTACCTCATGGCTTACATCGCAGTTCC-3′ Reverse 5′-GGCTCGAGGTCACGGATCACGCCAAGTCAGA-3′
M2 Forward 5′-AACTCGAGATGGGGAACGCTTCCTCTATCG-3′ Reverse 5′-GGGATATCTTAACGTGTGTACCCACGTTTG-3′
M3 Forward 5′-AAGGTACCATGGCTTCATTCAAGGGATTCT-3′ Reverse 5′-GGCTCGAGCTATCACCTACAATTCATCAGT-3′

Materials and Methods

Construction of DNA vaccines. The reovirus, R4, was isolated from cells cultured from throat swabs of the first SARS patient identified in Beijing, China (He *et al.*, 2005, 2006; Song *et al.*, 2008). Viral RNA was extracted from 140 μl of cell culture lysate with a QIAamp Viral RNA Mini Kit (Qiagen, Germany) following the manufacturer′s instructions. The S1-S4 and M1-M3 genes were respectively amplified using RT-PCR. The identities of the genes were confirmed by sequence analysis (Table 1), and then the genes were inserted into pcDNA3.1+ (Invitrogen, USA). In the subsequent text, plasmids are designed according to their encoded genes. Restriction enzyme and sequence analysis indicated that all seven plasmids were correctly constructed. The plasmids were purified using the Qiagen Plasmid Mega Kit (Qiagen) according to the manufacturer′s protocol. DNA concentrations were determined using a SmartSpec™ Plus Spectrophotometer (Bio-Rad, USA).

Immunization of mice. Female BALB/c mice (6–8-week-old) were purchased from the Academy of Military Medical Sciences, China, and divided randomly into 9 groups. Mice in each group (n = 6) were immunized i.m with 100 μl (1 μg/μl) of one of the 7 DNA vaccines or controls. Mice were immunized 4 times at 2-week intervals. Control vector pcDNA3.1+ and PBS (Sigma) were used as negative controls.

ELISA of IgG antibodies. Sera were collected prior to each immunization. Anti-R4 antibody levels were measured by ELISA. Briefly, 96-well microtiter plates (Costar, USA) were coated with 100 $TCID_{50}$ R4 (cultured from L929 cell lines and centrifuged after freeze-thawing) and incubated at 4°C overnight. Plates were then blocked and incubated with diluted sera (1:10) for 2 hrs, followed by a 1 hr incubation with alkaline phosphatase-conjugated goat anti-mouse IgG (1:5,000; Sigma) at 37°C. Color reaction was determined at A450. Results from 3 independent experiments were expressed as means ± SD.

ELISA of IgG subclass antibodies. A similar ELISA protocol was followed to quantify the IgG subclasses (IgG1, IgG2a, and IgG2b) using ADI ELISA kits (Alpha Diagnostic International Inc., USA). One hundred μl of the samples, standards, and controls, were added to the wells and incubated for 1 hr. HRP-conjugated antibodies were then added to each well followed by washing and pat drying. After incubation with substrate, A450 was determined. Results from 3 independent experiments were expressed as means ± SD.

ELISpot assay of IFN-γ induction. Cellular immune responses to R4 were assessed using an IFN-γ ELISpot assay (Dakewe Biotech, China) according to the manufacturer′s instructions. Mouse splenocytes were harvested 10 days after the final immunization

and incubated in RPMI 1640 medium with 1×10^5 cells/well in triplicates. Specific peptides targeting seven different genes were designed (Table 2) and synthesized by Life Technologies Corporation (USA). These peptides (2 μg/ml) were used as the stimulating antigens. The plates were read with an ELISpot reader (Bioreader 4,000, Bio-sys, Germany). The number of spot-forming cells (SFC) per 10^5 splenocytes was calculated. Medium backgrounds were consistently lower than 10 SFC/10^5 splenocytes. Results from 3 independent experiments were expressed as means ± SD.

Flow cytometry of CD4+ and CD8+ T-cells. T cell surface markers were analyzed by flow cytometry. Briefly, 10 days following the final immunization, splenocytes were isolated and stained using the following monoclonal antibodies: allophycocyanin-labeled anti-mouse CD3, FITC-labeled anti-mouse CD4, peridinin chlorophyll protein-labeled anti-mouse CD8 (BD PharMingen™, USA), or the corresponding isotype controls. Flow cytometry was performed on 10^5 cells using a FACS Calibur flow cytometer (BD Biosciences), and data were analyzed using FlowJo version 7.6 software. Results from 3 independent experiments were expressed as means ± SD.

