Screening of novel malaria DNA vaccine candidates using full-length cDNA library

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Abstract

No licensed malaria vaccine exists, in spite of intensive development efforts. We have been investigating development of a DNA vaccine to prevent malaria infection. To date, we have established a full-length cDNA expression library from the erythrocytic-stage murine malaria parasite, *Plasmodium berghei*. We found that immunization of mice with combined 2000 clones significantly prolonged survival after challenge infection and that splenocytes from the immunized mice showed parasite-specific cytokine production. We determined the 5'-end one-pass sequence of these clones and mapped a draft genomic sequence for *P. berghei* for use in screening vaccine candidates for efficacy.

In this study, we annotated these cDNA clones by comparing them with the genomic sequence of *P. falciparum*. We then divided them into several subsets based on their characteristics and examined their protective effects against malaria infection. Consequently, we selected 104 clones that strongly induced specific IgG production and decreased the mortality rate in the early phase.

Most of these 104 clones coded for unknown proteins. The results suggest that these clones represent potential novel malaria vaccine candidates.

1. Introduction

Approximately 250 million people are infected with malaria each year worldwide, and about 800 thousand people die (WHO 2011). The vast majority of patients are infants and children under 5 years old. Development of effective malaria vaccines is an area of intensive research, but it has proved difficult because of the complicated life cycle and antigenic polymorphism of malaria parasites (Good and Doolan 2010).

Research and development of malaria vaccines have been carried out using a variety of malaria parasite antigens (Hill 2011). For example, viral vector-based DNA vaccines, including the prime-boost approach, aim to induce cellular immunity against liver-stage malaria parasites (Schuldt and Amalfitano 2012; Limbach and Richie 2009; Hill et al. 2010; Hill 2011). Blood-stage antigen-targeted vaccines are aimed at direct reduction of malaria symptoms (Richards and Beeson 2009; Ellis et al. 2010; Hill 2011). Mosquito-stage vaccines aim to interrupt transmission (Hill 2011; Lavazec and Bourgooin 2008). Multi-stage combination-vaccines have also been studied (Hill 2011; Doolan and Hoffman 2001). Among such various vaccine candidates, only whole-parasite vaccines, using mosquito-stage parasites called sporozoites, provide a complete protective effect (Hoffman et al. 2002; Roestenberg et al. 2011). However, they are not practical because their delivery requires thousands of mosquito bites (Schuldt and Amalfitano 2012; Hill 2011). To date, the most effective vaccine candidate is RTS,S, an S subunit vaccine that targets the circumsporozoite protein (CSP). RTS,S

1 has progressed to phase III clinical trials (A phase 3 trial of rts,s/as01 malaria vaccine in
2 african infants 2012), but it has been unable to block infection completely. Therefore,
3 a more effective vaccine needs to be developed.

4 Recently, genome analysis demonstrated a high degree of homology between
5 the human malaria parasite, *Plasmodium falciparum* (*P. falciparum*), and the murine
6 malaria parasite, *P. berghei* (Hall et al. 2005). That finding suggests that orthologues of
7 murine malaria parasite-derived cDNA clones may induce an immune response against
8 human malaria.

9 We previously reported that immunization of mice with the G2000 subset,
10 which contains about 2000 cDNA clones from a full-length cDNA expression library
11 from erythrocytic-stage *P. berghei*, using the Gene Gun significantly prolonged the
12 survival time after challenge infection and that splenocytes of the immunized mice
13 produced parasite-specific IFN- γ , IL-2, IL-4 and IL-10, (Shibui et al. 2005).
14 Determination of the 5'-end-one-pass sequences of those clones and comparisons with
15 parasite and mouse genomes revealed that 1518 clones represented parasite transcripts
16 (Shibui et al. 2005). In this study, we further narrowed down the effective cDNA clone
17 candidates from those 1518 by examining their prolongation of survival after challenge
18 infection.

2. Materials and Methods

2.1. Mice, rats and parasites

Female BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). Female ICR mice and female Wistar rats were purchased from Japan SLC (Shizuoka, Japan). The animals were housed under specific-pathogen-free conditions at The Institute of Medical Science, The University of Tokyo, and the animal protocol was approved by the Institutional Review Board of the Institute.

