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Plasmodium falciparum sporozoite invasion is inhibited by naturally acquired or experimentally induced polyclonal antibodies to the STARP antigen

Antibody(Ab)-mediated inhibition of sporozoite invasion of hepatocytes is a mechanism that has been clearly demonstrated to act upon *Plasmodium falciparum* pre-erythrocytic stages in humans. Consequently we have analyzed the Ab response to a recently identified *P. falciparum* sporozoite surface protein, STARP, in malaria-exposed individuals and tested the inhibitory effect of these Ab upon hepatocyte invasion *in vitro*. STARP-specific IgG were detected in 90 and 61 % of sera from regions where individuals were exposed to 100 and 1–5 infectious bites per year, respectively. These IgG were predominantly of the cytophilic IgG1 or IgG3 type. STARP and the major sporozoite surface protein, CS, elicited equivalent IgG levels in adults. When affinity purified from either African immune sera or the serum of an individual experimentally protected by irradiated sporozoite immunization, STARP-specific Ab prevented up to 90 % of sporozoites from invading human hepatocytes. The dose-dependent and reproducible inhibition was more pronounced than that observed with human CS-specific Ab affinity purified under identical conditions. Substantial reduction of sporozoite invasion was also observed with Ab induced by artificial immunization with recombinant STARP protein and reactive with the native protein. Taken together with recent findings of human cytotoxic T lymphocytes specific for this antigen, these results promote the interest of studying the efficacy of STARP as a target for immune effector mechanisms operating upon pre-erythrocytic stages.

1 Introduction

Malaria caused by the protozoan parasite *Plasmodium falciparum* represents a major cause of morbidity and mortality in many tropical areas in the world and is therefore the focus of efforts aimed at artificially inducing an effective immunity. Experimentally, it has been reproducibly shown that sterile, stage-specific immunity can be acquired in humans immunized with *P. falciparum* irradiation-attenuated sporozoites (IrrSpz) [1–4]. Therefore, a major goal has become the identification of the critical effector mechanism(s) and Ag responsible, with the aim of developing a recombinant vaccine against pre-erythrocytic stages [sporozoites (Spz) and/or liver stages (LS)].

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Abbreviations: **AU:** Arbitrary unit **CS:** Circumsporozoite **IFAT:** Immunofluorescent Ab test **ILSDA:** Inhibition of liver stage development assay **IrrSpz:** Irradiated sporozoites **IS-Vol:** IrrSpz-immunized volunteer **ISI:** Inhibition of sporozoite invasion **LS:** Liver stage **LSA-1:** Liver stage antigen-1 **STARP:** Sporozoite threonine- and asparagine-rich protein **Spz:** Sporozoite

Key words: *Plasmodium falciparum* / STARP / Antibody inhibition of sporozoite invasion / Seroepidemiology

Work conducted mainly in rodent malaria models has uncovered a plethora of Ab- and cell-mediated immune mechanisms operating upon malaria pre-erythrocytic stages, including Ab acting upon Spz and CTL, cytokines and free radicals targeting infected hepatocytes (reviewed in [5–7]). While the critical effector role of $CD8^+$ or $CD4^+$ CTL operating on the liver stages has been clearly demonstrated for certain rodent malaria systems such as BALB/c-*Plasmodium yoelii* or A/J-*Plasmodium berghei* [8–10], in other combinations such as B10.BR-*P. yoelii* or BALB/c-*P. berghei*, these have been shown to have little if any effect [11]. Non-stimulated human hepatocytes do not express detectable levels of MHC class I or class II molecules, although MHC class II expression can be induced by IFN-γ [12, 13]; indeed human liver transplants are routinely performed using non-MHC-matched donors. Thus, in the absence of evidence demonstrating whether *P. falciparum*-infected human hepatocytes are indeed up-regulated in their MHC gene expression, caution must be exercised in extrapolating rodent malaria data to the human context. One mechanism, however, which has been clearly demonstrated for *P. falciparum* is that of Ab-mediated inhibition of Spz invasion of human hepatocytes [14–16]. Moreover, comparisons of *P. falciparum* and *P. yoelii in vitro* and *in vivo* suggest that the "Inhibition of Liver-Stage Development Assay" (ILSDA), which employs the natural host cell, the hepatocyte, can be predictive of the effect *in vivo* of the Ab tested [15, 17, 18].

In terms of the critical target *P. falciparum* Ag, for many years the focus was exclusively on the major Spz surface protein, the CS. However, CS vaccine trials conducted in endemic areas have yet to show that protective immunity comparable to the IrrSpz vaccines can be induced [19].

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While more immunogenic experimental CS vaccines look promising, there are several arguments in favor of analyzing additional pre-erythrocytic stage antigens. First, there is documented immunological non-responsiveness and antigenic diversity within T cell epitopes of the CS protein (reviewed in [20]). Second, the presence of CTL and Ab to the CS protein is not indicative of protective immunity induced in humans or mice by immunization with IrrSpz ([21] and references within). Third, experiments with *P. yoelii* have indicated that combining additional pre-erythrocytic stage Ag with the CS protein give far broader protection than was possible with the CS alone [21, 22]. Last, it is now clear that the CS is only one of many Ag present in *P. falciparum* pre-erythrocytic stages [5, 23, 24]. Of these, the recently discovered STARP Ag has the attractive property of being found on the surface of all Spz strains and isolates tested to date (using Ab directed to the repeats of a single parasite clone) as well as being detected at high levels in liver stages [25]. Expression of this gene in sporozoites has been conclusively demonstrated by Northern (RNA) blot and reverse transcriptase PCR. The 78-kDa STARP protein contains three internal repeat regions, denoted Mosaic (containing multiple small degenerate repeats), Rp45 and Rp10 (encoding 45- and 10-amino acid repeats, respectively); this structure is highly conserved in *P. falciparum* [26]. Recently, the STARP Ag was found to contain an HLA-A*0202-restricted epitope for CTL in humans naturally exposed to malaria infection [27], suggesting that it may be a target of cell-mediated immune mechanisms operating upon infected hepatocytes. Subsequently, 75 % of individuals from a high-transmission region were found to have a CTL response to this epitope, exceeding the frequency of response to all other CTL epitopes studied from other *P. falciparum* pre-erythrocytic stage Ag [28].

Consistent localization of STARP at the Spz surface [25] also raises the possibility that this Ag may constitute an effective target for Ab capable of preventing Spz invasion of hepatocytes. This question has been addressed by examining Ab responses to STARP in the serum of malaria-exposed individuals and assessing the capacity of these Ab to inhibit *P. falciparum* Spz invasion of human hepatocytes, using the ILSDA *in vitro* assay. Results show a strong biological effect of both STARP-specific Ab purified from the sera of either African individuals exposed to *P. falciparum* malaria or an individual experimentally protected by immunization with IrrSpz, and Ab produced in animals immunized with the recombinant protein.

2 Materials and methods

2.1 Sero-epidemiological study areas and serum donors

Donsé is a rural village of 500 inhabitants situated 50 km north of the capital in a Savannah area of Burkina Faso. Malaria there is seasonal with parasite transmission rates averaging 100 infectious bites per individual per year [29]. Podor (Northern Senegal), situated 10 km south of the Senegal river, is a dry Sahel area with a brief transmission season characterized by only one to five infectious bites per individual per year [30] which is low by African standards. After informed consent, plasma samples were obtained from donors whose age ranged from 1 to 75 years. Sample groups for serological studies were chosen to have an even distribution over the different age groups studied.

