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# Optimization of Antibody Responses of a Malaria DNA Vaccine in *Aotus* Monkeys

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## ABSTRACT

**We have demonstrated that intramuscular (IM) immunization with a plasmid DNA vaccine encoding the *Plasmodium yoelii* circumsporozoite protein (PyCSP) induces high antibody (Ab) levels and cytotoxic T cells against PyCSP and induces a CD8+ T-cell-dependent protection in 85% of BALB/C mice. In preparation for development of DNA vaccines designed to produce protective antibodies against *P. falciparum* erythrocytic-stage antigens (Ag), we conducted studies to optimize Ab responses in *Aotus* monkeys after immunization with the PyCSP DNA vaccine. Groups of 3 monkeys were immunized four times at weeks 0, 3, 6, and 47 with either 125-, 500-, or 2000-$\mu$g PyCSP plasmid-encoded DNA. Monkeys were injected either IM, with and without muscle pretreatment with bupivaciane, or intradermally (ID). Only monkeys injected ID produced antibodies against sporozoites as measured by both immunofluorescent assay test (IFAT) and enzyme-linked immunosorbent assay (ELISA). After the first three immunizations Ab titers in the ID groups peaked briefly at week 9 and titers declined to 50% their peak values by week 14. There was a general trend toward a dose response in the ID-injected groups with peak geometric mean IFAT titers at week 9 of 806, 508, and 403 for the 2000-, 500-, and 125-$\mu$g doses respectively. By week 46 anti-PyCSP Ab titers in the groups immunized IM remained negative, and anti-PyCSP Ab titers in the ID immunized groups declined to 20, 2, and 6% of the week 14 peak values for the 2000-, 500, and 125-$\mu$g doses, respectively. The monkeys were immunized with a fourth dose at week 47. At week 49 anti-PyCSP Ab titers in the ID immunized groups reached geometric mean IFAT titers of 28,963, 10,240, and 6,451 for the 2000-, 500-, and 125-$\mu$g doses of plasmid DNA respectively. This increase in Ab titer was at least a 4-fold increase over the week 14 mean peak titers and was equivalent to Ab titers generated with a PyCSP multiple Ag peptide (MAP) vaccine delivered with an adjuvant. No significant Ab titers were detected after the fourth dose in any of the IM immunized groups. These data clearly demonstrate that ID immunization of *Aotus* monkeys with a PyCSP plasmid DNA vaccine generated Ab responses equivalent to a MAP/adjuvant based vaccine, and support the use of the ID route for initial studies of the efficacy of DNA vaccines in inducing protective antibodies against *P. falciparum* erythrocytic stage Ags.**

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## INTRODUCTION

Each year there are 300–500 million new infections and 2–5 million deaths attributable to malaria that occur primarily in countries in the tropics, particularly in sub-Saharan Africa.[1] During the past 10–20 years the malaria problem has intensified in some parts of the world because parasites have developed resistance to drugs used for treatment and prevention;[2] the *Anophles* mosquito, which transmits the parasite to humans, has developed resistance to insecticides,[2] and control efforts have been reduced as resources have diminished in some developing countries. Many now believe that vaccines will be required to optimally control this disease.

All of the symptoms, signs, and pathology of malaria can be attributed to the asexual, erythrocytic, stage of the parasite life cycle, and thus the asexual stage of the life cycle is a major focus of vaccine development efforts.[3] One of the primary strategies for development of asexual, erythrocytic stage vaccine is to design a vaccine that induces protective antibodies against multiple parasite proteins expressed at this stage of the life cycle. To date, this has been a formidable task using traditional vaccine development methods and has resulted in limited success. However, DNA vaccine technology now offers the unique opportunity to rapidly construct and test multiple vaccines against many Ags.[4–9] This technology circumvents the need to express and purify recombinant proteins from prokaryotic or eukaryotic cells; there is no need to emulsify the vaccine with an adjuvant; vaccine production costs are likely to be a fraction of the cost of a recombinant vaccine, and the DNA is heat stable and does not require refrigeration. These vaccines have been shown to be highly immunogenic,[5,7,8,10–14] and we have been actively pursuing the development of a multivalent, multistage antimalaria plasmid DNA vaccine suitable for human use (6,13,15–18). To date, we have demonstrated the immunogenicity and protective efficacy in rodents of several antimalarial DNA vaccines by utilizing the rodent malaria *P. yoelii* model.[13,17] To translate our success in protecting mice from malaria to protecting humans from malaria requires the demonstration of the applicability of what we have learned in the *P. yoelii* model to *P. falciparum*.