Statistical analysis. We performed statistical analyses using SPSS software (version 12.0; SPSS Corp., USA). Quantitative variables were tested for normality of distribution. Normal quantitative variables such as mean and SD were calculated and analyzed by parametric tests. The median and interquartile ranges were also calculated and analyzed by non-parametric tests. The figures were generated using Microsoft Excel software. Differences with P-values ≤0.05 were considered statistically significant.

Results

Humoral immune response

Mice were immunized with DNA vaccines containing S1-S4 and M1-M3 genes four times at 2-week intervals. To determine the humoral responses elicited by these plasmids, the titers of mouse sera collected at 1-week intervals were tested by ELISA using inactivated R4 as the captured antigen. As shown in Fig. 1, all DNA vaccines induced significantly higher levels of anti-R4 IgG compared with the controls (pcDNA3.1+ and PBS), and titers were highest in mice immunized with S3. A continuous increase in antibody levels was observed in groups immunized with S1, S3, M2, and M3. The S4 group titer peaked at week 5 and then dropped precipitously at week 6. In contrast, S2 and M1 group titers peaked at 4-th week. However, following the final immunization, the M1 group titer dropped at the fifth week and then rose the sixth week to the same level as in the fourth week. In contrast, the third immunization made no difference in M1 and M3 group titers.

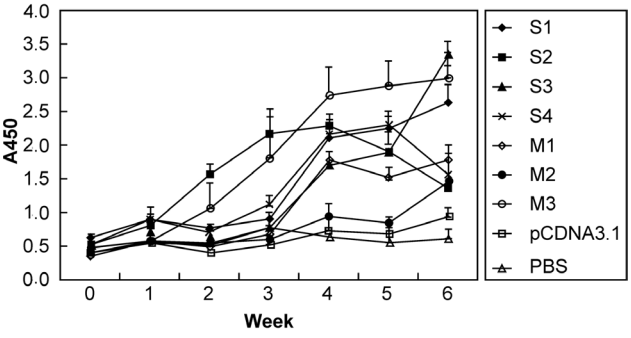

Fig. 1

Humoral IgG antibodies induced by DNA vaccines

Antibodies assayed by ELISA at 0–6 weeks post immunization.

The relevant IgG subclass and T-helper (Th) type could be critical for protection against a particular disease. The production of IgG1 is representative of the Th2 response, and IgG2a is typical for the Th1 response. Therefore, 10 days after the final immunization, R4-specific antibody subclasses in mice sera were determined by ELISA to evaluate the types of Th cell responses associated with DNA vaccination (Fig. 2).

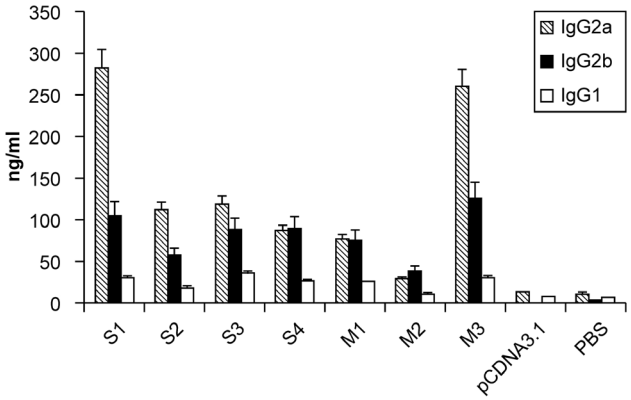

Fig. 2

Humoral IgG subclass antibodies induced by DNA vaccines

Antibodies assayed by ELISA 10 days after final immunization.

Table 2. Peptides targeting individual reovirus genes

Gene	Peptide
S1	QTGSTQPSSTTDPMS
S2	RTKPFTNAQWGRGN
S3	RERLLGQRNLERISTRD
S4	EGWDKTISAQPDMMVC
M1	GWHVPREQLMQDGWC
M2	KPDCPTSGDSGESSNRR
M3	KNVELDALNQRQAKS

The IgG2a, IgG2b, and IgG1 responses were significantly different (P <0.001) between the 7 immunized groups and the 2 negative groups, excluding the IgG1 response in the S1/M3 and S4/M1 groups and the IgG2b response in the S3/S4 group (P >0.05). Antibodies produced in all immunized animals except for the M2 and S4 groups were primarily of the IgG2a subclass. Moreover, the S1 and M3 groups had nearly 2 and 8–10 times higher IgG2a levels than IgG2b and IgG1, respectively. The results of the IgG1 assays were consistent with those of anti-R4 IgG; however, the IgG2a and IgG2b responses of the S3 group were much lower than those of the S1 and M3 groups.

