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ORIGINAL PAPER

Protective immunity against *Naegleria fowleri* infection on mice immunized with the rNfa1 protein using mucosal adjuvants

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Abstract The free-living amoeba, *Naegleria fowleri*, causes a fatal disease called primary amoebic meningoencephalitis (PAM) in humans and experimental animals. Of the pathogenic mechanism of *N. fowleri* concerning host tissue invasion, the adherence of amoeba to hose cells is the most important. We previously cloned the *nfa1* gene from *N. fowleri*. The protein displayed immunolocalization in the pseudopodia, especially the food-cups structure, and was related to the contact-dependent mechanism of the amoebic pathogenicity in *N. fowleri* infection. The cholera toxin B subunit (CTB) and *Escherichia coli* heat-labile enterotoxin B subunit (LTB) have been used as potent mucosal adjuvants via the parenteral route of immunization in most cases. In this study, to examine the effect of protective immunity of the Nfa1 protein for *N. fowleri* infection with enhancement by CTB or LTB adjuvants, intranasally immunized BALB/c mice were infected with *N. fowleri* trophozoites for the development of PAM. The mean time to death of mice immunized with the Nfa1 protein using LTB or CTB adjuvant was prolonged by 5 or 8 days in comparison with that of the control mice. In particular, the survival rate of mice immunized with Nfa1 plus CTB was 100 % during the experimental period. The serum IgG levels were significantly increased in mice immunized with Nfa1 protein plus CTB or LTB adjuvants. These results suggest that the Nfa1 protein, with CTB or LTB adjuvants, induces strong protective immunity in mice with PAM due to *N. fowleri* infection.

Keywords *Naegleria fowleri* · Nfa1 protein · Cholera toxin · Heat-labile enterotoxin · Immunization · PAM

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Introduction

The free-living amoeba, *Naegleria fowleri*, is commonly found in widespread areas, including moist soil, sewage water, and sediment (Culbertson 1970; Willaert 1971). *N. fowleri* also exists as a virulent pathogen, causing fatal primary amebic meningoencephalitis (PAM) in experimental animals and humans (Carter 1968; Marciano-Cabral and Cabral 2007). PAM is a fulminating disease, causing death within 1 to 2 weeks after hospitalization (Visvesvara et al. 2007). It occurs mainly in healthy young adults and children, and has been associated with swimming or bathing in contaminated warm bodies of water (Marciano-Cabral and Cabral 2007). Adherence of the amoeba to host cells is the critical initial step in the infection process (Ma et al. 1990). Trophozoites of *N. fowleri* enter the central nervous system through the olfactory bulb by penetrating the nasal epithelium. *N. fowleri* has developed mechanisms to evade the host immune system. There are many reports which have demonstrated the immune responses against *N. fowleri*, such as IgA antibody, innate immunity, humoral immunity, the complement system,

neutrophils, macrophages, and cell-mediated immunity (Rivera-Aguilar et al. 2000; Marciano-Cabral and Cabral 2007).

Previously, to identify the pathogen-related molecules in *N. fowleri* infection, we cloned the *nfa1* gene from an *N. fowleri* complementary DNA (cDNA) library by means of immunoscreening using infected and immune mouse sera (Shin et al. 2001; Kang et al. 2005). This gene had a 360 bp coding sequence and was expressed as a recombinant Nfa1 protein (rNfa1) with a molecular weight of 13.1 kDa. By confocal microscopy, Nfa1 could be immunolocalized to the pseudopodia, especially to the food-cups, of the amoebic trophozoites (Shin et al. 2001; Kang et al. 2005). Anti-Nfa1 antibody reduced the cytotoxicity of *N. fowleri* in Chinese hamster ovary cells and rat microglial cells in a dose-dependent manner, suggesting that the Nfa1 protein is involved in the pathogenic mechanism of *N. fowleri* (Jeong et al. 2004; Oh et al. 2005; Lee et al. 2007).

According to previous study, mice immunized with nfa1 antigen had significant increase of the IgG levels as well as mean time to death compared with the control group (Lee et al. 2011). Lee et al. (2011) suggested that intranasal inoculation of the rNfa1 protein does not induce immune hypersensitivity, and that the mice immunized intranasally with rNfa1 protein demonstrated high levels of Nfa1-specific IgA antibody, along with prolonged survival time. Nfa1-specific IgA antibody may prevent the adherence of the *N. fowleri* trophozoites to the nasal mucosa and delay trophozoite invasion. Previous data indicate that immunization with the rNfa1 protein can induce partially protective immune responses, leading to prolonged survival times of the host upon *N. fowleri* infection. Moreover, the intranasal route of immunization would appear to be suitable for treatment or vaccination against *N. fowleri* infection. However, although rNfa1 protein increased the mean time to death of *N. fowleri*-infected mice, the immunization already infected mice was not found to increase survival. Consequently, further improvement of the immunization strategy is required to maintain protective immunity and increase the survival rate of mice. It is possible that the use of mucosal adjuvants such as cholera toxin (CT) or *Escherichia coli* heat-labile enterotoxin (LT) B subunit may induce a more effective immune response and stronger protection against *N. fowleri* infection upon intranasal immunization.

