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Process Development and Analysis of Liver-Stage Antigen 1, a Preerythrocyte-Stage Protein-Based Vaccine for *Plasmodium falciparum*

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Plasmodium falciparum **liver-stage antigen 1 (LSA-1) is expressed solely in infected hepatocytes and is thought to have a role in liver schizogony and merozoite release. Specific humoral, cellular, and cytokine immune responses to LSA-1 are well documented, with epitopes identified that correlate with antibody production, proliferative T-cell responses, or cytokine induction. With the goal of developing a vaccine against this preerythrocyte-stage protein, we undertook the good manufacturing practices (GMP) manufacture of a recombinant LSA-1 construct, LSA-NRC, incorporating the N- and C-terminal regions of the protein and two of the centrally placed 17-amino-acid repeats. To improve the protein yield, a method of codon harmonization was employed to reengineer the gene sequence for expression in *Escherichia coli*. A 300-liter GMP fermentation produced 8 kg of bacterial cell paste, and a three-step column chromatographic method yielded 8 mg of purified antigen per g of paste. The final bulk protein was >98% pure, demonstrated long-term stability, and contained <0.005 endotoxin units per 50 μg of protein. To accomplish the initial stages of evaluation of this protein as a human-use vaccine against malaria, we immunized rabbits and mice with LSA-NRC in Montanide ISA 720. New Zealand White rabbits and A/J (*H-2*^{K}) mice produced high-titer antibodies that recognized liver-stage parasites in infected cultured human hepatocytes. Gamma interferon-producing cells, which have been associated with LSA-1-mediated protection, were detected in splenocytes harvested from immunized mice. Finally, sera taken from people living in a region where malaria is holoendemic recognized LSA-NRC by Western blotting.**

The protozoan parasite *Plasmodium falciparum* is responsible for the most debilitating form of malaria, and several antigens from different stages of the parasite's development are being considered as vaccine candidates. For prophylaxis, a vaccine targeting the preerythrocyte stage would be advantageous, as it would prevent or reduce clinical symptoms of disease. The ability to successfully immunize people against the preerythrocyte stage has been demonstrated with either irradiated sporozoites or the recombinant circumsporozoite protein vaccine RTS,S (17).

Liver-stage antigen 1 (LSA-1), from current evidence, is one of the few antigens exclusively expressed in hepatocytes. The *lsa-1* gene encodes a 230-kDa protein that is characterized by a large central repeat region varying in length (86.5 degenerate repeats of 17 amino acids in strain NF54) flanked by two highly conserved N- and C-terminal regions (20, 21). The nonrepeat regions have been shown to contain B- and T-cell-stimulating epitopes (3, 7, 10, 13). Expression of LSA-1 commences after sporozoite invasion of the liver hepatocyte and increases throughout hepatic stage development. LSA-1 is localized within the parasitophorous vacuole as a flocculent material but separate from the developing parasites, suggesting its involvement in liver schizogony and merozoite release (11, 18). Merozoites released from ruptured hepatic schizonts are encased in LSA-1 as they traverse through the liver sinusoid into the bloodstream (18), suggesting that LSA-1 adhering to the surface of merozoites may play a crucial role in liver schizogony, perhaps protecting the merozoite (11).

Although the exact function of LSA-1 for the parasite remains unknown, there is still evidence that this antigen is an attractive target for vaccine design at both the T-cell and B-cell level. This is especially true for the protein's nonrepeat regions, which are known to contain B- and T-cell epitopes (3, 7, 13). Individuals exposed to either natural or experimental malaria infection produce immune responses (proliferative T-cell, cytokine, or antibody) to LSA-1 protein or peptides that have been associated with complete protection or reduced parasitemia upon subsequent exposure (1, 3–5, 8–15).

The objective of this work was the scalable production, under good manufacturing practices (GMP), of a recombinant protein product based on LSA-1 from the *P. falciparum* 3D7 strain (PfLSA-1) capable of stimulating a cellular immune response in animals and humans and inducing the production of antibodies able to recognize the native protein. A synthetic gene construct was designed that incorporated regions known to contain previously identified T-cell epitopes in the N- and C-terminal regions and 2 of the 17 amino acid repeats (Fig. 1). A new algorithm of codon harmonization was employed to engineer a gene resulting in high-level expression in *Escherichia coli*. This protein, LSA-NRC, was manufactured as a clinical-grade product, falciparum malaria protein 011 (FMP011), by the methods described here.

MATERIALS AND METHODS

Cloning and expression. A synthetic gene containing modified codons to encode the N terminal (residues 28 to 154), the C terminal (residues 1630 to 1909), and two 17-amino-acid repeats of LSA-1 of the *P. falciparum* 3D7 clone

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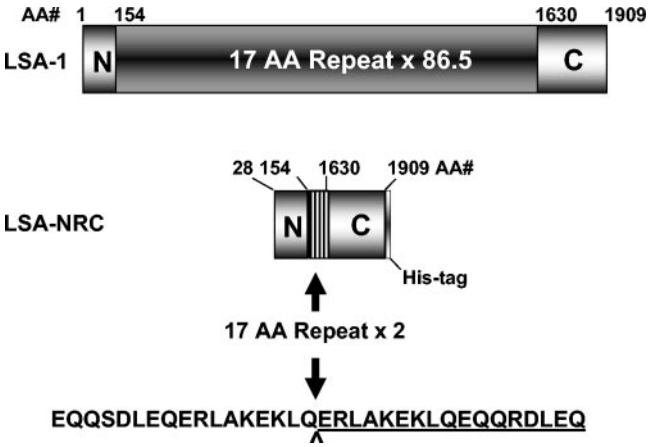

