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A conserved matrix epitope based DNA vaccine protects mice against influenza A virus challenge

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ABSTRACT

DNA vaccination represents a unique strategy to overcome the limitations of immunization with conventional vaccines which is restricted by the high variability of influenza viruses. We evaluated the protective efficacy of a plasmid DNA (pDNA), encoding an evolutionarily conserved epitope of viral matrix protein, against the influenza A virus infection. It was found that the mice immunized via the intra-muscular route purely elicited cell mediated immune response to the pDNA, with enhanced level of Th1 cytokines viz. IL-12 and IFNγ production in the stimulated splenocyte supernatant. The cytotoxic T lymphocytes in the spleen of immunized mice significantly lysed the virus-infected MDCK cells. A significant decrease in virus replication was also observed in the lungs of immunized mice and 83% of the mice were protected against the lethal challenge of influenza A viruses. These findings suggest that the plasmid DNA expressing a single matrix epitope may serve as a promising vaccine candidate to provide effective immunity in the susceptible (mouse) population.

1. Introduction

Influenza virus (IV) has been a major cause of upper respiratory tract infection leading to millions of cases of severe illness every year. As a prophylactic measure, inactivated seasonal vaccines are formulated every year which are effective against the currently circulating strains only. The virus genome exhibits a very rapid rate of mutation due to lack of 3′–5′ exo-nuclease activity in their RNA polymerase. These mutations lead to the emergence of new strains as demonstrated by the sudden appearance of the lethal forms of the virus leading to the pandemic situations (Brown et al., 2001; Khanna et al., 2006; Layne et al., 2009). Consequently, the vaccines formulated annually are rendered ineffective every next season [Vaccine composition recommended by WHO in 2009 – A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2) and B/Brisbane/60/2008; Vaccine composition in 2011 – A/California/7/2009 (H1N1) – like virus, A/Perth/16/2009 (H3N2) and B/Brisbane/60/2008].

The eight segmented viral genome code for eleven proteins. The matrix protein (M1) plays a major role in the assembly of virus particles inside the infected host and being the surface protein, they elicit immune response in infected host (Bright et al., 2008). Although the gene coding for this protein is also prone to mutation, there is a certain immunogenic region ($M_{58–66}$: GILGFVFTL) that is evolutionarily conserved (Thomas et al., 2006) and is 100% conserved in almost all the strains of influenza virus including H1N1, H5N1, H3N2 and pandemic H1N1 (2009) (Webster and Hinshaw, 1977; Braciale, 1977; Reiss and Schulman, 1980).

The traditional vaccines developed against influenza virus include either attenuated or killed virus vaccines which are rendered ineffective with the emergence new viral strains. Therefore, there is an urgent need to develop new approaches for the design and production of vaccines that can reduce the severity of the disease and protect the infected individuals. The more focused and effective vaccines against various pathogens have been developed which involves the use of immuno-dominant parts of pathogens to elicit an epitope-specific $CD8^+$ T cell response. These vaccines consist of either synthetic peptide, recombinant peptides expressed in bacterial system or the plasmid DNA. The vaccines based on the plasmid DNA offer several advantages, viz. simplicity and de novo synthesis of endogenous protein in the host (Robinson and Pertmer, 2000). The DNA vaccines that elicit $CD8^+$ T cell response have been developed and the effectiveness of these vaccines has also been studied. The $CD8^+$ T cells recognize the viral antigens in the form of short peptides associated with the MHC Class I molecule on the surface of virus infected cells (Mackaness and Blanden, 1967; Doherty, 1997) and this recognition triggers either the specific lysis of infected cells (McFarland, 1974) or the intracellular inactivation of the virus by cytokines such as IFNγ (Christensen et al., 1996). Although the $CD4^+$ T cells also have a role

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in the resisting the virus infection, some studies have revealed that the $CD8^+$ T cell-depleted host shows delayed recovery from the influenza virus infection (Epstein et al., 1998). The humoral immunity induced by the $CD4^+$ T cells plays a role in providing protection against the virus infection, but is mostly directed against the surface HA and NA proteins which are subtype-specific and is ineffective against the newly emerging strains. By contrast, the cellular immunity is type-specific and therefore, it provides better protection against virus infection (Werner, 1993).

We developed a plasmid DNA (pDNA) encoding a conserved epitope of matrix protein of influenza A virus and investigated its role in the inhibition of virus replication in the mice infected with influenza A viruses.

2. Materials and methods

2.1. Mice and viruses

BALB/c mice were obtained from INMAS (Institute of Nuclear Medicine and Allied Sciences), New Delhi. Six to eight weeks old mice were used in all the experiments and the animals were housed under specific pathogen-free conditions, as validated by screening of sentinels. All the animal experiments in this study were done as per the guidelines of Indian National Science Academy (INSA). Experimental use of mice was approved by the Institutional Animal Ethical Committee (IAEC) of Vallabhbhai Patel Chest Institute, University of Delhi and efforts were made to minimize suffering.

The virus strains [A/PR/8/34 (H1N1) and A/Udorn/307/1972 (H3N2)] used in this study were obtained from CDC (Atlanta, GA) and propagated in embryonated chicken eggs in our laboratory as per the standardized protocol.

2.2. Generation of vaccine construct

The vaccine construct was generated by cloning the DNA encoding a nine amino acid long conserved epitope (M_{58-66}: GILGFVFTL) of M1 protein of influenza A virus in pSecTag2B (Invitrogen, Carlsbad, CA). The two commercially synthesized complementary oligonucleotides (having the sites of Hind III and Kpn I, respectively, at their 5′ end) encoding the matrix epitope was obtained from MWG Biotech, Hyderabad, India. The oligonucleotides were annealed as described earlier (Zhu et al., 2005). Briefly, the oligonucleotides were mixed in an equal amount (10 μg each) and heated at 80 °C for 5 min followed by incubation at room temperature till the temperature reaches 25 °C. The oligonucleotide was digested and cloned between Hind III and Kpn I in pSecTag2B. The recombinant plasmid DNA having the vaccine construct (pDNA) was sequenced and purified by maxi-prep using Plasmid midi kit (Qiagen, GmbH, Hilden, Germany) and the stock was stored at −80 °C for further use.