The most widely used mucosal adjuvants for experimentation in animals are CT and the closely related LT. CT and LT consist of a homo-pentamer of cell-binding B subunits associated with a single toxic-active A subunit. Both CT and LT are powerful mucosal adjuvants (Rappuoli et al. 1999). They strongly potentiate the immunogenicity of most other antigens, whether linked to or simply admixed with the toxins, provided that the antigen is given at the same time and at the same mucosal surface as the toxins. To avoid toxicity, the B units isolated from CT and LT (CTB and LTB) have been explored for their ability to augment immune responses against co-administered antigens (Holmgren et al. 2003a). In this study, these adjuvants were tested for ability to increase the immune responses in intranasal immunization, and observation was carried out on the Nfa1-specific IgG, IgA, and various cytokines, which may effectively prevent the adherence and invasion of *N. fowleri* trophozoites.

The present study was undertaken in an attempt to induce protective immunity in experimental *N. fowleri*-PAM mice by immunization with the rNfa1 protein using effective mucosal adjuvants, such as cholera toxin B (CTB) and heat-labile enterotoxin B (LTB). To evaluate the host immune response, the Nfa1-specific serum IgG (including IgG subclasses) and IgA were analyzed, and the levels of cytokines (IFN-γ, IL-2, IL-10, and IL-4) produced by splenocytes were measured after intranasal immunization with rNfa1 protein using mucosal adjuvants. Furthermore, the survival rate and mean time to death of the immunized mice after *N. fowleri* infection were estimated.

Materials and methods

N. fowleri culture

N. fowleri trophozoites (Cater NF69 strain; ATCC No. 30215) were cultured at 37 °C in axenic Nelson's medium supplemented with 10 % fetal bovine serum (FBS). Before use, *N. fowleri* was tested for the ability to induce PAM experimentally in mice.

Expression and purification of recombinant Nfa1 protein

The rNfa1 protein was produced according to the method previously described (Lee et al. 2011). Purified DNA (5 μg/ul) obtained from a PCR-T7/NT TOPO expression vector (Invitrogen, Groningen, Netherlands) containing the *nfa1* gene was subsequently transferred to the BL21(DE3)-pLysS *E. coli* strain using the heat-shock method. Cells were cultured at 37 °C in Luria-Bertani media containing 100 mg/ml of ampicillin and 34 mg/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 isopropylthiogalactose, the cells were harvested by centrifugation (8000 rpm for 15 min). Cell extracts from transformed and non-transformed BL21(DE3)-pLysS *E. coli* were analyzed by SDS-PAGE, and the presence of the expressed gene product (a his-tag fusion rNfa1 protein) was confirmed by Western blot using sera from both immunized and infected mice. The rNfa1 protein was purified by metal affinity chromatography using a Ni-NTA agarose column (QIAGEN Inc., Hilden, Germany), as described previously (Lee et al. 2011). Elution was carried out using imidazole buffer (5 M urea,

20 mM Na_2HPO_4, 5 M NaCl, and 500 mM imidazole). The protein was dialyzed in PBS (pH 7.4), and the purity of rNfa1 protein was evaluated by SDS-PAGE and Western blot.

Intranasal immunization

For the intranasal immunization, BALB/c mice (7-weeks old, female; KIST, Daejeon, Republic of Korea) were divided into six experimental groups, each containing ten animals. All mice were anesthetized with a mixture of 5 mg/kg ketamine, 0.5 mg/ml Rompun, and 0.9 % saline. Mice in the control group were immunized by intranasal exposure to 20 μl of phosphate-buffered saline (PBS, pH7.4) three times at 2-week intervals. In the rNfa1 only group, mice were first immunized with 50 μg of rNfa1 protein mixed with 20 μl of PBS, after which the second and third immunizations contained 25 μg rNfa1 mixed with 20 μl PBS. For adjuvant only groups (CTB or LTB alone), mice were immunized 5 μg of CTB or LTB (Sigma Chemical Co., St. Louis, MO, USA) mixed with 20 μl of pure distilled water, respectively. Finally, in the rNfa1 plus adjuvant groups (rNfa1 plus CTB or rNfa1 plus LTB), mice were immunized with 50 μg of rNfa1 protein mixed with 5 μg of CTB or LTB in a total volume of 20 μl. Second and third immunizations were carried out with 25 μg of rNfa1 protein mixed with 5 μg of CTB or LTB, respectively, in a total volume of 20 μl at 2-week intervals.

