

Effects of immunization with the rNfa1 protein on experimental *Naegleria fowleri*-PAM mice

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SUMMARY

Free-living Naegleria fowleri causes primary amoebic meningoencephalitis (PAM) in humans and animals. To examine the effect of immunization with Nfa1 protein on experimental murine PAM because of N. fowleri, BALB/c mice were intra-peritoneally or intra-nasally immunized with a recombinant Nfa1 protein. We analysed Nfa1-specific antibody and cytokine induction, and the mean survival time of infected mice. Mice immunized intra-peritoneally or intra-nasally with rNfa1 protein developed specific IgG, IgA and IgE antibodies; the IgG response was dominated by IgG1, followed by IgG2b, IgG2a and IgG3. High levels of the Th1 cytokine, IFN- γ , and the regulatory cytokine, IL-10, were also induced. The mean survival time of mice immunized intra-peritoneally with rNfa1 protein was prolonged compared with controls, (25.0 and 15.5 days, respectively). Similarly, the mean survival time of mice immunized intra-nasally with rNfa1 protein was 24.7 days, compared with 15.0 days for controls.

Keywords cytokine, *Naegleria fowleri*, primary amoebic meningoencephalitis

INTRODUCTION

The free-living amoeba, *Naegleria fowleri*, is found in water and soil and is the causal agent of primary amoebic meningoencephalitis (PAM) in animals and humans (1,2). PAM is a fulminating disease, causing death within 1–2 weeks after hospitalization (3). Although some 30 species of *Naegleria* have been recognized based upon

sequencing data, *N. fowleri* is the only one that has been isolated from cases of PAM. Adherence of amoeba to host cells is the critical initial step in the infection process. The ability of trophozoites to attach themselves to the nasal mucosal, an increased rate of locomotion, the formation of food-cup structure and a chemotactic response to nerve cell components, may all be important in disease progression (4–7).

In an attempt to identify antigen- or pathogen-related molecules with a potential use as immunogens and diagnostic agents in *N. fowleri* infection, we previously cloned an *nfa1* gene from an *N. fowleri* cDNA library by means of immunoscreening using infected and immune mouse sera. 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 pseudopodia, especially to the food-cups, of amoebic trophozoites (8,9). Anti-Nfa1 polyclonal antibody treatment reduced the cytotoxicity of *N. fowleri* for Chinese hamster ovary cells and rat microglial cells in a dose-dependent manner, suggesting that the Nfa1 protein is involved in a pathogenic mechanism of *N. fowleri* (7,10). An anti-Nfa1 monoclonal antibody also decreased the cytotoxicity of *N. fowleri* trophozoites on rat microglial cells (11).

Human sera from healthy individuals as well as from PAM patients were found to contain antibodies to *N. fowleri* antigen, indicating that exposure to the amoeba is common (12,13). Furthermore, a number of studies have been carried out to determine the role of particular antibody isotypes, such as IgA, IgG and IgM, in host defense to *Naegleria* infection (14–16). In mice experimentally infected with *N. fowleri* trophozoites via the nasal route, circulating IgG was observed from day 7 after infection demonstrated by ELISA (15). Additional studies of experimental infections in mice and of PAM patients have been limited to the analysis of serum antibody responses (17–19).

Host protective immunity to *Naegleria* infection has been studied in the mouse model of PAM following

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repeated administration of formalinized amoebae, freeze-thawed amoebic extracts, amoeba culture fluid and sublethal doses of live amoebae (19,20). Furthermore, mice immunized by intra-peritoneal inoculation of live or killed trophozoites of *N. fowleri* showed variable levels of partial protective immunity (15,21). Although marked humoral responses were induced with all these immunization regimens, only a modest protection against *N. fowleri* infection was observed (2).