The *Aotus* monkey has served as an animal host/model for the study of *P. falciparum*.[19] Despite the limitations of this model, *Aotus* monkeys have been successfully used for decades in the development of antimalarial drugs and, relatively recently, to study the efficacy of candidate antimalaria vaccines.[20–24, 26–37] We report here a series of studies in which we tested the ability of a plasmid DNA vaccine encoding the *P. yoelii* CSP protein to induce Ab generation in *Aotus* monkeys. Plasmid DNA encoding the *P. yoelii* CSP Ag was chosen to define the optimal dose and route of immunization in *Aotus* because we have no prior information on the immunogenicity of a DNA vaccine in these monkeys, yet we had a large database of information on the immunogenicity and efficacy of this vaccine in the rodent malaria model. Furthermore, using a *P. yoelii* Ag allowed us to make efficient use of our animal resources by using monkeys that had been previously infected and cured of *P. falciparum* and *P. vivax* rather than malaria-naive monkeys.

## MATERIALS AND METHODS

### *Immunogens: P. yoelii plasmid DNA vaccine*

The nkCMVintPyCSP.1 (PyCSP) plasmid was constructed as previously described.[13] Briefly, the Dra-I-EcoRV fragment of the PyCSP gene, cloned into the *Hinc*II site of pBluescript II SK(+) (Strategene), was transferred into the *Sal* I/Klenow-filled and *Bam*HI sites of kCMVinBL vector (modified pUC18-based plasmid pCMVintBL),[38] where the ampicillin-resistant gene has been replaced with a kanamycin-resistant gene by using the pBluescript *Xho*I/Klenow-filled and *Bam*HI restriction endonuclease sites located 5′ and 3′, respectively, to the PyCSP coding sequence. Expression of CSP was tested by in vitro transfection of COS cells and immunoblot analysis of cell lysates.

### P. yoelii *multiple Ag peptide (MAP) vaccine*

A previously described MAP vaccine, MAP4, $(QGPGAP)_4$ P2P30 was used.[39] This vaccine consists of a central lysine core and four branched chains, each containing the B-cell epitope $(QGPGAP)_4$ from the PyCSP major repeat, and two T-cell helper epitopes, P2 and P30,[40,41] from tetanus toxin.

### *Monkeys*

Panamanian adult (male and female) *Aotus lemurinus lemurinus* monkeys were maintained in the animal facility of the Gorgas Memorial Laboratory in Panama City, Panama. All monkeys used for these experiments had prior exposure to both erythrocytic stage *P. falciparum* and *P. vivax*. Prior to use in these experiments all monkeys were screened by IFAT and were negative for antibodies to PyCSP.

### *Injection of plasmid DNA*

Monkeys were injected with 29.5-gauge tuberculin needles either IM or ID. IM injections were administered at two sites, right and left tibialis anterior muscles. At each site injections were with plasmid DNA dissolved in 100-$\mu$l (Experiment 1) or 500-$\mu$l (Experiment 2) sterile normal saline. When the muscle was pretreated with bupivacaine the left and right tibialis anterior muscle was injected with 500 $\mu$l of 0.25% bupivacaine (Marcaine®) 24 hr prior to plasmid DNA injection.[42] For ID injections, monkeys were injected in the lower back approximately 2–5 cm above the tail with plasmid DNA dissolved in sterile normal saline. One hundred microliters were used per injection site, and there were six injection sites per dose per animal.

### *Injection of MAP peptide*

Monkeys were bilaterally injected IM with a 28-gauge needle in the quadriceps with a total of 100 $\mu$g of $MAP_4$ $(QGPGAP)_4$ P2P30 emulsified in 400 $\mu$l of Montanide ISA 51 (20 $\mu$l [50 $\mu$g] in each quadricep).

