Immune Responses of Mice with Different Genetic Backgrounds to Improved Multiepitope, Multitarget Malaria Vaccine Candidate Antigen FALVAC-1A

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FALVAC-1A is a second-generation multitarget, multiepitope synthetic candidate vaccine against Plasmodium falciparum, incorporating elements designed to yield a stable and immunogenic molecule. Characteristics of the immunogenicity of FALVAC-1A were evaluated in congeneric (H-2b, H-2k, and H-2d) and outbred strains of mice. The influences of four adjuvants (aluminum phosphate, QS-21, Montanide ISA-720, and copolymer CRL-1005) on different aspects of the immune response were also assessed. FALVAC-1A generated strong antibody responses in mice strains. The highest mean enzyme-linked immunosorbent assay (ELISA) antibody concentrations against FALVAC-1A were observed in the outbred ICR mice, followed by B10.BR, B10.D2, and C57BL/6 mice, though this order varied for the different adjuvants, with no statistical differences between mouse strains. In all mouse strains, the highest anti-FALVAC-1A antibody titers in ELISAs were induced by FALVAC-1A in copolymer and ISA-720 formulations, followed by QS-21 and AlPO4. These antibodies were of all four subclasses, though immunoglobulin G1 (IgG1) predominated, with the exception of FALVAC-1A with the QS-21 adjuvant, which induced predominantly IgG2c responses. Both sporozoites and blood stages of P. falciparum were recognized by anti-FALVAC-1A sera in the immunofluorescence assay. In addition to antibody, cellular immune responses were detected; these responses were studied by examining spleen cells producing gamma interferon and interleukin-4 in enzyme-linked immunosorbent assays. In summary, FALVAC-1A was found to be highly immunogenic and elicited functionally relevant antibodies that can recognize sporozoites and blood-stage parasites in diverse genetic backgrounds.

One approach to an effective vaccine against Plasmodium falciparum malaria may be an artificial molecule comprised of key antigens and/or epitopes from different stages of the life cycle that induces both humoral and cellular immune effector mechanisms capable of mediating protection (9, 14, 15). The development of such a multicomponent subunit vaccine may also circumvent the problems associated with host genetic restriction and parasite antigenic variability sometimes noted with vaccines based on a single antigen. This approach also allows a number of distinct epitopes to be assembled to a size sufficient to be immunogenic (12, 15, 16, 36, 40). A further advantage is the ability to manipulate the construct, as new information regarding protective epitopes and antigen diversity becomes available.

Our laboratory previously developed a multistage, multivalent antigen, FALVAC-1, which comprised 21 different B-cell, T-cell, and cytotoxic T-lymphocyte (CTL) epitopes from seven different antigens of P. falciparum and a universal helper epitope from tetanus toxoid (37). Although FALVAC-1 showed promising results by inducing antibodies in both rabbits and mice that had in vitro antiparasitic activity (33, 37, 38), it was unsuitable for further clinical development because of concerns about the stability of the molecule, potential homology of an epitope with human sequences, and product yield. Thus, in the context of a vaccine development program, the molecule was redesigned to redress these limitations. The new molecule, termed FALVAC-1A, retained most of the FALVAC-1 epitopes and their order in the molecule but included some replaced or modified epitopes and, most critically, two types of spacer sequences intended (i) to promote molecular conformation and folding and (ii) to facilitate antigen processing (23, 42). The design and construction of the FALVAC-1A gene, as well as the expression, purification, and physicochemical characteristics of FALVAC-1A and its immunogenicity in rabbits, have been described previously (45). The amino acid sequence of FALVAC-1A, including the origin of the epitopes, is shown in Table 1 (45).

Because a vaccine construct must be immunogenic in genetically diverse populations, analysis of major histocompatibility (MHC)-restricted immune responses in experimental animal systems is of interest to characterize novel antigens. Thus, in the present study, FALVAC-1A was tested for its immunogenicity against different genetic backgrounds of both H-2 (H-2b, H-2d, and H-2k) congenic and outbred (ICR) mice. Subunit antigens will almost certainly require adjuvants to maximize their potential; therefore, FALVAC-1A was formulated with four different adjuvants: aluminum phosphate (AlPO4), the saponin QS-21, Montanide ISA-720, and copolymer CRL-1005 in a water-in-oil (w-o) emulsion. Because both humoral and
cellular responses are believed to be important to the development of immunity to malaria, antibody responses to the molecule were determined by enzyme-linked immunosorbent assays (ELISAs) and immunofluorescence assays (IFAs), and T-cell responses were determined by enzyme-linked immunospot assays (ELISPOTS) measuring interleukin-4 (IL-4) and gamma interferon (IFN-γ).