Cellular immune response

Cellular immune responses to R4 were assessed by determination of IFN-γ levels, a marker for Th1 responses. Splenocytes were collected 10 days after the final immunization and assayed for IFN-γ production. As shown in Fig. 3, only a low number of non-specific IFN-γ spots were observed in the control groups (<10 of 10^5 cells). All of the recombinant plasmid groups induced at least a 6-fold increase of antigen-specific IFN-γ-secreting cells compared with the control groups (P <0.001). Interestingly, S1 and M2 gene immunization induced a much higher number of IFN-γ-specific spots, which may indicate a stronger cellular response. Although the humoral response elicited by M2 was the weakest, the cellular response was the most robust of all groups.

As activated CD4+ and CD8+ T lymphocytes are among the most crucial components of antiviral effectors, peripheral blood mononuclear cells in all immunized groups were assessed by flow cytometry. Unstimulated cells were used to standardize the background responses, and there was little variation in unimmunized mice. The numbers and percentages of CD4+- and CD8+-activated cells increased in all immunized groups (data not shown). The ratio of CD4+/CD8+ was higher in the immunized than in the control groups (P <0.05) (Fig. 4), and these results were parallel with those of the IFN-γ ELISpot assay. Furthermore, S1-immunized mice showed the highest CD4+/CD8+ ratio. Overall, the S1 group had much stronger humoral and cellular responses, while the M2 group exhibited the weakest humoral and highest cellular responses.

Discussion

Reoviruses are ubiquitous viruses that have been isolated from a wide variety of mammalian species including humans, but they are not associated with any known diseases and are considered benign (Tyler *et al.*, 1996). Reovirus replication and assembly are thought to occur within cytoplasmic viral inclusions where viral and cellular proteins, viral RNAs, and immature and mature viral particles are concentrated (Tyler *et al.*, 1996). In the present study, we successfully immunized mice against reovirus antigens using a DNA vaccine and demonstrated the presence of humoral and cell-mediated immune responses.

IFN-γ is the principal macrophage-activating cytokine and mediates critical functions in innate immunity and adaptive cell-mediated immunity. IFN-γ can also promote the differentiation of naive CD4+ T cells to the Th1 subset and inhibit the proliferation of Th2 cells (Whitmire *et al.*, 2005). Indeed, we observed much higher IFN-γ secretion in response to immunization with M2 and S1; M2 elicited a Th1-based immune response in mice, whereas S1 elicited a mixed Th1/Th2 response. Th1 cells can elicit phagocyte-mediated defense against infections; therefore, Th1-dominated immune responses elicited by the M2 gene may be

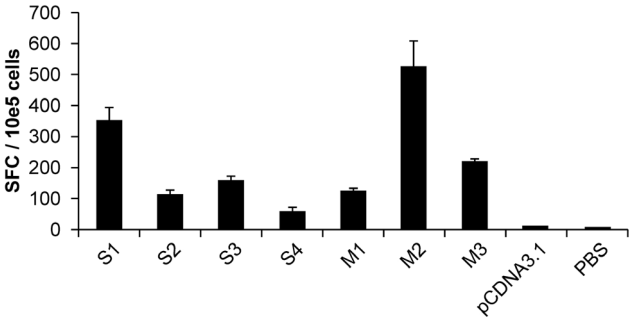

Fig. 3

Induction of IFN-γ by DNA vaccines

Mice were sacrificed 10 days after final immunization, their splenocytes were stimulated with gene-specific peptides and IFN-γ levels were assayed by ELISpot.

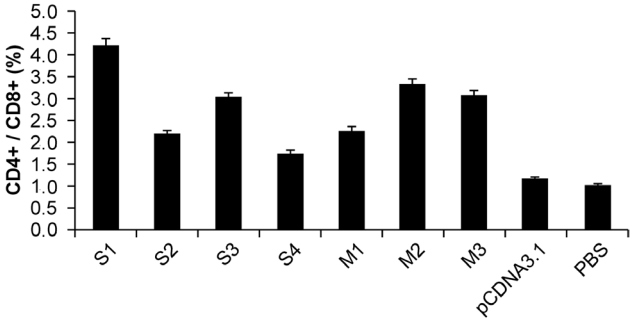

Fig. 4

Induction of CD4+ and CD8+ T-cells by DNA vaccines

Peripheral blood mononuclear cells were isolated from mice 10 days after final immunization and were assayed for CD4+/CD8+ T-cell ratio (%) by flow cytometry.

important for virus control. M2 is a virulence gene and could contribute to the marked differences found between reovirus strains (Coffey *et al.*, 2006; Danthi *et al.*, 2008; Hrdy *et al.*, 1982; Rubin and Fields, 1980); M2 could also directly affect the ability of reovirus to cause disease after introduction into the gastrointestinal tract. The S1 and M2 genes are involved in receptor binding and penetrating the host cell membrane and could determine the capacity of different reovirus strains to induce apoptosis (Clarke *et al.*, 2001). In agreement with this assumption, in our present study, the vectors expressing μ1C (encoded by M2) and σl proteins (encoded by S1) were highly immunogenic and could be the most promising vaccine candidates against R4.