### *Measurement of antibodies to PyCSP: ELISA*

Ab titers were determined by ELISA as previously described[39] with slight modification. In brief, 50 $\mu$l of 0.3 $\mu$g/ml PyCS.1 (a recombinant fusion protein PyCS.1 produced in *Escherichia coli* that includes aa 64-321 or PyCSP fused to 81 aa from the nonstructural protein of influenza A) was added to wells of flat-bottom, Immunlon II ELISA plates (Dynatech Laboratories, Chantilly, VA) and incubated for 6 hr at room temperature. The Ag wells were blocked overnight with a blocking buffer (PBS containing 0.5% Tween 20, 3% nonfat dried milk) and washed three times with a washing buffer (PBS containing 0.05% Tween 20). A diluted volume of sera in PBS from each monkey was added to the wells, and the plates incubated for 2 hr at room temperature. The wells were washed three times with PBS-Tween 20 before incubating for 1 hr with 50 $\mu$l of a 1:200 dilution of horseradish peroxidase-conjugated goat anti-*Aotus* immunoglobulin (V. Sang, CDC, Atlanta, GA). The wells were washed again after incubation, and 100 $\mu$l of a 1:1 dilution of ABTS substrate, (2,2-azino-di-3-ethyl-benzthiazoline sulfonate; Kirkegaard and Perry, Gaithersburg, MD), and hydrogen peroxide was added. Color reaction was measured on a Dynatech micro-ELISA reader model MR5000 at an OD of 310 nm. The assays were performed in quadruplicate, and the results shown are the OD of a 1:100 dilution of the sera.

### *IFAT*

Sera were tested in the IFAT as previously described[39,43] with slight modification. In brief, diluted sera from each individual monkey was added to each well of multispot Ag slides containing 5,000 air-dried *P. yoelii* sporozoites. The slides were incubated in a moist chamber for 30 min at 37°C and washed once in PBS. A 1:200 dilution of fluorescein-labeled goat anti-*Aotus* immunoglobulin (V. Sang, CDC, Atlanta, GA) in PBS was added to the wells, and slides were incubated for an additional 30 min, washed with PBS and mounted in 10% glycerol in PBS, and examined under an Olympus UV microscope. IFAT values reported are the last dilution in which the sporozoites on the multispot Ag slides had fluorescence.

## RESULTS

### *IM immunization with a PyCSP DNA vaccine (Experiment 1)*

Previous work in mice had demonstrated that the PyCSP plasmid gave consistently high Ab responses when 50 $\mu$g of PyCSP plasmid DNA was injected IM.[13,17] To determine an optimal IM dose of plasmid

DNA in *Aotus* monkeys we performed a pilot experiment in which nine monkeys (three groups of three monkeys) were IM injected with 500, 50, or 5-$\mu$g doses (50% of each dose in each *tibialis anterior* muscle) of the PyCSP plasmid DNA. Animals received a total of three doses at 4-week intervals. Unlike our experience in mice[13] (100% of mice positive for antibodies with three doses of 50 $\mu$g at 3-week intervals), none of the monkeys developed an Ab titer greater than 1/80 over a 32-week period following the first dose, even at doses as high as 500 $\mu$g (data not shown).

### *IM immunization with a PyCSP DNA vaccine (Experiment 2)*

Due to the lack of an Ab response in our pilot experiment, we tried altering several immunization variables to determine the optimal dose and route of administration in *Aotus* monkeys. To this end, we designed an experiment in which we (a) increased the dose of plasmid DNA, (b) pretreated the muscle with bupivacaine prior to plasmid DNA injection, (c) injected the plasmid DNA ID,[44] (d) shortened the time between the initial three doses and included a fourth dose at week 47, (e) increased the volume of IM-injected plasmid DNA fivefold, and (f) compared Ab generation after immunization with the plasmid DNA-based vaccine with a MAP vaccine.

A total of 30 monkeys were used for this experiment. Twenty-seven monkeys received three doses of 2000-, 500-, or 125-$\mu$g of plasmid DNA at 3-week intervals with a final dose at week 47. Of the 30 monkeys 9 received the plasmid DNA IM; 9 were pretreated with bupivacaine at the site of muscle injection 24 hr prior to IM plasmid DNA injection; and 9 received the plasmid DNA ID. In addition, three monkeys were injected with the MAP PyCSP vaccine to compare the ability of a plasmid DNA-based vaccine and the peptide-based vaccine to induce antibodies.