Serum collection

Bloods were collected from immunized mice 2 weeks after the third immunization. Sera containing the anti-Nfa1 polyclonal antibody were collected from the mouse blood by centrifugation at 13,000 rpm for 15 min at 4 °C. Immune sera were used for antibody titer analysis.

Antibody detection by enzyme-linked immunosorbent assay

In each group, three mice were used for antibody detection. Sera were tested for the presence of Nfa1-specific IgG, IgG subclass (IgG1 and IgG2a), and IgA by enzyme-linked immunosorbent assay (ELISA). Briefly, the experiments were performed using 96-well ELISA plates (Nunc, Roskilde, Denmark) in a reaction with 100 μl/well, containing 1 μg/ml rNfa1 protein mixed with coating buffer (0.05 M carbonate-bicarbonate, pH 9.6). After incubation and washing, the reaction was blocked with 3 % bovine serum albumin (Bovogen, Essendon, Australia) in PBS (pH 7.4) at 4 °C for 2 h. After washing, serum samples diluted in PBS were added to each well. After incubation and washing again, 100 μl of alkaline phosphatase-conjugated goat anti-mouse IgA, IgG, and IgG subclass (Sigma Chemical Co., St. Louis, MO, USA) were added to each appropriate well. After a final incubation and washing step, antibodies were detected with 4-nitrophenyl phosphate disodium salt hexahydrate substrate solution (pNPP; Sigma Chemical Co., St. Louis, MO, USA). The plate was read at 405 nm in a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Cytokine release assay

Spleens were removed from immunized mice under aseptic conditions 2 weeks after the final immunization. Single splenocyte suspensions were prepared by mashing the tissue through sterile metal screens in RPMI-1640 medium (Well gene, Daegu, Republic of Korea) supplemented with 2 mM L-glutamine, 100 U of penicillin/ml, and 100 μg of streptomycin/ml (complete RPMI-1640 medium). After lysis of red blood cells with lysis buffer and washing, splenocytes were resuspended in complete RPMI-1640 medium containing 10 % FBS. Splenocytes were adjusted to the density of 4×10^6 cells/ml and seeded in 24-well tissue culture plates (Nunc, Roskilde, Denmark). For cytokine induction, splenocytes were stimulated in vitro with 10 μg/ml of rNfa1 protein. After cultivation for 72 h at 37 °C in a humidified atmosphere with 5 % CO_2, culture supernatants were harvested and stored at −80 °C until assayed for cytokine production. Blood samples were allowed to clot for 2 h at room temperature or overnight at 2–8 °C before centrifuging for 20 min at 2000*g*. Serum was removed and assayed immediately, or aliquoted and stored at −80 °C until assayed for cytokine production. Cytokines produced by activated splenocytes and serum were measured by sandwich ELISA. Monoclonal antibodies specific for mouse IFN-γ, IL-2, IL-4, and IL-10 were used as the capture antibodies, and enzyme-linked polyclonal antibody was used as the reporter antibody, according to the manufacturer's instructions (R&D systems, Inc., NE, USA). Tetramethylbenzidine microwell peroxidase was used as substrate, and the reaction was terminated with 1 M H_3PO_4. The plate was read at 405 nm in a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Duration of the Nfa1-specific IgG levels in vaccinated mice

The Nfa1-specific IgG responses were evaluated by ELISA in each group at 1, 4, 8, and 12 weeks after the last vaccination. Saline and rNfa1 only groups were used as negative and positive controls, respectively, and received saline and rNfa1 protein under the same conditions.

Experimental PAM in mice model

In each group, all mice were infected with *N. fowleri* 2 week after the final immunization, and the development of experimental PAM was monitored. The infection dosage was described in a previous study (Lee et al. 2011). Briefly, *N. fowleri* trophozoites cultured for 3 days in Nelson's medium at 37 °C were harvested by centrifugation at 1500 rpm for 3 min, and the suspensions were adjusted to 1×10^4 trophozoites per 20 μl

using a hemocytometer. The *N. fowleri* trophozoites were then used for intranasal inoculation of 7-week-old female BALB/c mice (KIST, Daejeon, Republic of Korea), which were under anesthesia with a mixture of ketamine and Rompun. The suspension was slowly instilled into the nostril of each mouse. The dates of disease onset and death of the mice were recorded. Survival rate and mean time to death of experimental PAM mice were compared between groups using the Student's *t* test. Animal card and experimental procedures were carried out under approval from the animal care committee of Ajou University School of Medicine (AMC66).