Recently, the immunity which can be induced by immunization with IrrSpz was re-assessed in a group of four volunteers [4, 31]. For the purpose of our study, a large volume of serum was very kindly donated by one of these volunteers (referred to as WR4 in [4] and 5H in [31]; in this study as IS-Vol). This volunteer had been repeatedly immunized by IrrSpz and was protected against multiple challenges by both homologous and heterologous *P. falciparum* strains.

2.2 STARP peptide selection and design of the mixotope

On the basis of preliminary serological studies using various STARP recombinants, which localized immunodominant B cell epitopes to the Rp10 region (D. Fidock, unpublished data), this region was chosen to characterize and evaluate STARP-specific Ab responses. The 10-amino acid encoding Rp10 region alone accounts for 40 % of the entire STARP sequence. The localization of immunodominant epitopes to this region agrees with previous reports that repetitive regions of *P. falciparum* antigens frequently contain immunodominant B cell epitopes [32, 33]. From the Rp10 region we designed two peptide Ag, *i.e.* Rp10-A and a convergent, combinatorial, multi-epitope peptide, the mixotope Rp10-Mixo (Table 1).

2.3 Peptide synthesis and recombinant antigens

The synthesis of both STARP peptides was performed on a N^{α}-Boc N^{ε} palmitoyl lysine-MBHA. The C-terminal lysine was introduced on the MBHA resin (0.5 mmol; loading of the starting resin at 0.55 mmol/g) as N^{α}-Boc, N^{ε} Fmoc lysine. After piperidine deprotection of the Fmoc group, the N^{ε} function was palmitoylated using palmitic acid activated with dicyclohexylcarbodiimide/1-hydroxybenzol-triazole (DCC/HOBt). The synthesis of Rp10-A was performed in an automatic peptide synthesizer (Applied Biosystems model 430 A, Foster City, CA) using the conventional N-tert-Butoxy-carbonyl-trifluoro-acetic acid (BOC-TFA) strategy and a DCC-HOBt activation method [34]. After assembly of the complete protected peptide chain, the terminal t-BOC group was removed with 50 % TFA. Following neutralization, the N-terminal group was then acetylated using acetic anhydride. The resin was dried, cleaved and deprotected in HF, r-cresol and thiocresol (90.0:7.5:2.5) for 2 h at 0 °C. Initially, the crude peptide was dissolved in 30 ml neat TFA, then precipitated by pouring this into 300 ml dry ice-cooled diethylether. This peptide was found to be totally water-insoluble. Thus, to prevent aggregation, 100 mg crude peptide was solubilized with TFA in the presence of 600 mg SDS [35]. This product, when precipitated with ether, could then be solubilized with water. Excess SDS was eliminated by extensive dialysis.

The synthesis of the mixotope Rp10-Mixo was performed on 0.5 mmol of N^{α}-Boc, N^{ε} palmitoyl lysine-MBHA as described [36]. Precise equimolecular amounts of appropriate protected amino acids were used in coupling reac-

Table 1. STARP Rp10 region – amino acid distribution and synthetic peptides

Rp10	1	2	3	4	5	6	7	8	9	10
region[a]	S_{14}	T_{23}	D_{25}	N_{25}	N_{14}	N_{23}	T_{21}	N_{10}	T_{18}	I_{12}
	L_5	I_1	T_1	S_1	S_3	K_2	I_2	D_6	I_4	K_8
	A_4	N_1			T_3	S_1	A_2	K_6	V_2	N_3
	E_1	P_1			D_3		N_1	T_3	K_1	T_2
	P_1				I_1			G_1	Q_1	V_1
	T_1				K_1					
					Y_1					
Rp10-A[b]	STDNNNTKTISTDNNNTKTI-(pam)K-COOH									
Rp10-Mixo[c]	STDNNNTNTISTDNNNTNTI-(pam)K-COOH L T NTIKA S IT N D D NL D T K K K									

a) The STARP Rp10 (10-amino acid repeat) region is composed of 26 repeat units that show considerable sequence diversity [25]. These units were aligned vertically and for each position (1–10) we have listed the amino acids utilized and the number of times that each amino acid was present, in order of decreasing use. The top row indicates the most frequently used amino acids.

b) Rp10-A is a synthetic linear 20-mer peptide representing two copies of the only repeat unit present more than once in this region (as three tandem copies in the T9/96 clone [25].

c) Rp10-Mixo is a convergent combinatorial peptide designed from the Rp10 region following alignment of the sequence as a string of 20 amino acids. In Rp10-Mixo, any residues found at least twice at a given position in the repeat sequence string were incorporated in that position during synthesis. Equimolar amounts of the appropriate subset of amino acids (shown vertically) were introduced in each degenerate position. This theoretically produced 55296 closely related combinatorial sequences, collectively representing not only the observed degeneracy in the Rp10 repeats [25], but indeed all possible Rp10 sequences that the parasite could generate using this set of amino acids.

tions when degenerated sites were required. The first coupling was performed with 0.5 nmol total amount of the introduced BOC amino acids. A second coupling, using 2 mmol total amount, was then systematically performed. The activation procedure was the DCC/HOBt method, with long coupling reaction (90 min) periods. The completeness of the coupling reaction was tested by a qualitative ninhydrin assay and a third coupling performed when necessary. After synthesis, the resin was dried, cleaved and deprotected in HF, r-cresol and thiocresol (90.0:7.5:2.5) for 2 h at 0 °C. The cleaved deprotected peptide was precipitated, extensively washed with cold diethylether, dissolved in 5 % acetic acid and lyophilized. Final amino acid compositions, determined 24 h and 48 h after acid hydrolysis, accurately reflected the theoretical composition, calculated on the basis of an equimolecular amount of each amino acid introduced in the degenerated positions.

The LSA-Rep peptide, previously described [37], represents 2.5 units of the 17-amino acid immunodominant repeat of the liver stage Ag LSA-1. The CS peptide $(NANP)_6$ was purchased from Bachem (Bubendorf, Switzerland). The CS peptide $[(NANP)_2\text{-}NVDP]_2$ was a kind gift of the late R. L. Beaudoin. The R32tet32 recombinant protein ($[(NANP)_{15}NVDP)]_2\text{-}tet_{32}$) was an aliquot of the highly purified CS protein described in [38], a kind gift of Mitch Gross (SmithKline Beecham).