Similar to our pilot experiment (above; data not shown), even at the highest dose (2000 $\mu$g) no monkey injected IM with the plasmid DNA developed an Ab titer above 1/80, by IFAT, or an optical density (O.D.) at a 1:100 serum dilution greater than 0.624 by ELISA over a 48-week period following the first dose (data not shown). The highest Ab response, as determined by IFAT, was a titer of 1/80 (2 weeks after the first dose) in a monkey that received 125 $\mu$g of PyCSP plasmid. The IFAT titer was at 1/80 after the second dose, but it dropped to 1/40 after the third dose, and remained 1/40 after the fourth dose at week 47. The monkeys that received either 2000 or 500 $\mu$g of PyCSP by IM injection had IFAT Ab titers of 1/20 or less throughout the experiment. ELISA values of monkeys injected IM were in concordance with the IFAT data.

### *IM immunization of a PyCSP DNA vaccine with bupivacaine (Marcaine®) pretreatment*

Bupivacaine pretreatment of murine muscle prior to plasmid injection had been demonstrated to increase immunogenicity of plasmid DNA immunization.[42,45] The mechanism by which bupivacaine increases immunogenicity is unknown but has been thought to be due to the ability of bupivacaine to cause minor myofiber damage, thereby allowing the regenerating muscle to be more susceptible to in vivo plasmid transfection.[42,46–50] To test the capacity of bupivacaine to augment IM administration of PyCSP plasmid DNA in *Aotus* monkeys each *tibialis anterior* muscle was pretreated with 500 $\mu$l of 0.25% bupivacaine 24 hr prior to plasmid DNA injection at the same site. Nine *Aotus* monkeys (three groups of three) were immunized bilaterally in the *tibialis anterior* with 1000 $\mu$l (500 $\mu$l per site) of either 2000-, 500-, or 125-$\mu$g of the PyCSP plasmid. Similar to the IM results without bupivacaine pretreatment none of the pretreated, IM-injected monkeys had more than a twofold increase in IFAT titer over baseline, and no monkey had an IFAT titer greater than 1/40.

### *ID immunization with a PyCSP DNA vaccine*

Nine *Aotus* monkeys (three groups of three) were immunized ID on the lower back with 600 $\mu$l (100 $\mu$l per site; 6 sites per dose) of either 2000, 500, or 125 $\mu$g of the PyCSP plasmid. In remarkable contrast to animals injected IM, all monkeys injected ID developed antibodies to PyCSP within 3 weeks after the first dose (Fig. 1a and 1b). Ab titers in the ID groups peaked 3 weeks after the third dose (week 9) and declined to 50% their peak values by week 14 (5 weeks after the third dose). There was an apparent dose response