The present study was undertaken in an attempt to induce protective immunity in experimental *N. fowleri*-PAM mice by immunization with the rNfa1 protein. To evaluate the host immune response, the Nfa1-specific serum IgG (including IgG subclasses), IgA and IgE were analysed, and the levels of cytokines (IFN- γ , IL-2, IL-10 and IL-4) produced by splenocytes were measured after immunization with the rNfa1 protein intra-peritoneally (i.p.) or intra-nasally (i.n.). Furthermore, we determined the survival rate and mean time to death of immunized mice after lethal challenge with *N. fowleri* trophozoites.

MATERIALS AND METHODS

Culture of *Naegleria fowleri* trophozoites

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% foetal bovine serum (FBS) (22).

Expression and purification of recombinant Nfa1 protein

The rNfa1 protein was produced according to the method previously described (9). Purified DNA (5 μ g/ μ L) obtained from a PCR-T7/NT TOPO expression vector (Invitrogen, Grohingen, 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 media containing 100 μ g/mL of ampicillin and 34 μ g/mL of chloramphenicol (LAC) for selection. A transformed colony was selected and cultured in the LAC broth at 37°C. After 4 h incubation with 1 mM isopropyl thiogalactose, the cells were harvested by centrifugation (6000 $\times$ *g* for 15 min). Cell extracts from transformed and non-transformed BL21(DE3)pLysS *E. coli* were analysed 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 (11). Elutions were car-

ried out using imidazole buffer (5 M urea, 20 mM Na₂HPO₄, 5 M NaCl and 500 mM imidazole). The protein was dialysed in PBS (pH 7.4), and the purity of rNfa1 protein was evaluated by SDS-PAGE and Western blot.

Intraperitoneal immunization

For intra-peritoneal immunization, BALB/c mice [6-week-old female, purchased from Samtaco (Suwon, Korea)] were divided into four experimental groups of 60 animals each. Mice in group I were injected intra-peritoneally with 200 μ L of PBS (phosphate-buffered saline, pH 7.4) three times at 2 weeks intervals. In group II, as another control, mice were first injected with 100 μ L of Freund's complete adjuvant (Sigma Chemical Co., St. Louis, MO, USA) mixed with 100 μ L of PBS, and then injected with two doses of Freund's incomplete adjuvant (Sigma) mixed with PBS in a total volume of 200 μ L; injections were given at 2-week intervals. Mice in group III were intra-peritoneally injected with 50 μ g of rNfa1 protein mixed with Freund's complete adjuvant in a total volume of 200 μ L. Second and third immunizations were carried out with 25 μ g of rNfa1 protein mixed with Freund's incomplete adjuvant in a total volume of 200 μ L at 2-week intervals. In group IV, mice were first injected with 50 μ g of rNfa1 protein mixed with PBS in a total volume of 200 μ L. Second and third immunizations were carried out with 25 μ g of rNfa1 protein mixed with PBS in a total volume of 200 μ L at 2 weeks intervals.

Intranasal immunization

For the intra-nasal immunization, BALB/c mice (6-week-old female) were divided into two experimental groups, each of 60 animals. All mice were anesthetized with a mixture of 5 mg/kg of ketamine, 0.5 mg/mL of rompun and PBS (pH 7.4). In group V, as another control, 20 μ L of PBS was slowly instilled into the nostril of each mouse. Mice in group VI were intra-nasally immunized with 50 μ g of rNfa1 protein mixed with PBS in a total volume of 20 μ L. Second and third immunizations were carried out with 25 μ g of rNfa1 protein mixed with PBS in a total volume of 20 μ L at 2 weeks intervals.

Detection of antibodies

Two weeks after the final immunization, blood samples of nine mice from each group were analysed for antibodies. Serum collected from the tail vein was centrifuged and tested for the presence of the Nfa1-specific total IgG, IgG subclasses (IgG1, IgG2a, IgG2b and IgG3), IgA and IgE, by ELISA. The rNfa1 protein (2 μ g/mL in coating buffer:

0.05 M carbonate-bicarbonate, pH 9.6) was placed into 96-well ELISA plates (Nunc, Roskilde, Denmark) in 100 µL/well reaction. After incubation and washing, the reactions were blocked with 3% bovine serum albumin (Sigma) 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, 100 µL of alkaline phosphatase (AP)-conjugated goat anti-mouse IgE (Bethyl Laboratories, Montgomery, TX, USA) or 100 µL of horse-radish peroxidase-conjugated goat anti-mouse IgG or IgA, or IgG subclass antibodies (Bethyl Laboratories) were added to each well. After incubation and washing, the antibody was detected with 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) substrate solution (ABTS; Kirkegard and Laboratories, Gaithersburg, MD, USA) and the colouring reaction was terminated with 100 µL of 1% SDS. The plates were read at 405 nm in a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Duration of the Nfa1-specific IgG levels in immune sera

To observe the duration of the Nfa1-specific IgG levels in immunized mice sera, the Nfa1-specific IgG response was evaluated at 2, 6 and 12 weeks post-intra-peritoneal or intra-nasal immunization in three mice from each group.

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 (Gibco BRL; Life Technologies, Grand Island, NY, USA) 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 (Sigma) and washing, splenocytes were resuspended in complete RPMI-1640 medium containing 10% FBS. Splenocytes were adjusted at 4×10^6 cells/mL and seeded out on 24-well tissue culture plates (Nunc). 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% CO₂, culture supernatants were harvested and stored at -80°C until being assayed for cytokine production. Cytokine produced by activated splenocytes were measured by sandwich ELISA. Purified anti-IFN-γ, IL-2, IL-10 and IL-4 were used as capture antibody, and biotinylated antibody was used as reporter antibody according to manufacturer's instructions (Biosource International, Camarillo, CA, USA). Tetramethylbenzidine microwell peroxidase was used as substrate and the reaction

was terminated by 1 M H₃PO₄. The plates were read at 450 nm in a microplate reader (Bio-Rad).

Development of experimental PAM by inoculation of *N. fowleri* trophozoites

Two weeks after the final immunization, mice were infected with *N. fowleri* and monitored for the development of experimental PAM (Fig. 1). In a preliminary experiment, to determine the lethal dose of amoebic trophozoites, mice were intra-nasally inoculated with 1×10^3 , 1×10^4 or 1×10^5 trophozoites of *N. fowleri*, respectively. A lethal dose was found to be 1×10^4 trophozoites, as at this dose, all control mice died by day 17 post-infection. Thus, trophozoites of *N. fowleri* were harvested from the culture system and suspended in sterile PBS (pH 7.4). Using a hemacytometer, the suspensions were adjusted to 1×10^4 trophozoites per 20 µL. Under anaesthesia 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 mice were recorded. Survival rates and mean time to death of experimental PAM mice were compared between groups using Student's *t* test. Animal care and experimental procedures were carried out under approval from the Animal Care Committee of Ajou University School of Medicine (AMC66). The onset of PAM was confirmed by histopathological examination of brain tissue. Tissue samples were fixed in 10% neutral-buffered formalin (4% formaldehyde in PBS) and embedded in paraffin. Thick sections (5 µm) were placed on albuminized glass slides, stained with haematoxylin and eosin and examined by light microscopy.

RESULTS

Production of the recombinant Nfa1 protein

A 17-kDa recombinant His-tag fusion Nfa1 protein was produced according the method described previously (11). After enzymatic digestion, the final 13.1 kDa-rNfa1

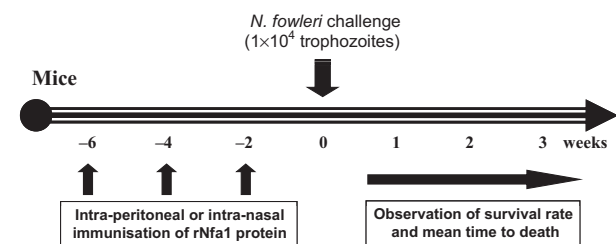


Figure 1 Flow-chart of the schedule for mice immunization and experimental primary amoebic meningoencephalitis development.

protein was purified on a nickel resin column (data not shown) and used for immunization.