**MATERIALS AND METHODS**

**Adjuvant preparations.** FALVAC-1A was adsorbed onto aluminum phosphate (AlPO₄) (ADJU-PHOS; Accurate Chemicals & Scientific Corporation, Westbury, NY) in the proportions 10 µg FALVAC-1A per 40 µg aluminum by overnight incubation at 4°C on a rocker (6). Greater than 90% of the antigen was bound (data not shown). The aqueous saponin adjuvant QS-21 (a gift from Antigenics, Inc., Lexington, MA) and FALVAC-1A were appropriately diluted and mixed in phosphate-buffered saline (PBS) (pH 6.8). Equal volumes of the oil phase adjuvant Montanide ISA-720 (a gift from SEPPIC SA, Paris, France) and FALVAC-1A in PBS (pH 7.2) were emulsified using two glass syringes and a double-hubbed needle to yield a w-o emulsion. The nonionic block copolymer CRL-1005 (43) (a gift from Robert Hunter) was formulated in an adjuvant oil phase consisting of 89% squalene, 10% sorbitan monooleate (Span 80; Sigma), and 1% CRL-1005 by weight. Equal volumes of the CRL-1005 oil phase and FALVAC-1A in PBS (pH 7.2) were emulsified using two glass syringes and a double-hubbed needle to yield a w-o emulsion. The nonionic block copolymer CRL-1005 (43) (a gift from Robert Hunter) was formulated in an adjuvant oil phase consisting of 89% squalene, 10% sorbitan monooleate (Span 80; Sigma), and 1% CRL-1005 by weight. Equal volumes of the CRL-1005 oil phase and FALVAC-1A in PBS (pH 7.2) were emulsified using two glass syringes and a double-hubbed needle to yield a w-o emulsion. The nonionic block copolymer CRL-1005 (43) (a gift from Robert Hunter) was formulated in an adjuvant oil phase consisting of 89% squalene, 10% sorbitan monooleate (Span 80; Sigma), and 1% CRL-1005 by weight. Equal volumes of the CRL-1005 oil phase and FALVAC-1A in PBS (pH 7.2) were emulsified using two glass syringes and a double-hubbed needle to yield a w-o emulsion.

**Mice and immunizations.** H-2 congenic mice, C57BL/6 (H-2b), B10.BR (H-2d), and B10.D2 (H-2k) were used in AAALAC-accredited Centers for Disease Control and Prevention (CDC) facilities in accordance with the guidelines in the National Research Council manual (30). The study protocol was approved and monitored by the CDC Animal Care and Use Committee. For analysis of antibody responses, mice were immunized subcutaneously on days 0, 14, and 28 with 10 µg of FALVAC-1A at each time point. For each of the four mouse strains, five groups

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**TABLE 1. Comparison of the components and amino acid sequences of FALVAC-1 and FALVAC-1A**

| Epitope origin | Immune reactivity | Epitope code | Amino acid sequence | Epitope origin | Immune reactivity | Amino acid sequence |
|---------------|------------------|--------------|---------------------|---------------|------------------|---------------------|
| **Start**     | **FALVAC-1**     | **FALVAC-1A**|                     | **Start**     | **MA**           |                     |
| Melittin      | M                | p865         | KPKHKKLQPGDGNP      | CSP           | B                | KPKHKKLQPGDGNP      |
| His tag       |                  |              |                     |               |                  |                     |
| CSP           | B                | p847         | NANPNAPNAPNAP       | AMA-1         | B                | GNGCIPDIPHVEFSAIDL  |
| TRAP          | B                | p846         | CSP MSHCSCTG        | AMA-1         | B                | GNGCIPDIPHVEFSAIDL  |
| CSP           | Th/Tc            | p843         | KPKDELQDFGKIMECCKS  | AMA-1         | B                | GNGCIPDIPHVEFSAIDL  |
| MSP-1         | B                | p842         | NSGFHRDLEECKL       | RAP-1         | B                | NSGFHRDLEECKL       |
| MSP-1         | B                | p845         | EDGSSNGKIKCTECKPDS  | LSA-1         | Tc               | KPIVQYDNF           |
| LSA-1         | Tc               | p846         | KPIVQYDNF           | MSP-2         | B                | SNTFINNA            |
| MSP-2         | B                | p853         | SNTFINNA            | EBA-175       | B                | LIKMHEHLAIYIESRILKR |
| EBA-175       | B                | p854         | GQHGHMGH            | MSP-2         | B                | GQHGHMGH            |
| AMA-1         | B                | p856         | NSGFHRDLEECKL       | EBA-175       | B                | EMTYNSGFNGQNYEWPYQKS|
| MSP-1         | B                | p857         | NSGFHRDLEECKL       | EBA-175       | B                | EMTYNSGFNGQNYEWPYQKS|
| LSA-1         | Tc               | p851         | KPKNDKSLY           | AMA-1         | Tp               | KPKNDKSLY           |
| MSP-2         | B                | p857         | DOPKQYEQLTLDYEKKEG  | EBA-175       | B                | DOPKQYEQLTLDYEKKEG  |
| EBA-175       | B                | p864         | TLPDEELY            | EBA-175       | B                | TLPDEELY            |
| MSP-1         | B                | p865         | GSYEYKVLAKYDDLE     | EBA-175       | B                | GSYEYKVLAKYDDLE     |
| LSA-1         | Tc               | p852         | GSYEYKVLAKYDDLE     | EBA-175       | B                | GSYEYKVLAKYDDLE     |

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*a* Adapted from reference 45 with permission of the publisher. Sequences deleted during the design of FALVAC-1A are shown in bold italic type; sequences added are shown in boldface type.

*b* The origin of the epitope is shown as follows: CSP, circumsporozoite protein; TRAP, thrombospodin-related protein; MSP-1, merozoite surface protein-1; LSA-1, liver stage antigen-1; AMA-1, apical membrane antigen-1; RAP-1, rhoptry-associated protein-1; P2-T, tetanus toxoid universal helper; MSP-2, merozoite surface protein-2; EBA-175, erythrocyte binding antigen-175; P27, P. falciparum gametocyte 27-kDa antigen.