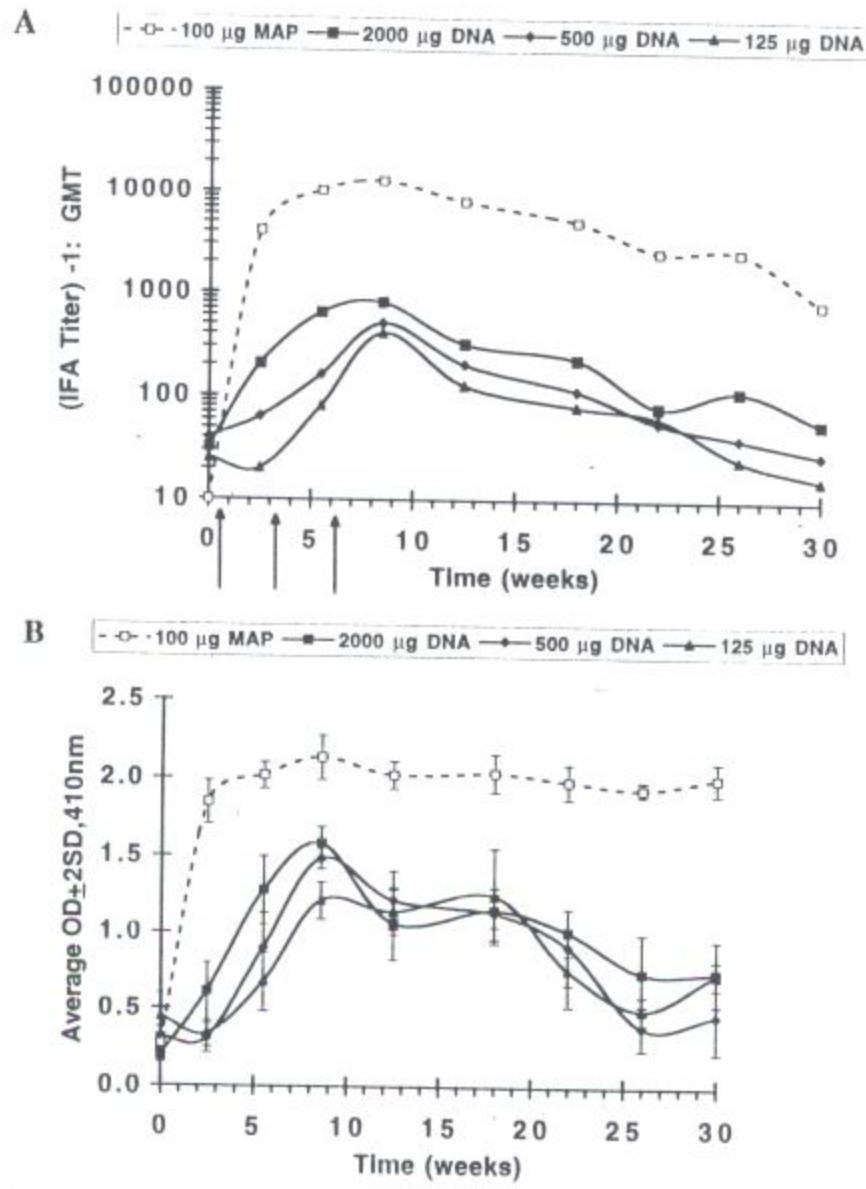


FIG. 1. Antibodies (Abs) after intradermal (ID) immunization of *Aotus* monkeys with a PyCSP DNA vaccine; first three doses. (A) Geometric mean IFAT titers of *Aotus* serum to air-dried *P. yoelii* sporozoites. (B) Geometric mean optical densities ± SEM of *Aotus* serum to the recombinant PyCSP protein, PyCS.1. Arrows indicate the day of each dose. Solid squares, diamonds, and triangles indicate Ab titers from monkeys injected with 2000-, 500-, or 125-$\mu$g PyCSP plasmid, respectively. Open squares with dotted line indicate Ab titers from monkeys injected with the PyCSP MAP vaccine in Montanide ISI 51.

in the ID-injected groups with peak geometric mean IFAT titers at week 9 of 1/806, 1/508, and 1/403 for the 2000-, 500-, and 125-$\mu$g doses, respectively. Because of the small sample size and variation in response among animals within groups, there was no significant difference between these peak antibody levels ($p$ = 0.30, Kruskal-Wallis one-way ANOVA). When taking into account all IFA data collected over the first 30 weeks of study, we found a positive, albeit weak association between IFA titer and dose, such that high titers tend to be found in monkeys injected with a high dose of the DNA, plasmid (correlation coefficient = 0.37, 95% confidence limits 0.15–0.55).

By week 46 anti-PyCSP Ab titers had declined to 20%, 2%, and 6% of the week 9 peak values for the 2000-, 500-, and 125-$\mu$g doses, respectively (Figures 1a and 1b). The monkeys were immunized with a

