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Vaccine 20 (2002) 1675–1680

Vaccine

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Absence of antigenic competition in *Aotus* monkeys immunized with *Plasmodium falciparum* DNA vaccines delivered as a mixture

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Received 28 June 2001; received in revised form 21 September 2001; accepted 23 October 2001

Abstract

Aotus lemurinus lemurinus monkeys were immunized four times with one of three DNA plasmids expressing important *Plasmodium falciparum* blood stage vaccine candidate proteins or with a mixture containing all three vaccines. The three vaccines encoded sequences from apical merozoite antigen-1 (AMA-1), erythrocyte binding protein-175 (EBA-175) and merozoite surface protein-1 (MSP-1). Antigen-specific enzyme-linked immunosorbant assays (ELISAs) showed no significant differences in antibody titer induced to the three antigens by a single vaccine compared with the titer induced to that same antigen by the trivalent preparation. Results of immunofluorescent antibody assays against erythrocytes infected with asexual blood stage *P. falciparum* indicated that each of the three monovalent vaccines induced significant antibody responses to whole parasites. The trivalent vaccine mixture induced, after four immunizations, an antibody titer to whole parasites that was 3–12-fold higher than those induced by any of the single vaccines. The fourth immunization with the trivalent vaccine increased the mean antibody in IFAT by more than five-fold. Published by Elsevier Science Ltd.

Keywords: Malaria; Immunization; DNA vaccines

1. Introduction

Efforts to develop DNA vaccines for a variety of diseases are increasing. To improve the effectiveness of the immune response, bivalent and multivalent vaccines are being evaluated [1–3]. Other types of vaccines, for example polysaccharide vaccines, have shown evidence of antigenic interference when multiple antigens are delivered together [4]. This study evaluates the humoral immune response in *Aotus* monkeys to three DNA vaccines encoding well-characterized *Plasmodium falciparum* blood stage antigens, apical merozoite antigen-1 (AMA-1) [5,6], erythrocyte binding protein-175 (EBA-175) [7–9] and merozoite surface protein-1 (MSP-1) [10,11]. These vaccines were administered individually and in a trivalent mixture.

2. Materials and methods

2.1. Plasmid construction

2.1.1. EBA-175

The production of the plasmid encoding EBA-175 has been described [12]. In brief, *P. falciparum* EBA-175 RII (amino acids 145–760, 1848 bp for FVO) was amplified using Vent DNA polymerase (New England BioLabs, Beverly, MA) from 100 ng of genomic DNA using the forward primer 5′-ATCGAGATCTGGAAGAAATACTTCATCT-3′. Reverse primer 5′-ATCGAGATCTTTACGAAGTTTGTTCATTATT-3′ was used for cloning into the expression plasmid vector VR1050 [2]. This plasmid vector uses the human cytomegalovirus promoter and intron A, human tissue plasminogen activator (tPA) as the secretory signal and the bovine growth hormone transcriptional terminator/polyadenylation signal. To enhance immunogenicity, the expression plasmid vector contained the gene sequences encoding the universal T-epitopes from tetanus toxin P2P30 [13] which were located 5′ to the *RII* gene in the DNA vaccine.

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0264-410X/02/$ – see front matter. Published by Elsevier Science Ltd.
PII: S0264-410X(01)00513-8

2.1.2. AMA-1

The full-length *AMA-1* gene (FVO) was cloned at the 3′ end and in fusion with the tPA leader sequence of the VR1020 plasmid as described previously [14]. The *AMA-1* gene was amplified with the following oligonucleotides: forward 5′-CCCGGATCCATGAGAAAATTATACTGCGTA-3′ and reverse 5′-CCCAGATCTTTAATAGTATGGTTTTTC-CAT-3′ and cloned into the Bam HI and Bgl II restriction sites of the VR1020 plasmid.