*c* The immune reactivity of each epitope (synthetic peptide) is shown as follows: B, B cell; Th/Tc, T helper and cytotoxic T cell; Th, T helper cell; Tc, cytotoxic T cell; Tp, T proliferative cell.

*d* Code number assigned to each epitope (synthetic peptide).

*e* The sequences of each epitope and spacer are indicated. The entire sequence runs continuously down each row in this column.
of 10 female mice, 6 to 8 weeks old, received the antigen either in PBS or formulated with one of the adjuvants, AlOPO4 (40 μg/mouse), Montanide ISA-720 (50:50), or copolymer CRL-1005 (50:50), respectively. Blood was collected from the retro-orbital plexus on days 0, 21, 42, 63, 84, and 147. Serum samples were stored at −20°C until used for serological assays. To evaluate the T-cell responses, groups of four mice were immunized on days 0 and 14 with 10 μg of FALVAC-1A at each time point in the same formulations as mentioned above, and spleen cells were harvested and assayed on either day 21 or 23.

ELISA. Antibody concentrations were determined by ELISAs (34). Briefly, Immulon 2HB plates (Thermo Electron, Franklin, MA) were coated overnight at 4°C with either 1 μg/ml FALVAC-1A or 10 μg/ml of a component peptide in PBS, blocked with 5% nonfat dried milk in PBS containing 0.05% Tween 20 (diluent), and washed with PBS containing 0.05% Tween 20. Serum diluted in binary series with diluent from 1:200 to 1,163,800 for anti-FALVAC-1A and from 1:100 to 1,204,800 for antipeptide antibody. Aliquots (100 μl) of these dilutions were added per well, and the plates were incubated overnight at 4°C. The plates were washed between additions with PBS containing 0.05% Tween 20. To determine the titers of total anti-FALVAC-1A immunoglobulin G (IgG), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Southern BioTech, Birmingham, AL) was used at a dilution of 1:1,000. IgG subclasses were measured using 1:5,000 dilution of HRP-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3, respectively (Caltag Laboratories, Burlingame, CA). Following washing, the final color reaction was developed using 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD). For 5 min. The reaction was stopped by the addition of 50 μl of 1.0 M phosphoric acid, and the optical density at 450 nm (OD450) was read on a Spectramax 340 PC microplate reader (Molecular Devices, Sunnyvale, CA). Serum titers were determined as the geometric mean of the reciprocal dilution where the OD450 of the titration curve equaled 0.1 (the maximum value of preimmune sera at 1:100).

IFA. An immunofluorescence assay was used to determine anti-FALVAC-1A reactivity with sporozoites (P. falciparum, Santa Lucia strain) and axenial blood-stage parasites (P. falciparum FVO strain). Sera from individual mice in each group were pooled before IFA determination. Methanol-fixed parasites on multispot slides were incubated (30 min, room temperature, 100% relative humidity) with fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody (Kirkegaard & Perry Laboratories [KPL], Gaithersburg, MD). Following three further PBS washes, the slides were dried, mounted under buffered glycerin (pH 9), and examined at ×40 with a fluorescence microscope. Mouse preimmune serum was used as a negative control. Reactions to the parasites were scored as follows: + (extremely bright) + 3 (yellow-green brightness), + 2 (medium intensity), + 1 (less intense but with fluorescence), and − (no fluorescence). Serum titers were determined as the reciprocal of the highest serum dilution giving a positive reading (+ 1). The slides were read independently by two individuals.

ELISPOT. An enzyme-linked immunospot assay was used to detect IFN-γ-producing spleen cells producing FALVAC-1A-specific IFN-γ and IL-4. Mice were immunized on days 0 and 14, two mice from each group were sacrificed on either day 21 or 23, and spleen mononuclear cells were isolated at each time point (4). The ELISPOT procedure described in the BD ELISPOT set instruction manual (BD Biosciences Pharmingen, San Diego, CA) was followed. Briefly, the wells of Immunosoft M200 96-well plates (BD Biosciences Pharmingen, San Diego, CA) were coated with the capture antibody (purified anti-mouse IFN-γ or anti-mouse IL-4; BD Biosciences Pharmingen) at a final concentration of 5 μg/ml in PBS and incubated overnight at 4°C. The following day, the plates were washed once with complete RPMI medium (RPMI with 10% heat-inactivated fetal bovine serum) for 2 h. After three washes with PBS (FALVAC-1A) on days 0, 14, and 28 with 10 μg FALVAC-1A formulated with PBS, AlPO4, QS-21 (QS-21/FAL), aluminum (AlPO4/FAL), or PBS (FALVAC-1A) on days 0, 14, and 28. Total IgG antibody titers (geometric mean of 10 mice) were determined as the geometric mean of the reciprocal dilution where the OD450 of the titration curve equaled 0.1 (the maximum value of preimmune sera at 1:100).