2.4 ELISA assays

These were performed essentially as described in [39]. Briefly, peptides Rp10-A or Rp10-Mixo were coated onto ELISA plates (Nunc-Immuno Plate II, Nunc) overnight at 4 °C (100 µl/well of a 10 µg/ml solution in 50 mM Na_2CO_3, pH 9.5). Sample sera, tested in duplicate, were diluted either 1 : 100 for IgG detection or 1 : 25 for isotype studies (detection of IgG1–4, IgA and IgM). Bound IgG or IgA were quantified using peroxidase-conjugated goat anti-human IgG or IgA, respectively (1 : 2000 dilution; Biosys, Compiègne, France). IgG1–4 isotypes were detected using anti-human isotype-specific mAb followed by peroxidase-labeled goat anti-mouse IgG (1 : 1000 dilution; Biosys) as described [40]. These mAb had been previously selected for their ability to recognize equally well the corresponding isotype in sera from Caucasians, Africans or Asians and were employed at empirically determined concentrations that gave isotype-specific results and similar absorbance readings for each subclass. The mAb employed were: mouse anti-IgG1 (NL16, diluted 1 : 20000; Boehringer Mannheim); -IgG2 (HP6002, diluted 1 : 10000; Sigma); -IgG3 (ZG4, diluted 1 : 10000; Immunotech); -IgG4 (RJ4, diluted 1 : 1000; Immunotech); and -IgM (MB11, diluted 1 : 30000; Sigma).

Prior to conducting the sero-epidemiological assays, each peptide was initially tested on a control group of 80 serum samples from healthy French blood donors with no history of malaria exposure. Five serum samples reproducing the mean and standard deviation of the control group were selected and included on each subsequent assay plate, along with two positive control African adult sera.

Antibody levels of test serum were defined in arbitrary units (AU), where the number of AU was calculated by dividing the mean absorbance (OD_{492}) value of the test serum by the mean + 3 SD of the five control serum samples from the same plate. AU values ≥ 1 were considered as indicative of the presence of specific Ab. This definition is the more stringent of the two commonly employed in malaria sero-epidemiology studies for defining a positive Ab response [37, 41, 42], the other one using 2 SD instead of 3 SD in the formula shown above [43, 44]. Use of exactly the same definition, sera samples and protocol enabled comparison of these results with our previously published data on the LSA-1 and CS Ag [37].

2.5 Affinity purification of human specific antibodies

Specific human antibodies were prepared from immune serum by affinity purification using ELISA plate-bound synthetic peptides or purified recombinant R32tet32 protein bound to ELISA plates, as described in [37]. Briefly, peptides or protein were coated onto ELISA plates by overnight incubation at 4 °C (200 µl/well of 10 µg/ml solu-

tions). After washing, wells were incubated for 1 h at 22 °C with human immune serum (diluted 1 : 100; 200 µl/well). Following extensive washes, Ag-bound Ab were eluted using 0.2 M glycine, pH 2.5 and immediately neutralized by the addition of 2 M Tris, pH 11. Eluted Ab samples were dialyzed extensively first against PBS, pH 7.4, then William's E culture medium (Gibco BRL). Samples were concentrated over a Centricon 30 (Amicon) membrane and protein concentrations calculated using a Bradford protein assay (Bio-Rad). The purity and concentrations of specific Ig in eluted Ab samples were estimated by scanning known amounts of stained PAGE Ig preparations with a laser densitometer (LKB Bromma Ultroscan XL). Samples were adjusted to final working concentrations in freshly supplemented William's E medium and filter sterilized (0.22 µm Millex filters, Millipore, Bedford, MA). The specificity of the Ab preparations for the STARP Ag was verified by ELISA on STARP peptides, by immunofluorescent antibody test (IFAT) on *P. falciparum* Spz, revealing the characteristic heterogeneous STARP Spz surface staining [25], and by immunoblot analysis of cultured asexual early ring forms (which express STARP). Affinity-purified anti-STARP Ab were never found to cross-react with the CS Ag, nor were anti-CS Ab found to cross-react with STARP.

2.6 Mouse antisera

Polyclonal anti-STARP antisera were obtained from Swiss outbred mice (Charles River, France) immunized by three injections at 2-week intervals of 15 µg of the entire repeat region of the STARP Ag (amino acids 81-528, T9/96 clone) expressed and purified as a glutathione-S-transferase (GST) fusion protein (referred to as GST+STARP/Rep), as described [25].

2.7 IgG preparations

IgG was purified from eluted human Ab or polyclonal mouse antisera by ion exchange chromatography on DEAE-Trisacryl (IBF, Biotechnics, Villeneuve-la-Garenne, France), according to the manufacturer's specifications. This procedure, which is used to separate IgG (eluted with 35 mM NaCl, 25 mM Tris, pH 8.8) from IgM and IgA (eluted with 500 mM NaCl, 25 mM Tris, pH 8.8), was later found to efficiently purify IgG1, 2 and 3, and to leave contaminating IgG4 in the IgM and A fraction. The effective separation of the Ig (sub)classes was verified in dot blot and ELISA assays using (sub)class-specific secondary Ab. Prior to use in the *in vitro* assays, Ig samples were first dialyzed against supplemented William's E medium, then adjusted to the final working concentrations and filter-sterilized as described above. The Ab activity of the recovered fractions was ascertained by testing serial sample dilutions in IFAT assays on *P. falciparum* Spz and by ELISA assays.

2.8 Immunofluorescent antibody tests

The presence and titer of purified Ab specific to *P. falciparum* sporozoite surface antigens was assessed using a previously described "wet" IFAT on NF54 sporozoites that had been very lightly glutaraldehyde fixed (0.05 %, 2 min) and deposited on slides coated with 50 µg/ml poly-L-lysine [29].

2.9 Assessment of the biological effect of Ab: inhibition of Spz invasion into human hepatocytes

Ab were tested in triplicate for their capacity to inhibit Spz invasion of human hepatocytes using the ILSDA assay with *P. falciparum* liver stage cultures essentially as described [45]. Briefly, human hepatocytes were isolated from < 1 cm^3 liver sections by collagenase (Sigma) perfusion and further purified over a 40 % Percoll (Pharmacia) gradient. Sections were obtained with informed consent from organ donors or from biopsies taken for diagnostic purposes. Hepatocyte purity and viability were > 95 % as assessed by trypan blue dye exclusion. Monolayers were obtained by seeding 12×10^4 hepatocytes per chamber in 8-chamber Lab-Tek dishes (Nunc) in William's E medium supplemented with 5 % FCS (ATGC, France), 1 × penicillin-streptomycin (Gibco BRL), 2 mM glutamine (Gibco BRL). After 24 h at 37 °C in 5 % CO_2, the medium was removed and test Ab or control reagents were added to each chamber. Thereafter, *P. falciparum* (strain NF54) Spz, which had been freshly dissected from infected salivary glands of *Anopheles gambiae*, washed and suspended in fresh supplemented medium, were added at 6×10^4 sporozoites per Lab-Tek chamber. After 3 h at 37 °C, the medium containing Ab and non-invaded Spz was discarded and replaced by normal supplemented medium. After 3–5 days of culture, hepatocytes were fixed in methanol and developing liver stages were counted following identification by IFAT with the anti-CS mAb NFS1 [46] (IgG1, diluted 1 : 1000; a kind gift from Yupin Charoenvit).

All test and control Ab assays were performed as triplicate cultures and each Lab-Tek dish contained additional duplicate control wells without Ab. The percentage of inhibition of sporozoite invasion was calculated as follows: % inhibition = [1 – (geometric mean no. of infected hepatocytes in control chambers – geometric mean of no. of infected hepatocytes in test chambers from that same slide) / geometric mean no. of infected hepatocytes in control chambers)] × 100. All experiments included the anti-CS mAb NSF1 as a positive control at 1 µg/ml final concentration (corresponding to an IFA titer of 10^5) which, in accordance with previous studies [14, 17], consistently gave 90–97 % inhibition.