2.3. Expression of the cloned construct

The recombinant plasmid (pDNA) was transfected in Chinese Hamster Ovary (CHO) cells [purchased from National Centre For Cell Science (NCCS), Pune, India] using Lipofectamine (Invitrogen, Carlsbad, CA) as per the Manufacturer's protocol. The transfected cells were cultured in DMEM (Sigma, St. Louis, MO) containing 10% FCS (Sigma, St. Louis, MO) and 200 μg/ml zeocin (Duchefa Biochemie) for 2 weeks till a complete monolayer of recombinant cell line was obtained. The recombinant cells were plated in 96 well plates with 2×10^4 cells per well and were maintained in complete DMEM medium containing 200 μg/ml zeocin for 72 h at 37 °C/5% CO_2. The cells were harvested and analyzed for the expression of pDNA by Western blotting of the cell supernatant and whole cell lysate.

2.4. SDS–PAGE and western blotting

The cells (bearing pDNA) plated in 96 well plates were harvested after 72 h. The cell supernatant was stored at 4 °C and the cells were lysed in mammalian cell lysis buffer [0.1 M NaCl, 0.01 M Tris Cl, 0.001 M EDTA, 1 mM protease inhibitor (PI) cocktail and 1 mM phenyl methyl sulfonyl fluoride (PMSF)] at 4 °C. The normal CHO cells and the cells transfected with vector backbone were taken as a control. The cell supernatant and the whole cell lysate were separated in 16% denaturing polyacrylamide gel followed by staining with coomassie blue (R-250). The protein bands obtained on the polyacrylamide gel were transferred to polyvinylidene difluoride (PVDF) membrane using Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, CA) and analyzed using 1:100 dilution of goat polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) against M1 protein of influenza A virus as primary antibody and 1:1000 dilution of HRP conjugated bovine anti-goat IgG as secondary antibody (Kumar et al., 2010).

2.5. Animal immunization (ex vivo experiments)

BALB/c mice (14–16 g) were used in this study. They were distributed into two groups:

(i) Group I ($n = 6$): control group having mock immunized (immunized with either PBS or vector backbone) healthy mice.
(ii) Group II: mice were divided into six subgroups:
  (a) subgroup I ($n = 6$) – mice immunized with two intramuscular doses of pDNA (50 μg per mice in 50 μl) at three weeks interval and spleen cells obtained from this sub group were stimulated with influenza virus-infected syngeneic spleen cells.
  (b) subgroup II ($n = 6$) – mice immunized in the same manner as subgroup I and spleen cells obtained from this sub group were stimulated with epitope peptide pulsed syngeneic spleen cells (cells bearing epitope peptide on their surface).
  (c) subgroup III ($n = 6$) – mice immunized with two intramuscular doses of formalin fixed virus [A/PR/8/34 (H1N1)] at three weeks interval and spleen cells obtained from this sub group, after one week of second immunization, were stimulated with influenza virus [A/PR/8/34 (H1N1)] infected syngeneic spleen cells.
  (d) subgroup IV ($n = 6$) – mice immunized with two intramuscular doses of formalin fixed virus [A/PR/8/34 (H1N1)] at three weeks interval and spleen cells obtained from this subgroup, after one week of second immunization, were stimulated with epitope peptide-pulsed syngeneic spleen cells.
  (e) subgroup V ($n = 6$) – mice immunized with two intramuscular doses of plasmid DNA encoding complete matrix protein of influenza A virus [A/PR/8/34 (H1N1)] at three weeks interval and spleen cells obtained from this subgroup, after one week of second immunization, were stimulated with influenza virus [A/PR/8/34 (H1N1)]-infected syngeneic spleen cells.
  (f) subgroup VI ($n = 6$) – mice immunized with two intramuscular doses of plasmid DNA encoding complete matrix protein of influenza A virus [A/PR/8/34 (H1N1)] at three weeks interval and spleen cells obtained from

this subgroup, after 1 week of second immunization, were stimulated with epitope peptide pulsed syngeneic spleen cells.

2.6. Stimulation of splenocytes and cytokine release assay

Twenty-one days after the second immunization, the mice were sacrificed and their spleens were taken out in PBS for *in vitro* assessment of epitopic peptide and influenza A virus-specific CTL activity. The splenocytes were re-stimulated for 6 days with the syngeneic spleen cells infected with A/PR/8/34 (H1N1) and with the syngeneic spleen cells transfected with pDNA to generate the CTLs which acted as the effector cells (Zhang et al., 2009; Jamali et al., 2010). The media for re-stimulation was enriched with IL-2 (10 U/ml) on the second day. After 6 days, the splenocyte proliferation was tested by the MTT assay.

On the third day of stimulation, the culture supernatants were collected and assayed for the secretion of gamma interferon, IL-12 and IL-4 by ELISA using commercial cytokine assay kit (BD Pharmingen™, NJ) (Tamizifar et al., 1997).

