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Original Article

Intranasal Administration of *Schistosoma japonicum* Paramyosin Induced Robust Long-Lasting Systemic and Local Antibody as well as Delayed-Type Hypersensitivity Responses, but Failed to Confer Protection in a Mouse Infection Model

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SUMMARY: To investigate intranasal (i.n.) immunization efficacy of *Schistosoma japonicum* 97-kDa myofibrillar protein paramyosin (PM), a vaccine candidate for Asian schistosomiasis, BALB/c mice were i.n. immunized with *Escherichia coli*-expressed recombinant PM (rPM). I.n. immunization using rPM mixed with cholera toxin (CT) was more potent than subcutaneous (s.c.) immunization with rPM emulsified in incomplete Freund's adjuvant for induction of serum (IgG, IgE, and IgA) and mucosal (IgA in nose, lung, and intestine) antibody and delayed-type hypersensitivity (DTH) responses. The second i.n. immunization was sufficient to induce maximal serum IgG and DTH responses, which were almost completely maintained for more than 6 months. Next, to evaluate protective efficacy of the rPM against *S. japonicum* infection, immunized mice were infected with *S. japonicum* cercariae at 2 weeks after the second immunization. At 7 weeks after infection, we observed no reduction in worm burden or fecundity in both i.n. and s.c. immunized groups. Results showed that i.n. immunization with rPM/CT failed to provide protection against parasite infection, albeit the antigen was a very potent mucosal immunogen. These results may emphasize the need to innovate new mucosal adjuvants or delivery molecules to overcome such hurdles in the construction of a mucosal antiparasite vaccine platform.

INTRODUCTION

Schistosomiasis is the most significant human helminth infection caused by trematode blood fluke worms belonging to the genus *Schistosoma*; five species are known to infect humans, i.e., *Schistosoma mansoni* (intestinal schistosomiasis), *S. haematobium* (urinary schistosomiasis), *S. intercalatum*, *S. mekongi*, and *S. japonicum* (Asian intestinal schistosomiasis). This parasitic disease, which is one of the 14 neglected tropical diseases (NTDs) currently listed by the World Health Organization (WHO), is endemic in remote, rural areas and urban slums in 74 countries in Africa, South America, and Asia, infecting more than 200 million people, with approximately 650 million people estimated to be living in endemic areas (1-3). There are continual reports of transmission of schistosomiasis japonica, a zoonosis, in southern China and the Philippines (3,4). Since *S. japonicum*, unlike *S. mansoni* and *S. haematobium*, infects nonhuman vertebrates (e.g., cattle, water buffalo, sheep, goats, pigs), a transmission blocking veterinary vaccine development, for example for water buffaloes in southern China, should provide an additional and a unique approach to *S. japonicum* control (4-8). Therefore, human vaccines and/or veterinary transmission blocking vaccines targeting worm numbers, parasite fecundity, or egg viability would constitute an indispensable component for future control campaigns of schistosomiasis japonica, while vaccine-linked drug chemotherapy is believed to become a basis for future Asian schistosomiasis control campaigns (9). Although, praziquantel (PZQ) is effective against all forms of schistosomiasis with few side effects, a total eradication of the parasite solely based on PZQ chemotherapy is considered difficult and impractical for the following reasons: (i) chronic infection and frequent reinfection are observed in people living in endemic areas even after successful drug chemotherapy (3,8), and (ii) increasing concern has been raised over the emergence of PZQ-resistant parasite strains in endemic regions where large-scale use of the drug is practiced (10).

Despite the existence of various practical difficulties regarding the control of schistosomiasis, there is considerable support concerning the possibility that anti-schistosome vaccines can be developed, based on several reasons; (i) radiation-attenuated cercariae con-

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fers significant levels of prophylactic protection from reinfection in experimental animals (11,12); (ii) age-related resistance to infection was observed in humans and also in animals such as buffaloes (13–15); (iii) naturally resistant individuals are seen in endemic populations despite years of exposure to the parasites (16).