RESULTS

Antibody responses to FALVAC-1A in different strains of mice with different adjuvant formulations. To investigate antibody responses to FALVAC-1A and evaluate the effects of MHC differences, three inbred strains (H-2b, H-2k, and H-2d) and one outbred strain of mice were immunized on days 0, 14, and 28 with 10 μg FALVAC-1A formulated with PBS, AlPO4, QS-21, Montanide ISA-720, and copolymer (CRL-1005), respectively. The kinetics of the mean anti-FALVAC-1A IgG responses measured in ELISAs are shown in Fig. 1. The majority of the responses peaked at day 42, for all adjuvant/strain combinations, followed by a slow decline. All the adjuvants enhanced antibody responses significantly compared to FALVAC-1A used without an adjuvant. Responses stimulated by the two w-o emulsion adjuvants, Montanide ISA-720 and copolymer CRL-1005, were the highest, and equivalent in all
In general, C57BL/6 mice were the least responders, regardless of the IgG subclass or adjuvant used. The ratios between different subclasses were determined to better understand the relative levels of these antibodies. The ratios differed between outbred and inbred mice. The most notable change in the ratios of IgG subclasses was observed with QS-21 adjuvant, which induced antibodies with the highest IgG2c/IgG1 and IgG2c/IgG2b ratios in all strains of mice. The IgG2b/IgG1 ratio was the least in all the mouse strains irrespective of the adjuvant used: (IgG1/IgG3 > IgG2c/IgG3 > IgG1/IgG2c > IgG2b/IgG3 > IgG2c/IgG2b > IgG2b/IgG1). The IgG1/IgG3 ratio was highest in C57BL/6 mice followed by outbred ICR, B10.D2, and B10.BR mice. AlPO4 induced antibodies with the highest IgG1/IgG3 ratio in all strains except for the B10.BR strain. These results indicate that both adjuvant and genetic background influenced the levels of various IgG subclasses to FALVAC-1A.

Antibody responses to vaccine components (peptide ELISA). Analyses of epitope-specific antibody responses by ELISAs showed that the vaccine-induced antibodies recognized several epitopes, including both B- and T-cell epitopes incorporated into FALVAC-1A (Table 2). Six of the B-cell epitopes (merozoite surface protein-1 [MSP-1]/p848, MSP-1/p845, apical membrane antigen-1 [AMA-1]/p849, CSA/VP1/p859, and rhoptry-associated protein-1 [RAP-1]/p866) induced epitope-specific antibody responses in all the mouse strains irrespective of the adjuvant used, and three B-cell epitopes (MSP-1/p853, erythrocyte binding antigen-175 [EBA-175]/p863, and EBA-175/p864) completely failed to induce antibody responses in any of the mouse strains (Table 2). As was observed in antibody responses to the whole-vaccine antigen, the antibody titers against the individual epitopes were also higher in outbred ICR mice (1 \times 10^5 to 1 \times 10^6) than those in the inbred mice (1 \times 10^5 to 5.1 \times 10^5). ICR mice also elicited antibodies to at least 17 of the 21 epitopes in the vaccine, whereas inbred mice generated antibodies to only 13 of the epitopes. ICR mice responded to all but 1 of the 9 T-cell epitopes and 9 out of 12 B-cell epitopes, while inbred mice responded to only 5 (CSP/p843, liver stage antigen-1 [LSA-1]/p846, AMA-1/p857, RAP-1/p859, and CSP/ p867) of the T-cell epitopes and 8 B-cell epitopes. Outbred ICR mice were the best responders, followed by B10.BR and B10.D2 mice and then by C57BL/6 mice, regardless of the adjuvant formulation used. The highest antibody titers were seen in the copolymer CRL-1005 vaccine formulation group, followed by Montanide ISA-720, QS-21, and AlPO4 vaccine formulation groups, CRL-1005, ISA-720, and QS-21 vaccine formulation groups induced moderate to high anti-FALVAC-1A antibody titers to 5 of 12 B-cell epitopes (CSP/p865, MSP-1/p844, AMA-1/p848, and RAP-1/p866) and only 1 of 9 T-cell epitopes compared to the low to moderate antibody titers induced by the AlPO4 vaccine formulation for the same epitopes. All the adjuvant formulations failed to induce antibodies to 3 (EBA-175/p863, EBA-175/p864, and MSP-1/p853) of 15 blood-stage epitopes, 1 (CSP/p867) of 4 sporozoite stage epitopes, and 1 (LSA-1/p851) of 2 liver stage epitopes.
**TABLE 2. Mouse anti-FALVAC-1A peptide titers found in ELISAs**