FIG. 1. Diagrammatic representation of the construction of *P. falciparum* LSA-NRC. Amino acid numbers (AA#) are given to denote N-terminal, repeat, and C-terminal regions in the native protein and the recombinant product. All numbers are based on the LSA-1 (NF54) sequence in GenBank. The sequence of the two 17-amino-acid (AA) repeats is given, and the ^ symbol denotes the delineation between the two subunits in this construct. The major and minor (underlined) subunits are repeated 31 and 4 times, respectively, in the native LSA-1 (NF54 isolate).

(residue numbers refer to the GenBank protein sequence for 3D7 clone, no. A45592) were synthesized commercially (Retrogen, San Diego, Calif.). The gene, *lsa-nrc*^{h}, was synthesized using codon harmonization, meaning the gene was designed to replicate, as closely as possible, *P. falciparum* codon frequency preferences rather than *E. coli* frequency preferences. Cloning of the gene into the expression plasmid resulted in a hexa-histidine affinity tag at the C terminus of the LSA-NRC protein.

The central repeats of PfLSA-1 are all 17 amino acids in length but show a slight degeneracy in their sequence (7). Overall, they still maintain conserved positional glutamine residues and complete two alpha-helical turns in their secondary structure. We chose one copy of the major repeat (EQQSDLEQER LAKEKLQ) and one copy of a minor repeat (EQQRDLEQERLAKEKLQ) that are found 31 and 4 times, respectively, in the native protein to represent the repeats in the recombinant LSA-NRC. In LSA-1 a copy of the minor repeat is found at the hinge region between the end of the repeats and the nonrepeat C-terminal end of the protein, an area that also has been described as a T-cell epitope, J (7). In the LSA-NRC recombinant construct this minor repeat begins at the ninth amino acid of a 17-amino-acid repeat unit in order to be in frame with the C-terminal portion of LSA-NRC, as it is in the native LSA-1 protein, and still maintain the alpha-helical turn phasing consistent with the first repeat (Fig. 1).

For protein expression the synthetic gene was ligated into the NdeI and NotI sites of pET(AT) (2). The new plasmid, pETK, was made acceptable for human use protein expression by replacement of the Tet^{r} and Amp^{r} genes with a single Kan^{r} gene. The resultant plasmid construct was designated pETK LSA-NRC^{h}, and the resultant protein was designated LSA-NRC. The recombinant plasmid was transformed into a nonexpression host cell, *E. coli* DH5α, for amplification. The gene insert in the plasmid recovered from this host was sequenced on both strands for verification. For protein expression, the plasmid was transformed into an *E. coli* host strain, Tuner (DE3) (Novagen, Madison, Wis.). The transformations were plated onto Luria-Bertani (LB) agar plates containing 50 μg of kanamycin ml^{-1} and 1% glucose. Expression of LSA-NRC was confirmed by isopropyl-β-D-thiogalactopyranoside (IPTG) induction (final concentration of 1 mM) in shake flask cultures. Before cell banks were created the cells were passaged three times through Select APS Superbroth medium (Difco, Becton Dickinson, Sparks, Md.), a plant-derived medium. A master seed (lot number 1158) was prepared following batch production record (BPR) 708-00 by inoculating select APS Superbroth medium supplemented with 1% glucose and 35 μg of kanamycin/ml. Cultures were grown to an optical density at 600 nm of 1.0, cryopreserved in 8% glycerol, and stored at −80°C. A production seed (lot number 1159) was made following BPR 709-00 from a vial of the master seed lot by the same method. Vials of the production seed lot were used to prepare the inoculum for bulk fermentation. All BPRs are permanently kept at the Walter Reed Army Institute of Research (WRAIR) Pilot Bioproduction Facility, Silver Spring, Md.

Fermentation (GMP production). The process for expression of LSA-NRC was developed in a 10-liter bioreactor (New Brunswick Scientific, Edison, N.J.) and scaled up to a 300-liter bioreactor (New Brunswick Scientific) for GMP production at the WRAIR Department of Biologics Research, Pilot Production Facility. To prepare *E. coli* cell paste containing LSA-NRC, select APS Superbroth medium containing 35 μg of kanamycin ml^{-1} and 1% glucose was inoculated with a late-log-phase culture in accordance with BPR 721-00. The culture was grown at 37 ± 1°C to an optical density at 600 nm of 8 to 10 and induced with a final concentration of 1 mM IPTG for 2 ± 0.25 h. The cell paste (lot number 1184) was harvested by centrifugation and stored frozen at −80 ± 10°C in 250-g aliquots.

Plasmid stability. The presence of recombinant plasmid for Kan^{r} in *E. coli* Tuner (DE3) cells after fermentation was determined by plating an appropriate dilution of cells on LB agar plates containing kanamycin (50 μg ml^{-1}; selective plates) and on LB agar plates containing no antibiotic (nonselective plates). The percent plasmid retention [(number of colonies on selective plates)/(number of colonies on nonselective plates)] was calculated using colony counts on appropriate dilution plates containing between 30 and 300 colonies.

