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Expression, Extracellular Secretion, and Immunogenicity of the *Plasmodium falciparum* Sporozoite Surface Protein 2 in *Salmonella* Vaccine Strains

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Deleting transmembrane α-helix motifs from *Plasmodium falciparum* sporozoite surface protein (SSP-2) allowed its secretion from *Salmonella enterica* serovar Typhimurium SL3261 and *S. enterica* serovar Typhi CVD 908-*htrA* by the Hly type I secretion system. In mice immunized intranasally, serovar Typhimurium constructs secreting SSP-2 stimulated greater gamma interferon splenocyte responses than did nonsecreting constructs ($P = 0.04$).

Combining antigens from the sporozoite, intrahepatic, and asexual erythrocytic stages of *Plasmodium falciparum* into a multivalent vaccine should increase the prospect of achieving protective efficacy (26, 29). Immunologic intervention against the preerythrocytic stages should reduce the number of merozoites emerging from the liver cells, thereby allowing other immune mechanisms to more successfully attack the asexual erythrocytic stages. We have embarked on a program to express in a suitable attenuated *Salmonella enterica* serovar Typhi strain protective antigens derived from the various stages in the life cycle of *P. falciparum* and, ultimately, to determine whether the live vector vaccine can stimulate relevant immune responses in humans (14).

SSP-2, also designated thrombospondin-related adhesive protein, which is expressed by the sporozoite once it reaches the mosquito salivary gland, contains a sulfated glycoconjugate-binding peptide sequence needed for parasite invasion (34). Immunization with *P. yoelii* SSP-2 protects mice against experimental malaria by induction of cytotoxic lymphocytes (CTL) specific to two independent T-cell epitopes (21, 36). Analogously, the specific SSP-2 CTL responses induced following immunization of volunteers with irradiated *P. falciparum* are thought to contribute to protection (42). Humoral responses may also play a protective role since antibodies to SSP-2 prevent sporozoites from invading human hepatocytes in vitro. Thus, SSP-2 should be included in a multivalent vaccine to prevent *P. falciparum* malaria (35).

The feasibility of using attenuated serovar Typhi expressing *P. falciparum* antigens as an oral live vector vaccine was demonstrated in a clinical trial in which attenuated serovar Typhi strain CVD 908 carrying a recombinant *P. falciparum* circumsporozoite protein gene integrated in the chromosome stimulated serum antibodies and cytotoxic lymphocytes in several vaccinees (14). Serovar Typhi live oral vaccine strain CVD 908-*htrA*, an improved live vector that harbors attenuating deletion mutations in *aroC, aroD*, and *htrA* (3, 23), is well tolerated and elicits antibody and cell-mediated immune responses to serovar Typhi following a single oral dose (40). CVD 908-*htrA* also functions well in humans as a live vector. One of three seronegative subjects who ingested a single $\sim 10^9$ CFU dose of CVD 908-*htrA* expressing fragment C of tetanus toxin mounted a strong serum tetanus antitoxin response (39).

The immune response to foreign antigens expressed by *Salmonella* live vectors, particularly CTL, is significantly enhanced if the heterologous proteins are secreted externally from the bacteria (11, 18, 20). However, whereas many bacterial proteins are readily expressed in attenuated *Salmonella* as either cytoplasmic, periplasmic, or secreted moieties (1, 9, 13, 30), expression of eukaryotic *P. falciparum* proteins, particularly as secreted proteins, has been much more problematic (24). Factors responsible include differences in codon usage between bacteria and *Plasmodium*, protein sequences (e.g., hydrophobic) deleterious to bacteria, and apparent posttranslational destruction of foreign proteins by bacterial proteases. A tactic to circumvent some problems and achieve adequate expression and secretion of plasmodial proteins in serovar Typhi is the use of a plasmid-based expression-secretion system such as the type I hemolysin (Hly) secretion system of uropathogenic *Escherichia coli* (12, 18). Although this system increases the number of copies of the foreign gene (thereby potentially elevating expression and increasing deleterious effects on the live vector), the likelihood of toxicity to the bacterial host is decreased since the foreign antigen is secreted. This system requires three membrane proteins, HlyB, HlyD, and TolC, and a signal sequence located at the C terminus of the wild-type HlyA (28). TolC, which is not part of the *hly* operon, is encoded in the *Salmonella* chromosome. Herein we describe the