Statistical analysis

Statistical differences between groups or samples were determined with the Student's *t* test. The differences were considered significant when P was <0.05.

Results

Production of recombinant Nfa1 protein and flow-chart of in vivo study

A 17-kDa recombinant His-tag fusion Nfa1 protein was produced, and then the final 13.1 kDa protein was purified on a nickel resin column (data not shown) and used for immunization. A flow-chart presenting the schedule for immunization, *N. fowleri* inoculation, and check of immune responses in immunized mice is provided in Fig. 1. Mice were infected with *N. fowleri* trophozoites 1 week after the final immunization and monitored for the development of experimental PAM.

Specific antibody formation in immunized mice

To determine the specific immune response in mice immunized with rNfa1 protein plus mucosal adjuvants (CTB or LTB), all sera were tested using ELISA, which measured Nfa1-specific IgG, IgG subclass (IgG1 and IgG2a), and IgA antibodies. In ELISA experiments, the group immunized with PBS, hereafter referred to as immunized (saline), was used as a negative control. The immunized (CTB), immunized (LTB), immunized (rNfa1), immunized (rNfa1 + CTB), and immunized (rNfa1 + LTB), groups represented mice immunized with CTB, LTB, rNfa1, rNfa1 plus CTB, and rNfa1 plus LTB, respectively. The immunized (rNfa1), immunized (rNfa1+CTB), and immunized (rNfa1+LTB) groups showed significantly higher levels of IgG than the control groups (immunized (saline), immunized (CTB), and immunized (LTB)) (P<0.001). In the case of IgG, the optical density (OD) values of the immunized (rNfa1+CTB) and immunized (rNfa1+LTB) groups were 1.344 and 1.178, while the OD values of the immunized (saline), immunized (CTB), and immunized (LTB) groups were 0.112, 0.114, and 0.127, respectively (Fig. 2). To determine whether Th1 type or Th2 type immune response was elicited in the immunized mice, the levels of IgG subclass (IgG1 and IgG2a) were also measured. The levels of both IgG1 and IgG2a in the immunized (rNfa1), immunized (rNfa1+CTB), and immunized (rNfa1+LTB) groups were significantly higher than in the control groups (P<0.001). Moreover, in the case of IgA, the immunized (rNfa1), immunized (rNfa1+CTB), and immunized (rNfa1+LTB) groups also showed significantly higher levels than the controls (P<0.001).

Duration of Nfa1-specific IgG in immunized mice

To evaluate the duration of the Nfa1-specific IgG antibody responses, Nfa1-specific IgG antibody was evaluated by ELISA in each group at 1, 4, 8, and 12 weeks after the last immunization. As shown in Fig. 3, in the immunized (rNfa1), immunized (rNfa1+CTB), and immunized (rNfa1+LTB) groups, high levels of Nfa1-specific IgG antibody were detected at 1 week, which continued until 12 weeks post-immunization, in contrast to the immunized (saline), immunized (CTB), and immunized (LTB) controls (P<0.001) (Fig. 3).

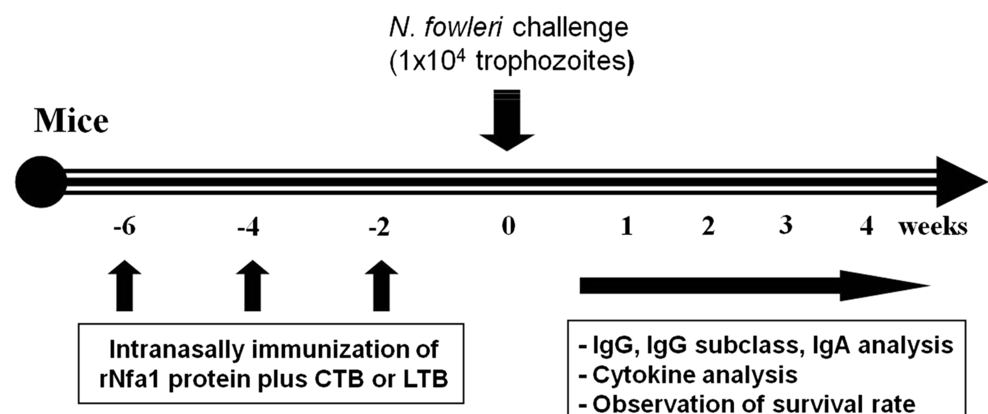

Fig. 1 Flow-chart of the schedule for mice immunization and experimental PAM development. The flow-chart presents the schedule for mice vaccination, *N. fowleri* infection, and in vivo experiments in this study