2.10 Statistical methods

All statistical tests were performed on log(e)-transformed data. The Pearson test was used to examine the correlation between Ab responses and age and between quantitative responses to pairs of peptides. The analysis of variance (ANOVA) F-test was used to compare mean Ab responses between age groups. The χ^2 test was used to examine the relationship between presence or absence of positive response to pairs of peptides.

3 Results

3.1 STARP-specific IgG is present at high frequencies and levels in malaria-endemic populations

To determine the prevalence and level of IgG responses to the STARP Ag in individuals exposed to *P. falciparum* under natural conditions, the STARP peptides Rp10-A and Rp10-Mixo were used in ELISA assays on individual sera from two regions, Donsé and Podor, representing nearly opposite ends of the spectrum of malaria transmission in sub-Saharan Africa (100 and 1–5 infectious bites per individual per year, respectively). Results indicate the presence of B cell epitopes in the STARP Rp10 region that are frequently recognized by *P. falciparum* malaria-exposed individuals. Indeed, 90 % and 61 % of the individuals spanning all age groups of Donsé and Podor, respectively, showed a positive IgG response to epitopes contained in the Rp10-A consensus sequence (Fig. 1). Among the adults (≥ 21 years), prevalence reached 93 % and 82 % in the two areas, respectively. When using the STARP mixotope Rp10-Mixo, which contains over 50 000 closely related combinatorial Rp10 repeat sequences, detectable STARP-specific IgG responses were slightly less prevalent, being found in 88 % and 49 % of the total Donsé and Podor samples, respectively.

To evaluate differences in the levels of IgG specific to the various Ag, positive individual responses were classified into four strata, *i.e.* low (1.00–2.49), medium (2.50–4.99), high (5.00–9.99), and very high (≥ 10.00) IgG levels (Fig. 1). In an interesting contrast to the prevalence data, the use of the combinatorial peptide Rp10-Mixo detected

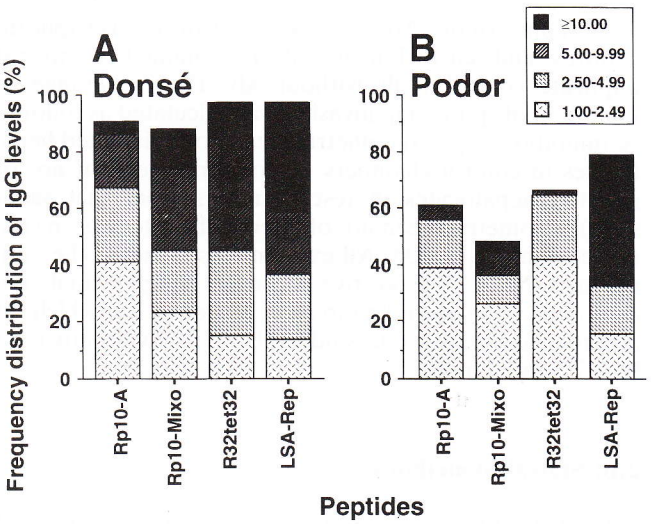

Figure 1. IgG prevalence data for STARP (Rp10-A, Rp10-Mixo) *vs.* CS (R32tet32) and LSA-1 (LSA-Rep) Ag in individuals from (A) the high-transmission area of Donsé (Burkina Faso, $n = 73$) and (B) the low-transmission area of Podor (Northern Senegal, $n = 106$). Individual IgG levels were calculated in AU; AU values ≥ 1 were considered indicative of a positive Ab response. The overall percentage of positive Ab responses equals the sum of the percentage of individuals whose IgG levels fell into one of the four strata indicated (1.00–2.49; 2.50–4.99; 5.00–9.99; ≥ 10.00). The data for the CS and LSA-1 Ag shown in Figs. 1 and 2 are reproduced from [37].

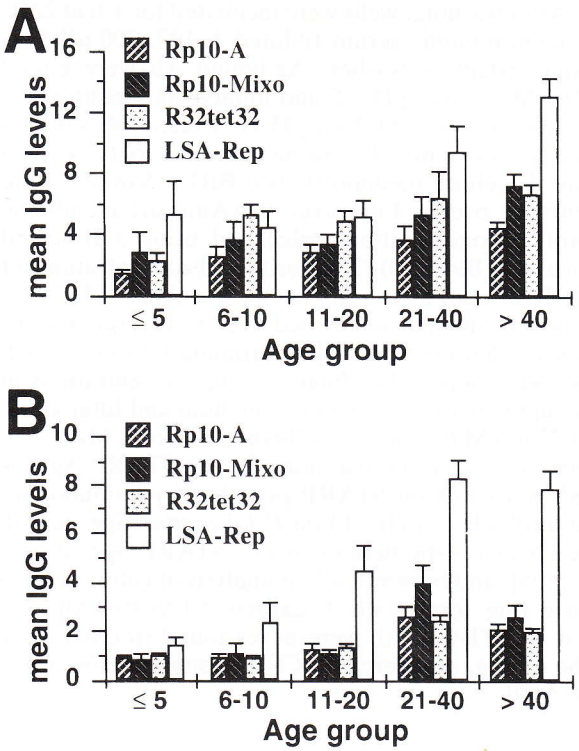

Figure 2. Arithmetic mean IgG levels (in AU), per age group, in the (A) Donsé and (B) Podor population samples. SEM bars are indicated. Note that scale bars differ between the two graphs. The arithmetic means ± SD for the total population samples were (A) 3.4 ± 2.7 for Rp10-A, 5.2 ± 4.3 for Rp10-Mixo, 5.6 ± 3.7 for R32tet32 and 8.9 ± 6.7 for LSA-Rep; and (B) 1.7 ± 1.5 for Rp10-A, 2.2 ± 2.7 for Rp10-Mixo, 1.7 ± 1.0 for R32tet32, and 5.6 ± 4.7 for LSA-Rep.

STARP-specific IgG at levels higher than those found using the single-sequence peptide Rp10-A. For example, when using the mixotope, IgG levels falling into the high stratum (5.00–9.99) were detected in 29 % and 9 % of the Donsé and Podor samples, respectively, as compared to 19 % and 5 %, respectively, when using Rp10-A. Individual responses to these two STARP peptides were nevertheless highly correlated, both qualitatively ($\chi^2 = 25.0$ and 42.6 for the Donsé and Podor sera, respectively; $p < 0.001$ for both) and quantitatively ($r = 0.84$ for Donsé and $r = 0.82$ for Podor).

In comparison, the prevalence of positive IgG responses to the immunodominant regions of the CS and LSA-1 proteins, determined in the same individual sera using R32tet32 and LSA-Rep, reached 97 % for both antigens in Donsé and 70 % and 77 %, respectively, in Podor. Interestingly, in both regions the levels of STARP-specific IgG were very close to those for the CS. These levels were nevertheless far below those specific for the LSA-1 Ag (for example, 25 % of the Podor samples had very high LSA-Rep-specific IgG levels (*i.e.* levels ≥ 10), as compared to 4 % and 0 % having very high levels of IgG specific for Rp10-Mixo and R32tet32, respectively).

