Vaccination with Lentiviral Vector Expressing the nfa1 Gene Confers a Protective Immune Response to Mice Infected with Naegleria fowleri

Jong-Hyun Kim, Hae-Jin Sohn, Jinyoung Lee, Hee-Jong Yang, Yong-Joon Chwae, Kyongmin Kim, Sun Park, Ho-Joon Shin
Department of Microbiology, Ajou University School of Medicine, Suwon, Republic of Korea

Naegleria fowleri, a pathogenic free-living amoeba, causes fatal primary amoebic meningoencephalitis (PAM) in humans and animals. The nfa1 gene (360 bp), cloned from a cDNA library of N. fowleri, produces a 13.1-kDa recombinant protein which is located on pseudopodia, particularly the food cup structure. The nfa1 gene plays an important role in the pathogenesis of N. fowleri infection. To examine the effect of nfa1 DNA vaccination against N. fowleri infection, we constructed a lentiviral vector (pCDH) expressing the nfa1 gene. For the in vivo mouse study, BALB/c mice were intranasally vaccinated with viral particles of a viral vector expressing the nfa1 gene. To evaluate the effect of vaccination and immune responses of mice, we analyzed the IgG levels (IgG, IgG1, and IgG2a), cytokine induction (interleukin-4 [IL-4] and gamma interferon [IFN-γ]), and survival rates of mice that developed PAM. The levels of both IgG and IgG subclasses (IgG1 and IgG2a) in vaccinated mice were significantly increased. The cytokine analysis showed that vaccinated mice exhibited greater IL-4 and IFN-γ production than the other control groups, suggesting a Th1/Th2 mixed-type immune response. In vaccinated mice, high levels of Nfa1-specific IgG antibodies continued until 12 weeks postvaccination. The mice vaccinated with viral vector expressing the nfa1 gene also exhibited significantly higher survival rates (90%) after challenge with N. fowleri trophozoites. Finally, the nfa1 gene vaccination effectively induced protective immunity by humoral and cellular immune responses in N. fowleri-infected mice. These results suggest that DNA vaccination using a viral vector may be a potential tool against N. fowleri infection.

The pathogenic free-living amoeba Naegleria fowleri is commonly found in water, soil, and sediment (1). N. fowleri is the causal agent of primary amoebic meningoencephalitis (PAM) in animals and humans (2, 3). PAM is a fulminating disease, causing death within 1 to 2 weeks from the onset of symptoms (4). It occurs mainly in children and young adults and has been associated with swimming or water activities in contaminated waters (3, 5). The adherence of the amoeba is the critical initial step in the infection process, and N. fowleri enters the central nervous system (CNS) through the olfactory bulb (6). Amphotericin B is the only known agent for the treatment of N. fowleri infection (7–9). However, not all PAM patients treated with amphotericin B have survived, and amphotericin B has side effects (10, 11). Unfortunately, until now, there have been no satisfactory therapeutic agents for the treatment of PAM.

In a previous study, we cloned an antigenic gene, nfa1, from the N. fowleri cDNA library, which had a coding nucleotide sequence of 360 bp, producing a recombinant protein of 13.1 kDa (12). The nfa1 gene, which is involved with amoebic pseudopodial activity and especially with food cup formation, plays an important role in the pathogenicity of N. fowleri infection (13, 14). Moreover, an anti-Nfa1 antibody caused a decrease in the cytotoxicity of N. fowleri against target cells (15). Therefore, because the nfa1 gene is the key molecule concerned with cytotoxicity against host cells in regard to contact-dependent pathogenesis of N. fowleri, the nfa1 gene is an appropriate candidate for DNA vaccination.

In 1990, DNA vaccination was first introduced, and the induction of protein expression upon direct intramuscular injection of plasmid DNA into myocytes was demonstrated (16). DNA vaccination has been shown to be the most effective way of inducing specific humoral and cellular immune responses; this represents a promising strategy for protecting humans against pathogenic microorganisms, such as human immunodeficiency virus, mycobacteria, and parasites (17–19). Recently, lentiviral vectors have emerged as very promising vaccination tools. Lentiviral vectors have been widely used for the development of DNA vaccines to deliver genes effectively. Lentiviral vectors have been evaluated in various preclinical models of gene therapy and immunization because they can infect dividing and nondividing cells (20, 21). These vectors elicit both specific cytotoxic and strong humoral immune responses in animal models (22). Lentiviral vectors are regarded as promising vaccine vector candidates for the treatment of infectious disease and cancer (23).