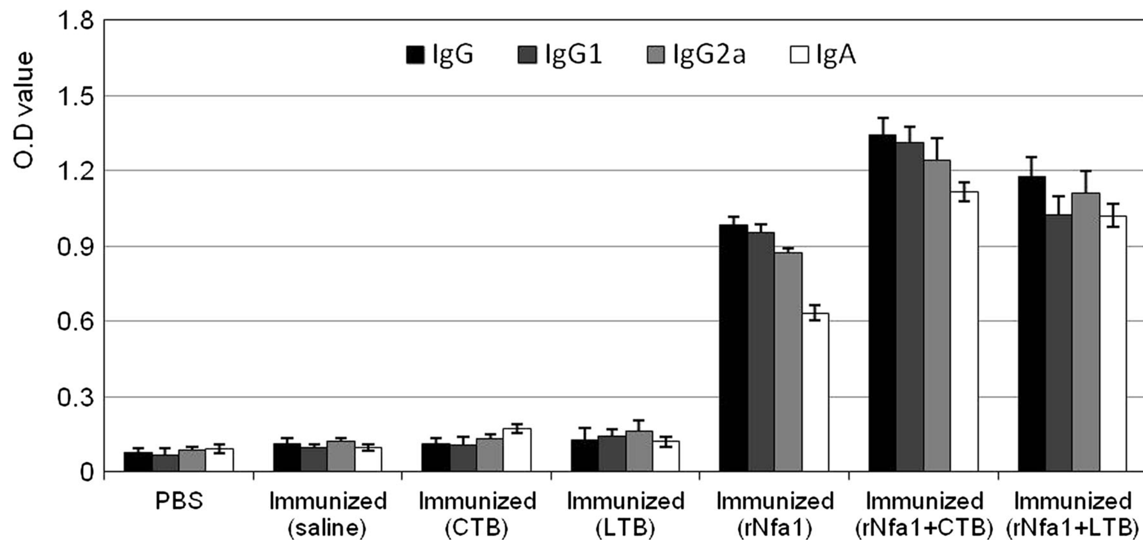

Fig. 2 Nfa1-specific immune response in mice after intranasal immunization with the rNfa1 protein. The OD values of specific anti-nfa1 IgG, IgG1, IgG2a, and IgA antibodies were measured at 405 nm using indirect ELISA. Sera of mice were collected 1 week after the last immunization. The OD values are expressed as mean±SD with three independent experiments. *PBS* phosphate-buffered saline

Cytokine response of immunized mice

To elucidate the T cell response in mice immunized with rNfa1 protein, the levels of cytokines (IFN-γ, IL-2, IL-4, and IL-10) from rNfa1-stimulated splenocytes were analyzed by ELISA (Fig. 4). The rNfa1-stimulated splenocytes in all groups except immunized (saline) produced high levels of type-1 cytokine (IFN-γ), and type-2 cytokine (IL-4), as well as the regulatory cytokines (IL-2 and IL-10). In all cases, the levels of cytokines were significantly increased in comparison with the control group (immunized (saline)) ($P<0.001$) (Fig. 4). In particular, the splenocytes of mice in the immunized (rNfa1+CTB) group showed the highest levels of all kinds of cytokines tested. These results show that the immunization of rNfa1 protein with addition of CTB or LTB leads to a Th1/Th2/Treg mixed-type immune response in mice.

Protective immunity of immunized mice

In order to determine whether the immunization with rNfa1 protein in the presence of mucosal adjuvants (CTB or LTB) could induce protection against *N. fowleri* infection, mice from each group were challenged by intranasal infection with 1×10^4 trophozoites of *N. fowleri* 1 week after the last immunization. Mortality of the mice was observed, and the cumulative percentage was recorded on a daily basis (Fig. 5; Table 1). 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). Mice of the immunized (saline) control group began dying on day 11, and all the mice were dead by day 19 post-infection (Fig. 5), resulting in a mean time to death (MTD) of were 14.3 days (Table 1). In the case of the immunized (rNfa1) group, although prolonged MTD was observed (24.4 days), the survival rate was 0 %. In contrast, the survival rates of the immunized (CTB) and immunized (LTB) groups were 60 and 40 %, respectively. In the case of the immunized (rNfa1+CTB) and immunized (rNfa1+LTB) groups, the survival rates were particularly high, demonstrating stable maintenance at 100 and 80 %, respectively, throughout the experimental period (1 month).