2.1.3. MSP-1

The 42 kDa region MSP-1 of *P. falciparum* (FVO strain) was cloned into the mammalian expression plasmid VR1050 (Kumar, unpublished data). The $MSP\text{-}1_{42}$ gene fragment was PCR-amplified from asexual stage genomic DNA and restriction digested with Bam HI and Bcl I enzymes and cloned into Bam HI/Bgl II sites in VR1050.

2.2. Monkeys

Forty Panamanian adult (male and female) *A. lemurinus lemurinus* (karyotype VIII or IX) monkeys [15] were maintained in the animal facility of the Gorgas Memorial Laboratory in Panama City, Republic of Panama. *A. lemurinus lemurinus* monkeys were obtained in western Panama. Upon arrival at the laboratory, each animal was given a physical examination, weighed and sexed, identified by a metal neck tag with an accession number, administered thiabendazole orally for treatment of endoparasites (100 mg base/kg) and vaccinated against *Herpes simplex* and *H. tamarinus* (New England Regional Primate Research Center, Southborough, MA) and *Klebsiella pneumoniae* [16]. The animals were housed and cared for as previously described [16]. About 1 month after arrival, each monkey was tattooed with its identification number and a thick blood film examined to exclude naturally occurring plasmodial infections. The animals remained in quarantine for a minimum of 90 days before being transferred to areas devoted to housing monkeys for malaria studies. The weight of the monkeys when inoculated ranged from 700 to 800 g. All monkeys used in these experiments were wild caught adult monkeys and had no history of experimental infection with *Plasmodium*.

2.3. Immunization

The 40 monkeys were randomly assigned to one of five groups, each group containing eight monkeys. The animals received four immunizations on weeks 0, 5, 10 and 21. Groups 1, 2 and 3 received 500 μg of plasmid per dose in a total volume of 400 μl per dose delivered intradermally (ID), approximately 100 μl per site, at four sites along the flanks. Group 4 received a mixture of 500 μg of each of the three plasmids encoding EBA-175, AMA-1 and MSP-1 in a volume of 400 μl delivered ID at four sites. Group 5 received 500 μg of plasmid VR1020 and 1000 μg of plasmid VR1050 in a total volume of 400 μl delivered ID at four sites along the flank. In all cases, insulin syringes with hubless 29 gauge needles were used for the ID injections.

Table 1
The schedule for immunizations, serum collections and immunologic assays

Event	Day	Test
Pre-immune bleed	0	All ELISAs, IFA
First immunization	2	
Post 1 bleed	12	EBA ELISA
Post 1 bleed	28	AMA and MSP ELISAs, IFA
Second immunization	36	
Post 2 bleeds	55	All ELISAs, IFA
Third immunization	64	
Post 3 bleeds	83	All ELISAs, IFA
Fourth immunization	113	
Post 4 bleeds	160	All ELISAs, IFA

2.4. ELISAs

IgG responses to the three immunogens were measured in ELISA as previously described [17–19]. *Escherichia coli*-produced recombinant *P. falciparum* AMA-1 ectodomain protein [20], purified baculovirus recombinant EBA-175 RII protein and *Saccharomyces cerevisiae*-produced recombinant $MSP\text{-}1_{19}$ were used as the capture antigens. The ELISA results are reported as the serum dilution estimated to give an OD of 0.5 which was determined by linear interpolation from the log-transformed dilutions yielding values bracketing OD 0.5.

2.5. Immunofluorescent antibody test

IFAT tests were conducted as described elsewhere [21]; fluorescence detection was done with FITC-labeled goat anti-human IgG (ICN Biomedical, Costa Mesa, CA). Positive control antibody was mouse polyclonal serum raised against an AMA-1 DNA vaccine.

2.6. Schedule

The schedule of immunizations, serum collections and assays is shown in Table 1.