Antibody formation in immunized mice

Nfa1-specific IgG, IgA, IgE and IgG subclass antibodies were measured by ELISA. As shown in Fig. 2, mice immunized either intra-peritoneally or intra-nasally with rNfa1 protein mixed with Freund's complete adjuvant (group III) or rNfa1 protein alone (group IV) displayed significantly higher levels of IgG and IgA than control mice (group I or II) (t test, $P < 0.005$). In contrast, however, Nfa1-specific IgE antibody was not detected in any of the experimental groups.

Figure 3 shows the results of the Nfa1-specific IgG subclass (IgG1, IgG2a, IgG2b and IgG3) ELISA. Mice in group III (rNfa1 + adjuvant, intra-peritoneal immunization), IV (rNfa1 only, intra-peritoneal) and VI (rNfa1 only, intra-nasal) developed significantly higher levels of

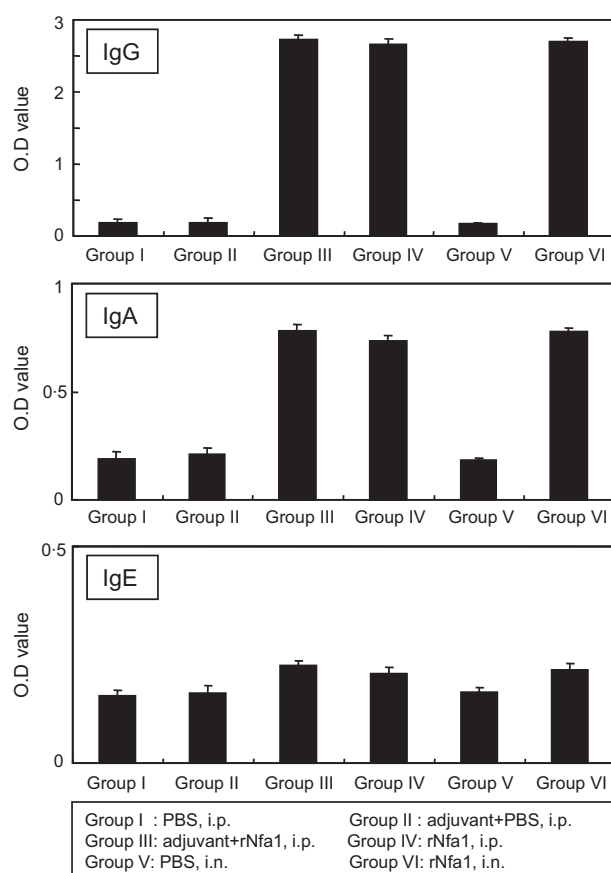


Figure 2 Nfa1-specific IgG, IgA and IgE antibody levels in sera of mice intra-peritoneally (i.p.) or intra-nasally (i.n.) immunized with the rNfa1 protein. Antibodies were measured 2 weeks after last immunization. The O.D. values are expressed as mean $\pm$ SD with nine mice per group.

these antibodies than control mice (group I, II and V) (t test, $P < 0.005$). Interestingly, neither the route of immunization nor the presence or absence of adjuvant had any significant effect on the antibody response to rNfa1.

Duration of Nfa1-specific IgG in immunized mice

To evaluate the duration of the Nfa1-specific IgG antibody responses, rNfa1-specific IgG antibody was evaluated by ELISA in each group at 2, 6 and 12 weeks following third immunization. As shown in Fig. 4, in both the intra-peritoneally immunized mice and intra-nasally immunized mice, high levels of rNfa1-specific IgG antibody were detected at 2 weeks and continued until 6 and 12 weeks post-immunization.

Cytokine response of immunized mice

To elucidate the T-cell response in mice immunized with an rNfa1 protein, the levels of cytokines in culture supernatants from rNfa1-stimulated splenocytes were analysed by ELISA (Fig. 5). In both the intra-peritoneal and intra-nasal immunization groups, the *in vitro* rNfa1-stimulated splenocytes of mice in group III and IV (rNfa1 protein immunized mice) produced high levels of a type 1 cytokine (IFN- γ), a type 2 cytokine (IL-4) as well as IL-2 and IL-10; in all cases, these were significantly higher than cytokine responses of control mice (group I and II) (t test, $P < 0.005$) (Fig. 5). Again, neither the route of immunization nor the presence or absence of adjuvant had any significant effect on the cytokine response to rNfa1.