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TABLE 1. Plasmids used in this study

Plasmid	Insert	Determinants[a]	Source or reference
pBluescript II KS		Amp^r, β-gal	Stratagene
pKS-SSP-2	Wild-type *ssp*-2	Amp^r, β-gal	This study
pBluescript II Sk		Amp^r	Stratagene
pMOhly1		Amp^r, Hly	12
pSK-2023	Wild-type *ssp*-2	Amp^r	This study
pSK-2123	*ssp*-2 with 5′deletion	Amp^r	This study
pSK-2027	*ssp*-2 with 3′deletion	Amp^r	This study
pSK-3027	*ssp*-2 with 5′ and 3′deletions	Amp^r	This study
pMO-2023	Wild-type *ssp*-2	Hly, Amp^r	This study
pMO-2123	*ssp*-2 with 5′deletion	Hly, Amp^r	This study
pMO-2027	*ssp*-2 with 3′deletion	Hly, Amp^r	This study
pMO-3027	*ssp*-2 with 5′ and 3′ deletions	Hly, Amp^r	This study
VR2519	Wild-type *ssp*-2 preceded by the translation initiation complex and leader peptide sequences of human tissue plasminogen activator.	Km^r	17

[a] β-gal, β-galactosidase.

expression and secretion of *P. falciparum* SSP-2 in attenuated serovar Typhi CVD 908-*htrA* and serovar Typhimurium and demonstrate the immunogenicity of the serovar Typhimurium construct in mice immunized mucosally.

E. coli DH5α and *aroA* mutant serovar Typhimurium SL3261 were grown in Luria broth (LB) or agar supplemented with 100 μg of ampicillin per ml when required (19). Serovar Typhi CVD 908-*htrA* was grown in LB supplemented with 0.0001% 2,3-dihydroxybenzoic acid (Sigma, St. Louis, Mo.). The plasmids used in this study are described in Table 1. In adapting pMOhly1, which carries the *E. coli* hemolysin secretion system, the gene encoding the protein to be exported is inserted into the unique *Nsi*I site located within a truncated *hlyA*, immediately upstream of the C-terminal secretion signal and downstream of the initiation codon. *P. falciparum ssp-2* and truncated *ssp-2* derivatives were inserted into pBluescript II SK and pMOhly1, and the plasmid constructions were transferred to *E. coli* DH5α, serovar Typhimurium SL2361, and Serovar Typhi CVD 908-*htrA* strains by electroporation. Genomic DNA from the 3D7 clone (41) of *P. falciparum* strain NF54 was amplified with primers 1 and 2 to obtain full-length *ssp-2* (35), which was subsequently cloned into pBluescript II KS. The resulting pKS-SSP-2 plasmid served as the template DNA for all amplification reactions (which utilized Deep Vent DNA polymerase with proofreading enzymatic activity). The primers used for PCR amplification are described in Table 2.

A *Pst*I site was incorporated at the 5′ end of each oligonucleotide primer to allow cloning into the compatible *Nsi*I site of pMOhly1. The 1.7-kb PCR product obtained with primers 20 and 23 was cloned in pBluescript II SK to get pSK-2023. From this plasmid a *Pst*I insert carrying the entire wild type *ssp-2* was cloned into pMOhly1 to obtain pMO-2023. The same strategy was used to construct pMO-2123, pMO-2027, and pMO-3027 (Table 1; also see Fig. 3).

Whole-cell lysates prepared from centrifuged pellets of late-logarithmic-phase aerated cultures of *E. coli* and *Salmonella* grown in LB at 37°C were boiled and then separated by sodium dodecyl sulfate-polyacylamide gel electrophoresis (SDS-PAGE) on a 12% polyacrylamide gel. Separated proteins were electrotransferred to nitrocellulose membranes, labeled with the SSP-2.1 monoclonal antibody for recognition of SSP-2 protein (2), and detected by chemiluminescence using the ECL kit (Amersham-Pharmacia-Biotech, Piscataway, N.J.).