Host protective immunity to N. fowleri infection has been studied in an in vivo model of PAM following administration of amoebic extracts, culture fluid, and amoebic trophozoites (24). Mice immunized with an intraperitoneal inoculation with live or killed trophozoites of N. fowleri showed variable levels of partial protective immunity (25). According to our previous studies, the nfa1 gene may be a proper candidate for DNA vaccination against N. fowleri infection (12–14, 26). Based on these findings, to evaluate the effect of our lentiviral vector systems expressing the nfa1 gene in the in vivo mouse model, vaccinated mice were tested for the development of specific immunity against N. fowleri infection, measured by humoral and cellular immune responses and by survival rates.
MATERIALS AND METHODS
Cultivation of *Naegleria fowleri*. Trophozoites of *N. fowleri* (Carter NF69 strain; American Type Culture Collection no. 30215) were cultured at 37°C in axenic Nelson’s medium supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Gaithersburg, MD) (27).

Expression and purification of recombinant Nfa1 protein. The recombinant Nfa1 (rNfa1) protein was produced according to the method previously described (12). Purified DNA (5 μg/μl) obtained from a PCR-T7/NT TOPO expression vector (Invitrogen, Groningen, Netherlands) containing the nfa1 gene was subsequently transferred to the BL21(DE3)-pLysS Escherichia coli strain using the heat shock method. Cells were cultured at 37°C in Luria-Bertani medium containing 100 μg/ml of ampicillin and 34 μg/ml of chloramphenicol (LAC) for selection. A transformed colony was selected and cultured in the LAC broth at 37°C. After 4 h of incubation with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), the cells were harvested by centrifugation (6,000g for 15 min). Transformed and nontransformed BL21(DE3)pLysS E. coli cells extracts were analyzed by SDS-PAGE, and the presence of the expressed gene product (a His tag fusion rNfa1 protein) was confirmed by Western blotting using sera from both immunized and infected mice. The rNfa1 protein was purified by metal affinity chromatography using a nickel-nitrilotriacetic acid (Ni-NTA) agarose column (Qiagen, Hilden, Germany), as described previously (12). The purified protein (5 μg/μl) was dialyzed in phosphate-buffered saline (PBS) (pH 7.4), and the purity of the rNfa1 protein was evaluated by SDS-PAGE and by Western blotting.

Construction of the nfa1 lentiviral vector and collection of viral particles. The nfa1 gene (GenBank accession no. AF230370) was previously cloned from an nfa1 gene-cloned vector, PCR-T7/NT TOPO (Invitrogen) (12). For the construction of the lentiviral vector expressing the nfa1 gene, the amplified egfp-nfa1 vector was digested with Nhel and EcoRI restriction enzymes and was cloned into the lentiviral vector pCDH (System Biosciences, Mountain View, CA) according to the method previously described (26). The egfp-nfa1 lentiviral vector was transformed into a ligation mixture into E. coli DH5α. The egfp-nfa1 vector was purified from transformed E. coli DH5α using a plasmid kit (Qiagen, Hilden, Germany). The lentiviral vector pCDH/egfp-nfa1 was packaged to 293T packaging cells with the pPACK packaging mix using a lentivector expression system (System Biosciences) according to the manufacturer’s instructions. At 48 h after transfection to packaging cells, we collected the medium from packaging cells, centrifuged the medium at 3,000 × g for 15 min at room temperature to pellet cell debris, and filtered the medium through a 0.45-μm filter. Each viral particle (VP) was aliquoted in cryogenic vials and stored at −70°C until ready for use. The titer of the viral particles was determined with a QuickFilter kit (Cell Biolabs, Inc., San Diego, CA) according to the manufacturer’s instructions.

DNA vaccination in mice. For the DNA vaccination, 6-week-old female BALB/c mice (Samtaco, Suwon, South Korea) were divided into five experimental groups, each consisting of 10 animals. All mice were anesthetized for the intranasal injection with a mixture of 5 mg/kg of body weight of ketamine and 0.5 mg/ml xylazine. In the uninfected groups, 50 μl of saline was intranasally instilled into the mice to verify the cause of death. Trophozoites, and the cumulative percentage of death was recorded on a daily basis. The mean time to death was also determined for each group. In order to verify the cause of death, brain tissue from dead mice was cultured at 37°C in Nelson’s medium, and *N. fowleri* parasites were observed microscopically.

Statistical analysis. Statistical differences between groups or samples were determined using the Student t test. The difference was considered statistically significant when the P value was <0.05.

RESULTS
Construction of a lentiviral vector expressing the nfa1 gene. To establish viral vector systems for DNA vaccination, we used a pCDH lentiviral vector as a backbone. In order to construct the viral vectors, amplified egfp-nfa1 was digested with restriction en-
zymes and was inserted into the pCDH vector (Fig. 1A). The flow-chart in Fig. 1B presents the schedule for vaccination, *N. fowleri* trophozoite inoculation, and immune responses of vaccinated mice. One week after the final vaccination, the mice were infected with *N. fowleri* trophozoites and monitored for the development of experimental PAM.