2.7. Statistics

All statistical analyses were performed using SPSS for Windows version 8.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. Antibody to AMA-1

Sera collected at the five collection dates (Table 1) from groups 1 (AMA-1 only), 4 (trivalent) and 5 (trivalent control)

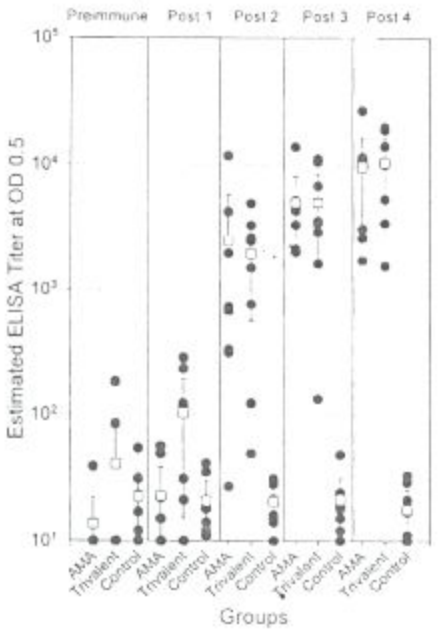

Fig. 1. Pre-immune and post-immunization serum samples were collected from *Aotus* monkeys (eight per group) immunized with a DNA vaccine encoding AMA-1 (AMA) or a pool of three DNA vaccines encoding EBA-175, AMA-1 and MSP-1_{42} (trivalent) or with a pool of the plasmids lacking the sequences encoding antigen (control). Individual estimated antibody titers producing an OD of 0.5 (●) and mean titers for each group with 95% confidence intervals (□) against recombinant AMA-1 protein are shown below. Each of the five panels shows the titers for each of the five serum collections, as labeled at the top of the graph.

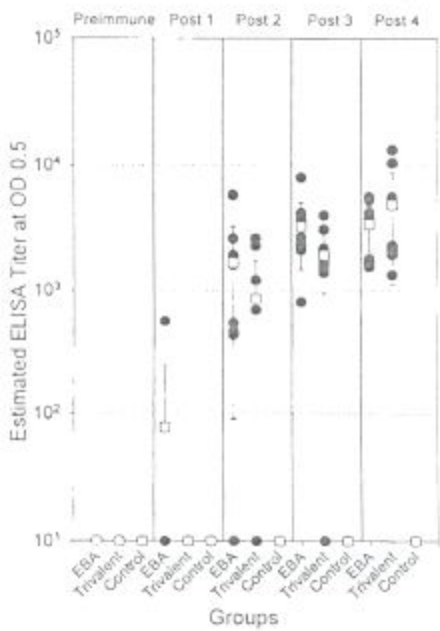

Fig. 2. Pre-immune and post-immunization serum samples were collected from *Aotus* monkeys (eight per group) immunized with a DNA vaccine encoding EBA-175 (EBA) or a pool of the DNA vaccines encoding EBA-175, AMA-1 and MSP-1_{42} (trivalent) or with a pool of three plasmids lacking the sequences encoding antigen (control). Individual estimated antibody titers producing an OD of 0.5 (●) and mean titers for each group with 95% confidence intervals (□) against recombinant EBA-175 RII protein are shown below. Each of the five panels shows the titers for each of the five serum collections, as labeled at the top of the graph.

were tested for antibody activity to AMA-1 in ELISA. The results are displayed in Fig. 1. The mean ELISA titer in the group immunized with AMA-1 alone was compared with the mean titer of the group immunized with the trivalent vaccine at each of the five sera collection time points. At no time point were the mean titers from the two groups significantly different from one another (P values ranging between 0. 26 and 0.99, Student's t-test, two-tailed). Power calculations based on the post-fourth immunization ELISA indicates that if the true difference between groups 1 and 4 is the difference we measured (10,430 (trivalent) − 9589 (monovalent) = 841), we had a 6% probability of detecting that difference ($P < 0.05$). We would have had a power of 80%, if the true difference between the means were 11,210.