PAM survival rate and mean time to death of immunized mice

To determine whether the immune response induced by intra-peritoneal or intra-nasal immunization with the rNfa1 protein was protective against subsequent *N. fowleri* infection, mice from each group were challenged by intra-nasal infection with 1×10^4 trophozoites of *N. fowleri*, and the survival rate and mean time to death were monitored (Fig. 6; Table 1). The cause of death was confirmed by culturing brain tissue of dead mice in Nelsen's medium prior to the examination under a light microscope to identify *N. fowleri* parasites (data not shown).

Control, unimmunized mice [group I (PBS) and II (PBS + adjuvant)] began dying on day 12 or 13 and all mice were dead by day 18 post-infection. The mean time to death was 15.5 days for group I and 15.0 days for group II. On the other hand, mice immunized by either the intra-peritoneal or intra-nasal route survived until at least day 18 and some mice survived until day 30 post-infection.

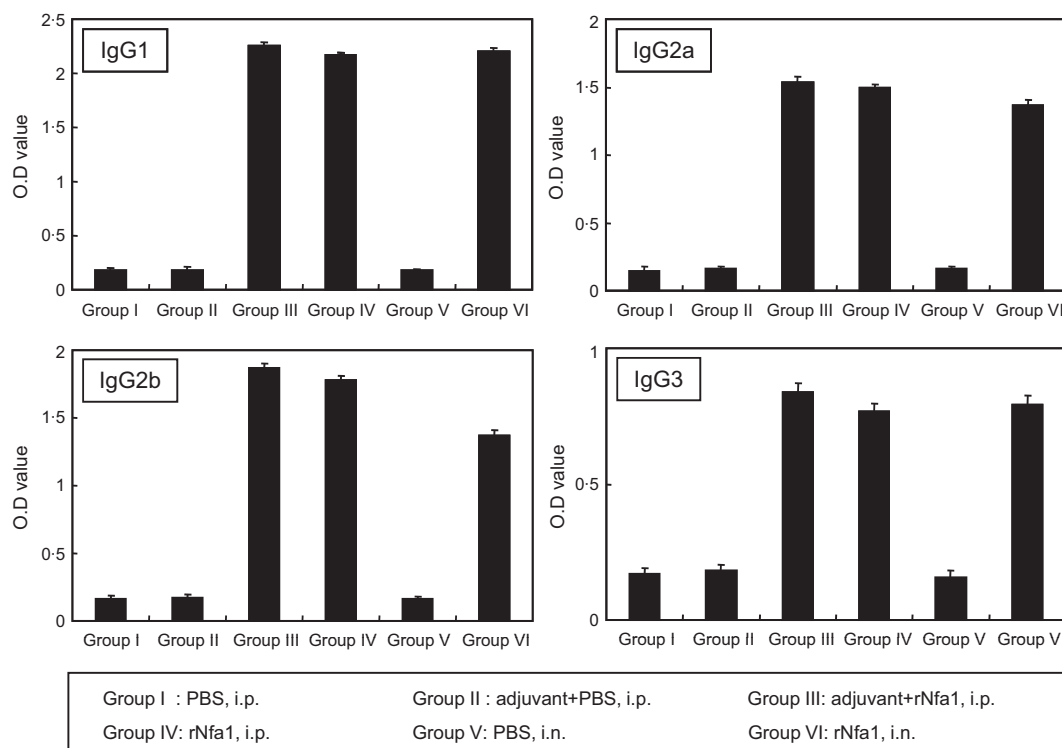


Figure 3 Nfa1-specific IgG subclass antibody levels in sera of mice intra-peritoneally (i.p.) or intra-nasally (i.n.) immunized with the rNfa1 protein. Antibodies were measured 2 weeks after last immunization. The O.D. values are expressed as mean $\pm$ SD with nine mice per group.