Metal affinity purification. All buffers were made with endotoxin-free water, and all chemicals used during purification were USP certified or the next best grade available. All reagents and materials were quarantined from the time of arrival until time of use and were only utilized for this project. Purification was carried out at room temperature (RT) following BPR 733-00. Briefly, cell paste was thawed, and 125 g was suspended in cold buffer A (100 mM sodium phosphate, 2 M NaCl; pH 6.2 ± 0.1) at a ratio of 25 ml of buffer per g of paste and mixed until homogenous. The bacterial cells were disrupted by high-pressure microfluidization (model 1109; Microfluidic Corp., Newton, Mass.), the cell lysate was clarified by centrifugation (12,000 × *g*), and buffer B (20% sodium lauroyl-sarcosinate [Sarkosyl]) was added to the supernatant to achieve a final concentration of 0.5% Sarkosyl. This mixture was incubated, with stirring, at 4°C for 30 min before loading onto a column containing 2 liters of packed Ni^{2+}-nitrilotriacetic acid (NTA) Superflow resin (QIAGEN, Valencia, Calif.; 16 ml of packed resin per g of starting bacterial cell paste). The Ni^{+2} resin was preequilibrated with buffer C (buffer A that contained 0.5% Sarkosyl; pH 6.2 ± 0.1). After loading the lysate, the Ni^{2+} resin was washed with 15 column volumes (CV) of buffer C followed by 29 CV of buffer D (100 mM sodium phosphate, 5 mM imidazole, 75 mM NaCl; pH 7.0 ± 0.1). Bound proteins were eluted from the resin in buffer E (100 mM sodium phosphate, 300 mM imidazole, 75 mM NaCl; pH 7.0 ± 0.1).

Ion-exchange purification. Ion-exchange column resins were sanitized with 0.2 N NaOH before use and then equilibrated under initial binding conditions. The protein was diluted 1 in 3 into buffer F (20 mM sodium phosphate; pH 7.0 ± 0.1) and then concentrated on a DEAE Sepharose anion-exchange column (Amersham Pharmacia Biotech, Piscataway, N.J.) containing 375 ml of packed resin (3 ml of packed resin per g of initial bacterial cell paste). The column was preequilibrated with buffer G (20 mM sodium phosphate, 25 mM NaCl; pH 7.0 ± 0.1). After loading the protein, the resin was washed with 10 CV of buffer H (20 mM sodium phosphate, 200 mM NaCl; pH 7.0 ± 0.1). The protein was eluted in buffer I (20 mM sodium phosphate, 280 mM NaCl; pH 7.0 ± 0.1). This elution was diluted 1 in 6 to 50 mM NaCl, pH 7.0 ± 0.1, in buffer F (20 mM sodium phosphate, pH 7.0 ± 0.1) and loaded on an SP Sepharose cation-exchange column containing 250 ml of packed resin (Amersham Pharmacia Biotech; 2 ml of packed resin per g of initial bacterial cell paste) preequilibrated with buffer J (20 mM sodium phosphate, 50 mM NaCl; pH 7.0). The resin was washed with 20 CV of buffer J. LSA-NRC bulk protein was eluted in buffer K (20 mM sodium phosphate, 150 mM NaCl; pH 7.0 ± 0.1). The parameters of the binding and elution of the protein on and off the ion-exchange resins were determined empirically. The pI of LSA-NRC is 5.07 and therefore should have shown quite different bindings to anion and cation exchangers. However, by maintaining the charge of the buffer at pH 7.0 and only modifying the NaCl concentration we were able to establish excellent reproducible and scalable binding and elution conditions for LSA-NRC over host cell proteins (HCPs).

Formulation, lyophilization, and storage. Purified LSA-NRC protein was quantified by Bio-Rad protein assay (Bio-Rad, Richmond, Calif.) and vialed following BPR 740-00 at 100 μg ml^{-1}. A total volume of 0.6 ± 0.06 ml (60 ± 0.06 μg of protein) was added to 3.0-ml vials in a final formulation buffer containing 23.5 mM sodium phosphate, 30 mM NaCl, and 3.15% sucrose (pH 7.0 ± 0.1) and lyophilized. A single vialing (lot 1204) yielded ~2,000 vials that passed all quality control release specifications (>99%). Excess purified bulk was stored at −80°C for future vialing.

Endotoxin content determination. Endotoxin content was estimated using the *Limulus* amebocyte lysate gel clot assay (Associates of Cape Cod, Falmouth, Mass.). The assay was performed according to the manufacturer's instructions.

Purity and stability analysis. LSA-NRC was evaluated for purity on precast polyacrylamide gels (4-to-12% gradient bis-Tris; Invitrogen, Carlsbad, Calif.), with 0.1 to 10 μg of protein loaded per well. Gels were stained with Coomassie blue R250 or GelCode SilverSNAP stain (Pierce, Rockford, Ill.), destained, and scanned on a laser scanning densitometer, and the acquired data were analyzed with ImageQuant 5.1 software (Molecular Dynamics, Sunnyvale, Calif.). Residual HCP content was assessed by enzyme-linked immunosorbent assay (ELISA) and Western blotting, using commercially available kits (Cygnus Technologies, Plainville, Mass.). The HCP standard recommended by the manufacturer was used. In addition to this control, a lysate of the *E. coli* Tuner (DE3) host was tested as a standard at concentrations between 15 and 1,000 ng of protein ml^{-1} to determine if the kit was capable of detecting proteins from this specific host *E. coli*. Stability of LSA-NRC was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting of aliquoted protein samples stored at −80, 4, and 37°C.