To date, several vaccine candidates against *S. japonicum* have demonstrated their potential to reduce worm burden and/or egg numbers in infected mice and other animal models (7,8). These candidates include: (i) 26-kDa glutathione S-transferase (Sj26GST), an enzyme isoform that catalyzes redox reaction; (ii) paramyosin (Sj97 or PM), a 97-kDa myofibrillar protein with a coiled-coil structure found only in invertebrates; (iii) calpain, a calcium-activated neutral proteinase found in the tegument of adults and penetration glands of cercariae; (iv) triose-phosphate isomerase (SjTPI), an enzyme in the glycolytic pathway; (v) a 23-kDa tetraspanin integral membrane protein (Sj23); (vi) SjFABP (Sj14), a fatty acid binding protein, an essential parasite protein in the take up of fatty acids from host blood as nutrients. Among these *S. japonicum* antigens, PM, a leading candidate for the schistosomiasis japonica vaccine (17–23), was first cloned as a full-length cDNA and then recombinantly expressed in *Escherichia coli* (20), with pilot-scale production recently reported (19). The PM is located on the surface of the tegument and in the secretory glands of the larvae (24–26), and can induce protective immunity, for example, in domestic pigs, conferring 40–50% reduction in worm recovery when immunized intradermally with recombinant PM (rPM) (18). Its vaccine efficacy for egg reduction in the liver of immunized water buffaloes was also reported (17). Further, its potential has also been demonstrated in other immunization methods including DNA vaccine (27).

The potent mucosal adjuvanticity of cholera toxin (CT) and its related heat-labile enterotoxin from *E. coli* (LT) in inducing systemic and mucosal antibody responses against otherwise weakly immunogenic antigens have been demonstrated in experimental animal models for parasitic diseases such as malaria (28–30). However, it has been well documented that recombinantly expressed nonreplicating inert antigens with mucosal adjuvants often induced immune responses with a clear tendency of bias toward Th2-type in mice, inducing primary IgG1 in serum and antigen-specific IL-13 in local draining lymph nodes without induction of IFN-γ (31). Therefore, if the induction of Th2-type of immunity is able to provide protective immunity against the target infectious diseases without any particular risk, recombinant antigens with a mucosal adjuvant administered through the nasal route would be free from any disadvantages. Internasal (i.n.) immunization frequently induces an antigen-specific IgE response, therefore, such an immunization regimen would be expected to be suitable for a PM-based vaccine. Furthermore, the recent trend to search for safer and more effective new mucosal adjuvants devoid of toxicity problems, a serious concern in the clinical use of CT, may add another promising dimension to anti-parasitic mucosal vaccine development research (21,22,24).

In this study we investigated an *E. coli*-expressed rPM-based i.n. immunization regime in a mouse model. rPM antigen administered with a potent mucosal adjuvant CT induced both mucosal and systemic immune responses of mixed Th1/Th2-type with PM-specific serum IgG, IgE as well as secretory IgA. We also examined the effects of immunization on the worm burden and/or the fecundity of female worms.

MATERIALS AND METHODS

Mice and immunization with rPM: Full-length rPM was expressed and purified as described previously (18). Six-week-old female BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). Five mice per group were immunized with rPM subcutaneously (s.c.) or by i.n. route. For s.c. immunization, 30 μg of rPM emulsified with incomplete Freund's adjuvant (IFA), 100 μl in total, was administered to the dorsal skin using a 28-gauge needle syringe. Mice were administered three times at weeks 0, 3, and 5. The same volume of phosphate-buffered saline (PBS) emulsified with IFA was administered to mice as a negative control. For i.n. immunization, 30 μg of rPM with or without 1 μg of CT (Sigma-Aldrich, St. Louis, Mo., USA) was administered three times at weeks 0, 3, and 5 to external nares using a micropipet. As a negative control, a group of mice was i.n. immunized with 1 μg of CT or PBS only. Mice were bled from the tip of the tail at weeks 2, 4, and 6 for antibody analysis. All animal experimental protocols were approved by the Animal Ethical Committee of the University of the Ryukyus and Nagasaki University.