| Epitope origin | Peptide | Immune reactivity | Anti-FALVAC-1A peptide titer of pooled sera (10 mice) |
|----------------|---------|-------------------|------------------------------------------------------|
|                |         |                   | C57BL/6 | B10.BR | B10.D2 | ICR |
|                |         |                   | CRL-1005 | ISA-720 | QS-21 | AIPO₄ | No adj. | CRL-1005 | ISA-720 | QS-21 | AIPO₄ | No adj. | CRL-1005 | ISA-720 | QS-21 | AIPO₄ | No adj. |
| CSP            | p865    | B                 | 6,400   | 3,200  | 3,200  | 800  | 100  | 51,200 | 6,400  | 12,800 | 3,200  | 200   | 6,400  | 6,400  | 6,400  | 200   | 100   | 25,600 | 12,800 | 12,800 | 3,200  | 1,600  |
| CSP            | p843    | Th/Tc             | 800     | 400    | –      | –    | 100  | 25,600 | 25,600 | 51,200 | 1,600  | –      | –      | –      | –      | –     | –     | 12,800 | 12,800 | 6,400  | 1,600  |
| CSP            | p867    | Th                | –       | –      | –      | –    | –    | –      | –      | –      | –      | –      | –      | –      | –      | –     | –     | –      | –      | –      | –      |
| MSP-1          | p844    | B                 | 3,200   | 6,400  | 3,200  | 3,200 | 100  | 51,200 | 3,200  | 25,600 | 6,400  | 1,600  | –      | –      | –      | –     | –     | 12,800 | 12,800 | 6,400  | 1,600  |
| MSP-1          | p845    | B                 | 800     | 400    | 800    | 400  | 100  | –      | 12,800 | 200    | 1,600  | 400    | –      | –      | –      | –     | –     | 6,400  | 6,400  | 3,200  | 1,600  |
| LSA-1          | p846    | Tc                | –       | –      | –      | –    | –    | 6,400  | 1,600  | 100    | –      | –      | 400    | –      | –      | –     | –     | 800    | 800    | 400    | 100    |
| CSP            | p847    | B                 | –       | –      | –      | –    | –    | –      | 200    | –      | –      | –      | –      | 400    | –      | –     | –     | 800    | –      | –      | –      |
| AMA-1          | p848    | B                 | 6,400   | 1,600  | 6,400  | 1,600 | 100  | 6,400  | 800    | 25,600 | –      | –      | 1,600  | 800    | 1,600  | 100  | 800   | 1,600  | 1,600  | 1,600  | 100    |
| AMA-1          | p849    | B                 | 3,200   | 3,200  | 12,800 | 3,200 | 100  | 6,400  | 400    | 25,600 | 6,400  | –      | 1,600  | 6,400  | 6,400  | 400  | 1,600  | 12,800 | 12,800 | 12,800 | 1,600  |
| RAP-1          | p866    | B                 | 12,800  | 6,400  | 12,800 | 6,400  | 800  | 6,400  | 400    | 25,600 | 6,400  | 25,600 | 51,200 | 25,600 | 6,400  | 400  | 1,600  | 12,800 | 12,800 | 12,800 | 1,600  |
| LSA-1          | p851    | Tc                | –       | –      | –      | –    | –    | –      | –      | –      | –      | –      | –      | –      | –      | –     | –     | –      | –      | –      | –      |
| EBA-175        | p863    | B                 | –       | –      | –      | –    | –    | –      | –      | –      | –      | –      | –      | –      | –      | –     | –     | –      | –      | –      | –      |
| MSP-2          | p853    | B                 | –       | –      | –      | –    | –    | –      | –      | –      | –      | –      | –      | –      | –      | –     | –     | –      | –      | –      | –      |
| MSP-2          | p854    | B                 | 100     | –      | –      | –    | 100  | –      | –      | –      | –      | –      | –      | 100    | –      | –     | –     | –      | –      | –      | –      |
| EBA-175        | p862    | B                 | –       | –      | –      | –    | –    | –      | –      | –      | –      | –      | –      | –      | 6,400  | 400  | 3,200 | –      | –      | –      | –      |
| AMA-1          | p856    | Tp                | –       | –      | –      | –    | –    | –      | –      | –      | –      | –      | –      | 1,600  | 1,600  | 1,600 | 1,600 | –      | –      | –      | –      |
| AMA-1          | p857    | Tp                | 1,600   | 1,600  | 12,800 | 1,600 | 100  | 25,600 | 3,200  | 3,200  | 3,200  | 1,600  | 6,400  | 6,400  | 3,200  | 400  | 1,600  | 12,800 | 12,800 | 12,800 | 1,600  |
| EBA-175        | p864    | B                 | –       | –      | –      | –    | –    | –      | –      | –      | –      | –      | –      | –      | –      | –     | –     | –      | –      | –      | –      |
| RAP-1          | p859    | Tp                | 400     | –      | –      | –    | 100  | –      | –      | –      | –      | 100    | –      | –      | –      | 12,800 | 800   | 1,600  | 100   | 100    |
| RAP-1          | p860    | Tp                | –       | –      | –      | –    | –    | –      | –      | –      | –      | –      | –      | –      | 100    | 200  | –     | –      | –      | –      | –      |
| MSP-1          | p861    | Tp                | –       | –      | –      | –    | –    | –      | –      | –      | –      | –      | –      | 100    | 100   | 100  | –     | –      | –      | –      | –      |

*Four strains of mice were immunized on days 0, 14, and 28 with 10 μg FALVAC-1A formulated with the indicated adjuvants. Sera collected on day 42 (serum with peak antibody titer) were used for peptide ELISA. ELISA titers against each epitopic peptide were determined by titration. The order of epitopes is the same as in Table 1. In CSP, the first epitope, Th/Tc, KPKDELDYENDIEKKICKMEKCSSVFNVVNS. Antibody responses were determined jointly against the peptide KPKDELDYENDIEKKICKMEKCSSVFNVVNS. The origin of the epitope is shown as follows: CSP, circumsporozoite protein; MSP-1, merozoite surface protein-1; LSA-1, liver stage antigen-1; AMA-1, apical membrane antigen-1; RAP-1, rhoptry-associated protein-1; EBA-175, erythrocyte binding antigen-175; MSP-2, merozoite surface protein-2.