Primary structure analysis. For N-terminal sequencing, purified LSA-NRC was subjected to SDS-PAGE and Western blot transferred to a 0.2-μm polyvinylidene difluoride membrane (Invitrogen). After transfer the membrane was washed three times in 50% methanol, two times in distilled water, and then briefly in 100% methanol. The membrane was stained for 1 min in Coomassie blue stain, rinsed five times in 50% methanol, and finally rinsed in distilled water. The visible stained bands on the membrane were excised and sent to ProSeq, Inc. (Boxford, Mass.) for sequencing.

For determination of the mass of the product, 2 μl of bulk protein lot 1198 was diluted 1:10 in sinapini acid matrix and analyzed by matrix-assisted laser desorption ionization–time of flight (mass spectrometry) (MALDI-TOF; Voyager biospectrometry RP system; Applied Biosystems, Foster City, Calif.). Lysozyme and cytochrome *c* were used as mass standards.

HPLC. High-performance liquid chromatography (HPLC) analysis of purified protein was carried out with an Agilent 1100 series HPLC apparatus, an 1100 quaternary pump connected to an Agilent 1100 series autosampler, and Agilent 1100 column thermostat and controlled by Chemstation software (Agilent Technologies Inc., Alpharetta, Ga.). An Agilent 1100 series diode array detector was used to monitor the elution profile at 280 nm. The separation matrix was a TosoHaas column, G3000SWXL, 7.8 mm by 30 cm, 6 μm, with column guard PWXL, 6.0 mm by 4.0 cm, 7 μm, regulated at 25 ± 5°C. The column was calibrated with molecular weight standards (Bio-Rad). The sample was analyzed at a flow rate of 1.0 ml min^{-1} with 50 to 60 μg of protein per load. Solvent A contained 100% filtered H_2O; solvent B contained 50 mM morpholinepropanesulfonic acid (MOPS), 200 mM sodium sulfate, 0.02% sodium azide; solvent C contained 100% column storage solution (20% sodium azide); and solvent D contained wash solution (20% methanol). The run time (20 min) was used with an isocratic flow of solvent A (MOPS running buffer).

Animal immunization. A/J ($H\text{-}2^K$) female mice, 6 to 8 weeks old, were immunized with formulations containing 0.1, 1.0, or 10 μg of LSA-NRC adjuvanted with 70% (vol/vol) Montanide ISA 720 (Seppic Inc., Paris, France). Each formulation was prepared in a 0.1- or 0.2-ml volume for subcutaneous (s.c.) or intraperitoneal (i.p.) injection, respectively. Mice were immunized three times s.c. or twice i.p. at 4-week intervals and euthanized 14 days after the last immunization for collection of serum samples and spleens. Four-month-old NZW rabbits were immunized s.c. with 100 μg of LSA-NRC adjuvanted with 70% (vol/vol) Montanide ISA 720 and boosted twice s.c. with 50 μg of the antigen 4 and 8 weeks after the first immunization. Rabbits were bled 14 days after each immunization. Control groups for both species were immunized with the same amount of adjuvant in saline using similar immunization routes and volumes. All animal procedures were reviewed and approved by WRAIR's Institute Animal Care and Use Committee, and they were conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the *Guide for the Care and Use of Laboratory Animals* (15a).

ELISA. Antibody responses against LSA-NRC were evaluated in an ELISA. Briefly, 96-well microplates (Dynax, Chantilly, Va.) were coated with 100 ng of LSA-NRC per well, kept overnight at 4°C, and then blocked for 1 h with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 5% casein (Sigma, St. Louis, Mo.). Plates were washed three times (PBS, 0.05% Tween 20) and incubated for 2 h at RT with individual or pooled mouse sera. Plates were washed again and incubated for 1 h at RT with 1:4,000-diluted secondary anti-mouse immunoglobulin antibodies labeled with horseradish peroxidase (Southern Biotechnology Associates, Birmingham, Ala.). Plates were washed and developed with 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate]–peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) and examined at 405 nm.

IFN-γ ELISpot. Production of LSA-NRC-related gamma interferon (IFN-γ) by immunized mice was determined by using an ELISpot^{plus} kit for mouse IFN-γ (Mabtech, Mariemont, Ohio) following the manufacturer's guidelines. Briefly, spleens were surgically removed from four or five euthanized mice and homogenized in Hank's balanced salt solution (Invitrogen) to obtain a pooled spleen cell suspension. Leukocytes were resuspended at 2×10^6 cells ml^{-1} in Iscove's modified Dulbecco's medium (BioWhittaker, Walkersville, Md.) supplemented with 0.5% normal mouse serum, 2 mM L-glutamine, 55 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 100 U of penicillin-streptomycin (Invitrogen) ml^{-1}. Aliquots (100 μl) of the cell preparation were added to wells of a polyvinylidene difluoride-bottom 96-well plate previously coated with 1.5 μg of mouse IFN-γ capture antibody $well^{-1}$. Cultures of cells (200 μl per well) were grown in the absence or presence of 1.0 or 10.0 μg of LSA-1 ml^{-1} or a control protein, 0.5 μM AMA-1/e (6), for 48 h at 37°C under a humidified atmosphere with 5% CO_2. Plates were washed five times with PBS and incubated with a biotinylated mouse IFN-γ antibody, 0.1 μg $well^{-1}$, for 2 h at RT. Plates were washed again and incubated with streptavidin-alkaline phosphatase, 0.1 μg $well^{-1}$, for 1 h at RT. Plates were developed using a substrate solution containing 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium. Spots, representing IFN-γ-secreting splenocytes, were inspected and counted using an AID ELISpot reader system and software (Autoimmun Diagnostika GmbH, Strassberg, Germany).