3.2. Antibody to EBA-175

Sera collected at the five collection dates (Table 1) from groups 2 (EBA-175 only), 4 (trivalent) and 5 (trivalent control) were tested for antibody activity to recombinant EBA-175 in ELISA. The results are displayed in Fig. 2. The mean ELISA titer in the group immunized with EBA-175 alone was compared with the mean titer of the group immunized with the trivalent vaccine at each of the five sera collection time points. At no time point were the mean titers from the two groups significantly different from one another (P values ranging between 0.15 and 0.41, Student's t-test, two-tailed). The pre-immune sera could not be compared statistically because antibody activity was so low that an estimated titer giving an OD of 0.5 could not be calculated. Power calculations based on the post-fourth immunization ELISA indicates that if the true difference between groups 2 and 4 is the difference we measured (4925 (trivalent) − 3433 (monovalent) = 1492), we had a 13% probability of detecting that difference ($P < 0.05$). We would have had a power of 80% if the true difference between the means were 5167.

3.3. Antibody to MSP-1

Sera collected at the five collection dates (Table 1) from groups 3 (MSP-1 only), 4 (trivalent) and 5 (trivalent control) were tested for antibody activity to MSP-1 in ELISA. The results are displayed in Fig. 3. The mean ELISA titer in the group immunized with MSP-1 alone was compared with the mean titer of the group immunized with the trivalent vaccine at each of the five sera collection time points. At no time point were the mean titers from the two groups significantly different from one another (P values ranging between 0.12 and 0.53, Student's t-test, two-tailed). The mean titers after four immunizations were not similar (MSP-1 group (8756) versus trivalent group (1830)). This large but non-significant

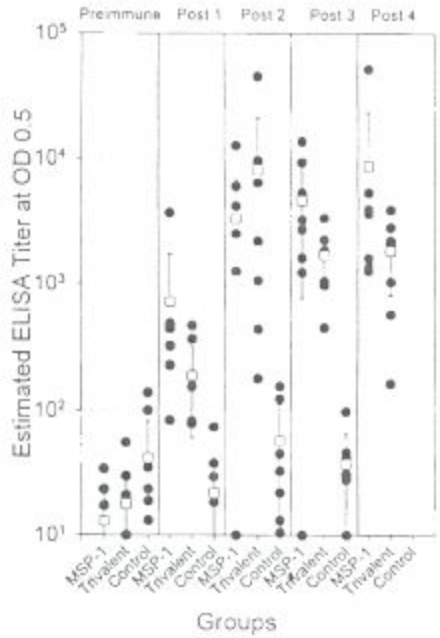

Fig. 3. Pre-immune and post-immunization serum samples were collected from *Aotus* monkeys (eight per group) immunized with a DNA vaccine encoding MSP-1_{42} (MSP-1) or a pool of three DNA vaccines encoding EBA-175, AMA-1 and MSP-1_{42} (trivalent) or with a pool of the plasmids lacking the sequences encoding antigen (control). Individual estimated antibody titers producing an OD of 0.5 (●) and mean titers for each group with 95% confidence intervals (□) against recombinant MSP-1_{19} protein are shown below. Each of the five panels shows the titers for each of the five serum collections, as labeled at the top of the graph.

difference was due entirely to a single monkey in the MSP-1 group with an extraordinarily high titer (51,579). Because of this very high single value, we did not perform power calculations on these data.

3.4. Antibody to whole blood stage parasites

Sera collected at the five collection dates (Table 1) from all five groups were tested for antibody activity to whole blood stage parasites in IFAT. The results are displayed in Fig. 4. The mean titers for each of the five groups were compared at each sera collection. There were no differences among the groups at the pre-immune collection. After one immunization, the mean end-point titers in groups 3 (MSP-1) and 4 (trivalent) were greater than group 5 (trivalent control), $P = 0.045$ and 0.042, respectively. After two immunizations, the mean end-point titer in group 4 (trivalent) was greater than all other groups (P values ranging from 0.001 to <0.0009); group 3 (MSP-1) was greater than groups 1 (AMA-1, $P = 0.029$), 2 (EBA-175, $P = 0.039$) and 5 (trivalent control, $P = 0.005$). After three immunizations, both groups 3 (MSP-1) and 4 (trivalent) had higher mean end-point titers than group 5 (trivalent control), $P = 0.001$ and 0.0156, respectively. Group 3 (MSP-1) also had a higher titer than group 1 (AMA-1), $P = 0.013$. After the fourth immunization, group 4 (trivalent, titer = 17,920) had a mean end-point titer greater than all other groups (all P values <0.0009; titers = 1480, 2880 and 6400 for AMA-1,