**Specific immune responses in vaccinated mice.** To determine specific immune responses in mice vaccinated with the lentiviral vector expressing the *nfa1* gene, all serum samples were tested using ELISAs. In the ELISAs, anti-Nfa1 monoclonal antibodies and PBS were used as the positive and negative controls, respectively. The uninfected (saline), uninfected (DMEM), and empty vector (5 \times 10^{11} VP) groups, representing mice vaccinated with saline, DMEM, or viral particles (lentiviral vector only), respectively, were used as controls for the *in vivo* mouse experiments. The pCDH-*nfa1* (2.5 \times 10^{13} VP) and pCDH-*nfa1* (5 \times 10^{11} VP) groups showed significantly higher levels of IgG than the control groups (uninfected [saline], uninfected [DMEM], and empty vector [5 \times 10^{11} VP]) \((P < 0.001)\). In the case of IgG, the optical density (OD) values for the pCDH-*nfa1* (2.5 \times 10^{13} VP) and pCDH-*nfa1* (5 \times 10^{11} VP) groups were 1.287 and 1.442, while the OD values for the uninfected (saline), uninfected (DMEM), and empty vector (5 \times 10^{11} VP) groups were 0.114, 0.127, and 0.154, respectively (Fig. 2). To determine whether Th1-type or Th2-type immune responses were elicited in the vaccinated mice, we also measured the levels of IgG subclasses (IgG1 and IgG2a). The levels of IgG1 as well as IgG2a in the pCDH-*nfa1* (2.5 \times 10^{13} VP) and pCDH-*nfa1* (5 \times 10^{11} VP) groups were significantly higher than those of the other control groups \((P < 0.001)\). These data show that the *nfa1* vaccination using a lentiviral vector efficiently induced Th1-type and Th2-type immune responses in the mice.

**Duration of IgG in vaccinated mice.** To evaluate the duration of the Nfa1-specific IgG antibody responses, Nfa1-specific IgG antibodies were evaluated by ELISAs in each group at 1, 4, 8, and 12 weeks after the last vaccination. As shown in Fig. 3, in the pCDH-*nfa1* (5 \times 10^{11} VP) group, higher levels of Nfa1-specific IgG antibodies were detected at 1 week and continued until 12 weeks postvaccination than those in the uninfected (saline) and empty vector (5 \times 10^{11} VP) groups. Moreover, the level of IgG in the pCDH-*nfa1* (5 \times 10^{11} VP) group was higher than that of the mice immunized with recombinant Nfa1 protein (rNfa1 group) used as a positive control \((P < 0.01)\) (Fig. 3).

**Cytokine responses of vaccinated mice.** To elucidate the T-cell responses in mice vaccinated with a viral vector expressing the *nfa1* gene, the levels of a type 1 cytokine (IFN-γ) and a type 2 cytokine (IL-4) in splenocytes stimulated with the Nfa1 protein were analyzed by ELISAs (Fig. 4). In the case of IFN-γ, the levels in the pCDH-*nfa1* (2.5 \times 10^{13} VP) and pCDH-*nfa1* (5 \times 10^{11} VP) groups were markedly enhanced compared with those of the other groups (uninfected [saline], uninfected [DMEM], and empty vector [5 \times 10^{11} VP]). In the case of IL-4, the levels were low in all groups, and no significant differences were observed among the groups.

**FIG 1** The map of the lentiviral vector and flow chart of the *in vivo* study. (A) The pCDH/egfp-*nfa1* vector was constructed. The egfp-*nfa1* gene was inserted into the multiple cloning site (MCS) of the lentiviral vector (pCDH). LTR, long terminal repeat; CMV, cytomegalovirus; SV40, simian virus 40; RSV, respiratory syncytial virus. (B) The schedule for mouse vaccination, *N. fowleri* infection, and *in vivo* experiments in this study.

**FIG 2** Specific immune responses in mice vaccinated with the lentiviral vector expressing the *nfa1* gene. The OD values of specific anti-Nfa1 IgG, IgG1, and IgG2a antibodies were measured at 405 nm using indirect ELISAs. Serum samples were collected from the mice 1 week after the last vaccination. The OD values are expressed as means ± SD from 3 independent experiments. Positive control, anti-Nfa1 monoclonal antibody; PBS, phosphate-buffered saline.