Immunofluorescence. A continuous cell line of human hepatocytes (HC-04) was infected with *P. falciparum* sporozoites, strain NF54, and cultured for 1 week. Cells were washed twice with PBS, fixed with cold methanol, and washed again with PBS. Immune rabbit serum at 1:200 was added (30 min, RT) and after washing with PBS, anti-rabbit immunoglobulin G peroxidase-conjugated secondary antibody was added (30 min, RT).

Nucleotide sequence accession number. The *lsa-nrc^h* gene sequence has been submitted to GenBank (accession number AY751501).

RESULTS

Fermentation of *E. coli* Turner (DE3) expressing the LSA-NRC protein at 10- and 300-liter scales. The synthetic gene, *lsa-nrc^h*, was cloned into the expression vector pETK and transformed into *E. coli* Tuner (DE3) cells, and it expressed soluble LSA-NRC at a level ~13% of the total bacterial cell protein as estimated by laser scanning densitometry of a Coomassie blue-stained SDS-PAGE gel (data not shown). Fermentation conditions were developed in a 10-liter fermentor typically yielding 22 to 25 g of wet cell paste $liter^{-1}$. Glucose was included in all cultures to down regulate the *lac* promoter that controlled T7 RNA polymerase expression and thus prevent premature LSA-NRC expression. A scaled up 300-liter fermentation process yielded 8 kg of bacterial cell paste after cell harvest. Plasmid stability tests showed no detectable loss of plasmid during fermentation.

Purification of LSA-NRC. The purification of LSA-NRC was developed in three chromatographic steps. After microfluidization at 4°C and centrifugation, the soluble fraction was incubated with 0.5% Sarkoysl, an anionic detergent, to facilitate the removal of endotoxin that may have bound to the LSA-NRC or the Ni^{2+}-NTA Superflow resin. The clarified lysate was applied to the Ni^{2+} resin for affinity purification and resulted in its increased purity from 13% to about 80% by densitometry (data not shown). The inclusion of Sarkoysl and the extensive washing of the bound protein in high salt (2 M NaCl) and low pH (pH 6.2) reduced the endotoxin levels to below the limits of detection in this first step. LSA-NRC was further purified to greater than 98% purity by anion-exchange chromatography (DEAE Sepharose) followed by cation-exchange chromatography (SP Sepharose) as shown by densitometric analysis of the purified bulk on Coomassie blue-stained

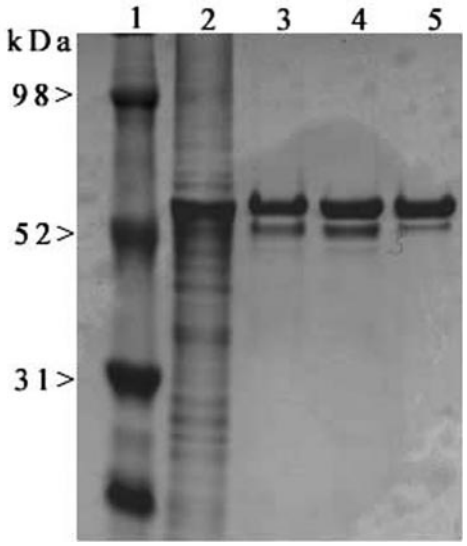

FIG. 2. SDS-PAGE analysis after each step in purification of LSA-NRC-purified bulk FMP011. Coomassie blue-stained gel lanes: 1, molecular mass marker; 2, soluble lysate; 3, Ni^{2+}-NTA Superflow elution; 4, DEAE-Sepharose elution; 5, SP-Sepharose elution.

(Fig. 2) and silver-stained (Fig. 3) SDS-PAGE gels and by HPLC analysis (Fig. 4). The final yield of LSA-NRC was 230 mg liter of fermentation culture^{-1}.