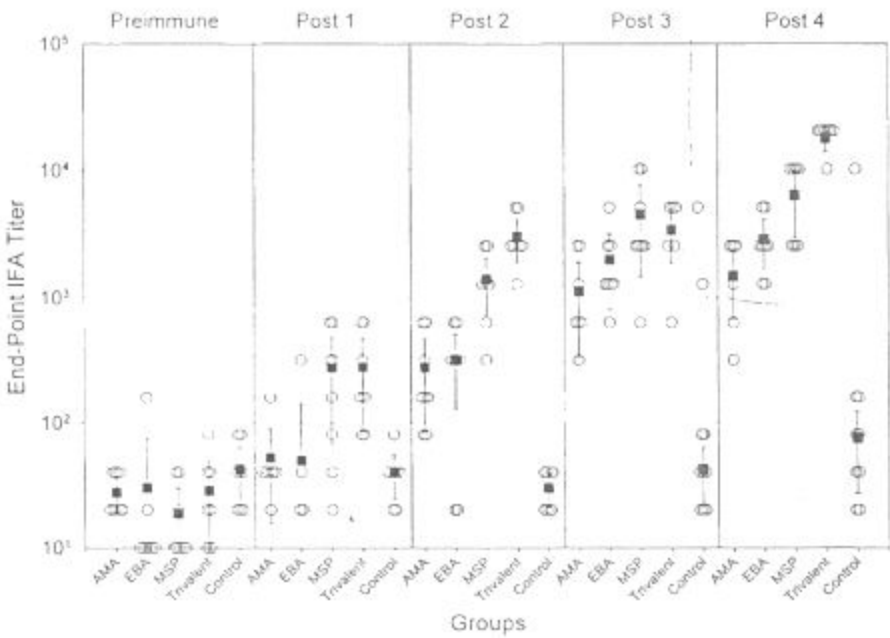

Fig. 4. Pre-immune and post-immunization serum samples were collected from *Aotus* monkeys (eight per group) immunized with a DNA vaccine encoding AMA-1 (AMA) or EBA-175 (EBA) or MSP-1_{42} (MSP) or a pool of three DNA vaccines encoding AMA-1, EBA-175 and MSP-1_{42} (trivalent) or with a pool of the plasmids lacking the sequences encoding antigen (control). Individual end-point titers in an immunofluorescent antibody test against erythrocytes infected with *P. falciparum* asexual parasites (○) and mean titers for each group with 95% confidence intervals (■) against erythrocytes infected with *P. falciparum* asexual parasites are shown below. Each of the five panels shows the titers for each of the five serum collections, as labeled at the top of the graph.

EBA-175 and MSP-1, respectively). In addition, group 3 (MSP-1) had a higher titer than groups 1 (AMA-1) and 5 (trivalent control), $P = 0.015$ and 0.001, respectively. All comparisons of mean IFAT end-point titers were made using one-way ANOVA with Tukey's HSD post-hoc test.

3.5. Antibody titers in ELISA after three versus four immunizations

The fourth monovalent AMA-1 and the fourth trivalent immunization increased the anti-AMA-1 titer by approximately two-fold (5041 to 9589, monovalent, $P = 0.17$; 5018 to 10,430, trivalent, $P = 0.07$). The fourth monovalent EBA-175 immunization did not change the anti-EBA-175 titer antibody titer (3302 to 3433, $P = 0.89$), while the fourth trivalent immunization increased the anti-EBA-175 titer by 2.5-fold (1956 to 4925, $P = 0.11$). The fourth monovalent MSP-1 immunization almost doubled the anti-MSP-1 titer (4628 to 8757, $P = 0.53$), while the fourth trivalent immunization did not change the anti-MSP-1 titer (1684 to 1830, $P = 0.79$). All comparisons of means described here were performed by Student's t-test (two-tailed).