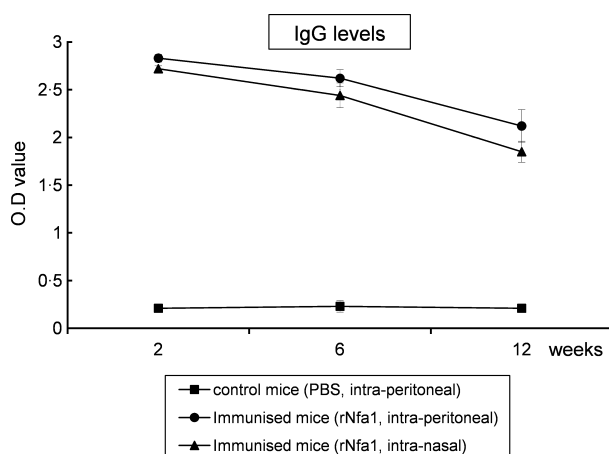


Figure 4 Duration of the Nfa1-specific IgG levels in the sera of immune mice. The Nfa1-specific IgG responses were evaluated by ELISA in each group at 2, 6 and 12 weeks after last immunization.

The mean time to death was 25.0 days for group III, 24.4 days for group IV and 24.7 days for group IV. Immunized mice in all groups survived significantly longer than unimmunized mice (groups III and IV vs. groups I and II, and group VI vs. group V, *t* test, $P < 0.005$ in all cases).

Neither the route of immunization nor the presence or absence of adjuvant had any significant effect on the survival time of immunized mice after challenge infection.

DISCUSSION

Naegleria fowleri is the causal agent of PAM in humans. Other species (*Naegleria italica*, *Naegleria philippinensis* and *Naegleria australiensis*) have also been suggested to be pathogenic in the mouse model of PAM, however, they have not been identified from any human cases (2,3). Adherence of *N. fowleri* trophozoites to host cells is the critical initial step in amoebic infection. Using *in vitro* cell culture systems, *N. fowleri* trophozoites have been shown to destroy nerve cells as well as other cell types by trophocytosis, using the food-cup structure on their surface (13,23,24), and also by the release of cytolytic molecules (25,26). Therefore, the role of the *nfa1* gene product (Nfa1), which is involved with amoebic pseudopodial activity and especially with food-cup formation, should carefully be considered in the pathogenicity of *N. fowleri* infection.

Humoral immunity in natural *N. fowleri* infection was recently reviewed by Marciano-Cabral and Cabral (2) who