ELISA: A flat-bottom 96-well microtiter plate (Immulon 4; Dynex Technology Inc., Chantilly, Va., USA) was coated with 50 μl of the rPM (3 μg/ml in bicarbonate buffer, pH 9.6) at 4°C overnight. The plate was blocked with 1% (or 5% for IgE antibody detection) bovine serum albumin (BSA) (Sigma-Aldrich) in PBS at 37°C for 2 h. Fifty microliters of mouse antisera diluted 50-fold (or 20-fold for IgE antibody detection) with PBS containing 0.5% BSA were applied to wells in duplicates and incubated for 2 h at 37°C. Secondary antibodies (i.e., specific for mouse IgG, IgG subclasses, IgM, and IgA) conjugated with alkaline-phosphatase were added to wells followed by its substrate. Plates were measured by microplate reader (Bio-Rad Laboratories, Redmond, Wash., USA) with the OD_{405} after 20 min incubation. For measurement of serum IgE, secondary (rat anti-mouse IgE monoclonal antibody [IM2992; Immunotech, Marseille, France]) and tertiary (rabbit anti-rat IgG conjugated with horseradish peroxidase [HRP] [SAB-200; Stressgen Biotechnologies, Victoria, Canada]) antibodies were applied followed by HRP substrate. Plates were measured with the OD_{405} after 20 min incubation. For analysis of secretory antibodies in nasal secretions, the nasal cavities of sacrificed animals were washed several times with 200 μl of PBS. Intestinal antibodies were collected by extensively washing 3-cm long intestinal tubes excised from the ileal region with 500 μl of PBS containing protease inhibitor cocktail (Sigma-Aldrich). Bronchoalveolar lavage fluid (BALF) was collected by repeated injections and withdrawal of fluid several times from the trachea into the lungs using an 18-gauge needle. The collected mucosal fluids were directly analyzed by ELISA as described above. Statistical significance of differences be-

tween antibody levels was determined by Student's t test ($P < 0.05$).

Delayed-type hypersensitivity (DTH) measurements: DTH responses were measured at weeks 6 and 36 (i.e., 1 week and 31 weeks after the third immunization) by injecting 2 μg of the full-length rPM into the footpad of a hind leg of immunized mice, with swelling measured after 24 h. The DTH response was calculated from the differences in thickness between the left and the right, administered with PBS and the rPM, respectively. Statistical significance of differences was determined by the Mann-Whitney U test ($P < 0.05$).

Experimental infection: Cercariae of *S. japonicum* Chinese strain (obtained from Jiangsu Provincial Institute of Parasitic Diseases in Wuxi, People's Republic of China) were released from the infected snails using light source. The cercariae which climbed up to the surface of the water were scooped up using a cover slide glass, and cercariae numbers were counted under a light microscope. The parasites were immediately used for infection experiments to avoid any reduction in infectivity. For experimental infection, 8–12 female BALB/c mice immunized twice at weeks 0 and 2 were challenged at week 4 through the abdominal skin with 30 cercariae per mouse. Briefly, mice were anesthetized by intraperitoneal injection of pentobarbital and the abdominal hair was shaved to expose the skin for infection. Animals were returned to cages after confirming the penetrations of all cercariae into the skin. At 7 weeks after the cercariae infection, mice were sacrificed and portal vein perfusion was conducted to count eggs in the liver and adult worms in the mesenteric veins. The eggs were isolated from the livers according to the standard procedure (32). Briefly, the chopped liver from each mouse was homogenized in 0.1% actinase in PBS, and digested at 37°C for 1 h. The digested sample was centrifuged (1,500 rpm, 5 min) and the pellet was resuspended in 0.01% actinase and 0.05% collagenase mixture in PBS, and additionally digested at 37°C for 1 h. After incubation, the digested sample was centrifuged again (1,500 rpm, 5 min), and the pellet was resuspended in PBS. The eggs were sieved with a steel mesh and counted under the light microscope. Adult worms were divided into male and female worms, and their numbers counted.

Fig. 1. Full-length recombinant *Schistosoma japonicum* paramyosin (rPM) expressed in *Escherichia coli* was detected by SDS-PAGE/CBB stain.