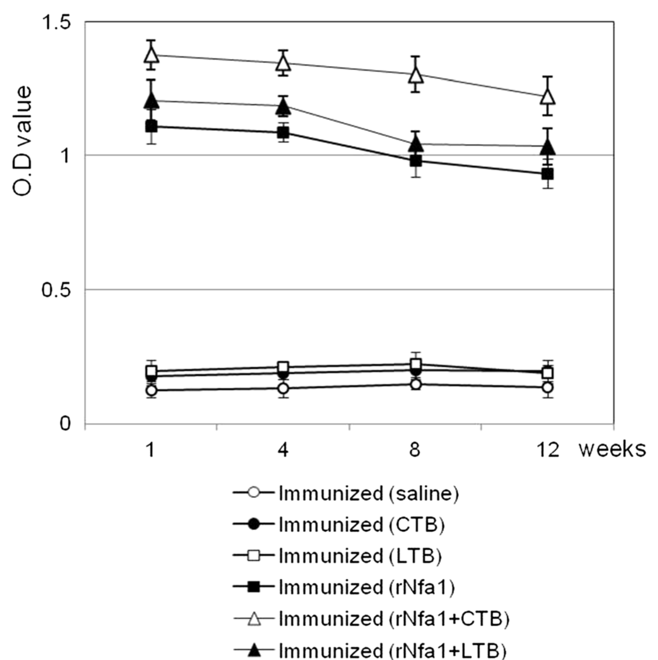

Fig. 3 Duration of the Nfa1-specific IgG levels in the sera of mice immunized with rNfa1. The Nfa1-specific IgG responses were evaluated by ELISA in each group at 1, 4, 8, and 12 weeks after the last immunization. The OD values are expressed as mean±SD with three independent experiments

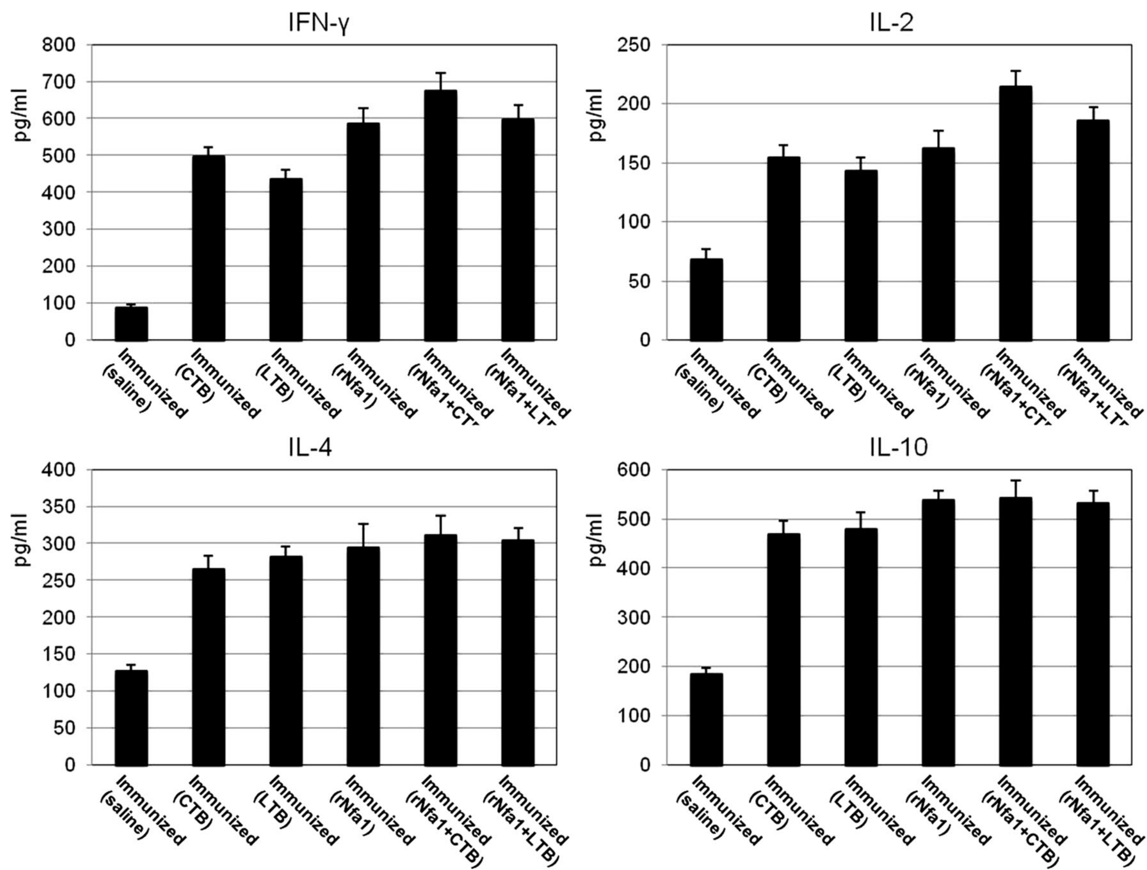

Fig. 4 Production of cytokines by splenocytes from mice immunized with rNfa1 protein. Splenocytes were collected 2 weeks after the last vaccination. The supernatants were collected 72 h after stimulation with 10 μg/ml rNfa1 protein. The values are expressed as mean±SD with three mice per group