Age-dependent changes in IgG levels were then examined in both the Donsé and Podor population samples, initially chosen for their evenly spread age distribution. For both populations, a gradual consistent increase in STARP-

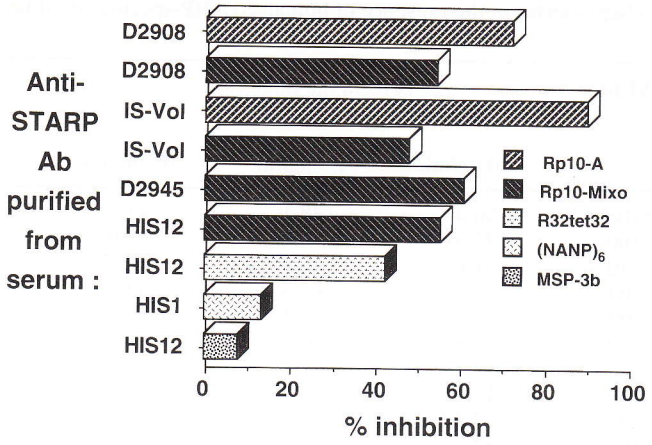

Figure 3. Inhibitory effect of human anti-STARP Ab on *P. falciparum* Spz invasion of human hepatocytes *in vitro*. The Y-axis shows the starting serum used for affinity purification: D2908 = Donsé serum No. 2908 (having titers of 12.4 and 16.7 AU of IgG specific for Rp10-A and Rp10-Mixo, respectively); IS-Vol (having titers of 2.0 and 1.8 AU of IgG specific for Rp10-A and Rp10-Mixo, respectively); D2945 = Donsé serum No. 2945 (titer of 17.0 AU of IgG specific for Rp10-Mixo); HIS12 = hyperimmune serum No. 12 (from a holoendemic area in the Congo; titers of 8.0 AU, 9.2 AU and 2.8 AU of IgG specific for Rp10-Mixo, R323tet32 and MSP-3b, respectively). HIS1 = Congo hyperimmune serum No. 1 [titer of 3.6 AU of IgG specific for $(NANP)_6$]. Ab were affinity purified against either the STARP peptides Rp10-A or Rp10-Mixo, the CS recombinant Ag R32tet32 or peptide Ag $(NANP)_6$, or the control asexual blood-stage merozoite Ag peptide MSP-3b [47]. All Ab samples were tested in triplicate at 2 μg/ml and percentage inhibition was calculated with respect to control wells in which no Ab were added. The STARP and CS Ab preparations had Spz IFAT titers of 1 : 100, while the MSP-3b-specific Ab had a Spz IFAT titer of < 1 : 10 (background).

specific IgG levels with age was demonstrated with both Rp10 peptides (Fig. 2). This was also reflected in the significant increase in percentage of positive responders to STARP in the older age groups (data not shown). This differed notably from the younger age groups in Podor (< 10 years old) where the majority of children (69 % and 90 % as measured by Rp10-A and Rp10-Mixo, respectively) did not show a positive Ab response to the STARP Ag. Interestingly, in adults of the high-transmission area and in all age groups of the low-transmission area, the levels of IgG specific for STARP were similar or greater than those for the CS protein. Again, the rise was much less rapid than that observed with levels of LSA-1-specific IgG. For all Ag tested, the age-dependent increase was statistically significant ($p < 0.02$, analysis of variance test).

3.2 Human anti-STARP antibodies dramatically reduce sporozoite invasion

We have assessed whether STARP-specific Ab present in the sera of individuals exposed to *P. falciparum* Spz can inhibit invasion of their natural host cell, the human hepatocyte, using the established ILSDA *in vitro* assay [45]. Using the RP10-A or Rp10-Mixo STARP peptides as solid phase Ag, human anti-STARP Ab were affinity purified from sera of African individuals, exposed to *P. falciparum* infection under natural conditions and highly reactive to STARP peptides (Fig. 3 legend) and, from a volunteer experimentally protected against multiple challenges by repeated immunization with *P. falciparum* IrrSpz ([4]; D. Haynes and W. R. Ballou, unpublished data).

Significant inhibition, measured as a reduction in the number of resulting *P. falciparum* liver stages 3–5 days post-invasion, was consistently found with polyclonal STARP-specific human Ab prepared from either African immune sera or the IrrSpz-immunized volunteer (IS-Vol) serum and tested at a concentration of 2 μg/ml (Fig. 3). Levels of inhibition ranged from 48 % to 90 % (with a mean of 64 %), depending on the serum and the STARP peptide used for affinity purification. No correlation was observed for this small number of sera between initial STARP Ab titers and levels of Spz inhibition. In contrast, human polyclonal anti-CS Ab prepared under exactly the same conditions gave only minimal (13 %) inhibition when Ab were purified against the $(NANP)_6$ peptide, and 43 % inhibition when using the R32tet32 recombinant protein (containing a greater number of repeats and the variant tetrapeptide NVDP) as the solid-phase Ag. Negative control Ab, similarly affinity purified against the asexual blood-stage merozoite Ag MSP3 which is not expressed in Spz [47], gave no significant inhibition (< 10 %). Using a 24-h assay, 2 μg/ml STARP-specific IgG purified from the D2945 and D2908 sera gave inhibition levels of 82 % and 70 %, respectively; this similar reduction in numbers of invaded Spz indicates that Ab were operating at the level of Spz invasion.

Subsequent experiments were performed to determine if this inhibition with anti-STARP Ab was reproducible, whether it was concentration dependent and which classes of Ig were implicated. These assays, performed with human hepatocytes from another donor and new Ab preparations, focused on the African immune serum D2908 and the IS-Vol serum, which had previously given the highest inhibition levels (Fig. 3). Following affinity purification, STARP-specific Ab were fractionated into either IgG or non-IgG by conventional chromatography and tested at 2.0, 1.0 and 0.5 μg/ml concentrations.

The results confirmed the significant inhibitory activity of anti-STARP Ab on Spz invasion of human hepatocytes and, furthermore, demonstrated that the level of inhibition was dependent on the concentration of STARP-specific Ab (Table 2). For the Donsé serum D2908, STARP-specific Ab prepared from unfractionated serum was found to inhibit Spz invasion by up to 90 % and 69 % when Rp10-Mixo and Rp10-A, respectively, were used as the peptides for affinity purification. A significant but lower level of inhibition (47–49 %) was obtained with Ab prepared from the IgG fraction, whereas little or no inhibitory activity was recovered from the non-IgG fraction. These results correlated well with the immunofluorescence titers of the various Ab samples, with end-point titers of 1 : 100 for Ab prepared from whole serum or the IgG fraction, as opposed to an essentially negative titer (< 1 : 10) for the non-IgG sample, suggesting that the only significant source of inhibitory anti-STARP Ab in this African serum was of the IgG class.