2.7. Cytotoxicity assay

The MDCK cells either transfected with pDNA or infected with 2×10^6 pfu/ml of influenza A viruses were used as the target cells and the cytotoxicity of the CTLs were assessed by LDH assay as described earlier (Yaya et al., 2007). Briefly, the target cells and the effector cells were suspended in the assay medium (RPMI 1640 with 1% BSA), and then the target cells (1×10^4 cells per well) were cocultured with the effector cells at different ratios in round bottom 96 well plates at 37 °C/5% CO_2. After 5 h incubation, the plates were centrifuged and the supernatants (100 μl/well) were collected in another ELISA plate (BD Biosciences). The LDH detection mixture was added to each well (100 μl/well) and incubated in dark for 30 min followed by addition of 50 μl stop solution to each well. The absorbance of the sample was measured with ELISA Reader (EL_X800_{MS}, Biotek Instruments, Inc., Winooski, VT) at 490 nm with a reference wavelength of 630 nm. The spontaneous release of the LDH by either the target cells or effector cells was measured by incubating the target cells without any effector cells and vice versa. The maximum release of LDH was measured by treating the target cells with 1% Triton X 100. The percentage of specific cytotoxicity was measured by the following equation:

$$\% \text{ cytotoxicity} = \frac{[\text{target and effector mixture} - \text{target spontaneous} - \text{effector spontaneous}] \times 100}{[\text{maximum} - \text{target spontaneous}]}$$

2.8. Animal infection

The mice were divided into four groups and the groups had six mice each for the challenge studies:

(i) Virus-infected/unimmunized mice
(ii) Virus-infected/mock immunized mice
(iii) Virus-infected/immunized mice
(iv) Mock-infected mice

The mice were immunized with pDNA as done earlier in this study. Three weeks after the second immunization, the mice were instilled intranasally with 2×10^6 p.f.u of influenza A viruses

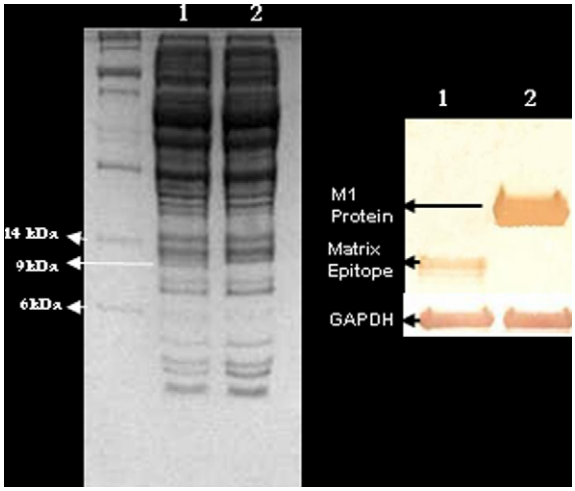

Fig. 1. Expression analysis of the M1 epitope: the oligo encoding the matrix epitope was cloned in pSecTag2B. The recombinant plasmid (pDNA) was transfected in CHO K1 cells and the cell lysate was analyzed for the expression of pDNA by SDS PAGE and western blotting at 72 h post transfection. A unique peptide of ~9 kDa was seen in SDS–PAGE of the lysate of cells transfected with pDNA (Lane 1) which was not observed in the lysate of non-transfected cells (Lane 2). The peptide of ~9 kDa (Lane 1) was also detected in western blot by the polyclonal antibody against M1 protein of influenza A virus which also detected the matrix protein (Lane 2) in the lysate of virus infected cells.

suspended in 100 μl sterile PBS. The lungs of mice were harvested at 4 days post-infection for various assays to quantify the effect of pDNA immunization on virus replication.

2.9. RNA isolation, RT-PCR and real-time RT-PCR

The immunized mice were sacrificed after 4 days of virus infection and their lungs were collected in RNA*later* RNA Stabilization Reagent (Qiagen, GmbH, Hilden, Germany) for the assessment of virus replication under the effect of pDNA immunization (Machado et al., 2010). The lungs were homogenized and total RNA was isolated using RNeasy kit (Qiagen, GmbH, Hilden, Germany) as per the Manufacturer's protocol. The RNA obtained from the lungs of all groups of mice was quantified by spectrophotometric analysis at 260 nm and their stability was determined by formaldehyde agarose gel electrophoresis. Lung RNA (1 μg) of each mouse was reverse transcribed using IM-PROM reverse transcription kit (Promega, Madison, WI) and 2 μl of each cDNA was used for the amplification of NS1 gene of influenza A virus using gene specific universal primers (Forward: 5′ accggtAGCAAAAGCAGGGTG 3′; Reverse: 5′ ctcgagAGTAGAAACAAGGGTGTTTT 3′). Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified simultaneously using gene specific primers (Forward: 5′ CCATGGAGAAGGCTGGGG 3′; Reverse: 5′ CAAAGTTGTCATGGATGACC 3′) as a control.

To further quantify the effect of pDNA immunization on virus replication in mice, a SYBR green-based real-time RT-PCR protocol was also employed by using an iScript one-step RT-PCR kit (Bio-Rad Laboratories) as described in our previous studies (Kumar