3.6. Antibody titers in IFAT after three versus four immunizations

The fourth monovalent immunizations had little effect on increasing the antibody titer as measured in IFAT against *P. falciparum*-parasitized *Aotus* erythrocytes. Monovalent AMA-1 immunization increased the mean titer from 1120 to 1480 ($P = 0.46$). Monovalent EBA-175 immunization increased the mean titer from 2000 to 2880 ($P = 0.25$). Monovalent MSP-1 immunization increased the mean titer from 4560 to 6400 ($P = 0.36$). The immunization with the trivalent vaccine, however, increased the mean antibody in IFAT by more than five-fold (3440 to 17,920, $P < 0.0009$). All comparisons of means described here were performed by Student's t-test (two-tailed).

4. Discussion

Immunization with one monovalent DNA vaccine and with a mixture of monovalent DNA vaccines offers an opportunity to examine antigenic competition among these vaccines when used to immunize non-human primates. In this study, the vaccines encode immunogenic regions of proteins expressed by blood stage *P. falciparum*. AMA-1 is originally located in the necks of the rhoptry organelles, but is transferred to the apical end of the merozoite around the time of schizont rupture [22,23]. EBA-175 is a 175 kDa erythrocyte-binding protein and is a merozoite ligand that binds its receptor, glycophorin A, the predominant *P. falciparum* receptor on red blood cells [7]. At schizont rupture, MSP-1 is proteolytically processed and a number of fragments form a complex on the merozoite surface [24,25]. Antibody titers were measured after each serum collection and no differences in antibody titer to any of the three immunogens in animals receiving a monovalent vaccine compared to the trivalent preparation. There was also no evidence of antigenic competition when the sera were tested for reactivity to whole parasites in IFAT. In addition, the collective activities of the three antibody specifies in the trivalent group produced an overall IFAT titer higher than seen in any of the monovalent groups.

Three earlier studies have evaluated the co-administration of DNA vaccines in mice. Doolan et al. [1] used DNA vaccines encoding *P. yoelii* circumsporozoite protein and hepatocyte erythrocyte protein-17 to induce protection from sporozoite challenge. No evidence of antigenic competition was seen. Grifantini et al. [26] gave, either alone or in combination, four DNA vaccines encoding *P. falciparum* antigens, to mice. They administered the four DNA vaccines as a mixture, just as we did, rather than as four different injections at different sites. They failed to find any evidence of suppression of response to one vaccine by another. In fact, they report that the response to MSP-1 was strongly potentiated by the presence of additional plasmids. We did not, however, observe this potentiation in the primates. Multivalent DNA vaccines to tuberculosis antigens were studied in mice [27]. The mice were immunized with plasmids encoding four different immunogens. Mice received either only one plasmid or all four. Unlike our study, it appears that the mice received the four vaccines in separate injections instead of as a mixture. In addition, the vaccines were delivered intramuscularly instead of ID. Nonetheless, they also did not see evidence of antigenic competition, as measured by the humoral or cell-mediated responses.

In a study of the immunogenicity in rhesus monkeys of multiple plasmids encoding pre-erythrocytic antigens from *P. falciparum*, Wang et al. [3] showed that administration of vaccines in a multivalent setting did not reduce vaccine-induced CTL responses. The effect on the humoral response may, however, have been reduced by co-administration, particularly in the group receiving the different plasmids at different injection sites rather than as a mixture at one site. At least one of the plasmids was more immunogenic when administered alone.