Strong inhibition was also observed with anti-STARP Ab purified from unfractionated serum of the IrrSpz-

Table 2. Percentage inhibition of *P. falciparum* Spz invasion of human hepatocytes in the presence of human STARP-specific Ab: dose dependency and Ig fractionation studies

Starting serum	Peptide used for affinity purification	Fraction tested[a]	Spz IFAT titer	Percentage inhibition[b]		
				2.0 μg/ml	1.0 μg/ml	0.5 μg/ml
D2908	Rp10-Mixo	whole	1:100	90.4	48.1	47.3
D2908	Rp10-Mixo	IgG	1:100	47.3	44.6	27.9
D2908	Rp10-Mixo	non-IgG	<1: 10	21.6	8.4	6.7
D2908	Rp10-A	whole	1:100	69.2	67.3	52.8
D2908	Rp10-A	IgG	1:100	48.7	35.1	13.7
D2908	Rp10-A	non-IgG	<1: 10	20.4	0[c]	0
IS-Vol	Rp10-Mixo	whole	1:100	80.2	71.4	47.6
IS-Vol	Rp10-Mixo	IgG	<1: 10	13.3	18.1	10.3
IS-Vol	Rp10-Mixo	non-IgG	1:100	58.4	28.2	28.2
IS-Vol	Rp10-A	whole	1:100	87.8	86.7	56.0
IS-Vol	Rp10-A	IgG	1: 10	6.1	11.4	5.9
IS-Vol	Rp10-A	non-IgG	1:100	73.5	61.2	42.3
HIS1	$[(NANP)_2$-NVDP$]_2$	IgG	1:100	56.0	33.7	28.6
HIS5	MSP-3b	IgG	<1: 10	22.3	3.1	0

a) Affinity purification of specific Ab was performed either using whole serum or serum previously fractionated into IgG and non-IgG. The sera employed were: D2908, Donsé serum no. 2908; IS-Vol; HIS, hyperimmune African serum.
b) Indicated are the STARP-specific Ab concentrations tested. The percentage inhibition was calculated with respect to the number of intra-hepatocytic forms obtained in the absence of Ab.
c) All values marginally lower or equal to 0 were expressed as 0.

Table 3. Inhibitory effect of mouse anti-STARP antisera on *P. falciparum* Spz invasion of human hepatocytes *in vitro*

IgG preparation[a]	STARP-specific IgG levels[b]	IgG test concentration (mg/ml)	Spz IFAT titer[c]	% inhibition
GST+STARP/Rep no. 1	17.4	0.1	1:1000	41.6
GST+STARP/Rep no. 2	24.8	0.1	1:1000	52.8
GST+STARP/Rep no. 3	17.0	0.05	1: 200	68.3
GST+STARP/Rep no. 4	8.9	0.05	1: 200	42.3
GST no. 1	<1.0 (negative)	0.1	<1: 10	0[d]
GST no. 2	<1.0 (negative)	0.05	<1: 10	11.1
pre-immunization	<1.0 (negative)	0.1	<1: 10	0

a) From a total of 27 mice immunized with GST+STARP/Rep, the 12 with the highest levels of STARP-specific IgG (as measured by ELISA using the Rp10-Mixo peptide) were chosen and split into 4 groups of three (No. 1–4). Serum samples taken from each of these 12 mice just prior to immunization were pooled for the negative preimmune control. Control GST-specific IgG were prepared from two separate groups of six mice immunized with GST alone.
b) For each mouse, STARP-specific IgG levels were calculated as the ratio of absorbances obtained with the post-immune *vs.* the pre-immune serum samples in ELISA assays using the peptide Rp10-Mixo as coating Ag. Shown above is the geometric mean of these levels for each group of mice. Values ≥ 1 clearly indicated the presence of STARP-specific IgG.
c) The IFAT Spz titer shown is that of the IgG preparation tested at the above concentration.
d) All values marginally lower or equal to 0 were expressed as 0.

immunized volunteer, reaching 80–88% inhibition with the Rp10-Mixo- or Rp10-A-specific Ab. Moreover, the percentage of inhibition remained elevated even at the lowest Ab concentrations (0.5 μg/ml). Surprisingly, such significant inhibitory activity was confined, for this experimentally protected individual, to the non-IgG fraction (reaching up to 74% efficacy) while the IgG fraction showed no effect. In agreement with this finding, elevated Spz IFAT titers (1:100) were found in the non-IgG STARP-specific Ab preparation, while the titers of the IgG samples were essentially negative (<1:10). The finding for both sera that inhibitory activities were lower in the fractionated versus unfractionated ("whole") Ig is possibly due to partial denaturation of the fractionated Ig recovered from the two-step process of ion-exchange chromatography and elution of affinity-purified Ab.

In this same experiment (Table 2), a 56% inhibition was observed with anti-CS IgG that had been affinity purified from a peptide containing the two major CS repeat sequences. Again, no significant inhibition was observed with affinity-purified Ab specific to the asexual blood stage merozoite Ag, MSP-3.

3.3 Anti-STARP Ab induced by immunization are also inhibitory

To address the question of whether inhibitory Ab could be produced by immunization with recombinant STARP protein, we immunized mice with either the central repetitive region of the STARP Ag (encompassing the Mosaic, Rp45 and Rp10 repeat sequences) fused to glutathione S-

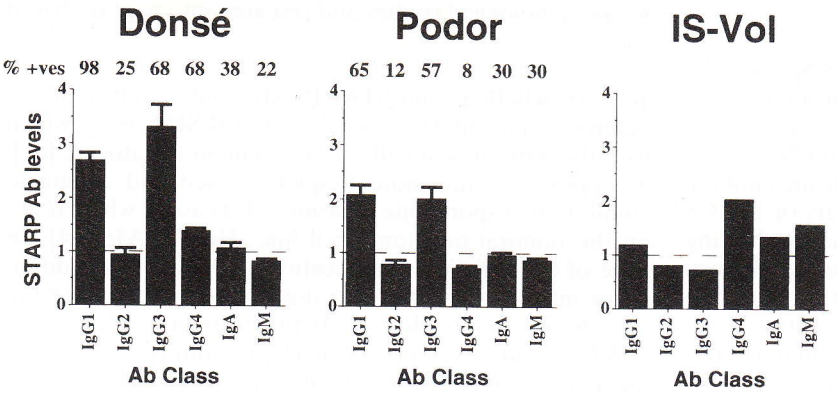

Figure 4. Isotype analysis of Ab responses to STARP in the serum of individuals from malaria-endemic areas or a volunteer experimentally immunized with IrrSpz. The arithmetic mean +SEM of the levels of STARP-specific IgG1-4, IgA or IgM, calculated for the high-transmission Donsé ($n = 63$) and low-transmission Podor ($n = 99$) sera samples is shown (left and center panel). Indicated above are the percentage of sera samples for which a positive Ab response to STARP was detected for each (sub)class. Note that a positive response was defined as one for which the Ab level, as calculated in arbitrary units, was ≥ 1.0. Data were retained only from those individuals for which all isotype studies were performed. (Right panel): Levels of anti-STARP Ab detected in the serum of an IS-Vol. This indicates that the STARP-specific Ab response in this individual was restricted to the IgG1, IgG4, IgA and IgM (sub)classes. Note that for all these ELISA assays (performed using the STARP Rp10-A peptide as coating Ag), total IgG was measured using 1 : 100 serum dilutions, whereas IgG1-4, IgA and IgM were measured using serum dilutions of 1 : 25.