Murine malaria parasite, *P. berghei* ANKA strain, was a gift from Drs. Chris Janse and Andrew Waters (Leiden University, Leiden, the Netherlands). Parasites were maintained by intraperitoneal injection into female ICR mice and propagated in female Wistar rats by intraperitoneal injection. Parasitemia was quantified by microscopic observation of Giemsa-stained smears of tail blood.

2.2. Plasmid DNAs used for immunization

Construction of the expression plasmid vector pCE-FL and the full-length cDNA library of murine malaria was previously described (Shibui et al. 2001). *E. coli* was transformed with a plasmid, single colonies were picked up and isolated in 96-well plates, and stored as glycerol stocks at -80°C . 5' end-one-pass sequencing of these clones was performed, and the results were published in the DNA Database of Japan (Shibui et al. 2005). The sequences were mapped onto the draft genomic sequence of *P. berghei* and compared with the homologous annotated genes of *P. falciparum*.

Subsets of plasmid DNAs were isolated using an EndoFree Plasmid Maxi Kit (QIAGEN, Chatsworth, CA) according to the manufacturer's protocol. As a control, an empty vector without any inserted cDNA was prepared in the same way.

A list of GenBank Accession number of EST clones contained in, G1518, UK, HP and HP3 is shown as supplementary data.

2.3. DNA vaccine immunization using a Gene Gun

The abdominal hair of mice was removed using a hair-removing cream (Kanebo, Tokyo, Japan) before immunization. Eight BALB/c mice of 6-week-old per group were immunized at 1-week intervals for a total of 3 times in the abdomen using a Helios Gene Gun System (Bio-Rad, Hercules, Richmond, CA). Plasmid DNAs were precipitated onto 1- μ m gold particles using CaCl_2 in the presence of spermidine at a loading rate of 2 μ g DNA/mg of gold according to the instructions of the manufacturer. Five shots, *i.e.*, a total of 5 μ g of DNA, were used to inoculate each mouse at a time. The helium pressure of bombardment was set at 400 psi.

One week after the last immunization, each mouse was injected intraperitoneally with 50,000 parasite-infected erythrocytes from pre-infected mouse blood freshly collected in heparinized saline by cardiopuncture, or sacrificed to collect spleen cells and serum for immunological studies.

2.4 Preparation of crude parasite antigens

BALB/c mice and Wistar rats were infected with *P. berghei* ANKA. When

1 parasitemia was about 20%, whole blood was drawn into a heparinized syringe by heart
2 puncture. The blood was washed in phosphate buffered saline (PBS) and then lysed
3 using 0.15% saponin (SIGMA) at room temperature for 5 min. Crude parasite antigens
4 were collected by centrifugation at 2,500 rpm for 7 min, washed twice in PBS, disrupted
5 using a sonicator, frozen in liquid nitrogen and kept at -80°C until use. Control red
6 blood cell membranes were similarly prepared from the blood of non-infected mice and
7 rats.

9 **2.5 Cytokine ELISA**

10 Spleen cells were prepared by a standard method, and cells from 4 mice were
11 pooled. Four million spleen cells were cultured with 50 µg of *P. berghei* crude antigens
12 in 1 ml of RPMI 1640 medium (SIGMA) containing 10% fetal bovine serum, 50 mM
13 2-mercaptoethanol, 2 mM L-glutamine and antibiotic-antimycotic (GIBCO) in 24-well
14 tissue culture plates. Cultures were performed in triplicate. Supernatants were collected
15 after 24 h (for IL-2 and IFN-γ) and 72 h (for IFN-γ, IL-4 and IL-10) and frozen at
16 -20°C.

17 The concentrations of IL-2, IFN-γ, IL-4 and IL-10 in cell culture supernatants
18 were assayed using the respective Mouse BD OptEIA Sets (BD Pharmingen, San Diego,
19 CA), in triplicate, according to the manufacturer's instructions.