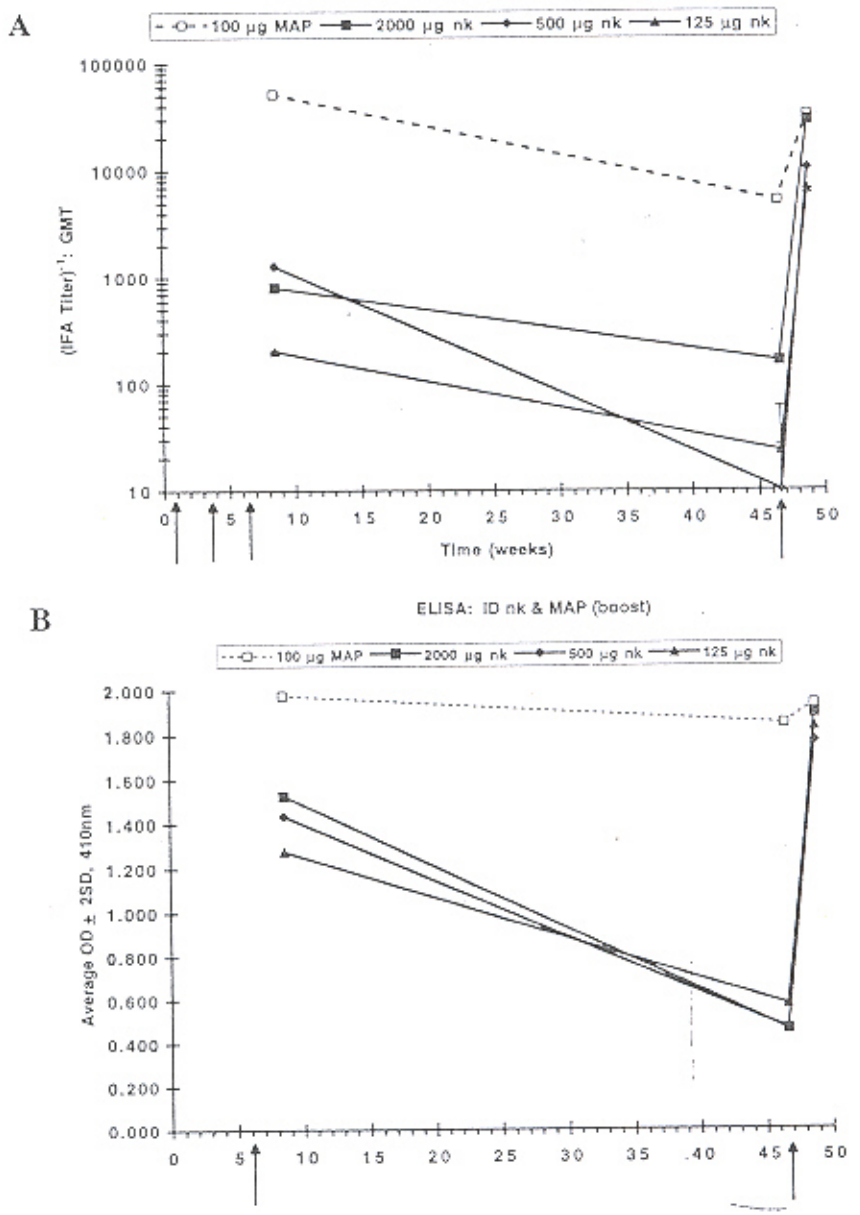


FIG. 2. Antibodies (Abs) after intradermal (ID) immunization with a fourth dose following a 41-week "rest" period. (A) Geometric mean IFAT titers of *Aotus* serum to air-dried *P. yoelii* sporozoites. (B) Geometric mean optical densities ± SEM of *Aotus* serum to the recombinant PyCSP protein, PyCS.1. Arrows indicate the day of each dose. Solid squares, diamonds, and triangles indicate Ab titers from monkeys injected with 2000-, 500-, or 125-$\mu$g PyCSP plasmid, respectively. Open squares with dotted line indicate Ab titers from monkeys injected with the PyCSP MAP vaccine in Montanide ISI 51.

fourth dose at week 47. At week 49 geometric mean IFAT titers were 1/28,963, 1/10,240, and 1/6,451 in sera from monkeys that received the 2000-, 500-, and 125-$\mu$g doses, respectively. These Ab titers were equivalent to Ab titers in the monkeys immunized with the PyCSP peptide vaccine delivered in an adjuvant (Figure 2a).

In concordance with and confirmation of the IFAT data, the ELISA values closely parallel the IFAT data (Figs. 1b and 2b).