transferase (GST+STARP/Rep), or with GST alone. ILSDA assays were performed using IgG purified from either post-immune or pre-immune sera (Table 3). No inhibition was seen either with control IgG from GST-immunized mice or IgG prepared from pooled preimmune sera from the mice subsequently immunized with recombinant STARP protein. However, purified post-immune anti-GST+STARP/Rep IgG did reduce Spz invasion of hepatocytes by 42–68 %. These data imply that inhibition was due to the effect of STARP-specific IgG. Indeed, IFAT and ELISA data indicated the presence of significant levels of STARP-specific IgG in the post-immune sera of GST+STARP/Rep-immunized mice, with Spz IFAT titers reaching 1 : 200 to 1 : 1000, whereas anti-GST or preimmune sera were negative. Interestingly, the highest level of inhibition (68 %) was not found with the preparations having the highest (1 : 1000) titers, but instead with a GST+STARP/Rep IgG preparation that had a Spz IFAT titer of 1 : 200. This would suggest that subtle though functionally important differences in Ab specificity between these IgG preparations were responsible for the greater efficacy of the IgG from mice with lower STARP-specific IgG titers, underlying the possible importance of qualitative factors (*i.e.* avidity or fine specificity) in determining the biological effect of these Ab.

3.4 Isotype analysis of humoral responses to the STARP Ag

In view of the known differences in the biological functions of separate Ab species [48, 49] and their distinct roles in defense against malaria [40], we investigated which subclasses were involved in STARP-specific Ab responses in Donsé and Podor. Interestingly, results showed that the cytophilic isotypes IgG1 and IgG3 clearly predominated in both populations (Fig. 4). In the Donsé group, the mean level of STARP-specific IgG was highest for IgG3, whereas the prevalence of positive responses was the highest for the IgG1 isotype, being detected in 98 % of the individual sera. This higher prevalence, compared to the 90 % prevalence of IgG responses, is likely related to the greater sensitivity of the double sandwich ELISA used and/or the lower serum dilution employed for the isotype analyses. Sixty-eight percent of the sera also contained STARP-specific Ab of the IgG3 and IgG4 isotypes, the latter in spite of weakly positive levels. STARP-specific IgG2 was both infrequent and of low levels when detected. Surprisingly, STARP-specific Ab of the IgA class were encountered in up to 38 % of the sera, although the levels were generally very low. IgM responses were detected in only 22 % of the population sampled, and levels were in general even lower than those of IgA.

In Podor, the overall picture of isotype distribution was essentially similar to that of Donsé, except that the mean titers and prevalences were lower (Fig. 4), in agreement with the lower exposure to pre-erythrocytic stages in this area, with again a net predominance of IgG1 and IgG3 specific for STARP.

Overall, only IgG1 and IgG3 showed a statistically significant increase in Ab levels with age for both population samples. The remarkable dominance of these two isotypes was reflected in the finding that there was not a single individual from either Donsé or Podor that was positive for only IgG2 and/or IgG4 (and negative for IgG1 and IgG3).

A similar isotype analysis, carried out for the IS-Vol serum, revealed unexpected differences. Most notably, among the IgG, the response to STARP was restricted mainly to IgG4, with a borderline IgG1 response and no IgG3 or IgG2. Though low, positive levels of STARP-specific Ab were also detected within the IgA and IgM classes. It was subsequently found that under our experimental conditions the chromatographic separation technique recovered IgG1-3 in the IgG fraction, while the non-IgG fraction was enriched in IgG4 as well as IgA and IgM (data not shown). Thus, in the case of the IS-Vol serum, the inhibitory effect of affinity-purified STARP-specific Ab correlated with the presence of IgG4, IgA and IgM.

4 Discussion

For many years, Ab responses to *Plasmodium* Spz and the abundant CS surface protein have been considered one and the same; indeed, many papers have measured Ab responses to Spz on the basis of reactivity to CS repeats, and one recent review stated that "all neutralizing antibodies to sporozoites are directed against repeats of the CS protein" [50]. Our data challenge this dogma by showing that Ab to a recently identified Spz surface protein known as STARP [25, 26] are frequently present in naturally exposed individuals and attain levels equivalent to those found specific for the CS protein. More importantly, STARP-specific Ab, affinity purified from the serum of individuals either naturally exposed to, or experimentally protected against pre-erythrocytic stages of *P. falciparum*, can significantly inhibit *P. falciparum* Spz invasion of human hepatocytes. This further demonstrates the surface location of the molecule and provides compelling evidence that immune responses to this Ag can play a biological role in limiting Spz invasion of hepatocytes. We note that there is growing evidence from bed-net and other studies that reducing the numbers of Spz entering the liver can lead to a reduction in parasite density and malaria-associated morbidity and mortality [51; 52 and references within]. Thus, elicitation of an Ab response that prevents most although perhaps not all Spz from entering the liver, by means of a vaccine incorporating STARP, may be of significant help in combating malaria.

The target of those neutralizing Ab to STARP, the Rp10 repeat region, is known to be highly conserved in *P. falciparum* [25, 26]. Its potent antigenicity is demonstrated by the finding that even in a region where transmission levels (1–5 infectious bites / individual / year) are amongst the lowest of sub-Saharan, malaria-endemic Africa, IgG specific for the conserved peptide Rp10-A was found in 82 % of adults. IgG responses to the Rp10 region were also measured using a "mixotope" Ag. The mixotope approach, based on combinatorial synthesis, represents a novel means of simultaneously generating a series of epitope motifs represented by a multiplicity of closely related sequences. This was initially developed to mimic the hypervariable V3 loop of the HIV-1 gp120 envelope protein [36]. Remarkably, the HIV-1 mixotope was able to prime an immune response which could be stimulated *in vitro* by a range of polymorphic MHC-restricted V3 sequences and to elicit Ab, $CD4^+$ and $CD8^+$ responses in multiple genetic backgrounds [53]. STARP was chosen as a good candidate for further studying the utility of this approach since the considerable degeneracy of the Rp10 region had been predicted to result in multiple closely related epitopes [25], thus creating a "natural" mixotope. Interestingly, when using the combinatorial Rp10 mixotope, the prevalence of STARP-specific IgG responses was only slightly reduced, while the mean levels of Ab detected in this way were higher, as compared to the conserved single-repeat sequence Rp10-A. A likely explanation is that the mixotope, which contains many related epitopes, is able to recruit an enlarged population of Ab directed to the Rp10 region, while the consensus Rp10-A peptide contains the major B cell epitope(s). Mixotopes may thus be more accurate than unique peptides in reflecting the true extent of polyclonal specificities to native repeat antigens of *Plasmodium* and provide an interesting alternative both for immunological studies and research into peptide-based vaccines.