The whole-cell lysate from *E. coli* DH5α(pSK-2023) revealed the expression of a 65-kDa protein (Fig. 1A), i.e., a heterologous protein with the expected molecular mass of SSP-2. Other proteins of 90, 75, and 52 kDa were also observed. The 90- and 75-kDa proteins show a molecular mass in SDS-PAGE migration slightly larger than expected, possibly due to the high proline content of SSP-2 (21). The 52-kDa protein apparently represents a degradation product of SSP-2 or a complete product of a truncated SSP-2 transcript. As expected, no SSP-2 was observed in the supernatant (Fig. 1B). DH5α(pMO-2023) expressed a 75-kDa SSP-2 in the cytosol and the smaller band of 52 kDa. The 75-kDa protein correlated very well with the expected molecular mass of the SSP-2–HlyA secretion signal fusion, whereas the 52-kDa protein may correspond to a degradation product of the same protein. Lower

TABLE 2. Primers used in this study for polymerase chain reaction amplifications

Primer	Sequence	ssp-2 position (nt)
1 sense	AATAATGAATCATCTTGGG	1–15
2 antisense	TATTTAATTCCACTCGTTTTC	1728–1708
20 sense	ACTAGTAGCATGCCTGCAGACCCAATAATGAATCATCTTG	1–13
21 sense	ACTAGTAGCATGCCTGCAGACGAATGGTCTCCATGTAG	742–761
23 antisense	TCTGCAGTGGATCCTCTAGATTTCCACTCGTTTTCTTCAG	1721–1703
27 antisense	AACTGCAGCTTCACTATTAGGTACGTGC	1387–1368
30 sense	TTCTGCAGATGTGCAAAACAATATAGTGG	78–100

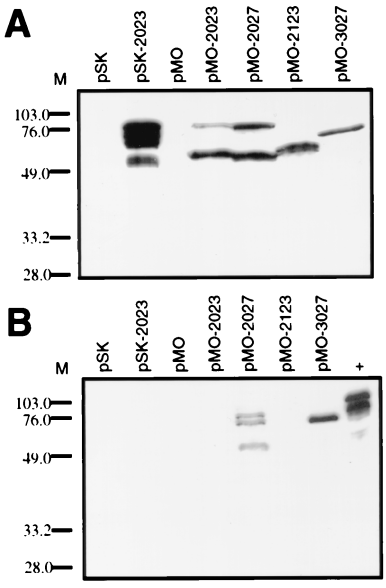

FIG. 1. Expression of the wild type and truncated SSP-2 by the type I secretion system. (A) Immunoblot of whole-cell lysates of *E. coli* DH5α carrying pSK or pMOhly1 (pMO) vectors (as controls) or pSK-2023, pMOhly1-2023, pMOhly1-2027, pMOhly1-2123, or pMOhly1-3027, encoding either full-length or truncated SSP-2. (B) immunoblot of culture supernatants from the above samples. M, molecular weight markers. The control lane (+) contains the bacterial lysate from *E. coli* DH5α(pSK-2023) which, expresses full-length SSP-2.