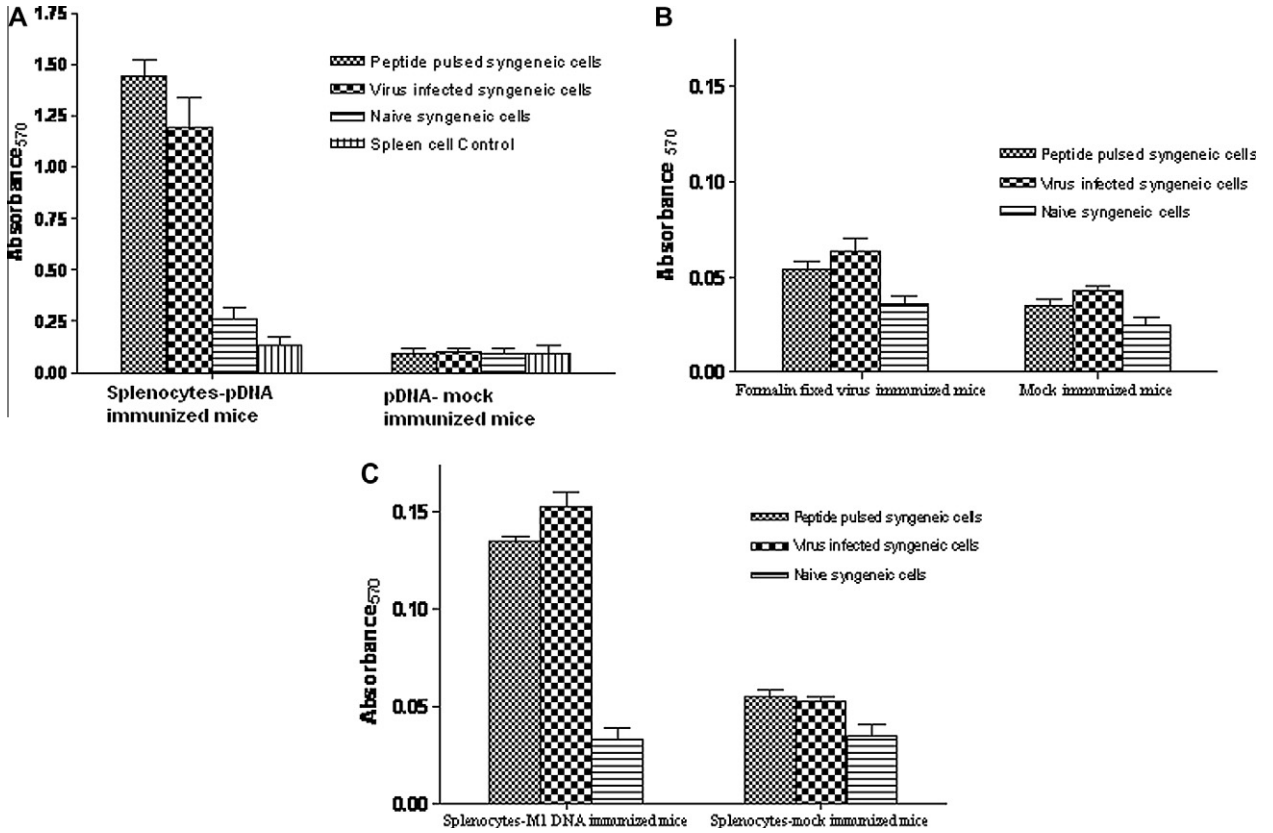

Fig. 2. Splenocyte proliferation with virus-infected and epitope peptide-pulsed syngeneic spleen cells. The BALB/c mice were immunized twice with pDNA, formalin-fixed virus [(A/PR/8/34 (H1N1)] and plasmid encoding matrix protein [(A/PR/8/34 (H1N1)] at 21 days interval by intra-muscular injection. Three weeks after second immunization with pDNA and one week after second immunization with formalin-fixed virus [(A/PR/8/34 (H1N1)] and plasmid encoding matrix protein [(A/PR/8/34 (H1N1)], the mice were sacrificed and their splenocytes were stimulated by co-culturing them with influenza A virus-infected and epitope peptide-pulsed syngeneic spleen cells. The splenocytes' proliferation was studied by MTT assay and the amount of proliferation of splenocyte from immunized mice was compared with that of the mock-immunized mice (*x*-axis). The sub-groups termed "naive syngeneic cells" and "spleen cell control" contained the splenocytes co-cultured with uninfected/unpulsed syngeneic cells and with splenocytes, respectively. The Ab_{570} (*y*-axis) corresponds to amount of proliferation and the error bars indicate the S.D. derived from triplicates. (A) The stimulation of splenocytes of pDNA immunized mice by epitope peptide-pulsed spleen cells and virus-infected spleen cells. (B) The stimulation of splenocytes of mice immunized with formalin-fixed virus by peptide pulsed and virus-infected syngeneic spleen cells. (C) The stimulation of splenocytes of mice immunized with plasmid encoding matrix protein by peptide-pulsed and virus-infected syngeneic spleen cells.

et al., 2010). Reaction mixtures containing 20 ng total lung RNA and appropriate amounts of reagents and primers (specific for matrix gene of influenza A virus) were first incubated at 50 °C for 30 min, heated at 95 °C for 8 min and then subjected to 40 thermal cycles (95 °C for 20 s, 50 °C for 30 s and 72 °C for 30 s) of PCR amplification followed by generation of melting curve with an iCycler iQ real-time PCR detection system (Bio-Rad Laboratories). The reactions were performed in duplicate.

2.10. Virus lung titration

The lungs of mice, harvested at 4 days post infection, were homogenized in PBS containing 1% BSA and centrifuged at 3000 RPM for 15 min at 4 °C. Tenfold dilutions of lung extract supernatants were prepared in PBS containing 1% BSA and used for plaque assay as described earlier (Lukacher et al., 1984). The results were expressed as mean plaque-forming unit (PFU) per ml ±SD in lung extracts of four mice.

2.11. Survival assay

The mice were immunized with pDNA as described earlier in this study. Three weeks after the second immunization, the mice were instilled intra-nasally with 2×10^6 p.f.u of influenza A viruses. The mice were monitored twice daily up to 21 days post infection for their survival and to analyze the protective effect of pDNA immunization against the influenza A virus infection.