21 **2.6 Assay of parasite-specific IgG after DNA immunization**

22 Immunized mouse sera were absorbed by non-infected rat red blood

cell-lysates overnight at 4°C. Parasite-antigen-specific IgG in mouse sera was assayed by ELISA using BD Opti-EIA Reagent Set A (BD Pharmingen). *P. berghei* crude antigens from rats (3.2 µg/ml) were coated onto ELISA plates (50 µl/well) overnight at 4°C. Horseradish-peroxidase-linked sheep anti-mouse IgG (Amersham Life Science) was used as the detecting antibody. Absorbance at 450 nm was measured using Sunrise R microplate reader (Tecan, Männedorf, Switzerland). Assays were performed in triplicate.

2.7. Statistical analysis

Production of cytokines and parasite-specific antibodies was analyzed using Student's t-test. Parasitemia data was analyzed using two-way analysis of variance (ANOVA). Mouse survival after infection was analyzed by the Kaplan-Meier product limit method using Prism 5 software (Graph Pad Software, La Jolla, CA). P values were calculated by the log-rank (Mantel-Cox) test.

3. Results and Discussion

For further screening, we mapped the sequences of 1518 parasite-derived cDNA clones on the *P. berghei* draft genomic sequence using the sim4 program and annotated those clones by comparing them with the *P. falciparum* genomic sequence and homologous annotated genes. We made a genome viewer and opened the results (Full-malaria: <http://fullmal.hgc.jp>). Then the 1518 clones were subdivided into three groups: SP, consisting of 525 clones (242 genes) that encode proteins with signal peptides; NSP, consisting of 382 clones (143 genes) that encode proteins without signal peptides; and UK, consisting of 611 clones which were not mapped onto the *P. berghei* draft genome and thus not further characterized at that time. Using the Gene Gun, mice were immunized intradermally in the inguinal region with combined clones from these three groups, the combined 1518 clones (G1518) as a positive control and an empty vector (pCE-FL4) as a negative control. Following immunization, splenocytes were collected and co-cultured with the crude parasite antigens. Cytokine assays (by ELISA) of culture supernatants revealed that the UK clones induced higher levels of IFN- γ , IL-2, IL-4 and IL-10, suggesting that this group contained potentially effective clones (Figure 1). Immunization with the NSP clones or the empty control vector induced no significant production of cytokines. The expression levels of parasite-specific IFN- γ , IL-2 and IL-4 in SP were significantly higher than V, but the production levels of them were lower than G1518. Therefore, SP was excluded from screening.

1 The UK clones were further subdivided into three groups based on further
2 characterization by BLAST analysis: HP, 312 clones (185 genes) that hit to hypothetical
3 proteins; NSP2, 155 clones (85 genes) that hit to different genes than HP; and NH, 144
4 clones showing no significant homology. Cytokine ELISA after immunization revealed
5 that only the HP group induced higher cytokine production (Figure 1), suggesting that
6 ineffective clones had been excluded. The HP clones were yet again randomly
7 subdivided into three groups consisting of 104 clones each and analyzed. HP3 showed
8 the most effective induction of cytokine production (Figure 1). However, except for
9 IL-4, HP3-induced cytokine production appeared to be lower than with HP, suggesting
10 that effective clones may consist of multiple components.

11 Cytokine assay using purified splenic CD4⁺ T cells showed the same results as the
12 whole splenocytes, but all cytokine production levels were lower (data not shown).

13 Parasite-specific IgG was also detected in the sera from vaccinated mice. In
14 agreement with the patterns of cytokine production, G1518, UK, HP and HP3 showed
15 significantly higher IgG levels than the empty-vector control group (V), and HP1, HP2
16 and other groups showed the same level as V (Figure 2, and data not shown).

17 To assess the protective effect of the subsets, we performed challenge
18 infection by intraperitoneal injection of 50,000 parasite-infected erythrocytes per mouse
19 after vaccination. Early deaths were caused by the cerebral-malaria-like symptoms, and
20 late deaths were caused by the high-parasitemia derived symptoms. Although the levels
21 of parasitemia in all groups were the same as V (Figure 3A), the survival test revealed
22 that the UK, HP and HP3 subsets showed reduced early death after challenge infection

(Figure 3B).