RESULTS

Detection of antibody levels after immunizations: The full-length rPM was expressed in *E. coli* and purified as previously described (18) (Fig. 1). To evaluate its immunogenicity, BALB/c mice were immunized through s.c. or i.n. route at weeks 0, 3, and 5, and specific antisera were collected at 1 week after the second and the third immunization, respectively. I.n. immunization with rPM/CT, but not with the antigen alone, induced robust serum IgG, which was higher than the response induced by s.c. immunization with

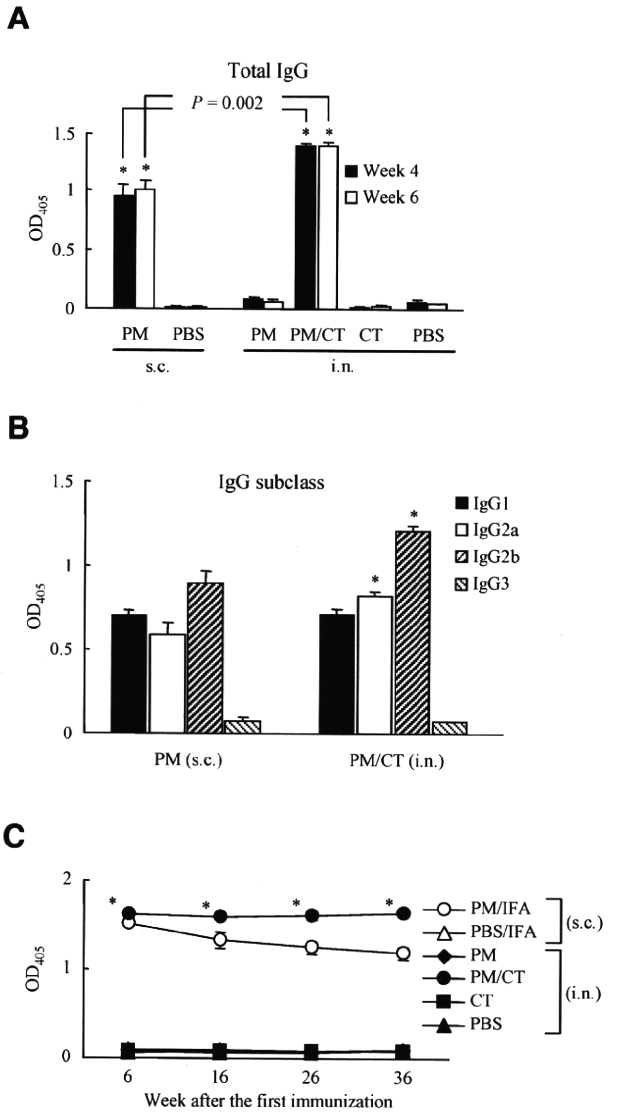

Fig. 2. Female BALB/c mice were immunized with rPM through subcutaneous (s.c.) or internasal (i.n.) route three times at weeks 0, 3, and 5, and antisera were collected at weeks 4 and 6. (A) For total serum IgG analysis, 50-fold diluted antisera collected at weeks 4 and 6 were reacted with rPM, and analyzed by ELISA. *, $P < 0.0001$ (as compared with PBS). (B) For serum IgG subclass analysis, 50-fold diluted antisera collected at week 6 were reacted with rPM. *, $P = 0.01$ (as compared with PM [s.c.]). (C) PM-specific serum IgG levels were monitored for up to 36 weeks from the first immunization. Levels of PM-specific antibodies were shown as average OD_{405} ± standard error of the mean. Statistical significance of differences was determined by the Student's t test.