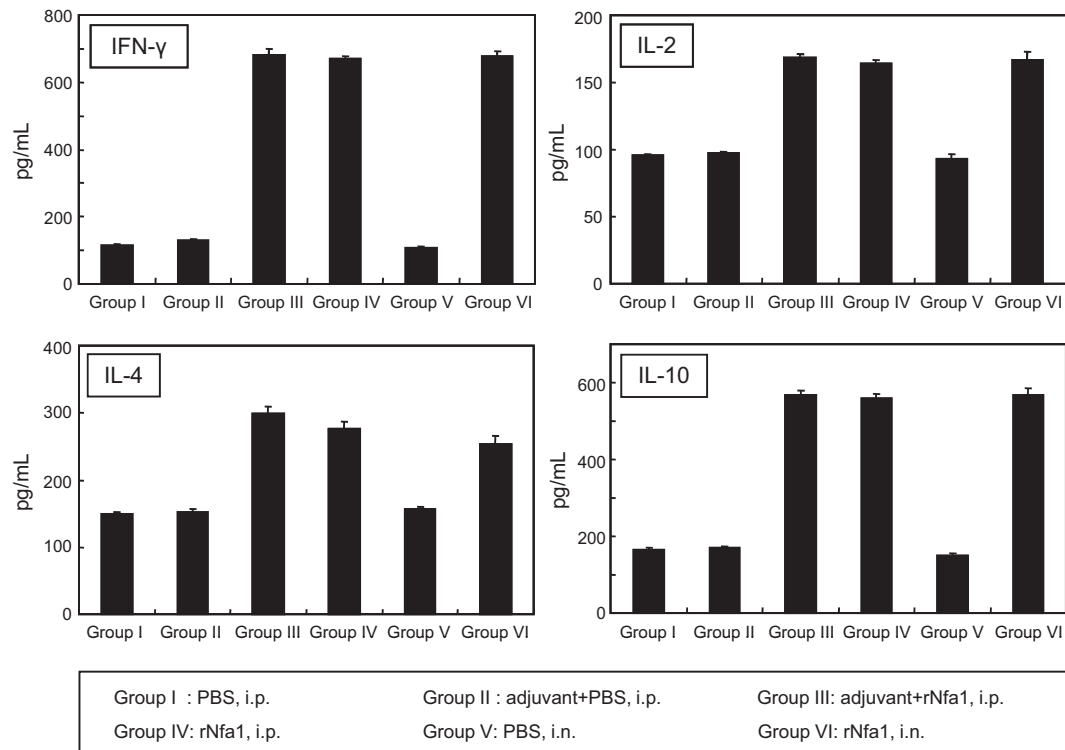


Figure 5 Production of cytokines by splenocytes from mice immunized with rNfa1 protein. The mice were sacrificed 2 weeks after last immunization, and the supernatants were collected after 72 h after stimulation with 10 µg/mL rNfa1 protein. The values are expressed as mean ± SD with nine mice per group.

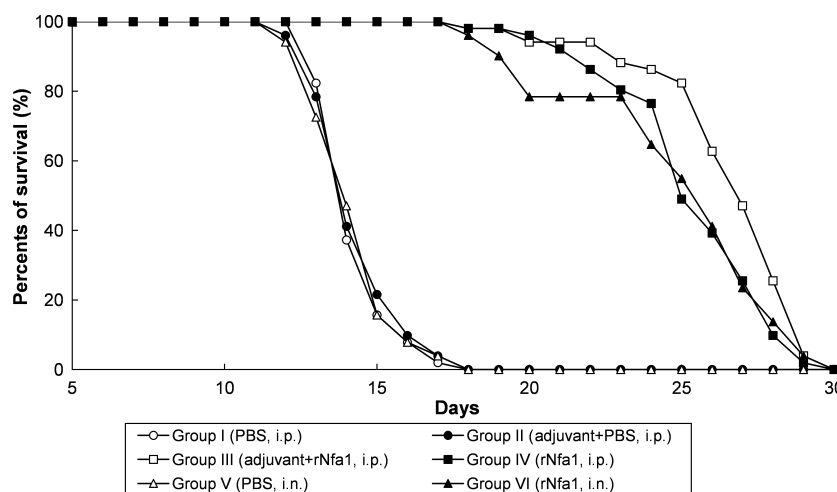


Figure 6 Survival curves after immunization and *Naegleria fowleri* trophozoite infection. Each group contained 51 mice.

concluded that there was no evidence of naturally acquired humoral immunity to *N. fowleri*, even though immunized mice develop species-specific agglutinating antibodies. For example, in ICR mice, circulating IgG antibodies have been detected by ELISA from day 7 after infection with *N. fowleri* and from day 14 after infection with *Naegleria*

jadini, whereas antibodies are not detected to the non-pathogenic species *Naegleria gruberi* (15,27).