**FIG 3** Duration of the Nfa1-specific IgG levels in the sera of mice vaccinated with the lentiviral vector. The Nfa1-specific IgG responses were evaluated by ELISAs in each group at 1, 4, 8, and 12 weeks after the last vaccination. The uninfected (saline) and rNfa1 groups were used as negative- and positive-control groups, respectively.
The levels of IFN-γ in the pCDH-nfa1 (2.5 × 10^11 VP) and pCDH-nfa1 (5 × 10^11 VP) groups were increased in a dose-dependent manner (P < 0.01). Similarly, increased IL-4 levels were detected in splenocytes from mice vaccinated with the nfa1 gene (Fig. 4). The levels of IL-4 in the pCDH-nfa1 (2.5 × 10^11 VP) and pCDH-nfa1 (5 × 10^11 VP) groups were increased on a dose-dependent basis (P < 0.01). These data show that vaccination with a lentiviral vector expressing the nfa1 gene leads to Th1/Th2 mixed-type immune responses in mice.

Effective protection of vaccination in mice. To evaluate whether nfa1 vaccination could induce protection against N. fowleri infection, nfa1-vaccinated mice were challenged intranasally with 5 × 10^4 trophozoites of N. fowleri at 1 week after the last vaccination. We observed the mortality rate of the mice, and the cumulative percentage was recorded on a daily basis. The cause of death was confirmed by culturing the brain tissue of dead mice prior to examination under a light microscope to identify N. fowleri (data not shown). Survival rates of the different groups of mice are shown in Fig. 5 and Table 1. Substantially increased survival rates were found in the mice vaccinated with the viral vector expressing the nfa1 gene. Mice in the control groups (uninfected [saline], uninfected [DMEM], and empty vector [5 × 10^11 VP]) began dying on day 8, and all mice were dead by day 16 postinfection (Fig. 5). The mean times to death (MTDs) were 12.3, 12.3, and 12.7 days for the uninfected (saline), uninfected (DMEM), and empty vector (5 × 10^11 VP) groups, respectively (Table 1). On the other hand, the survival rates of the pCDH-nfa1 (2.5 × 10^11 VP) and pCDH-nfa1 (5 × 10^11 VP) groups were stably maintained at 70% and 90% throughout the experimental periods (1 month). The MTDs of the pCDH-nfa1 (2.5 × 10^11 VP) and pCDH-nfa1 (5 × 10^11 VP) groups were 15 and 20 days, respectively.

DISCUSSION

Infection with N. fowleri occurs by inhalation into the nasal cavity, and subsequently, the invasive amoeba enters the CNS through the olfactory apparatus (2, 3, 5, 6). In the infection process of the amoeba, the adherence to host cells is the critical step (6). Previously, we reported that the nfa1 gene cloned from the N. fowleri cDNA library is a key molecule that mediates contact between the amoeba and host cells (13, 14, 29). Recently, we constructed lentiviral vector systems expressing the nfa1 gene and showed that our vector system delivered the nfa1 gene effectively into the target cells (26). Therefore, to find a potentially effective DNA vaccina-
describes that vaccinated mice had longer mean times to death and higher survival within 16 days after nfa1 mice exhibited high levels of both IgG1 and IgG2a, suggesting that in the control groups. Consequently, these results suggest that Nfa1-specific IgG antibodies in the vaccinated mice was pro-

cant levels of Nfa1-specific IgG2a and IgG1 that are characteristic significantly in comparison with those in the control groups. Moreover, we previously reported that the intraperitoneal or intranasal immunization of Nfa1 protein immunization does not induce immune hypersensitivity (29). Furthermore, our results show that nfa1 vaccination through the intranasal route effectively induced mucosal immune responses and significantly increased the survival rate of N. fowleri-infected mice. Collectively, the intranasal route appears to be suitable in comparison with other routes for vaccination against N. fowleri infection because it effectively inhibits the adhesion of amoebae via a contact-dependent pathway.

A successful vaccine requires an efficient adjuvant system as well as a vector system. The suitable adjuvant is one that induces a more effective immune response. Furthermore, improvements in the vaccine strategy are required to determine factors such as the adjuvant combination, vector selection, antigen presentation pathways, immune status of the mice, and protection against infection. In conclusion, these results show that nfa1 vaccination is able to strongly enhance specific IgG and IgG subclass antibody responses and significantly increased the survival rate of N. fowleri-infected mice. This is the first report to evaluate in vivo immune responses of DNA vaccination against N. fowleri infection. Finally, our results should be helpful for future investigations into the detailed pathogenesis of N. fowleri infection. Furthermore, we strongly suggest that nfa1 vaccination may be a new potential tool for the treatment of N. fowleri infection.

ACKNOWLEDGMENT
This work was supported by the Mid-career Researcher Program through an NRF grant (2011-0015429) funded by the MEST.

REFERENCES