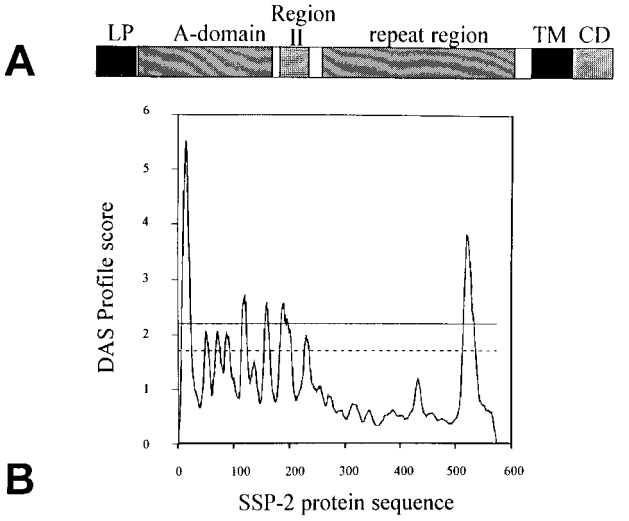

FIG. 2. Transmembrane α-helix regions within SSP-2. (A) Diagram of SSP-2 showing the main protein regions including the leader peptide (LP), the A-domain, region II with homology to the circumsporozoite protein, the repeat region, the transmembrane domain (TM), and the cytoplasmic domain (CD). (B) Dense alignment surface (DAS) analysis of the SSP-2 protein sequence. Sequences with scores above 2.2 are predicted to form transmembrane α-helices with a high degree of statistical significance.

expression of SSP-2 was observed with DH5α(pMO-2023) than with DH5α(pSK-2023), suggesting that the lower copy number of pMO-2023 may account for the difference. Protein analysis of bacterial culture supernatants of DH5α(pMO-2023) showed no export of SSP-2 to the extracellular space (Fig. 1B).

Identical protein analysis results were obtained with whole-cell and supernatants samples from serovar Typhimurium SL3261 and serovar Typhi CVD 908-*htrA* strains carrying the above plasmids (data not shown), further indicating that the full-length SSP-2 cannot be exported by the hemolysin secretion system and that further engineering of the protein was required.

We surmised that the lack of secretion of SSP-2 was most probably the result of interference with the hemolysin secretion machinery (28). Analysis of the SSP-2 sequence using the dense alignment surface method (4) revealed transmembrane α-helices located at the N terminus and at the C terminus which would be expected to inhibit secretion (Fig. 2). We hypothesized that obliteration of these transmembrane α-helices would eliminate interference with the Hly system and allow secretion of SSP-2. Accordingly, truncated versions of *ssp-2* were amplified with primers designed to eliminate the regions encoding either the N-terminal signal sequence, the C-terminal transmembrane domain, or both (Fig. 3), and the products were cloned into pBluescript II SK; *Pst*I cassettes carrying the truncated *ssp-2* derivatives were then introduced into pMOhly1 at the *Nsi*I site. Potential pMOhly1 clones were screened by PCR employing the same primers to construct the truncated versions of *ssp-2*. The constructs were initially recovered in *E. coli* DH5α and then transferred to serovar Typhimurium SL3261 and serovar Typhi CVD 908-*htrA* using electroporation.

Supernatant and whole-cell lysates of *E. coli* and *Salmonella* carrying pMOhly1 constructs were evaluated by immunoblotting using the SSP-2.1 monoclonal antibody. *E. coli* DH5α carrying pMOhly1 encoding wild-type or truncated SSP-2 showed expression in all whole-cell lysates (Fig. 1A). However, only two of the plasmid constructs, pMO-2027 and pMO-3027, were able to secrete the protein, as indicated by the detection of truncated SSP-2 in supernatants (Fig. 1B). *E. coli* DH5α (pMO-2027) expressed a modified SSP-2 that lacks the C-terminal transmembrane domain, whereas DH5α(pMO-3027) secreted the engineered SSP-2 lacking both the N-terminal signal sequence and the C-terminal transmembrane domain. Identical results were obtained with whole-cell lysates (data not shown) and supernatants from serovar Typhimurium SL3261 (Fig 4A), indicating that the SSP-2 C-terminal transmembrane region also interferes with protein secretion in serovar Typhimurium. Most importantly, in the serovar Typhi CVD 908-*htrA* background, only serovar Typhi CVD 908-*htrA*(pMO-3027), which encodes the SSP-2 truncated at both the N and C termini, achieved secretion of the malarial protein (Fig. 4B).

The effectiveness of the HlyA secretion system was highlighted by comparison of results with those obtained with pBluescript II SK, an otherwise excellent expression system that does not encode a secretion apparatus. Whereas whole-cell lysates from *E. coli* DH5α carrying pBluescript II SK encoding wild-type and truncated SSP-2 demonstrated expression of these proteins, in no supernatant from any construct was there evidence of secretion of SSP-2 (data not shown).