*The immune reactivity of each epitope (synthetic peptide) is shown as follows: B, B cell; Th/Tc, T helper and cytotoxic T cell; Th, T helper cell; Tc, cytotoxic T cell; Tp, T proliferative cell.

*Four strains of mice (C57BL/6, B10.BR, B10.D2, and ICR) were immunized with 10 μg FALVAC-1A formulated with adjuvants (copolymer CRL-1005, Montanide ISA-720, QS-21, and AlPO₄) or with no adjuvant (No adj.). Dashes indicated that the titer was <100.
Reactivity of FALVAC-1A antiserum with parasite antigens in IFAs. To determine whether FALVAC-1A-induced antibodies can recognize native antigens expressed on the parasites, IFA studies were performed with sporozoites and blood-stage parasites using pooled sera collected on day 42 after primary immunization. Sera from all immunized groups showed reactivity with both sporozoites and blood-stage parasites (Fig. 3). In general, IFA titers of the serum pools against both stages were correlated with the ELISA group mean titers against the whole molecule. Sera from mice immunized with FALVAC-1A alone had the lowest IFA titers (4 × 10^1 to 1.6 × 10^2) in all the mouse strains (Fig. 3). No correlation was found between the peptide ELISA titers and IFA titers.

Cellular immune responses to FALVAC-1A assayed by ELISPOTs. In order to evaluate the induction of T-cell responses to FALVAC-1A in mice, splenic IFN-γ- and IL-4-secreting cells were assayed by ELISPOTs. As shown in Fig. 4, T-cell responses against FALVAC-1A, as determined by the number of IFN-γ- and IL-4-secreting cells, were detected in all mouse strains, irrespective of the adjuvant used. The frequency of cytokine-secreting splenocytes from mice immunized with FALVAC-1A vaccine plus the different adjuvant formulations ranged from 222 to 609 (for IFN-γ) and 216 to 914 (for IL-4) spot-forming cells/10^6 cells in comparison to 11 to 35 IFN-γ spot-forming cells or 50 to 100 IL-4 spot-forming cells/10^6 cells in the PBS control group (Fig. 4). IFN-γ and IL-4 secretion detected in this assay was antigen specific, since very few spots (or cells) could be observed when these immune cells were incubated with medium only (4 to 100 IFN-γ spots/10^6 cells and 7 to 88 IL-4 spots/10^6 cells) or stimulated with bovine serum albumin BSA (a nonspecific antigen) (27 to 100 IFN-γ spots/10^6 cells and 19 to 100 IL-4 spots/10^6 cells (Fig. 4). A mitogen (ConA) that has the ability to activate all T-cell types was used as a positive control. ConA induced 269 to 657 IFN-γ spot-forming cells/10^6 cells and 216 to 914 (for IL-4) spot-forming cells/10^6 cells.

Outbred ICR mice responded with the highest mean number of antigen-specific IFN-γ-secreting splenocytes (448.3 ± 54.8 spots/10^6 cells), followed by B10. BR (396.3 ± 81.3 spots/10^6 cells), B10.D2 (320.8 ± 178.6 spots/10^6 cells), and C57BL/6 (264.8 ± 86.5 spots/10^6 cells) mice. Interestingly, the group receiving the QS-21 formulation exhibited the highest number of FALVAC-1A-specific IFN-γ-secreting spleen cells in B10.D2 mice and showed a similar trend in B10. BR mice (Fig. 4). In contrast, C57BL/6 mice were the best responders with regard to induction of IL-4-secreting cells, with an average of 643.8 ± 176.4 spots/10^6 cells, followed by outbred ICR mice with 584 ± 118.7 spots/10^6 cells, B10. BR mice with 463.3 ± 111.1 spots/10^6 cells, and B10.D2 mice with 258.8 ± 33.1 spot-forming cells/10^6 cells.

Adjuvants were critical to the induction of T-cell responses, as the numbers of IFN-γ or IL-4 spot-forming cells/10^6 cells in groups vaccinated with FALVAC-1A/adjuvant were significantly higher (P = 0.02) than in the groups vaccinated with FALVAC-1A alone (data not shown).

The influence of adjuvants on the overall response measured by ELISPOTs varied in different mouse strains. As measured by the number of IL-4 spot-forming cells/10^6 cells in ELISPOTs, the influence of adjuvant in the different mouse strains was ranked as follows: for C57BL/6 mice, CRL-1005 > QS-21 > AlPO4 > ISA-720; for B10. BR mice, QS-21 > AlPO4 > CRL-1005 > ISA-720; and for ICR mice, AlPO4 > QS-21 > CRL-1005 > ISA-720. In B10.D2 mice, no significant differences were observed among the numbers of IL-4 spot-forming cells/10^6 cells for the different adjuvants used in formulating FALVAC-1A. Although copolymer CRL-1005 and Montanide ISA-720 had the greatest effect in eliciting anti-FALVAC-1A antibodies, ISA-720 had the least effect on induction of IL-4-secreting cells in the ELISPOT. With respect to IFN-γ response, QS-21 generated the highest number of IFN-γ spot-forming cells/10^6 cells in both B10. BR and B10.D2 mice, followed by AlPO4. ISA-720 generated the lowest IFN-γ response in B10. BR mice, whereas CRL-1005 generated the lowest IFN-γ response in B10.D2 mice. In C57BL/6 mice, CRL-1005 induced the highest number of IFN-γ spot-forming cells, followed by AlPO4, QS-21, and ISA-720. In contrast, ISA-720 generated the highest number of IFN-γ spot-forming cells/10^6 cells and AlPO4 generated the lowest number in the outbred ICR mice. No significant differences were observed in the number of cytokine-secreting cells generated among the different mouse strains (P = 0.06). No positive correlation was observed between anti-FALVAC-1A titers in ELISAs and the numbers of IL-4-secreting cells in ELISPOTs.