*Immunization with the MAP PyCSP vaccine*

To compare the relative immunogenicity of the PyCSP plasmid DNA vaccine to a PyCSP peptide vaccine we immunized a group of three monkeys with a PyCSP peptide vaccine. The peptide vaccine consisted of a four-branched-chain multiple Ag peptide (MAP) vaccine that included the PyCSP repeat peptide, QGPGAP, as the B-cell epitope and two T-helper epitopes (P2 and P30) from tetanus toxin.[39] This PyCSP MAP vaccine had been shown in mice to produce high titer antibodies and protection from a *P. yoelii* challenge.[39] The PyCSP MAP peptide vaccine was administered by bilateral quadricep IM injections of a total of 100 $\mu$g of MAP vaccine emulsified in Montanide ISA 51 adjuvant. All monkeys developed high geometric mean titers of anti-PyCSP antibodies within 2 weeks after the first dose as measured by both IFAT (1/4,064; Figure 1a) and ELISA (O.D. of 1.845 at a serum dilution of 1:100; Fig. 1b). Anti-PyCSP antibodies reached a peak geometric mean IFAT titer at week 9 of 1/12,902 (Fig. 1a). The PyCSP MAP vaccine induced antibody levels at least 10-fold higher than any dose of the ID-injected plasmid DNA vaccine (Figures 1a and 1b). At week 14, when the plasmid DNA vaccine had fallen to 50% its week 9 peak value, the PyCSP MAP vaccine IFAT titer was 1/5120, a drop to 63% of its peak value. By week 46 the PyCSP MAP vaccine was at 40% its week 9 value (Figs. 1a,b and 2a,b). At week 49 (2 weeks after the fourth dose), the mean IFAT titers of the PyCSP MAP vaccine rose to 1/32,510, approximately a 2.5-fold increase from the week 9 mean peak value when tested simultaneously (Fig. 2a).

## DISCUSSION

These studies clearly demonstrate that ID administration of the PyCSP plasmid DNA vaccine is an effective way to elicit a potent humoral immune response in *Aotus* monkeys. After the fourth dose at week 47 the Ab titers were equivalent to the titers of the PyCSP MAP vaccine. The first three doses of PyCSP plasmid DNA vaccine produced a low, transient titer of Ab that reached a peak at week 9 and declined to 50% of the week 9 values by week 14. The magnitude of this Ab response at week 9 was 10-fold less than that elicited by the PyCSP MAP vaccine. It was not clear after the first three doses if the peak antibody titers achieved at week 9 represented the accumulation of multiple primary antibody responses or some degree of boosting after each successive dose. The fourth dose at week 47 produced a clear boost of antibody response resulting in high levels of Ab, indicating that optimal immunization and memory B-cell formation requires a significant "rest" period between initial immunization and the following "boost." The minimal number of doses and the minimal time interval between doses to achieve high, sustainable Ab generation is currently under investigation.

Of profound significance is the complete lack of an Ab response in all monkeys injected IM with plasmid DNA. In previous reports from our laboratory, and the laboratories of others, IM immunization with plasmid DNA vaccines in mice has resulted in Ab production to a variety of pathogens.[7,10–12,14,51–55] However, there have been very few studies of plasmid DNA vaccines in monkeys.[56,57] There has been a study documenting the expression of proteins encoded by reporter genes in the muscle cells of monkeys injected IM with plasmid DNA,[45] and one study that reported induction of neutralizing Ab responses in monkeys after IM DNA immunization.[57] Both of these studies reported far less expression of the injected plasmid, or in subsequent Ab production, in monkeys as compared to mice. The decreased expression of IM-injected plasmid DNA vaccines in monkeys, as compared to mice, may be due to any one, or a combination of several factors, which include, but are certainly not limited to, host differences in muscle physiology and anatomy, age and immunological maturity of the host, plasmid promoter activity in tissues of different hosts, differences in the immunogenicity of specific gene sequences in different hosts, host differences in the ability to present Ags in the muscle, and differences in the abundance or migration of professional Ag-presenting cells in the muscle of different species. To discuss all these possibilities is certainly not within the scope of this report and would be an interesting topic of a speculative review. However, regardless of the reason, it is clear from these data and the data of others that IM plasmid DNA immunization in nonhuman is less effective for induction of antibodies in monkeys than it is in mice.