Immunity to preerythrocytic-stage antigens of the malaria parasites, including SSP-2, is largely mediated by $CD8^+$ T cells and involves gamma interferon (IFN-γ) (shown to be involved

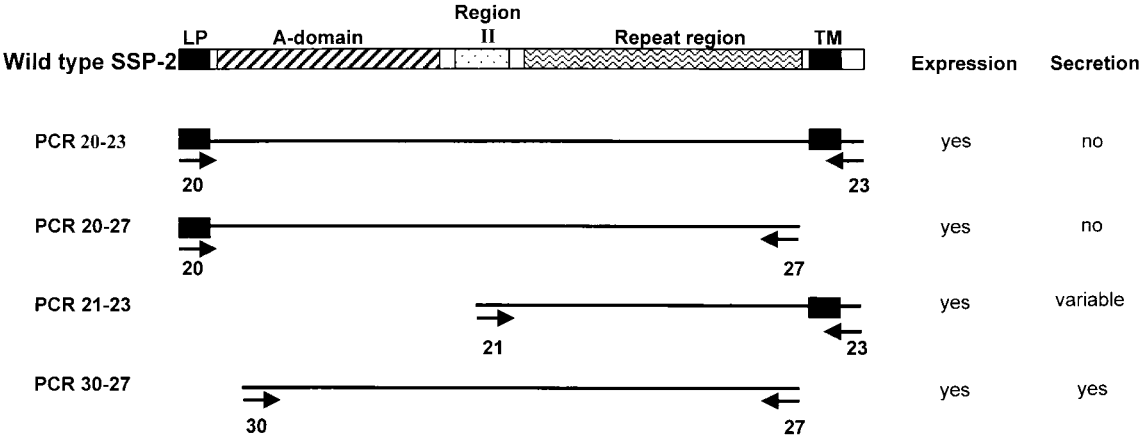

FIG. 3. Amplification of full-length and truncated versions of *ssp-2*. The wild-type *ssp-2* was amplified from DNA of *P. falciparum* clone 3D7 of strain NF54 using primers 1 and 2 and cloned into pBluescript II KS. This cloned plasmid DNA was thereafter used as the template for deriving different versions of *ssp-2* that were amplified by PCR and cloned into the *Nsi*I site of pMOhly1. Oligonucleotide primers 20 and 23 amplified the wild-type *ssp-2*, primers 20 and 27 amplified *ssp-2* without the C terminus, primers 21 and 23 amplified *ssp-2* without the N terminus, and the primers 30 and 27 amplified SSP-2 without the N and C termini. Summarizing data from Fig. 1 and 4, expression refers to the detection of SSP-2 in whole-cell lysates by immunoblotting with monoclonal antibody SSP-2.1; secretion results in detection of SSP-2 in immunoblots of culture supernatants. LP, leader peptide; TM, transmembrane domain.

in the killing of developing liver-stage parasites in infected hepatocytes), nitric oxide, and interleukin-12 (IL-12) production (6, 8, 16, 25, 43). To assess the immunogenicity of serovar Typhimurium strains carrying plasmids encoding SSP2, we measured IFN-γ production by effector cells from immunized mice in response to target cells infected with a vaccinia virus expressing PfSSP-2, using an enzyme-linked immunospot (ELISPOT) technique (15, 27). Groups of 8 to 10 female C57BL-6 (*H-2*^{b}) mice (Charles River Breeding Laboratories, Wilmington, Mass.) aged 6 to 8 weeks, were immunized intranasally (i.n.) with 10 μl containing 1×10^9 to 2×10^9 CFU of SL3261(pMO2023), SL3261(pMO3027), SL3261(pMO), or SL3261(VR2519). Mice serving as the positive control were inoculated intramuscularly with an SSP-2 DNA vaccine consisting of eukaryotic expression plasmid VR2519 encoding full-length SSP-2 under the control of a cytomegalovirus promoter (17); a total of 100 μg of DNA was injected into the tibialis anterioris, 50 μg in each leg. The mice were given a total of three doses, at 3-week intervals. The negative control mice received phosphate-buffered saline i.n.