Collectively, the results from human and animal studies suggest that humoral immunity is not a major line of defense against *Naegleria* infection. Nevertheless, a slight protective effect of antibody is attributed to immobilization

Table 1 Mean time to death of mice intraperitoneally (i.p.) or intranasally (i.n.) immunized with the rNfa1 protein due to experimental infection of *Naegleria fowleri* trophozoites

Group	No. of mice exam	Cumulative number of death on days after infection																			Mean time to death (Mean $\pm$ SD)
		12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
I	51		9	32	43	47	50	51													15.5 $\pm$ 1.7
II	51	2	11	30	40	46	49	51													15.0 $\pm$ 2.0
III	51							1		3			6	7	9	19	27	38	49	51	25.0 $\pm$ 3.7
IV	51							1		2	4	7	10	12	26	31	38	49	50	51	24.4 $\pm$ 3.6
V	51	3	14	27	43	47	49	51													15.0 $\pm$ 2.2
VI	51							2	5	11				18	23	30	39	44	49	51	24.7 $\pm$ 4.0

Group I: Normal (PBS) (i.p.), Group II: Adjuvant + PBS (i.p.), Group III: rNfa1 + Adjuvant (i.p.), Group IV: rNfa1 (i.p.), Group V: Normal (PBS) (i.n.), Group VI: rNfa1 (i.n.).

of amoebae and prevention of their rapid migration to the brain, enhanced complement-mediated lysis and neutralization of amoeba cytotoxins (20,28,29).

Using the present mouse model, several attempts have been made to show that mice can be immunized against *N. fowleri* using intra-peritoneal inoculation of either live amoeba or amoeba killed by formalin-fixation or freeze-thawing, however, these studies resulted in diverse outcomes with only partial protective immunity (21). Furthermore, studies of immunity in experimental infections and PAM patients have rather been limited to the analysis of serum antibody response (17–19). Therefore, in the present study, we attempted to describe the serological and cellular immune responses which confer partial protection against *N. fowleri* infection in Nfa1-immunized mice.

Mice immunized intra-peritoneally or intra-nasally with an rNfa1 protein, mixed in adjuvant or free of adjuvant, developed high levels of Nfa1-specific IgG, including IgG2b, IgG2a and IgG3, and IgA antibodies. Neither the route of immunization (intra-peritoneal or intra-nasal) nor the presence or absence of adjuvant made any difference to the serological response. Moreover, there was evidence of an antigen-specific IgE response in any of the mice, suggesting that intra-nasal inoculation of the rNfa1 protein does not induce immune hypersensitivity. Therefore, the intra-nasal route of immunization would appear to be suitable for future studies.

It is generally known that the inhibition of bacterial adherence by mucosal IgA is one of the most important defense mechanisms against mucosal invasion and attachment of bacteria to epithelial cells (30,31). The adherence of *N. fowleri* to host cells is the critical initial step in the infection process, and the Nfa1 protein is involved in pseudopodia and food-cup formation. In the present study, mice immunized intra-peritoneally or intra-nasally with the rNfa1 protein showed significantly

high levels of the Nfa1-specific IgA antibody and prolonged survival time. We hypothesise that Nfa1-specific IgA antibody may prevent the adherence of the *N. fowleri* trophozoites to the nasal mucosa and thus delay trophozoite invasion.

In terms of the cellular response, irrespective of the route of immunization or the presence of adjuvant, spleen cells from all rNfa1-immunized mice secreted Th1 type cytokines (γ -IFN), Th2 type cytokines (IL-4) and the immune regulatory cytokine IL-10 as well as IL-2. These data suggest that the rNfa1 protein may trigger a Th1/Th2/Treg mixed type of immune response, however, more detailed examination is required.

In line with the immunological data, all the immunized mice (irrespective of the route of immunization or presence of adjuvant) showed a prolonged survival time after challenge with a lethal dose of *N. fowleri* trophozoites; survival times were prolonged by 9–10 days in comparison with control mice. These data indicate that immunization with the rNfa1 protein can induce partially protective immune responses, leading to the host prolonged survival times in *N. fowleri* infection. However, overall survival of the mice was not enhanced by vaccination, indicating that further improvement of the vaccine strategy is required.

In conclusion, the present results demonstrate that the Nfa1 protein, which is involved in the food-cup formation, is a candidate antigen for inclusion in a *N. fowleri* vaccine. Further studies are required to determine the mechanism of partially protective immunity conferred by rNfa1 vaccination.

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