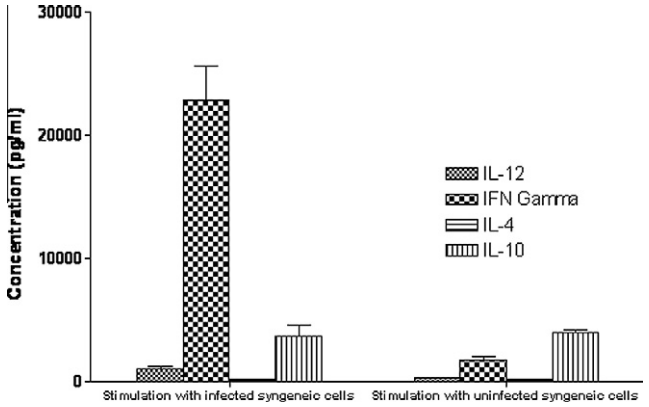

Fig. 3. Estimation of cytokines in the supernatants of proliferating splenocytes. The Balb/c mice were immunized twice with pDNA at 21 day interval by intra-muscular injection. Three weeks after second immunization, the mice were sacrificed and the spleens were taken out. Their splenocytes were stimulated by co-culturing them with influenza A virus infected and epitope peptide-pulsed spleen cells. The supernatants were collected at the third day of stimulation and concentrations of cytokines (IL-12, IL-4, IL-10 and gamma interferon) were estimated using Cytokine Assay kit from BD Pharmingen. The concentrations (pg/ml; *y*-axis) of the cytokines released by the proliferating cytokines during stimulation with influenza A virus infected syngeneic spleen cells were compared with that released during stimulation with uninfected syngeneic spleen cells (*x*-axis). The error bars indicate the S.D. derived from triplicates. The expression of gamma interferon was shown to be elevated significantly ($p < 0.001$) along with the increased level of IL-12 indicating the induction of Th1 and cell-mediated immune response.

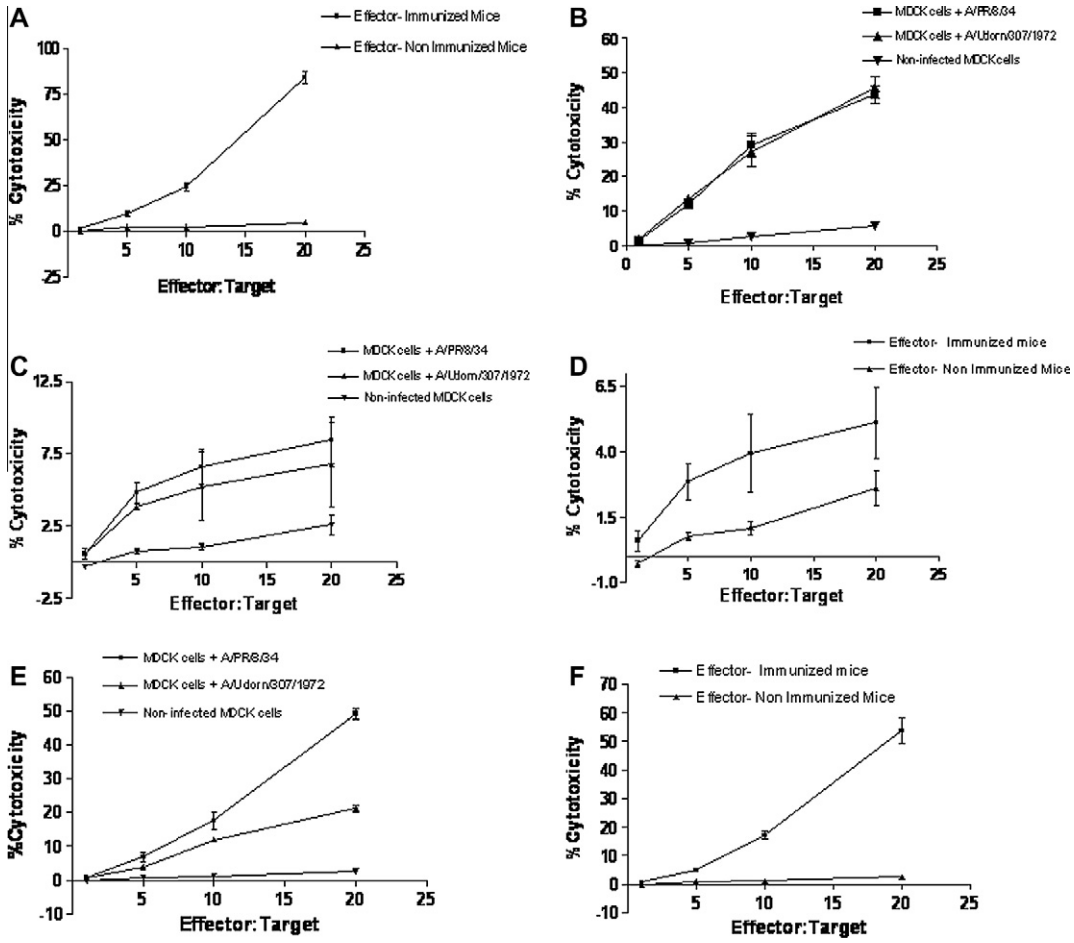

Fig. 4. Cytotoxic effect of stimulated splenocytes on influenza A virus-infected and epitope peptide bearing target cells. Cytotoxic effect of stimulated splenocytes on influenza A virus infected and epitope peptide bearing target cells: The MDCK cells were used as the target for the effector cells generated after the stimulation of mice splenocytes. The percent cytotoxicity (*y*-axis) was calculated with Ab_{490} for each sample. The error bars indicated the S.D. derived from triplicates. (A) % Cytotoxicity of the effector cells from pDNA immunized mice against the target cells transfected with pDNA at increasing effector: target ratio (range 5:1–20:1 on the *x*-axis). Cytotoxicity of the effectors generated from the splenocytes of immunized mice was compared with that of the mock immunized mice. (B) Cytotoxicity of the effector cells on the target cells infected with different strains of influenza A virus. % Cytotoxicity against virus-infected cells was also compared with the cytotoxicity against uninfected target cells at different effector: target ratios (ratio range 5:1–20:1 on *x*-axis). (C) Cytotoxicity of the effector cells from mice immunized with formalin-fixed virus on the target cells infected with different strains of influenza A virus. (D) Cytotoxicity of the effector cells from mice immunized with formalin-fixed virus on the target cells transfected with pDNA at an increasing effector: target ratio (range 5:1–20:1 on the *x*-axis). (E) Cytotoxicity of the effector cells from mice immunized with plasmid encoding matrix protein on the target cells infected with different strains of influenza A virus. (F) Cytotoxicity of the effector cells from mice immunized with plasmid encoding matrix protein on the target cells transfected with pDNA at an increasing effector: target ratio (range 5:1–20:1 on the *x*-axis).