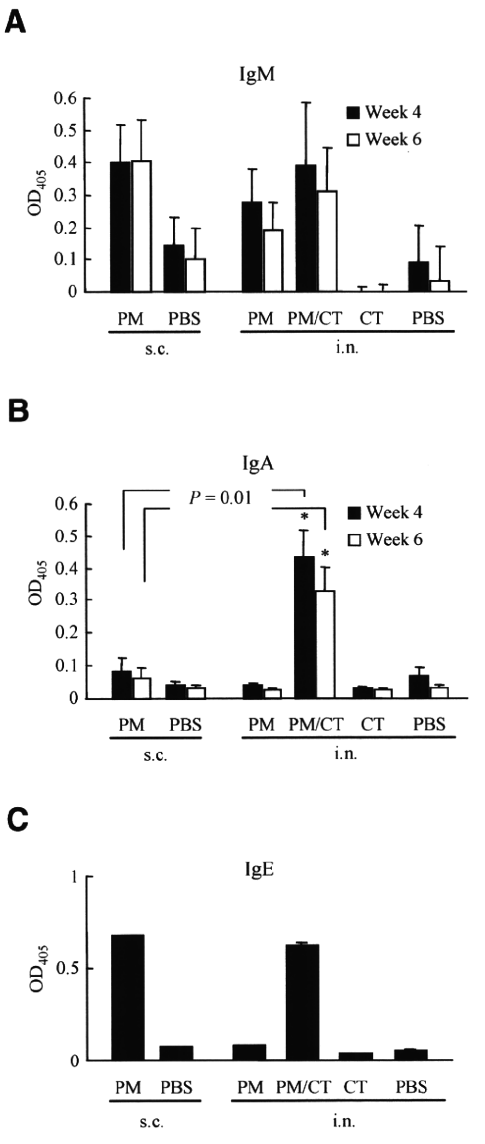

Fig. 3. Serum IgM (A) and IgA (B) levels were measured using antisera collected at weeks 4 and 6. *, $P < 0.01$ (as compared with PBS). (C) Serum IgE levels were measured using antisera collected at week 6. Levels of PM-specific antibodies were shown as average OD_{405} ± standard error of the mean. Statistical significance of differences was determined by the Student's t test.

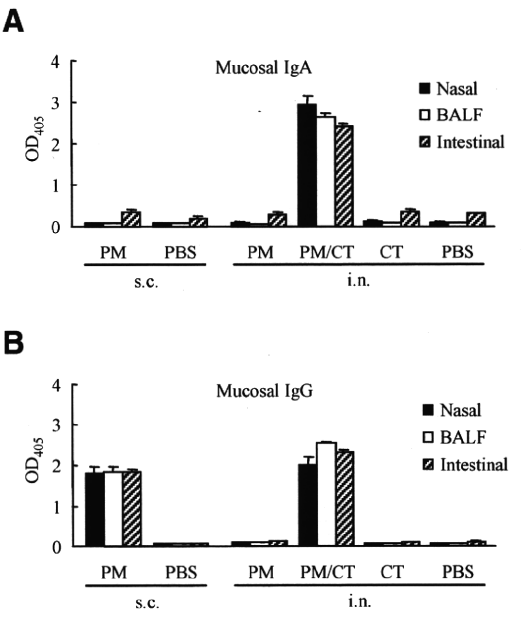

Fig. 4. Mucosal IgA (A) and IgG (B) levels were measured using mucosal samples (nasal washings, bronchoalveolar lavage fluid [BALF], and intestinal washings) collected at week 6. Data are shown as average OD_{405} ± standard error of the mean.

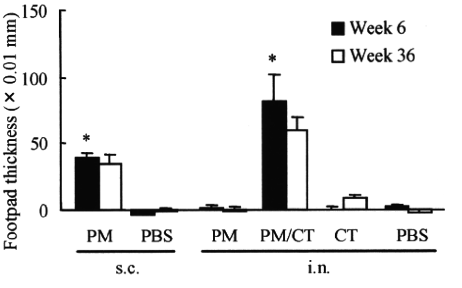

Fig. 5. A long term maintenance of PM-specific immune response was analyzed by measuring DTH. PM-specific DTH response elicited by rPM immunization by s.c. or i.n. route was analyzed at weeks 6 and 36 (i.e., 1 week and 31 weeks after the third immunization) by injecting 2 μg of rPM protein into the footpad of hind legs of immunized mice, and footpad thickness increments were measured after 24 h. Data are shown as average differences ± standard error of the mean in the thickness between the right and the left footpad injected with rPM and PBS, respectively.

rPM/IFA (Fig. 2A). The second immunization with each route was sufficient to induce the maximal levels of IgG. IgG subclass levels indicated that IgG1, IgG2a, and IgG2b were significantly elevated in rPM/CT and rPM/IFA groups (Fig. 2B). To determine the duration of antibody levels in serum, antigen-specific IgG levels were monitored for more than 6 months after the final immunization without additional booster immunization. IgG levels in serum were completely maintained in mice immunized with rPM/CT, but IgG levels in mice immunized with rPM/IFA gradually declined over the course of time (Fig. 2C). Weak but detectable serum IgM was also induced in rPM/CT and rPM/IFA groups (Fig. 3A), however, only rPM/CT immunization induced IgA in serum (Fig. 3B). Further, PM-specific serum IgE were detected in both rPM/CT and rPM/IFA immunizations (Fig. 3C).