DISCUSSION

FALVAC-1A is a synthetic recombinant malaria vaccine candidate antigen that contains 21 epitopes from Plasmodium falciparum. The antigen was redesigned from its parental molecule, FALVAC-1, for the purpose of improving its safety, immunogenicity, and stability. This antigen is fundamentally different from FALVAC-1 in two aspects. First, it contains several spacer sequences between the epitopes, inserted with the intent of facilitating antigen processing and increasing the stability of this molecule. Second, three epitopes were replaced with new ones (45). Because of these substantial differences, it requires immunological reassessment before it can be taken to clinical trial. The basic characteristics of FALVAC-1A have been recently reported (45), and in this study we systematically evaluated the immunogenicity of this antigen in various adju-
vant formulations in three inbred strains (C57BL/6, B10.BR, and B10.D2) and one outbred ICR strain of mice.

It is evident from this study that FALVAC-1A is a highly immunogenic protein capable of eliciting strong T-cell and antibody responses in both inbred and outbred mouse strains. FALVAC-1A induced antibody responses in all the strains of mice tested with some variation in the level of response. In general, the responses in outbred ICR mice were higher than in inbred (H-2b, H-2k, and H-2d) mice. Important differences in the response pattern were noticed in the specificity of antibody responses to individual peptide epitopes. Among the inbred strains, the C57BL/6 strain showed lower responses to peptides compared to those of the B10.BR and B10.D2 strains, and ICR mice showed the highest response. The antibodies showed functional reactivity to both sporozoites and blood-stage parasites, demonstrating that this multiepitope vaccine is capable of inducing antibodies that can recognize native antigenic determinants on the parasite surface. Overall, the re-

FIG. 4. Detection of antigen-specific IFN-γ-producing (A) and IL-4-producing (B) T cells in the spleen cells of mice immunized with FALVAC-1A formulated in different adjuvants. Mice were immunized with 10 μg FALVAC-1A/dose formulated in copolymer (Copol/FAL), Montanide ISA-720 (ISA-720/FAL), QS-21 (QS-21/FAL), aluminum (AlPO4/FAL), or PBS on days 0 and 14. Seven days after the last immunization, spleen cells were harvested, and ELISPOT was performed on the same day. A total of 10^6 spleen mononuclear cells/well were incubated in vitro for 24 h (for IFN-γ) or 48 h (IL-4) with FALVAC-1A. Cells incubated with ConA were used as positive control, while bovine serum albumin (BSA) and medium only served as negative controls. Each bar represents the mean number of spots plus standard error (error bars) from three separate experiments. In each experiment, quadruplicate wells per treatment were set up.
Response to FALVAC-1A has improved compared to that of FALVAC-1, and this may be due to the recent modifications made to FALVAC-1A using spacers. The spacer sequences have been used to facilitate epitope processing (24, 42), to preserve conformation-dependent immunogenicity (23), and to overcome reduced immunogenicity by junctional epitopes (23). We have shown that the FALVAC-1A is a more stable molecule than FALVAC-1 is (45).

As expected, adjuvant influenced the antibody responses significantly. The FALVAC-1A-elicited antibody titers among the various adjuvant formulations within each mouse strain were statistically different (CRL-1005 ≡ ISA-720 > QS-2 > AlPO4), suggesting that the adjuvant influenced the magnitude of the anti-FALVAC-1A antibody responses in these mice. These observations are consistent with our earlier observations (33, 37, 45) that copolymer CRL-1005 and Montanide ISA-720 adjuvants induced higher and long-lasting antibody titers to FALVAC-1A than QS-21 and AlPO4 adjuvants did. These adjuvants, CRL-1005 and Montanide ISA-720 (both water-in-oil), have been shown to induce both Th1 and Th2 responses and possibly CTL responses (2, 11, 19, 25, 27, 31, 32, 35, 39, 44). Outbred ICR mice induced epitope-specific antibodies with higher antibody titers than inbred mice did, and CRL-1005 and ISA-720 adjuvants were better at inducing epitope-specific antibodies in mice than QS-21 and AlPO4 adjuvants were. Besides the quantitative difference in the overall antibody response, adjuvants qualitatively influenced the antibody responses. CRL-1005 and ISA-720 adjuvants induced predominantly subclass IgG1 antibody, while QS-21 was the best adjuvant at inducing IgG2c subclass antibodies in all the mouse strains and had the highest IgG2c/IgG2b ratio in all strains of mice except for C57BL/6 mice. These observations are also consistent with the results obtained from our previous investigations showing that QS-21 modulated production of higher levels of IgG2a (analogous to IgG2c in other mouse strains) (33, 37, 38, 45). This property of QS-21 adjuvant has been generally attributed to the ability of this adjuvant to elicit higher levels of IFN-γ response.