2.12. Statistical analysis

The data were analyzed by Student's *t*-test and one way ANOVA and all the results were expressed as mean ± SD.

3. Results

3.1. Expression and purification of the recombinant plasmid (pDNA)

The recombinant plasmid (pDNA) was commercially sequenced by Ocimum Biosolutions and the clone was confirmed for the presence of vaccine construct in proper orientation. The cells with pDNA were cultured in the presence of zeocin (200 μg/ml) and their lysates were analyzed for the expression of cloned construct by western blotting using goat polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) against M1 protein of influenza A virus. Lysate of the cells infected with A/PR/8/34 (H1N1) was taken as the positive control and uninfected cells were taken as negative control. Peptides corresponding to the matrix epitope (~9 kDa) and matrix protein (~28 kDa) were captured during Western blotting in lysate of pDNA-transfected cells and virus-infected cells, respectively (Fig. 1).

3.2. Splenocyte proliferation response to M1 epitope

The BALB/c mice were immunized separately with pDNA, formalin-fixed virus and plasmid encoding complete matrix protein of influenza A virus, and generation of immunity against the virus was studied by stimulating their splenocytes with matrix epitope-bearing or whole virus-infected syngeneic spleen cells. The spleen cells from the mice immunized with pDNA proliferated significantly ($p \leqslant 0.001$) at sixfold higher rate as compared to the spleen cells from mock-immunized mice. The data presented in Fig. 2A shows that the splenocytes from the mice immunized with pDNA proliferated significantly ($p < 0.05$) on stimulation with epitope peptide-pulsed syngeneic cells as compared to stimulation with virus-infected cells. Significant proliferation was not observed in the splenocytes from mice immunized with formalin-fixed virus

when stimulated either with virus-infected or epitope peptide-bearing syngeneic spleen cells (Fig. 2B). The splenocytes from mice immunized with plasmid encoding complete matrix protein of influenza A virus proliferated at a higher rate ($p < 0.01$) when stimulated with virus infected syngeneic spleen cells as compared to stimulation with the epitope peptide bearing syngeneic spleen cells (Fig. 2C).

The cytokine profile in the supernatants of the co-culture showed a significant ($p < 0.001$) increase in gamma interferon as compared to IL-4 and IL-10 and the data also shows that there was a increase in the level of IL-12 (increase of 735.4 pg/ml) (Fig. 3). The change in level of IL-4 production was negligible while IL-10 production decreased by 110.4 pg/ml. The elevated levels of gamma-interferon and IL-12 confirmed the antiviral response of the mononuclear cells in the splenocytes and induction of cell mediated immune response against the viral epitope.

3.3. Induction of CTL response

The cytotoxic responses of the stimulated spleen cells (effector) against the target cells were examined by the Lactate Dehydrogenase (LDH) assay. The effector cells lysed the influenza virus-infected as well as the epitope-peptide pulsed target cells significantly ($p < 0.0001$) and there was increased lysis with increasing effector: target ratio. The cytolytic activity was more than 80% at effector:target ratio of 20:1 when the epitope peptide-pulsed MDCK cells and stimulated splenocytes from the pDNA immunized mice were taken as target cells and effector cells, respectively (Fig. 4A). The spleen cells from the mock-immunized mice were not able to recognize the target cells. The CTL response of the effector cells caused up to 50% lysis of the target cells infected with influenza A viruses (Fig. 4B). Significant lysis of the virus-infected or pDNA-transfected target cells was not observed when the stimulated splenocytes from the mice immunized with formalin-fixed virus was used as the effector cells (Fig. 4C and D). Stimulated splenocytes from the mice immunized with plasmid encoding M1 protein lysed the target cells infected with A/PR/8/34 (H1N1) (~50% cytotoxicity) but the target cells infected with A/Udorn/307/1972 (H3N2) were not recognized well by these effector cells (~20% cytotoxicity) (Fig. 4E). These effector cells also caused ~50% lysis of the target cells transfected with pDNA (Fig. 4F).

3.4. Animal protection

The immunized mice were sacrificed at four days post-infection for the evaluation of lung virus titer by plaque assay (Fig. 5). The residual virus titer in the immunized mice infected individually with A/PR/8/34 (H1N1) and A/Udorn/307/1972 (H3N2) was decreased significantly ($p < 0.005$) and a difference of more than 4 log was observed, while the titer in the mice given mixed infection with the two strains also decreased significantly ($p < 0.001$) and the virus titer was reduced by 3 log as compared to the mock-immunized mice.

The real time RT PCR was also performed using iScript SYBR Green from BioRad. The level of matrix RNA from lung homogenates in different groups was compared by calculating ΔΔCt value for each group. The RNA level of M1 in lungs of mock-immunized infected mice was taken as the reference whose value was "1" and compared with all the groups. It was shown that expression of matrix gene was decreased up to 70% ($p < 0.01$) (Fig. 6) in the immunized mice infected with A/PR/8/34 (H1N1) and A/Udorn/307/1972 (H3N2), while the expression was decreased by 45% ($p < 0.01$) in the immunized mice instilled with mixed infection with the two strains as compared to the mock-immunized mice.

The protective effect of pDNA immunization was also confirmed by animal survival assay. It was observed that 83% of mice receiving the pDNA recovered from lethal infection with A/PR/8/34 (H1N1) (Fig. 7A) and A/Udorn/307/1972 (H3N2) (Fig. 7B) while the unimmunized virus infected mice survived for not more than 9 days. The pDNA immunization also protected 65% of the mice given mixed infection with the two strains of virus and the un-immunized mice in this group did not survive for more than 7 days (Fig. 7C).