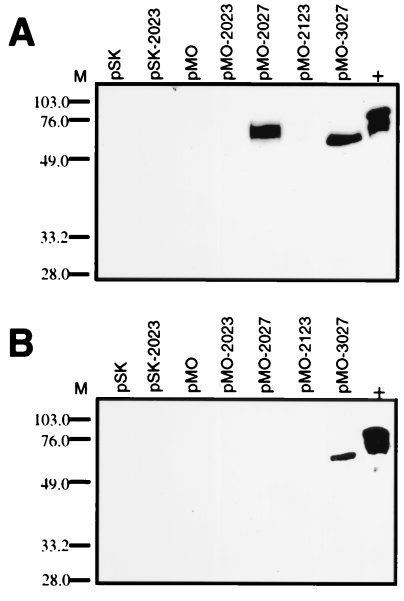

FIG. 4. Expression and secretion of SSP-2 by the hemolysin secretion system detected by immunoblot analysis of culture supernatants using monoclonal antibody SSP-2.1. (A) Culture supernatants of serovar Typhimurium SL3261 carrying pSK and pMOhly1 (pMO) constructs encoding full-length SSP-2 and truncated versions without the transmembrane α-helix domains. (B) Culture supernatants of CVD 908-*htrA* carrying pSK and pMOhly1 encoding full-length SSP-2 and truncated versions without transmembrane α-helix domains. M, molecular weight markers. Control lane (+) contains the bacterial lysate from *E. coli* DH5α(pSK-2023), which expresses full length SSP-2.

Ninety-six-well nitrocellulose plates (Multiscreen-HA; Millipore, Bedford, Mass.) were coated with 5 μg of anti-mouse IFN-γ monoclonal antibody (Pharmingen, San Diego, Calif.) per ml overnight at 4°C. After incubation, the plates were washed four times with RPMI and blocked with RPMI containing 2 mM L-glutamine, 10 mM HEPES, 50 μg of gentamicin per ml, and 10% heat-inactivated fetal calf serum (HyClone, Logan, Utah). Serial dilutions of effector splenocytes (1×10^6 to 1.25×10^5 cells/well) from immunized and control mice in culture medium supplemented with 20 U of recombinant murine IL-2 (R&D Systems Inc., Minneapolis, Minn.) per ml were incubated for 36 h at 37°C in 5% CO_2 in the presence of irradiated major histocompatibility complex-matched (*H-2*^{b}) EL4 cells (5×10^4 to 1×10^5 cells/well) that were previously infected with recombinant vaccinia virus carrying the PfSSP-2 gene (vP1254; WR-PfSSP-2) or parental vaccinia virus (WR) (Virogenetics, Troy, N.Y.). Vaccinia virus infection was performed the day before the assay. Briefly, EL-4 cells were centrifuged, resuspended in 0.5 to 1 ml of RPMI, and incubated for 2 h with vaccinia virus at 5 PFU per cell. The efficiency of vaccinia virus infection was assessed by flow cytometry using a