Discussion

The trophozoites of *N. fowleri* enter the nasal cavity and then migrate to the nasal mucosa and the olfactory nerve (Carter 1968, 1972; Ma et al. 1990). They cause an acute lethal central nervous system disease called PAM (Anderson and Jamieson 1972). Adherence to host cells is known to be the critical step in the invasion process of *N. fowleri*. Previously, we reported that the *nfa1* gene cloned from *N. fowleri* cDNA library is a key molecule involved in mediation of the contact mechanism to the host target cells. The Nfa1 protein was found to be localized in the pseudopodia, which plays an important role

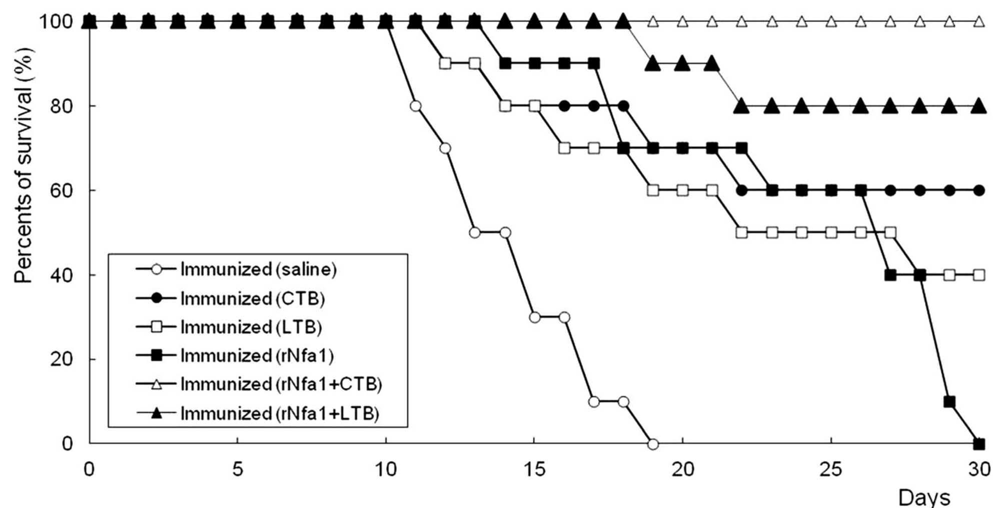

Fig. 5 Survival curves of immunized mice after *N. fowleri* infection. The mice were intranasally challenged with 1×10^4 *N. fowleri* trophozoites 2 weeks after the last immunization and observed for mortality. Each group had ten mice. Immunized (saline) was used as the control group

Table 1 Survival and mean time to death of *N. fowleri*-infected mice immunized with rNfa1 protein plus mucosal adjuvants

Group[a]	No of mice	No. of survived mice[b] (%)	MTD[c] (days)
Immunized (saline)	10	0 (0)	14.3
Immunized (CTB)	10	6 (60)	16.8
Immunized (LTB)	10	4 (40)	18.5
Immunized (rNfal)	10	0 (0)	24.4
Immunized (rNfa1+CTB)	10	10 (100)	0
Immunized (rNfa1+LTB)	10	8 (80)	20.5

[a] Immunized (saline) was used as control group and received saline under same conditions. All mice were intranasally inoculated with 1×10^4 trophozoites of *N. fowleri*

[b] Mice were held for 30 days after inoculation, and the cumulative percentage was recorded on a daily basis

[c] *MTD* mean time to death calculated by only dead mice

in the pathogenesis of *N. fowleri* (Cho et al. 2003; Kang et al. 2005; Lee et al. 2007, 2011). Therefore, the *nfa1* gene was considered as a strong candidate for treatment or vaccination, as the Nfa1 protein is a key factor involved in the contact and attack of host cells.

This study was carried out to determine the specific immune response in rNfa1-immunization carried out using mucosal adjuvants, such as CTB and LTB. Nfa1-specific IgG, IgG1, IgG2a, and IgA production, as well as the levels of cytokines, were measured after immunization. The levels of IgG and IgG subclass in the mice immunized with rNfa1 plus mucosal adjuvants were significantly increased in comparison with the control groups. Moreover, significant increase in the levels of rNfa1-specific IgA was also observed. These results suggest that the intranasal route of immunization appears to be suitable, and that immunization with the rNfa1 protein plus mucosal adjuvants can induce protective immune responses to *N. fowleri* infection.