Product analysis. The HCP content of the purified bulk product, as determined by quantitative ELISA, was 7 ng of LSA-NRC mg^{-1}. No contaminating *E. coli*-derived impurities were detected in 1 μg of purified bulk product by Western blotting of probe with antibodies made against a whole-cell

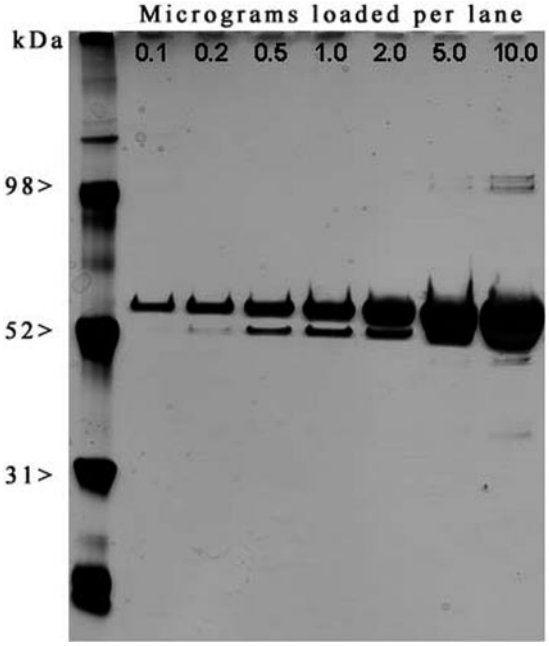

FIG. 3. SDS-PAGE analysis of LSA-NRC-purified bulk FMP011, lot 1198, as shown in a silver-stained gel with 0.1 to 10 μg of protein loaded into each lane as indicated. Multimark molecular mass standards are in the left lane.

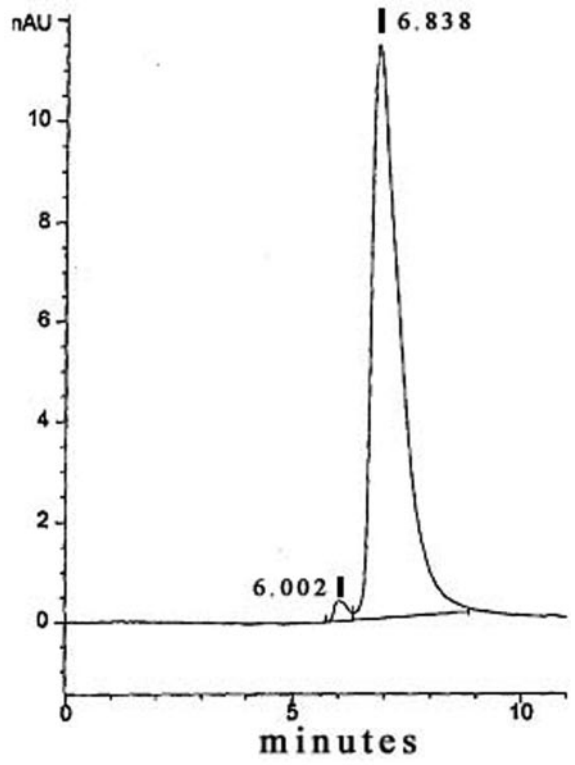

FIG. 4. HPLC results with LSA-NRC-purified bulk protein FMP011, lot 1198. Major and minor peaks are 98.3 and 1.7%, respectively, of total protein detected by this methodology. The buffer-only chromatograph had no measurable peaks in this time range (data not shown).

lysate of *E. coli* (Fig. 5d). Initial stability studies by SDS-PAGE showed the final purified LSA-NRC protein was stable for 1 month at −80, 4, and 37°C (data not shown). Endotoxin content was undetectable (lower limit of detection = 0.06 EU ml^{-1}) in the final bulk protein solution, which contained 698 mg of LSA-NRC protein ml^{-1}. This is equivalent to <0.005

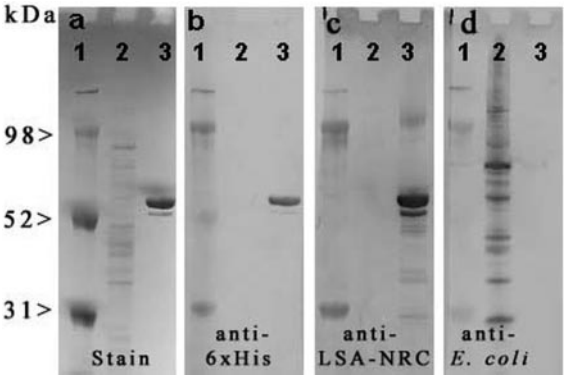

FIG. 5. Profile and identity of purified LSA-NRC by SDS-PAGE. (a) Coomassie blue-stained gel. (b to d) Western blots of the gel shown in panel a. Antibody probes for the Western blots were mouse anti-His_6 (b), rabbit anti-LSA-NRC (c), or rabbit anti-*E. coli* (d) antibodies. Lanes in each panel: 1, multimark protein size marker; 2, Tuner (DE3) bacterial lysate; 3, 1 μg of LSA-NRC-purified bulk FMP011.

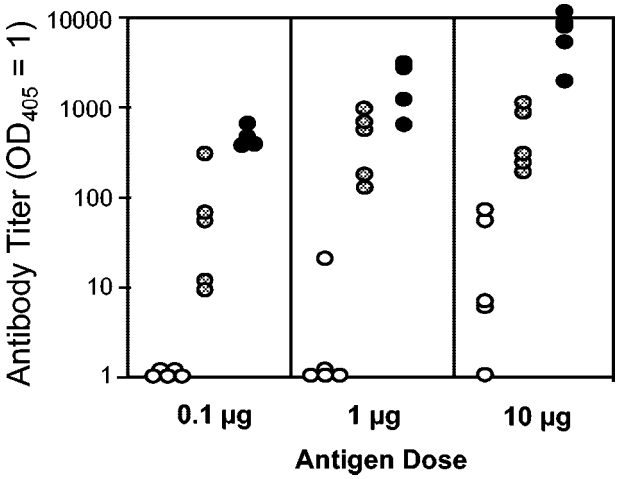

FIG. 6. LSA-NRC-specific antibody titers of sera of A/J ($H\text{-}2^K$) mice after s.c. immunizations with 0.1-, 1.0-, or 10-μg doses of LSA-NRC emulsified in 70% (vol/vol) Montanide ISA 720. White, gray, and black circles represent the titers of individual sera after one, two, or three immunizations, respectively.