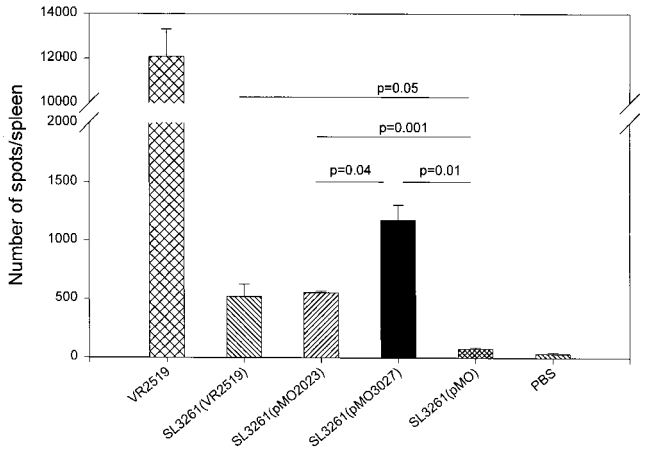

FIG. 5. Frequency of *P. falciparum* SSP-2-specific IFN-γ-secreting cells in mice immunized with serovar Typhimurium strain SL3261 carrying SSP-2 constructs, measured by ELISPOT. C57BL/6 mice were immunized i.n. with SL3261(pMO2023) (prokaryotic expression, nonsecreted full-length SSP-2), SL3261(pMO3027) (prokaryotic expression, secreted truncated SSP-2), SL3261(pMO) (prokaryotic expression negative control, carrying the Hly secretion system without an insert), SL3261(VR2519) (eukaryotic expression, full-length SSP-2) (negative control) and PBS (negative control). Positive control mice were immunized intramuscularly with DNA vaccine plasmid VR2519 encoding full-length SSP-2. Splenocytes were stimulated in vitro with irradiated major histocompatibility complex-matched ($H\text{-}2^b$) EL-4 cells infected with SSP-2 expressing recombinant vaccinia virus or native vaccinia virus for detection of IFN-γ secretion. Each bar represents the mean number of specific spots per spleen, and the error bars show standard deviation. Significant differences among the number of secreting cells measured in the different effector populations were determined by Student's *t* test. Data are representative of two independent experiments.

fluorescein isothiocyanate-labeled rabbit anti-vaccinia virus Lister strain antiserum (Virostat, Portland, Maine). Following undisturbed incubation, the plates were washed with PBS-Tween 20 (0.05%) and incubated with biotin-labeled anti-IFN-γ monoclonal antibody (Pharmingen) for 2 h at 37°C. The wells were washed, incubated with 100 μl of streptavidin-peroxidase (diluted 1:500 in PBS-Tween) for 1 h at 37°C, and then washed again, whereupon 50 μl of TrueBlue Peroxidase substrate (Kirkergaard & Perry Laboratories Inc., Gaithersburg, Md.) was added per well. The number of spots corresponding to IFN-γ-secreting cells in different spleen cell dilutions was counted using a stereomicroscope. The results were recorded as mean counts per 10^6 cells from quadruplicate wells per sample. The number of cells secreting IFN-γ per spleen was calculated based on the number of splenocytes obtained per mouse in each group. The results are shown in Fig. 5 as spot counts/spleen after subtraction of the number of spots in cultures containing EL-4 cells infected with vaccinia alone.

Mice immunized parenterally with the positive control preparation, the SSP-2 DNA vaccine plasmid VR2519, mounted the strongest ELISPOT response in terms of IFN-γ-secreting cells/per spleen (Fig. 5). Mice immunized mucosally with serovar Typhimurium SL3261(VR2519) carrying the identical SSP-2 DNA vaccine plasmid also exhibited a significant IFN-γ ELISPOT response, corroborating previous reports that *Salmonella* live vectors can successfully deliver eukaryotic expression plasmids and elicit immune responses (5, 32). However, the magnitude of the IFN-γ ELISPOT response in mice that received serovar Typhimurium SL3261(VR2519) i.n. was markedly lower than that in mice that received parenteral inoculations with VR2519 DNA. Mice immunized mucosally with the two Serovar Typhimurium constructs carrying the SSP-2 gene under a prokaryotic expression system also exhibited significant increases in the number of cells producing IFN-γ (Fig. 5). The magnitude of the response elicited by serovar Typhimurium construct SL3261(pMO2023), which encodes full-length SSP-2 that cannot be secreted, was very similar to that observed in mice immunized with serovar Typhimurium SL3261(VR2519) (Fig. 5). In contrast, in mice immunized i.n. with Serovar Typhimurium construct SL3261(pM3027), which encodes a truncated SSP-2 that is secreted, the IFN-γ cell ELISPOT response was significantly stronger than that elicited by the other serovar Typhimurium constructs ($P = 0.04$) (Fig. 5). These data support previous observations that with *Salmonella* live vectors, foreign antigens are more immunogenic if they are secreted, including the stimulation of cell-mediated immune responses (18).

Development of an effective malaria vaccine would provide a new tool to help control malaria. Because 90% of *P. falciparum* deaths and severe disease occur in sub-Sahara Africa among populations inhabiting some of the world's poorest countries, the characteristics of a malaria vaccine will affect its suitability in such venues. The strategy that we are pursuing, i.e., expressing protective antigens of *P. falciparum* in an attenuated serovar Typhi live vector, has several theoretical advantages, including oral administration and likely economy of manufacture. However, a drawback that has so far slowed the pace of vaccine development is the difficulty in expressing many eukaryotic proteins in bacterial live vectors.