Our study shows that antigenic competition among co-administered DNA vaccines does not occur with these blood stage *P. falciparum* antigens in *Aotus* monkeys. With the advent of prime-boost strategies where priming by a DNA vaccine is followed by recombinant protein or recombinant virus boost [8,28], the next important question to address will be whether priming with non-competing DNA vaccines will reduce the probability of competition among multiple antigens delivered as a protein or viral boost.

Acknowledgements

The authors thank Dr. Robin Anders of The Walter and Eliza Institute of Medical Research, Victoria, Australia for kindly providing the recombinant AMA-1 and Dr. Louis H. Miller of the National Institutes of Health, Bethesda for kindly providing the recombinant MSP-1_{19}. The authors also thank Ms. Gloria Cisneros for supervision of animal husbandry at the Gorgas Memorial Institute primate facility, Mr. Lionel Martinez and Mr. William Otero, also of the Gorgas Memorial Institute, for technical contributions and Ms. Maritza Brewer for secretarial support.

The experiments reported here were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals", Institute of Laboratory Animal Resources, National Research Council (Department of Health and Human Services, National Institutes of Health, publication 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 US Navy or the US Department of Defense.

This work was supported by the Naval Medical Research Center work unit 62787A.870.F.A0010 and US Army Contract DAMD17-91-C-1072.

References

[1] Doolan DL, Sedegah M, Hedstrom RC, Hobart P, Charoenvit Y, Hoffman SL. Circumventing genetic restriction of protection against malaria with multigene DNA immunization: $CD8^+$ T-cell-, interferon γ- and nitric oxide-dependent immunity. J Exp Med 1996;183:1739–46.

[2] Becker SI, Wang R, Hedstrom RC, et al. Protection of mice against *Plasmodium yoelii* sporozoite challenge with *P. yoelii* merozoite surface protein-1 DNA vaccines. Infect Immun 1998;66:3457–61.

[3] Wang R, Doolan DL, Charoenvit Y, et al. Simultaneous induction of multiple antigen-specific cytotoxic T-lymphocytes in non-human primates by immunization with a mixture of four *Plasmodium falciparum* DNA plasmids. Infect Immun 1998;66:4193–202.

[4] Fattom A, Cho YH, Chu C, Fuller S, Naso R. Epitopic overload at the site of injection may result in suppression of the immune response to combined capsular polysaccharide conjugate vaccines. Vaccine 1999;17:126–33.

[5] Deans JA, Knight AM, Jean WC, Waters AP, Cohen S, Mitchell GH. Vaccination trials in rhesus monkeys with a minor invariant *Plasmodium knowlesi* 66 kDa antigen. Parasite Immunol 1988;10:535–52.

[6] Waters AP, Thomas AW, Deans JA, et al. A merozoite receptor protein from *Plasmodium knowlesi* is highly conserved and distributed throughout *Plasmodium*. J Biol Chem 1990;265:17974–9.

[7] Sim BKL, Chitnis CE, Wasniowska K, Hadley TJ, Miller LH. Receptor and ligand domains for invasion of erythrocytes by *Plasmodium falciparum*. Science 1994;264:1941–4.

[8] Jones TR, Narum DL, Gozalo AS, et al. Protection of *Aotus* monkeys by *Plasmodium falciparum* EBA-175 region II DNA prime-boost immunization regimen. J Infect Dis 2001;183:308–12.

[9] Narum DL, Haynes JD, Fuhrmann S, et al. Antibodies against the *Plasmodium falciparum* receptor binding domain of EBA-175 block invasion pathways that do not involve sialic acids. Infect Immun 2000;68:1964–6.

[10] Chang SP, Case SE, Gosnell WL, et al. A recombinant baculovirus 42 kDa C-terminal fragment of *Plasmodium falciparum* merozoite surface protein-1 protects *Aotus* monkeys against malaria. Infect Immun 1996;64:253–61.

[11] Egan AF, Blackman MJ, Kaslow DC. Vaccine efficacy of recombinant *Plasmodium falciparum* merozoite surface protein-1 in malaria-naive, -exposed and/or -rechallenged *Aotus vociferans* monkeys. Infect Immun 2000;68:1418–27.