Mucosal immunization has attracted much interest for good reason, mainly as a means to induce protective immunity against infections, but also as a possible approach toward specific immunotherapy in conditions caused by harmful immune responses to autoantigens or selected foreign antigens resulting in tissue damage (Czerkinsky et al. 1999; Wu and Weiner 2003). When it comes to specific adjuvants, the best-studied and most potent mucosal adjuvants in experimental systems are CTB and LTB, and much effort has recently been made to generate toxicologically acceptable derivatives of these toxins (Holmgren et al. 2003b; Plant and Williams 2004). The adjuvanticity of CTB or LTB is much improved when coupled to antigens, possibly because of efficient presentation of the coupled antigen not only by DC and macrophages but also by naive B cells (George-Chandy et al. 2001). Most adjuvants exert at least some of their adjuvant activity through the induction of inflammatory or Th1-inducing cytokines and chemokines.

In a recent report, the intranasal co-administration of cholera toxin with amoeba lysates modulated the secretion of IgA and IgG antibodies, production of cytokines, and expression of polymeric Ig receptor (pIgR) in the nasal cavity of mice in the model of *N. fowleri* meningoencephalitis (Carrasco-Yepez et al. 2014). The influences of immunization and challenge on the production of inflammatory cytokines were also examined (TNF-γ and IL-1β). In the present study, nasal immunization with rNfa1 protein using mucosal adjuvants (CTB and LTB) was observed to result in induction of Nfa1-specific IgA and IgG subclasses (IgG1 and IgG2a) in *N. fowleri*-infected mice, compared to control mice. In comparison with the immunization carried out with rNfa1 protein alone, the utilization of mucosal adjuvants increased the immune response, as demonstrated by the levels of Nfa1-specific Ig antibodies and cytokines in *N. fowleri*-infected mice.

In the present study, mice immunized with rNfa1 protein using CTB demonstrated the survival rate of 100 % after *N. fowleri* infection. According to previous studies, CTB is an effective mucosal adjuvant for supporting the induction of Ag-specific mucosal and systemic immune responses (Berstad et al. 1997). It has been claimed that CTB primarily induces Th2 type immune responses characterized by $CD4^+$ T cells producing IL-4, IL-5, IL-6, and IL-10, and by the production of IgA, IgG1, and IgE antibodies. LTB, on the other hand, has been reported to induce a mixed Th1 and Th2 type immune response. However, other studies showed that CT can also induce mixed Th1 and Th2 type immune responses, in contrast to CTB, which appears to induce a more restricted Th2 type of immune response (Eriksson et al. 2003). Although the exact mode of the CT immunomodulator function is still not yet well understood, recent study suggests that mucosal co-administration of CTB is involved in the induction and regulation of Ag-specific Th1 and Th2 responses (Sanchez and Holmgren 2008). Therefore, it is possible that the coexistence of the Th1 and Th2 cytokines produced by the *N. fowleri* infection model herein may provide a highly effective immunological environment for the production of Ag-specific IgG and IgA response.

CTB and LTB can affect several steps in the induction of mucosal immune response. These adjuvants increase the permeability of the intestinal epithelium, leading to enhanced uptake of co-administered antigens and enhanced antigen presentation by various antigen-presenting cells. In the case of other parasites, mucosal administration of CTB conjugates can also prevent infection-induced pathologic changes. Conjugates of CTB and *Schistosoma mansoni* egg and/or worm antigen, or selected peptides from such antigens genetically fused to CTB, were found to reduce mortality and suppress hepatic granuloma formation following *S. mansoni* infection when given nasally to infected mice, in a process that

involves the appearance of TGF-β-producing T cells (Sun et al. 2001; Lebens et al. 2003).

In the present study, when mice were immunized with rNfa1 protein plus CTB or LTB, splenocytes from the immunized mice secreted Th1 type cytokines (IFN-γ), Th2 type cytokines (IL-4), and regulatory cytokines (IL-2 and IL-10). These results suggest that the immunization with rNfa1 protein using CTB and LTB triggered a Th1/Th2/Treg mixed-type immune response in *N. fowleri*-infected mice, although more detailed examination is required. Moreover, the survival rates of the rNfa1 plus CTB or LTB immunized groups were stably maintained to 100 and 80 % throughout the experimental period. All the immunized mice (rNfa1 protein or irrespective of presence of adjuvant) showed a prolonged survival time in comparison with the control mice. These data indicate that immunization with rNfa1 protein can induce protective immune responses, leading to the prolonged survival of *N. fowleri*-infected mice. Finally, the results demonstrated that the Nfa1 protein is a candidate antigen for treatment as well as vaccination against *N. fowleri* infection. Further studies are required to determine the mechanisms, including the antigen presentation pathways, combination of adjuvants, immune status of the mice, and protection against *N. fowleri* infection.

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