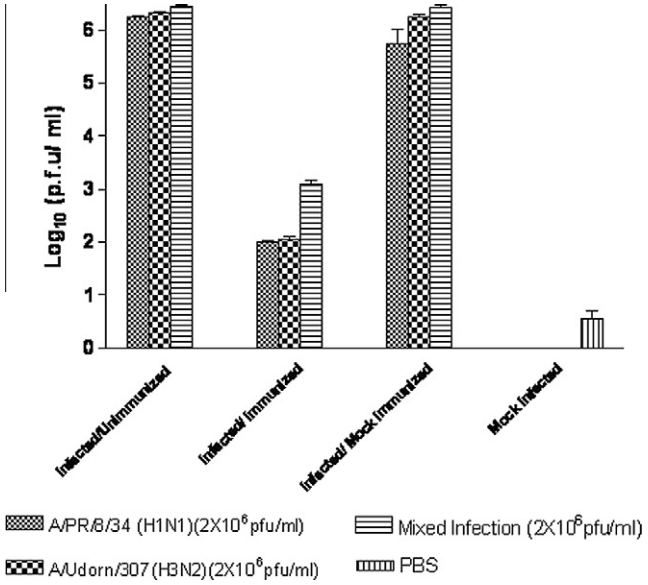

Fig. 5. Plaque assay for residual virus titer in the lungs of virus-infected immunized mice. The mice were immunized with pDNA twice at 21 days interval. Three weeks after the second immunization, they were instilled intra-nasally with 2×10^6 p.f.u of influenza A viruses suspended in 100 μl sterile PBS and were sacrificed at 4 days post-infection. Plaque assay was performed with the supernatants of their lung homogenates and the residual virus titer was calculated as $\log_{10}$ (p.f.u./ml) (*y*-axis). The virus titers in the lungs of immunized mice infected with different strains of influenza A virus were compared among themselves and with the titer in the lungs of unimmunized, mock-immunized and mock-infected mice (*x*-axis). The error bars indicate the S.D. derived from the replicates.

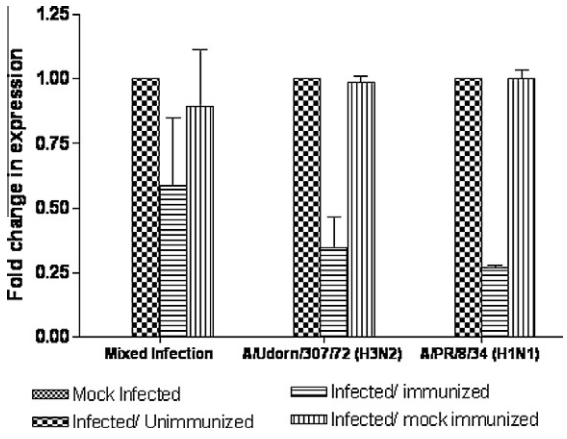

Fig. 6. Real Time RT-PCR for modulation of expression level of M1 gene of influenza virus in immunized mice. SYBR Green based real time RT PCR was performed in triplicate and the level of M1 RNA in different groups of immunized mice was compared by calculating ΔΔCt value for each group. The RNA level of M1 in the lungs of unimmunized was taken as the reference whose value was "1" and compared with other groups. The expression of M1 gene in immunized mice infected with A/PR/8/34 (H1N1) and A/Udorn/307/1072 (H3N2) was decreased by about 70% and was inhibited by about 45% in mice given mixed infection.