The high AT/GC ratio of *ssp-2* and the difference in codon usage with respect to *E. coli* and *Salmonella* strains did not prevent the expression of *ssp-2* in *E. coli* DH5α, serovar Typhimurium SL3261, and serovar Typhi CVD 908-*htrA* by two different expression systems. The Hly system cloned into pMOhly1 allowed secretion of a modified SSP-2 by serovar Typhi CVD 908-*htrA* once the transmembrane α-helices at the N terminus and C terminus were removed. Despite the high copy number of each plasmid, the level of SSP-2 protein expressed was not toxic in vitro based on a comparison of bacterial growth and colony morphology of *Salmonella* carrying or lacking these plasmids. The visualization of smaller moieties of SSP-2 in the protein gels suggests that differences in codon usage of *ssp-2* with respect to *E. coli* or *Salmonella* codon usage may stall or slow transcription.

The Hly secretion system incorporates a complex regulatory system that is dependent on a positive regulator that allows transcription of the *hly* operon. However, little is known about the in vivo induction of the promoter that drives expression of the *hly* operon. Therefore, one likely approach to improve the HlyA secretion system would be to substitute a promoter for which the in vivo inducing conditions are known.

So far, we have succeeded in expressing three preerythrocytic-stage antigens of *P. falciparum* in attenuated serovar Typhi, including circumsporozoite protein (14), SSP-2 (this work), and liver-stage antigen 1 (LSA-1) (unpublished data). We can now undertake practical steps to improve the expres-

sion of malarial antigens by serovar Typhi. These include (i) optimizing the codon usage of malarial genes to match *Salmonella* expression; (ii) utilizing regulated promoters that are activated by in vivo conditions, such as P*ompC* or P*dmsA*, to drive expression of the malaria gene as well as of the entire *hly* secretion system (31, 33); and (iii) utilizing expression plasmids that encode stabilization and plasmid maintenance functions (10).

Antigen-specific $CD8^+$ T lymphocytes and IFN-γ production are essential effector mechanisms that contribute to the protective responses against malaria infection in the murine model (7). $CD8^+$ CTL against preerythrocytic stages of the malaria parasites, including PySSP2, protect against sporozoite challenge (6, 22). It is believed that IFN-γ and $CD8^+$ T cells together contribute to the killing of developing liver-stage parasites either by regulating the production of nitric oxide in the liver or by stimulating mononuclear cells to produce IL-12, which in turn activates other lymphocytes and NK cells to further increase IFN-γ levels (6, 8, 16, 25, 38). Moreover, protection induced by previous vaccine strategies was associated with high levels of $CD8^+$ IFN-γ-secreting splenocytes (37). Thus, we assessed the immunogenicity of our vaccine constructs by measuring the frequency of IFN-γ secreting cells in short-term cultures of effector splenocytes incubated in the presence of MHC-matched *P. falciparum* SSP-2-expressing cells by ELISPOT. This technique has proven suitable to monitor antigen-specific $CD8^+$ T lymphocyte responses to malaria antigens. Furthermore, quantification of IFN-γ-secreting cells in short-term cultures at the single-cell level has been proposed as a reliable tool to assess the efficacy of malaria vaccines (15). The serovar Typhimurium strains encoding *P. falciparum* SSP-2 successfully delivered the foreign antigen and induced specific immune responses. Of particular note, the highest responses were observed in mice immunized with a live vector vaccine engineered to secrete *P. falciparum* SSP-2 extracellularly. The demonstration of the ability of the serovar Typhimurium constructs to elicit a relevant cell-mediated immune response (IFN-γ secreting cells) in a pre clinical mouse model provides a rationale for undertaking phase I clinical trials with the analogous serovar Typhi construct, bringing our ambitious quest to develop a mucosally administered, multivalent, live vector-based malaria vaccine one step closer.

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