Previously, we demonstrated that G2000 prolonged survival after challenge infection (Shibui et al. 2005). But this time, G1518, a subset consisting of only parasite-derived clones selected from G2000, failed to prolong the survival time. The results for the survival time with G2000 are not strictly reproducible, since G2000 consists of a pool of about 2,000 colonies of library-transformed bacteria formed on a plate. It is possible that the G1518 group includes both effective and exacerbating clones, and that it contains concentrated exacerbating clones more than G2000, resulting in no good effect on survival. This point needs to be clarified in future studies. In any case, the important point is that early death was reproducibly reduced by limited subsets of G1518.

One of the most important protective immune responses against malaria parasite infection is antibody-dependent cell cytotoxicity (ADCC) by macrophages (Good and Doolan 2010). All four cytokines, i.e., IL-2, IFN- γ , IL-4 and IL-10, specifically produced in response to the parasite are involved in ADCC. For example, IL-2 promotes T cell proliferation and differentiation (Kundig et al. 1993; Dooks et al. 2004; Saparov et al. 1999), IL-4 and IL-10 produced by Th2 cells are essential for B cell proliferation, expression of MHC-class II and antibody production (Freitas do Rosario and Langhorne 2012; Troye-Blomberg et al. 1994), and IFN- γ produced by Th1 cells is very important for macrophage activation (Schofield et al. 1987).

In summary, we used a murine model to screen for novel malaria DNA vaccine candidates from a *P. berghei*-derived full-length cDNA library. We succeeded in narrowing down the candidate clones from 1518 to 104 (Figure 4). Most of these 104

clones encode unknown hypothetical proteins, and they do not include already known vaccine candidate clones such as MSP or CSP (Schwartz et al. 2012; Schwartz et al. 2012; Holder 2009; Fowkes et al. 2010; Coppi et al. 2011). This suggests that novel malaria vaccine candidate molecules are included in the 104 clones. Further development of better screening methods will lead to identification of effective vaccine candidates. Our present study suggests that effective malaria DNA vaccine candidates can be found by elucidation of the immunological and physiological mechanisms involved in DNA vaccination.

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Figure legends

Figure 1. Parasite-antigen-specific cytokine production by spleen cells from immunized mice. Mice were immunized with pCE-FL control vector (V) or *P. berghei*-derived full-length cDNA subsets, as described in the Methods (n=4). The spleen cells from 4 mice were combined. Four million spleen cells were cultured with 50 µg of *P. berghei* crude antigens (black columns) or lysates of non-infected murine red blood cells (white columns) in 1 ml of RPMD1640 medium containing 10% fetal bovine serum in 24-well tissue culture plates. All cultures were performed in triplicate. Data show the mean ± SEM. *p<0.05, **p<0.01 vs. V. Data are representative of 3 to 5 independent experiments yielding similar results.

Figure 2. Parasite-antigen-specific serum IgG levels of immunized mice. Sera from subset-immunized mice were adsorbed with sonicated rat red blood cells, followed by reaction on plates coated with *P. berghei* crude antigens (black columns) or lysates of non-infected murine red blood cells (white columns). Absorbance at 450 nm was measured using Sunrise R microplate reader (Tecan, Männedorf, Switzerland). Data show the mean ± SEM (n=4). **p<0.01 vs. V. Data are representative of 3 to 5 independent experiments yielding similar results.

Figure 3. Protective effects of screened subsets after intraperitoneal *P. berghei* ANKA challenge infection (5×10^5 infected erythrocytes/mouse, n=8). A. Parasitemia was counted using Giemsa-stained blood smear preparations. Each point represents the mean

- 1 parasitemia $\pm$ SEM of the surviving animals of B. B. Survival after *P. berghei* infection.
- 2 The percentage survival was monitored daily (n=8).
- 3
- 4 **Figure 4.** Flow chart of the screening process in this study.
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Figure 1.

Figure 2.

Figure 3.

Figure 4.