Bupivacaine (Marcaine®) pretreatment of the muscle 24 hr prior to plasmid injection had no discernible augmenting effect on the immunogenicity of IM-injected PyCSP plasmid. Because no significant antibodies were detected in monkeys by IM injection without bupivacaine pretreatment, and the mechanism by which bupivacaine augments plasmid expression and immunogenicity in mice in unknown, it is difficult to discern why it failed to augment immunogenicity in *Aotus* monkeys. Wang et al. used 0.25% bupivacaine pretreatment to immunize mice and cynomolgus monkeys with a cloned fragment of the HIV gp160 gene in a RSV-enhancer/MMTV LTR-promoter plasmid[57] and demonstrated Ab production in both species. However, an IM-injected group without the use of bupivacaine was not reported, so it is difficult to tell what, if any, augmenting effect bupivacaine had. It is also possible that either the dose or time of muscle pretreatment was not optimal for *Aotus* monkeys. Danko et al.[42] demonstrated in mice that plasmid-encoded luciferase expression was optimal if the muscle was injected with bupivacaine 7 days prior to plasmid injection. Additionally, if the mechanism by which bupivacaine increases plasmid expression and immunogenicity in mice is due to myofiber damage and recovery as some have speculated,[42,46–50] then perhaps the dose of bupivacaine given to the *Aotus* monkeys was insufficient to cause significant myofiber damage.

The APCs in the skin that induce the humoral immune response to protein translated off the plasmid are unknown, but several obvious possibilities exist. When the plasmid is injected into the skin it may directly transfect "professional" APCs such as Langerhans cells. Alternatively, other cell types in the skin (fibroblasts) may be transfected with the plasmid and secrete protein, which in turn is taken up and processed by skin associated lymphoid tissues (SALT), the most obvious candidates being Langerhan's cells. It is well documented that Langerhan's cells, when loaded with foreign Ag, quickly migrate out of the skin and into regional lymph nodes to induce potent immune responses.[58–60] Certainly, potential differences in the abundance of APCs in the skin and muscle of *Aotus* monkeys may explain the stark differences in immunogenicity of plasmid DNA at these two sites. It should be emphasized, however, that we did not examine the ability of this plasmid to induce a class I restricted, CD8+ T-cell immune response in these animals. The primary reason for this is the lack of immuno reagents needed to quantitate such a response in *Aotus*. It is quite possible that IM administration of plasmid induced a potent class I MHC-restricted CD8+ T-cell response to PyCSP. We are currently examining this possibility in rhesus monkeys, where many of the reagents developed for human use cross-react with rhesus. Rhesus, however, is a poor host for *P. falciparum*, and nonhuman testing of the efficacy of *P. falciparum* vaccines against a parasite challenge will still have to be done in *Aotus*.

The difference between mice and monkey plasmid DNA immunization is important because of the implications these data have to human application of these vaccines. Because monkeys are phylogenically closer to humans it is reasonable to speculate that the optimal dose and route of immunization regimes in monkeys will more closely predict optimal regimens for humans than will mice. If this is true then based on these data, one must consider ID immunization as a possibility in the design of any human plasmid vaccine trial designed to induce protective Abs.

These data provide a strong foundation for beginning studies with erythrocytic stage vaccines in *Aotus* monkeys, and we plan to examine the immunogenicity and efficacy of several *P. falciparum* erythrocytic stage Ags, including merozoite surface protein-1 (MSP-1),[61] apical membrane Ag-1 (AMA-1),[62] exported protein-1 (Exp-1),[63] serine erythrocyte repeat Ag (SERA),[64] and erythrocytic binding antigen-175 (EBA-175)[65] by ID injection. Additionally, we have demonstrated the usefulness of *Aotus lemurinus lemurinus* as a *P. falciparum* (St. Lucia) preerythrocytic model (manuscript in preparation) and we have begun preerthryocytic multivalent plasmid immunization in these animals with *P. falciparum* circumsporozoite protein (CSP),[17] surface sporozoite protein-2 (SSP2),[66] and Exp-1 by both ID and IM immunization.

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Council (Department of Health and Human Services, Publ. National Institutes of Health 86-23, 1985). The opinions and assertions herein are the private ones of the authors and are not to be construed as official or as reflecting the views of the U.S. Navy or the Department of Defense.

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