Mucosal antibody levels in nasal, intestinal, and BALF were measured at 1 week after the third immunization. Mucosal IgA was induced only by i.n. rPM/CT immunization (Fig. 4A), but IgG was induced in both s.c. rPM/IFA and i.n. rPM/CT groups (Fig. 4B). With a clear contrast with serum IgG levels, which were found to last for at least 7 months (Fig. 2C), mucosal antibodies were completely diminished at week 40 post-immunization (data not shown), indicating much less efficient maintenance of the mucosal antibody response than that of the serum antibody response.

DTH measurement and IFN-γ production: PM-specific DTH was measured, and we found that the response was well maintained in mice immunized with both i.n. rPM/CT and s.c. rPM/IFA, up to 7 months (Fig. 5). rPM i.n. immunization without CT showed no

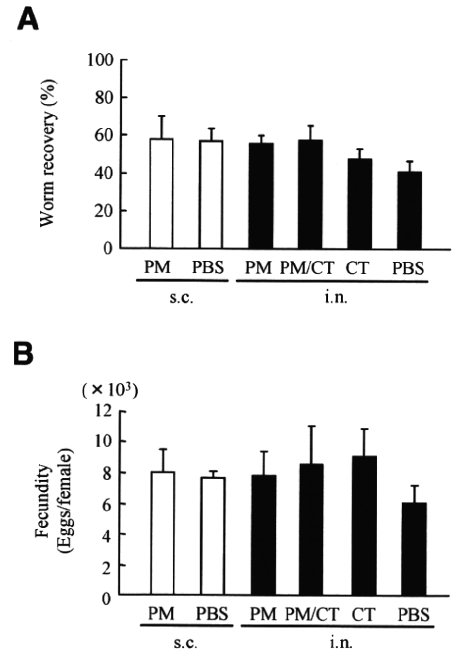

Fig. 6. Female BALB/c mice were immunized with rPM through s.c. (8 mice/group) or i.n. (12 mice/group) route twice at weeks 0 and 2. Mice were infected with approximately 30 *S. japonicum* cercariae per animal at week 4, and mice were sacrificed for parasite counts at week 11 for adult worms in the mesenteric veins and for eggs in the liver. (A) Worm recovery percents were calculated by the equation [(no. of adults recovered/no. of cercariae used for infection) × 100], and (B) fecundities were calculated by the equation (no. of eggs recovered from the liver/no. of female worms recovered/mouse). Data are shown as average numbers of adult worms or eggs ± standard error of the mean.

response at all, indicating a clear correlation between humoral and DTH responses. Despite strong humoral and DTH responses, no increment of antigen-specific IFN-γ production was observed in spleen or local draining lymph nodes collected from i.n. rPM/CT or s.c. rPM/IFA immunized mice (data not shown).

Vaccine trial against *S. japonicum*: Since the second immunization was sufficient to elicit the maximal antibody response (Fig. 2A), mice were infected with approximately 30 cercariae per animal through the abdominal skin at 2 weeks after the second immunization, and at 7 weeks post-infection mice were sacrificed and the parasite number was counted. We observed no reduction in the number of adult worms (Fig. 6A) or egg production from female worms (Fig. 6B).

DISCUSSION

PM has been demonstrated for its potential as a vaccine target, and thereby selected as one of several schistosomiasis vaccine candidates by the WHO (7). In this study we prepared the full-length rPM antigen in *E. coli* and evaluated its vaccine efficacy in a mouse infection model. The rPM induced robust humoral and DTH responses when administered mucosally or parenterally, however, the use of adjuvant was found to be essential for both immunization regimens. Although there is a report indicating that the combinations of adjuvants and routes in immunization influence the profile of immune response induction in the case of *S. mansoni* (33), we did not observe any significant difference in the quality of the response between i.n. and s.c. routes of immunization except for IgA induction by mucosal immunization (Figs. 3B and 4A). Furthermore, we found that the intensity of the immune response tends to be higher for i.n. than the s.c. route of immunization.