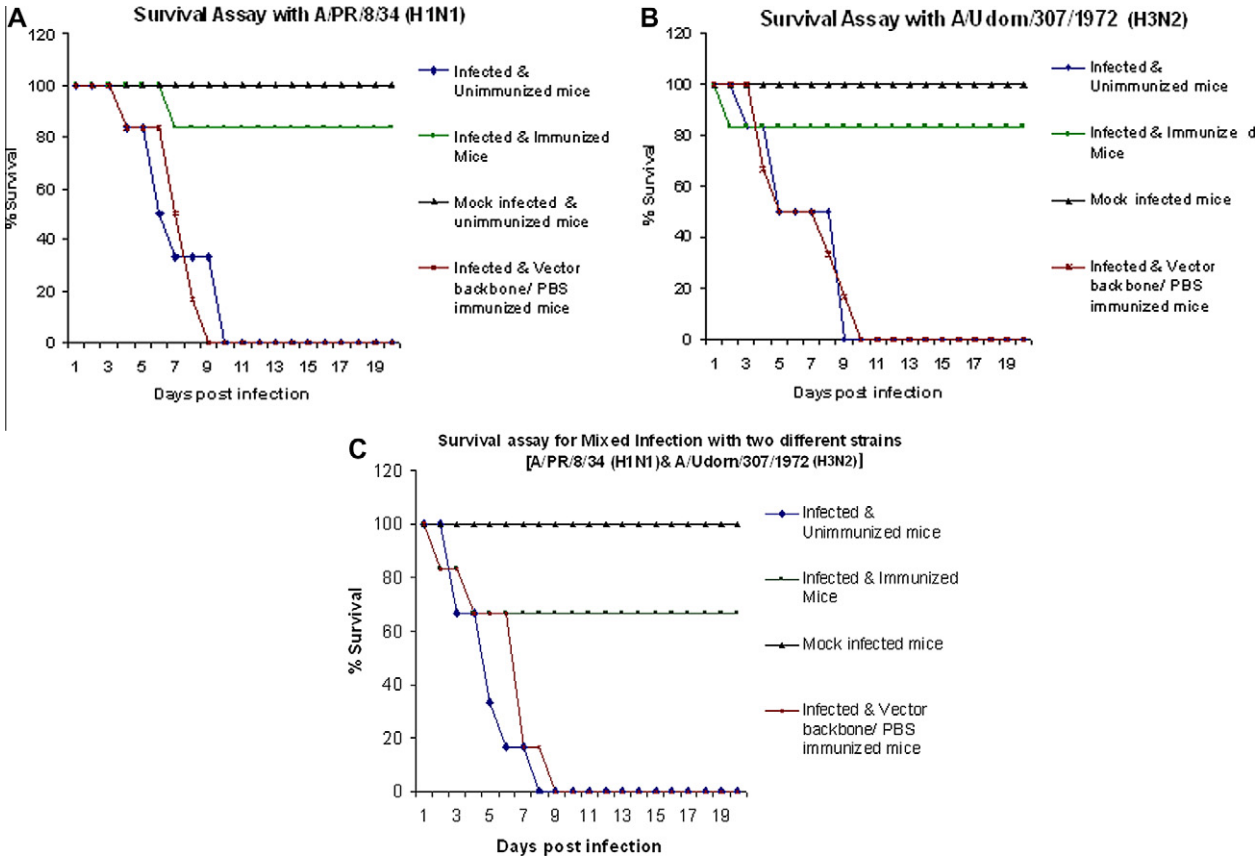

Fig. 7. Survival assay to assess the protective efficacy of pDNA immunization against the influenza A virus infection. The mice were immunized and infected as done for plaque assay. They were monitored twice daily up to twenty one days post infection. % Survival (*y*-axis) was calculated and the survival of immunized mice infected with influenza A viruses were compared with that of unimmunized, mock-immunized and mock-infected mice. (A) Comparison of % survival of immunized mice infected with A/PR/8/34 (H1N1) with that of unimmunized, mock-immunized and mock-infected mice. (B) Comparison of % survival of immunized mice infected with A/Udorn/8/34 (H3N2) with that of unimmunized, mock-immunized and mock-infected mice. (C) Comparison of % survival of immunized mice infected by A/PR/8/34 (H1N1) along with A/Udorn/8/34 (H3N2) with that of unimmunized, mock-immunized and mock-infected mice. The immunization with pDNA protected up to 83% of mice from the lethal effect of virus infection.

4. Discussion

Vaccines for influenza viruses are developed every year but the mortality caused by the virus is much higher than any other vaccine-preventable disease (Jansen et al., 2007). In spite of recent improvements including development of adjuvants to be used with traditional vaccines to enhance the antibody production (Vogel, 2000), the actual production capacity of these vaccines, their safety and efficiency in immunogenically naive populations are still not sufficient to control a pandemic situation. The DNA vaccines represent a powerful alternative to the conventional vaccine approach as they are non-infectious, non-replicating, extremely stable and can be produced at large scale at low cost (Kim and Jacob, 2009) and also elicit the cell-mediated immune response when inoculated via intra-dermal or intra-muscular route, thus giving better protection against the virus infection (Ulmer et al., 1994; Fu et al., 1999; Hu et al., 2006; Luo et al., 2008; Tao et al., 2009).

In the present study, we have generated a plasmid DNA (pDNA) encoding the conserved epitope of matrix protein of influenza A viruses for immunization and compared its immunogenicity with formalin-fixed virus and with the plasmid DNA encoding complete matrix protein of influenza A virus. The matrix epitope peptide was detected in lysate of the cells transfected with pDNA, which confirmed that the viral epitope could be successfully expressed by the pDNA in mammalian hosts. Earlier reports on DNA vaccination (Johnson et al., 2000; Okuda et al., 2001) mentioned the stimulation of cell-mediated immunity and induction of elevated levels of Th1 cytokines in response to virus infection. Our results lead to the assumption that pDNA construct and the plasmid encoding matrix protein may efficiently activate the host immune system. The pattern of cytokines secreted (Fig. 3) during the proliferation of splenocytes obtained from pDNA immunized mice point to the generation of cytotoxic T lymphocytes. The lysis of epitope peptide-pulsed MDCK cells by the stimulated splenocytes established that the epitope-specific CTLs were generated in the immunized animals.

Ramsay et al. (1997) reported that the mice immunized with plasmid DNA encoding full length nucleoprotein (NP) of influenza virus generated NP specific serum antibody along with memory CTLs (Ramsay et al., 1997). In contrast to the above study, the pDNA encoding the matrix epitope could not generate any detectable level of serum antibody before or after the booster immunization. Therefore, it could be inferred that the pDNA immunization purely elicited cell mediated immune response against the influenza virus. However, the immunization with formalin-fixed virus could stimulate a weak humoral immune response after one week of second immunization but the stimulation of cell-mediated immune response by these inactivated viruses was negligible.

Recently, it was reported that the live viral vaccines, but not the inactivated vaccines, could induce protective immunity against the highly pathogenic strains in mice (Kashima et al., 2009). The DNA vaccines encoding the full length viral genes (NP and HA) have also been shown to confer protection against the infection with influenza viruses (Xu et al., 2011). Our results are in agreement with

the published reports that there was significant reduction in virus replication in pDNA immunized mice as compared to the mock-immunized mice and the single epitope DNA immunization efficiently provided protection against two different strains [A/PR/8/34 (H1N1) and A/Udorn/307/1972 (H3N2)] of the pathogenic virus. The plasmid encoding the complete matrix protein could also stimulate the cell-mediated immune response but as shown in Fig. 4E, the CTLs generated against A/PR/8/34 (H1N1) could not efficiently lyse the cells-infected with A/Udorn/307/1972 (H3N2). These findings were significant as the cross-protective ability of the DNA vaccine based on the conserved viral epitopes may overcome the need of annual formulation of vaccines and could prevent the occurrence of pandemic situations.

Therefore, the vaccine based on minimal DNA constructs encoding the conserved viral epitope seems promising to provide efficient protection against the influenza A virus infection and resulted in broadened immune responses. This may be chosen as a suitable vaccination strategy against the virus infection. Further engineering of the plasmid DNA to encode more than one epitope of different viral proteins may result in better activation of immune response to provide cross-protection against the emerging viral strains. However, for the development of vaccines, the international surveillance and its formulations should never be overlooked.

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