The predominance of IgG2a in the sera of immunized mice in the present study is consistent with the results of Coutelier *et al.* (1987, 1988) and Nguyen *et al.* (1994), who demonstrated that serum antibody response to many viral infections (including infection with reovirus TD3) involves preferential production of IgG2a. Others have reported that immunization with many viral proteins or peptides often leads to strong IgG1 antibody responses (Balkovic *et al.*, 1987; Ben Ahmeida *et al.*, 1992, 1993; Markine-Goriaynoff *et al.*, 2000; Smucny *et al.*, 1995). In contrast, Pertmer *et al.* (1996) found that the IgG subclass elicited by DNA vaccination depends upon the route of DNA administration; i.m. inoculation leads to Th1-like responses due to elevated IgG2a levels, whereas gene gun responses tend to cause elevated levels of IgG1 (Pertmer *et al.*, 1996). Similarly, mice immunized i.m. with DNA vaccines expressing varicella-zoster virus also produce IgG2a (Stasikova *et al.*, 2003), and a DNA vaccine containing SARS coronavirus nucleocapsid (N) protein leads to increased levels of IgG2a in mice (Zhao *et al.*, 2005). Although the immune response generated by a DNA vaccine can be influenced via co-delivery of adjuvant containing cytokine genes or by different routes of injection (Chen *et al.*, 2003; Feltquate *et al.*, 1997; Gurunathan *et al.*, 2000; He *et al.*, 2005, 2006; Hung *et al.*, 2007; Kim *et al.*, 2001; Nobiron *et al.*, 2001; Osorio *et al.*, 1999; Rogers *et al.*, 1999; Saldarriaga *et al.*, 2006; Song *et al.*, 2000, 2008; Whitmire *et al.*, 2005). In this study, we injected mice with only DNA plasmids by the i.m. route without any adjuvant. Therefore, it is unclear whether the quality and specificity of immune responses in mice would be different with another immunization strategy.

Antibody responses to viruses often serve to neutralize the virus and possibly mediate virus inactivation by complement or antibody-dependent cellular cytotoxicity (Zinkernagel, 1993). Production of virus-specific IgG2a during virus infections could prove advantageous as IgG2a is efficient at fixing complement and mediating antibody-dependent cellular cytotoxicity (Heusser *et al.*, 1977; Klaus *et al.*, 1979). Moreover, herpes simplex virus glycoprotein D-specific monoclonal IgG2a is more effective at protecting infected mice than IgG1 of the same specificity (Ishizaka *et al.*, 1995).

The SARS epidemic occurred nearly 10 years ago. Since then, in addition to SARS coronavirus (Drosten *et al.*, 2003; Ksiazek *et al.*, 2003; Peiris *et al.*, 2003; Poutanen *et al.*, 2003), other pathogens, including human metapneumovirus, chlamydia, and poliovirus Sabin I (Chan *et al.*, 2003; Louie *et al.*, 2004; Shen *et al.*, 2012) have also been detected in some patients with SARS. Moreover, some groups identified bats as the reservoir host for a group of genetically diverse SARS-like coronaviruses (Li *et al.*, 2005; Ren *et al.*, 2006; Wang *et al.*, 2006). Reovirus was isolated from bats as well (Chua *et al.*, 2007). It is unclear whether microbial or other cofactors could enhance the severity or transmissibility of SARS.

In conclusion, we were able to induce humoral and cell-mediated immune responses to R4 proteins by immunizing mice with DNA vaccines. Expression of the M2 and S3 genes elicited Th1- and Th2-based immune responses, respectively. In contrast, S1 and M3 constructs induced a mixed Th1/Th2 immune response. To our knowledge, this is the first report characterizing mammalian reovirus DNA vaccines in a mouse model. Due to the safety and ease of manipulation of DNA vaccines, our study may provide a convenient strategy for studying the immunogenicity of mammalian reovirus genes in other animal models.

Acknowledgement. This work was supported by the grant No. 81000735 from The National Nature Science Fund, China.

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