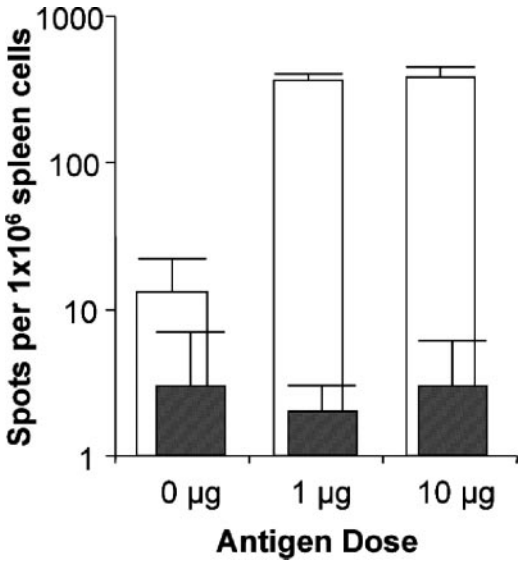

FIG. 7. LSA-NRC protein-specific IFN-γ responses as measured by ELISpot analysis of LSA-NRC-immunized A/J ($H\text{-}2^K$) mice. Bars represent the average number of IFN-γ-producing splenocytes per million recovered from mice 2 weeks after the second immunization and stimulated ex vivo with 10 μg of LSA-NRC ml^{-1} (open bars) or 10 μg of AMA-1 ml^{-1} (filled bars) for 48 h. Limits show SD of counts for three mice run in triplicate.

EU per 50 μg of LSA-NRC, a common dosage for malaria vaccines in initial testing.

After SDS-PAGE separation, a second protein band could be seen below the major LSA-NRC protein. Both proteins were recognized by an anti-His_6 antibody and therefore contained the His_6 tag at the C terminal (Fig. 5b). N-terminal sequencing showed the larger band had a sequence of GTNSEKDEIIKSNLRSGSSNSRNRINEEKH, which corresponded exactly to the 30 residues expected at the N terminus of LSA-NRC following the initial methionine residue, which is normally refractory to analysis. The smaller protein band started with SNVKNVSQTNFKSLLRNLGVSE, which corresponded to a truncated LSA-NRC starting translation at a second ATG codon located 186 nucleotides (62 amino acids) downstream from the initial methionine codon. This product would have a molecular mass 7 kDa smaller than the expected LSA-NRC. Purity evaluation by HPLC detected the two proteins in the final preparation, the major peak of 98.3% representing the full-length LSA-NRC protein and a minor peak of 1.7% representing the truncated LSA-NRC (Fig. 4). Mass spectroscopy of the protein by MALDI-TOF found the mass to be 53,573.6 Da. The calculated mass of LSA-NRC was 53,600 Da, the difference between the experimental mass and the calculated mass being 26.4 Da. The sensitivity of MALDI-TOF is not enough to detect the mass of the minor product in the sample.

Recombinant LSA-1 induces strong antibody responses. Immunization with LSA-NRC was well tolerated by mice and rabbits and induced strong antibody responses in both species. Individual mouse sera from each dose group were tested for anti-LSA-NRC immunoglobulin G titers. It was observed that antibody titers were proportional to antigen dose in mice immunized three times with protein emulsified in Montanide ISA 720 when given s.c., with titers of 530 (standard deviation [SD], 144), 2,060 (SD, 1,266) and 8,136 (SD, 4,365) when 0.1, 1.0, and 10 μg $dose^{-1}$, respectively, was injected (Fig. 6). The induction kinetics of anti-LSA-NRC antibodies was faster in animals injected i.p. than s.c. with the protein emulsified in Montanide ISA 720 (data not shown). Antibody titers after two i.p. immunizations with 1 or 10 μg of antigen were as high as after three s.c. immunizations with similar doses. In rabbits (n = 3) the average anti-LSA-NRC serum titer after three immunizations was 149,303 (SD, 24,558).

LSA-NRC stimulates a strong cellular response. LSA-NRC recombinant protein was used to induce antigen-specific IFN-γ-secreting splenocytes in an in vitro ELISpot assay. A high frequency of LSA-specific IFN-γ-secreting splenocytes was found in animals immunized i.p. with 1 or 10 μg of LSA-NRC (Fig. 7). The average response was 378 ± 60 spot-forming cells per million splenocytes, with no difference found between the two dosage groups. This response was substantially higher than in adjuvant-only control-injected mice or in vitro in immune splenocytes stimulated with a control protein.

Immunochemical identification. Kenyan pooled sera recognized LSA-NRC by both Western assay and ELISA (data not shown). Immune rabbit sera recognized the native *P. falciparum* LSA-1 expressed in infected HC-04 cells, giving a diffuse fluorescent pattern upon immunofluorescence (Fig. 8).