We do not know why some epitopes failed to elicit antibody responses. It is possible that subtle differences between the mouse and human MHC molecules could partly explain this observation, since most of the epitopes incorporated into FALVAC-1A were discovered in human-based studies. In general, only some segments of a protein are recognized as epitopes, and this recognition pattern varies for each protein, apparently influenced to a large extent by the MHC complex. It remains to be determined whether FALVAC-1A could induce antibody responses in humans to the epitopes nonimmunogenic in mice.

In mice, IgG1 responses are usually associated with Th2 responses, whereas high levels of IgG2a, sometimes associated with IgG2b and IgG3, are thought to reflect Th1 responses (1). Several parameters influence the IgG subclass responses to proteins, including the dose of antigen used, use of adjuvants or delivery systems, and the intrinsic immunogenicity of the protein itself (1, 3, 7, 10, 17, 21, 29, 34). The response elicited by FALVAC-1A with high levels of multiple IgG subclasses is thus likely to be a combined effect of several parameters. Nevertheless, adjuvants appeared to be stronger regulators of immune responses (in this study) and induced high IgG1 as well as considerable IgG2b, IgG2c, and IgG3 levels, reflecting adjuvant-dependent, mixed Th1/Th2 responses. The modulation of IgG subclass responses induced by FALVAC-1A suggests that T-cell responses to FALVAC-1A can, to some extent, override other factors determining the IgG subclass response, possibly reflecting a polarization toward Th1. Further studies of FALVAC-1A-induced cytokine response are required to confirm this hypothesis. This may also explain the decreased IgG1 (the predominant subclass generated by most adjuvant treatment groups within each mouse strain) levels observed in the inbred mice. IgG1 has been implicated in protection against Plasmodium yoelii (1, 13, 41). In addition to the modulatory effects of mouse strains and adjuvants on the Th1/Th2 balance of immune responses, evidence that intrinsic structures in the proteins themselves, termed modulotypes, may also contribute to this balance is accumulating (10).

IFN-γ plays a critical role in protection against preerythrocytic stages of malaria, while IL-4 response has been implicated in an antibody-dependent Th cell protection against the blood stages (9, 14, 28). Our results show that FALVAC-1A induced strong IFN-γ and IL-4 responses in all immunized mice. Spleen cells from mice immunized with the various FALVAC-1A-adjuvant formulations upon stimulation with FALVAC-1A secreted significantly larger amounts of IFN-γ and IL-4 (determined by the number of IFN-γ- or IL-4-secreting cells/10⁶ cells) than cells from mice immunized with PBS or medium or the negative-control mice. Each adjuvant group performed differently in each mouse strain with no clear pattern, as was observed in antibody responses with respect to the influence of adjuvant in T-cell responses to FALVAC-1A. QS-21 is known to induce predominantly IFN-γ response (5). However, in this study it induced distinctly high levels of IFN-γ only in B10.D2 mice. This adjuvant also induced higher IL-4 response, indicating that this adjuvant can also activate both Th1 and Th2 pathways; this observation is also consistent with the pattern of antibody subclasses observed in this study. Although copolymer CRL-1005 and Montanide ISA-720 facilitated predominantly IgG1 subclass production, they did not elicit high levels of IL-4 responses. Since we measured cytokine responses cross-sectionally, it may not reflect the full dynamics of in vivo cytokine responses, and this, in turn, may explain why we have failed to find direct correlations between cytokine responses and IgG subclass response patterns. It is of interest to note that whereas AlPO4 was not very potent in inducing antibody responses (Fig. 1), it was effective in inducing T-cell responses to FALVAC-1A. QS-21 was known to induce predominantly IFN-γ response (5). In this study, we have failed to find direct correlations between cytokine responses and IgG subclass response patterns. It is of interest to note that whereas AlPO4 was not very potent in inducing antibody responses (Fig. 1), it was effective in inducing T-cell responses to FALVAC-1A compared to the other adjuvants studied here (Fig. 4). Classical cell-mediated immunity measured by delayed-type hypersensitivity responses and induction of CD8+ CTL responses to a range of polypeptide and protein antigens is poorly induced by alum, because of a lack of cross-priming. However, proliferative responses of CD4+ T cells and Th2 cytokine production have been found to be enhanced in several murine and human studies, suggesting that alum boosts humoral immunity by providing Th2 cell help to follicular B cells (reviewed in reference 20). Although they have been used as a trusty aide to improve antibody responses to vaccines, the mechanism of alum adjuvanticity was only recently unveiled (20). Kool et al. (20) found that alum caused the production of uric acid, which stimulates dendritic cells. They reported that in response to uric acid, inflammatory monocytes and dendritic...
cells migrated to the injection site, took up antigens, and processed them down into T-cell-stimulating epitopes. These monocytes then migrated to lymph nodes where they matured into dendritic cells and activated CD4+ T cells. The NLRP3 inflammasome also plays a role during development of immune responses elicited by alum-enhanced vaccination (8, 22).

In conclusion, FALVAC-1A is a highly immunogenic vaccine candidate antigen capable of eliciting humoral and cellular responses in diverse genetic backgrounds. FALVAC-1A-elicted antibodies recognize sporozoites and blood-stage asexual parasites. FALVAC-1A immune responses are influenced by the choice of adjuvant: copolymer CRL-1005 and Montanide ISA-720 were the best adjuvants, followed by QS-21 and AlPO4. AlPO4 was effective in inducing T-cell responses to FALVAC-1A.

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