[12] Sim BKL, Narum DL, Liang H, et al. Induction of biologically active antibodies in mice, rabbits and monkeys by *Plasmodium falciparum* EBA-175 region II DNA vaccine. Mol Med, 2001;7:247–254.

[13] Valmori D, Sabbatini A, Lanzavecchia A, Corradin G, Matricardi PM. Functional analysis of two tetanus toxin universal T-cell epitopes in their interaction with DR1101 and DR1104 alleles. J Immunol 1994;152:2921–9.

[14] Luke CJ, Carner K, Liang X, Bardour AG. An OspA-based DNA vaccine protects mice against infection with *Borrelia burgdorferi*. J Infect Dis 1997;175:91–7.

[15] Ma NS, Rossan RN, Kelley ST, Harper JS, Bedard MT, Jones TC. Banding patterns of the chromosomes of two new karyotypes of the owl monkey (*Aotus*) captured in Panama. J Med Primatol 1978;7:146–55.

[16] Obaldia N. Detection of *Klebsiella pneumonia* in *Aotus lemurinus lemurinus* (Panamanian owl monkey) using an enzyme-linked immunosorbant assay (ELISA) test. Lab Anim 1991;25:133–41.

[17] Charoenvit Y, Fallarme V, Rogers WO, et al. Development of two monoclonal antibodies against *Plasmodium falciparum* sporozoite surface protein-2 and mapping of B-cell epitopes. Infect Immun 1997;65:3430–7.

[18] Liang H, Narum DL, Fuhrmann SR, Luu T, Sim BKL. A recombinant baculovirus expressed *Plasmodium falciparum* receptor-binding domain of the erythrocyte-binding protein EBA-175 biologically mimics native protein. Infect Immun 2000;68:3564–8.

[19] Kumar S, Yadava A, Keister DB, et al. Immunogenicity and in vivo efficacy of recombinant *Plasmodium falciparum* merozoite surface protein-1 in *Aotus* monkeys. Mol Med 1995;1:325–32.

[20] Riley EM, Wagner GE, Ofori MF, et al. Lack of association between maternal antibody and protection of African infants from malaria infection. Infect Immun 2000;68:5856–63.

[21] Aguiar JC, Hedstrom RC, Rogers WO, et al. Enhancement of the immune response in rabbits to a malaria DNA vaccine by immunization with a needle-free jet device. Vaccine, 2001;20:275–280.

[22] Peterson MG, Marshall VM, Smythe JA, et al. Integral membrane protein located in the apical complex of *Plasmodium falciparum*. Mol Cell Biol 1989;9:3151–4.

[23] Narum DL, Thomas AW. Differential localization of full-length and processed forms of PF83/AMA-1 an apical membrane antigen of *Plasmodium falciparum* merozoites. Mol Biochem Parasitol 1994;67:59–68.

[24] Holder AA. The precursor to major merozoite surface antigens: structure and role in immunity. Prog Allergy 1988;41:72–97.

[25] Blackman MJ, Holder AA. Secondary processing of the *Plasmodium falciparum* merozoite surface protein-1 (MSP-1) by a calcium-dependent membrane-bound serine protease: shedding of MSP-133 as a non-covalently associated complex with other fragments of the MSP-1. Mol Biochem Parasitol 1992;50:307–15.

[26] Grifantini R, Finco O, Bartolini E, et al. Multi-plasmid DNA vaccination avoids antigenic competition and enhances immunogenicity of a poorly immunogenic plasmid. Eur J Immunol 1998;28:1225–32.

[27] Morris S, Kelley C, Howard A, Li Z, Collins F. The immunogenicity of single and combination DNA vaccines against tuberculosis. Vaccine 2000;18:2155–63.

[28] Sedegah M, Jones TR, Kaur M, et al. Boosting with recombinant vaccinia increases immunogenicity and protective efficacy of malaria DNA vaccine. Proc Natl Acad Sci USA 1998;95:7648–53.