Humoral immunity, in addition to various inflammatory immune cells such as eosinophils, macrophages, and T-lymphocytes, may have a major importance in protection from schistosome infection (34). Although, T-cell-dependent cell-mediated immunity was found to be important for protection (34), IgE also appears as a major protective effector arm for resistance (21,22,24), and protective monoclonal IgE which recognizes B cell epitopes was determined to be located within the PM molecule (24,35). Analyses of IgG isotype profile indicated that relatively high IgG1, IgG2a, and IgG2b without noticeable levels of IgG3 were induced (Fig. 2B), suggesting the induction of mixed Th1/Th2 profile. These results were unexpected, particularly the observation of high IgG2b induction, because IgG1 is usually the only primary serum Ig isotype induced by i.n. immunization of recombinant antigens mixed with CT in mouse models (28,30). We do not have clear evidence to explain this observation, but it may suggest very high immunogenicity of recombinant PM/adjuvant vaccine formulation, and this notion is strongly supported by our observation that long-lasting serum IgG and DTH responses were induced in immunized mice (Figs. 2C and 5). We concluded that this strong immunogenicity was a result of the combination of PM and CT, because PM administration alone only induced weakly immunogenic effects.

Vaccine-induced IgA affects parasite fecundity in *S. mansoni* (36,37), and PM was determined as a target molecule of human IgA against *S. japonicum* in an epidemiological study conducted in the Philippines (13). Our results showed that antigen-specific IgA in serum and mucosa was induced only by mucosal immunization, not parenteral immunization, and this immunity may affect the migration of schistosomula in mucosal tissues such as the lungs. The advantage of mucosal administration was previously suggested by other researchers, who showed that oral administration of *S. japonicum* proteins induced specific anti-parasite antibodies and damaged adult worms (38). Given the fact that i.n. immunization is usually more effective in inducing secretory IgA than oral immunization, i.n. vaccine protocol is expected to provide better mucosal immunity than an oral vaccination regime.

After invasion by the free-swimming cercariae into the host skin of humans and animals, cercariae shed their tails and become schistosomula that migrate to the lungs. Therefore we proposed that the mucosal immunization may block larval migration into the lung and prevent them from reaching the portal vein. Furthermore, serum IgE and mucosal IgA responses induced only by i.n. immunization were expected to provide a reduction in worm burden and/or fecundity. A previous report by McManus et al. clearly demonstrated consistent protective effects of PM when immunized mice (both inbred and outbred) were challenged with *S. japonicum* cercariae (17). Further, the same study demonstrated PM's

vaccine effect on reducing liver egg numbers in water buffaloes. We supposed that their differences from our results were caused by the used mouse strains and adjuvants.

Effective antischistosome immunity seems to be dependent on a balance between protective and susceptibility-enhancing immune responses elicited by a particular vaccination regimen, rather than a mere induction of a high level of both types of immunity; for example, high IgE/IgG4 ratio to parasite antigens correlates to resistance to reinfection (21,39). Thus, it is presumably correct to draw a conclusion from our present study that the particular vaccination regime that we employed in our mouse study did not favor a protective immune response over a susceptibility-promoting immune response, though we do not know what factors critically contributed to the latter response. In humans, IgE and IgG4 responses were important in protection against *S. japonicum* infection (8,39). High IgG4 response causes sensitivity to *S. japonicum* (21). In our result, not only the IgE antibody but also all IgG subclasses apart from IgG3 increased. As one of the possibilities, the augmentation of IgG antibodies have masked an effect of IgE protection (40,41). Additionally, in our recent unpublished study with a swine infection model using miniature pigs, i.n. rPM/CT immunization failed to protect the immunized animals even though the immunization induced substantial levels of serum and mucosal antibody responses. Therefore, a failure to induce protective immunity in our i.n. immunization model may not be a host-specific phenomenon.