DISCUSSION

We have developed a process to manufacture a GMP quality recombinant protein product based on *P. falciparum* LSA-1 at a scale (230 mg liter of $culture^{-1}$) that is within industry advanced development standards for vaccine manufacture. Our previous attempts to express *P. falciparum* LSA-1 constructs in heterologous expression systems have resulted in poor yields or unstable plasmids. One of the first attempts at heterologous expression of LSA-1 was a repeatless LSA-1 gene cloned into the poxvirus vector, NYVAC-Pf7 (19), which resulted in low

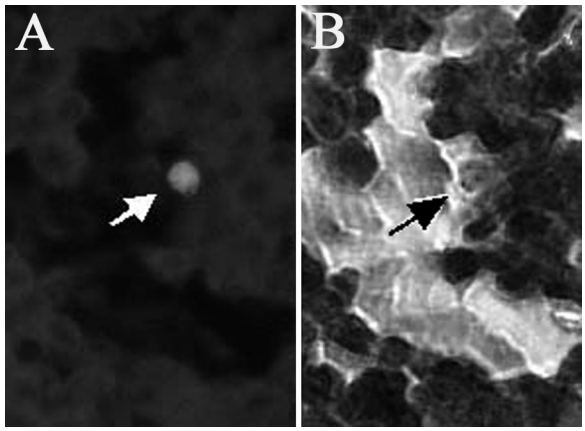

FIG. 8. Detection of anti-LSA-NRC rabbit antibody recognizing an infected cultured hepatocyte. (A) Immunofluorescence; (B) phase contrast of the same field. The arrow points to the infected cell in both panels.

expression levels in mammalian cells. Recently another poxvirus construct (MVA-L3SEPTL) was made that contains a repeatless LSA-1 gene, and expression of this protein is currently being evaluated (16). To address low expression levels in *E. coli*, we evaluated *E. coli* transformed with secondary plasmids encoding rare-frequency codon tRNA genes. While these cotransformants resulted in low, but acceptable, protein yields for the N-terminal or C-terminal polypeptides from LSA-1 (D. E. Lanar, unpublished data), the secondary plasmids required selection with antibiotics that are unsuitable for products developed for human use. We also synthetically constructed a gene (*lsa-nrc*^{e}) by using optimized *E. coli* codons, but the plasmid was unstable and quickly was lost or rearranged upon induction of protein expression (data not shown). Finally, the use of the codon harmonization approach (changing codons to ensure preservation of positional codon frequency in a heterologous expression system) instead of optimization (altering codons to always ensure usage of the most abundant codon in a heterologous system) resulted in a construct that had significantly enhanced efficiency of protein expression in *E. coli*. Codon harmonization is thought to permit translation in the heterologous host in the same way as the native host and thereby have an effect on protein folding. Unlike the product of an optimized gene, the LSA-NRC protein expressed from the codon-harmonized gene was not degraded, aggregated, or detrimental to cell growth.

The purification protocol developed resulted in a highly purified protein that is currently undergoing tests to determine if it meets Food and Drug Administration standards necessary for further evaluation in a phase I safety and immunogenicity clinical trial in humans. The residual levels of all chemicals used in the purification process have been evaluated and determined to be within acceptable levels for production specifications (data not shown). Endotoxin content in the final product was below the level of detection.

Although >99.9% of the protein in the final product, when analyzed by HPLC, was of LSA-NRC origin, the product was not homogeneous due to an alternative initiating methionine, 62 codons downstream of the initial ATG start site. The purification process removed a large proportion of this smaller protein, reducing its contribution to the final purified product to <2%. Preliminary immunogenicity data show that there may be some T-cell epitopes in the first 62 amino acids, but because the loss of this region is in only about 2% of the total protein it is not expected that there will be a significant effect on overall immunogenicity of the formulated product.

Both cellular and humoral immune responses have been implicated as critical in protection against preerythrocyte-stage malaria, and native LSA-1 is known to elicit both responses in humans (3–5, 7–10, 12–15). Fidock et al. (7) reported that most, but not all, of the antibodies to LSA-1 in individuals from areas where malaria is endemic recognize the central repeats. Recognition of LSA-NRC by human immune sera gives assurance that the recombinant construct, with only 2 of the 86 repeats, contains relevant antigenic epitopes. We have shown that LSA-NRC can, when injected into rabbits, induce antibodies that recognize the native parasite growing in cultured hepatocytes. We have also shown that this protein, when emulsified in Montanide 720, can induce protein-specific IFN-γ expression from lymphocytes in mice. Recently, John et al. (12) reported that in children living in a stable malaria transmission area, IFN-γ responses to LSA-1 peptides were associated with protection against clinical malaria and anemia. We are currently evaluating a wide variety of cellular immune responses in several different strains of mice, vaccinated with LSA-NRC in different adjuvants, to identify the relevant immuno-stimulatory sequences in the protein. With the highly purified LSA-1 material obtained using the protocols reported here, we should be able to more efficiently evaluate the immune response to the protein in small animals and nonhuman primates in anticipation of human trials. It is expected that this clinical-grade LSA-NRC (FMP011) formulated with a potent adjuvant should enable us to enter phase I and phase II clinical studies to determine whether a vaccine-induced immunological response to LSA-1 epitopes can protect vaccinees against mosquito-borne *P. falciparum* malaria.

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