To reemphasize the comments made by Bergquist et al. (9) and McManus (5) in their review articles, antiparasite vaccines present a formidable challenge and might not be possible without careful selection of a suitable adjuvant to promote stimulation to the desired levels of protective immunity. Further studies are strongly encouraged, as such new mucosal adjuvants or delivery molecules should be innovated and tested for their efficacy to be included as important components of PM-based vaccine platform technologies targeting Asian schistosomiasis.

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Purification and kinetic characterization of recombinant alternative oxidase from *Trypanosoma brucei brucei*

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ABSTRACT

The trypanosome alternative oxidase (TAO) functions in the African trypanosomes as a cytochrome-independent terminal oxidase, which is essential for their survival in the mammalian host and as it does not exist in the mammalian host is considered to be a promising drug target for the treatment of trypanosomiasis. In the present study, recombinant TAO (rTAO) overexpressed in a haem-deficient *Escherichia coli* strain has been solubilized from *E. coli* membranes and purified to homogeneity in a stable and highly active form. Analysis of bound iron detected by inductively coupled plasma-mass spectrometer (ICP-MS) reveals a stoichiometry of two bound iron atoms per monomer of rTAO. Confirmation that the rTAO was indeed a diiron protein was obtained by EPR analysis which revealed a signal, in the reduced forms of rTAO, with a *g*-value of 15. The kinetics of ubiquiol-1 oxidation by purified rTAO showed typical Michaelis–Menten kinetics (K_m of 338 μM and V_{max} of 601 μmol/min/mg), whereas ubiquinol-2 oxidation showed unusual substrate inhibition. The specific inhibitor, ascofuranone, inhibited the enzyme in a mixed-type inhibition manner with respect to ubiquinol-1.

1. Introduction

Trypanosoma brucei is a parasite that causes African sleeping sickness in humans and Nagana in livestock and is transmitted by the tsetse fly. There is an urgent need for further development of chemotherapy against African trypanosomiasis since current chemotherapeutic drugs are not entirely satisfactory [1].

Trypanosomal parasites are equipped with a unique energy metabolism, they live as the bloodstream form in the mammalian host and as the procyclic form in the vector. The procyclic form of *T. brucei* fulfills its ATP requirement from a cyanide-sensitive and cytochrome-dependent respiratory chain comparable to that observed in the host mitochondria, whereas in the bloodstream form, trypanosomes use the glycolytic pathway, which is localized in a unique organelle the glycosome, as their major source of ATP [2–5]. Once the parasites invade the mammalian host in the bloodstream form, both its cytochrome-dependent respiratory chain and ATP synthesis by oxidative phosphorylation disappear [2,5]. Instead a cyanide-resistant and cytochrome-independent trypanosomal alternative oxidase (TAO) functions as the sole terminal oxidase to re-oxidize NADH accumulated during glycolysis [5].

TAO is generally considered to be a good target for the anti-trypanosomal drugs because this oxidase is essential for their survival, since it reoxidises cytosolic NADH, and mammalian hosts do not possess this protein [5,6]. Indeed, we found that ascofuranone, isolated from the pathogenic fungus *Ascochyta visiae*, specifically inhibits the quinol oxidase activity of TAO and rapidly kills the parasites [7]. In addition, we have confirmed the chemotherapeutic efficacy of ascofuranone *in vivo* [8,9].

The alternative oxidase (AOX) is a non-protonmotive ubiquinol oxido-reductase catalyzing the 4-electron reduction of dioxygen to water [5,10–12]. Genes encoding AOX have been found in higher

Abbreviations: AOX, alternative oxidase; DM, *n*-dodecyl-β-D-maltopyranoside; EPR, electron paramagnetic resonance; ICP-MS, inductively coupled plasma-mass spectrometer; IPTG, isopropyl, β-D-1-thiogalactoside; k_{cat}, molecular activity; C10E8, octaethylene glycol-monododecylether; OG, *n*-octyl-β-D-glucopyranoside; rTAO, recombinant trypanosome alternative oxidase; SHAM, salycylhydroxamic acid; TAO, trypanosome alternative oxidase; Ubiquinol, reduced form ubiquinone

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