To test whether anti-STARP Ab could contribute to defense against malaria, we chose the ILSDA assay, which uses the natural host cell, *i.e.* the human hepatocyte [45], as opposed to the more frequently used and amenable "inhibition of sporozoite invasion" (ISI) assay, which relies on the tumoral hepatoma cell line, HepG2-A16 [54]. In spite of the logistical complications, several critical differences make it imperative to use primary human hepatocytes to obtain reliable data. In practical terms, the lack of *P. falciparum* development in HepG2 directly influences the assessment of results. In our experience, it has proven extremely difficult to distinguish between a uninuclear, blocked parasite inside a HepG2 cell and a rounded-up Spz that is immediately adjacent though still extracellular. In contrast, within hepatocytes, the normal development of the parasite leads to the production of liver schizonts that after 3–5 days are readily distinguishable from non-invaded Spz by their large size, intrahepatocytic localization and morphology. Moreover, HepG2 cells clearly differ functionally from host hepatocytes, as evidenced by the totally artificial receptivity of the former for the rodent malaria parasite *P. berghei* [55], contrasting with its inability to permit intrahepatocytic development of *P. falciparum* [54]. This demonstrates critical differences which may also concern their susceptibility to invasion. Indeed, multiple comparative studies have revealed major discrepancies in the inhibitory effect of Ab to Spz molecules, frequently being near-total for HepG2 while dramatically weaker for hepatocytes [56–59, 14]. Indeed, in one study, Ab found to significantly inhibit *P. falciparum* Spz invasion of HepG2 cells gave the opposite result, *i.e.* an increase in the numbers of invaded Spz, in human hepatocytes [57]. Furthermore, data from three human vaccine trials show a lack of correlation between levels of ISI in HepG2 and protection status [4, 60, 61]. In favor of the relevance of the ILSDA hepatocyte assay, a close correlation has been observed, for both *P. falciparum* and *P. yoelii*, between the levels of anti-Spz immunity *in vivo* and the inhibition found with the relevant anti-Spz Ab *in vitro* [15, 17, 18]. These findings lend weight to the assertion that data obtained using human hepatocytes will more faithfully reflect the true capacity of these Ab to limit Spz invasion *in vivo*.

Our ILSDA results show that human STARP-specific Ab can prevent up to 90 % of *P. falciparum* Spz from penetrating human hepatocytes. To our knowledge, this is the first demonstration that Ab present in the sera of naturally exposed or experimentally protected individuals have been affinity purified against a Spz Ag and shown to be so effective in reducing hepatocyte invasion. In comparison, the relatively few ILSDA results with polyclonal Ab raised to other Spz Ag have shown variable or lower inhibition (Pfs16: 44–67 % inhibition at 1 : 100 mouse serum dilution [58]; TRAP/PfSSP2: 44–52 % inhibition at 1 : 10–1 : 20 mouse serum dilution, no effect at 1 : 40 dilution, no prebleed control [16]; CS: −109 % [*i.e.* facilitation of invasion] to 4 % inhibition at 10 μg/ml purified human IgG [57]; 20–90 % inhibition at 1 : 10 mouse serum dilution [14]). In our study only limited (13–43 %) inhibition was recorded with human polyclonal anti-CS Ab. Inhibition levels with anti-STARP Ab were both reproducible and dependent on the Ab concentration (tested at 2.0, 1.0 and 0.5 μg/ml).

These are below concentrations obtainable in humans using highly immunogenic vaccine systems ([62] and references therein). Encouragingly, we did not observe a facilitation of Spz invasion rates at the lower Ab concentrations as has been reported with the ILSDA assay using Ab specific for the *P. falciparum* or *P. yoelii* CS protein [57, 63].

Similarly efficient inhibition was recorded with anti-STARP Ab purified from both the IrrSpz volunteer and African immune sera. While the former displayed complete protection against multiple Spz challenges (in the absence of CTL activity to the CS protein [31]), including one made 2 weeks after the serum was drawn, the level of pre-erythrocytic stage immunity of the African individuals is less defined. Nevertheless, several studies do suggest that individuals living in malaria-endemic areas can develop fairly effective, though not sterile, biological defenses that prevent a high proportion of Spz inoculations from reaching blood-stage infections ([64–66]; P. Druilhe, data not published). One could speculate that STARP-specific Ab contribute to these individuals' immune status.

We have found that immunization of mice with purified recombinant STARP expressed in *E. coli* led to the synthesis of IgG that recognized the native Ag and gave substantial inhibition (42–68 %) *in vitro*. While we now need to assess alternative Ag presentation systems and adjuvants, these results are encouraging in light of the experience with CS vaccines where it has generally been much more difficult to induce Spz-neutralizing Ab than had been anticipated by the results of passive transfer experiments with anti-CS mAb (reviewed in [50]).

In view of these results, experiments with anti-STARP Ab in *Aotus* monkeys are being initiated to compare inhibition of Spz invasion *in vitro* with the effect of passive transfer *in vivo, i.e.* to assess the extent to which inhibition *in vitro* translates to efficacy *in vivo*. These inhibition results also warrant further investigations to test whether STARP might be a ligand involved in hepatocyte invasion. Already, two molecules, CS and TRAP/PfSSP2, have been identified as components of the process of hepatocyte recognition and invasion [67, 68].

Our isotype analyses of the two malaria-endemic populations revealed that both the prevalence and mean levels of IgG1 or IgG3 responses to STARP far exceeded those seen with the IgG2 or IgG4 isotypes. A similar predominance of cytophilic IgG has also been observed with human Ab responses to the *P. falciparum* CS, MSP-1 and MSP-2 Ag [43, 44, 69]. For STARP, there was also a clear age-dependent increase in the ratio of cytophilic to non-cytophilic IgG responses. Interestingly, for malaria blood stages (as for other parasitic infections [70]), there is increasing evidence of the importance of a predominantly cytophilic Ab response for protection [42, 71–75] which is further strengthened by the apparent relationship between a progressive switch to cytophilic IgG and the gradual acquisition of protection [40], as well as by the critical role assigned to the monocyte-dependent, cytophilic Ab-mediated mechanism of ADCC [76]. In the case of immunity to pre-erythrocytic stages, there are data to support both direct and cell-mediated effects of Ab, with isotype distribution potentially being critical for the latter [77, 78]. Indeed, in *P. berghei* CS-immunized mice, it was found that titers of mouse (cytophilic) IgG1 to the Spz surface and Ab avidity paralleled acquisition of sterile immunity [77]. Also, infected hepatocytes have been found to be prone to Kupffer cell-mediated ADCC [78]. As a result of its expression by intrahepatocytic parasites [25], STARP may be a potential target of ADCC. There is, however, no evidence to indicate that isotype is important at the level of blocking Spz invasion and it is likely that all isotypes could be equally effective.

Unexpectedly, the IrrSpz-immunized volunteer's STARP-specific response was composed essentially of IgG4, IgM and IgA. This observation has recently been confirmed by isotype analyses of four additional volunteers' sera showing that two had similarly elevated levels of IgM, while increased levels of IgG4 and IgG3 were found in one and two individuals, respectively. One possible explanation is that the predominantly IgM response to STARP in the IrrSpz-immunized volunteers may be associated with exposure over a relatively short time frame to high Ag (Spz) doses, leading to a lack of maturation towards a mostly IgG response, while in endemic areas, people are exposed over many years to low Spz numbers and IgG response develops. Indeed, Spz Ag-specific immune responses have been found to differ depending on whether the vertebrate host was naturally exposed to malaria parasites or immunized with IrrSpz ([79, 80]; P. Druilhe, unpublished data). Whether IgM, by nature of its agglutinating ability, was primarily responsible for the powerful biological activity of this serum against Spz invasion deserves further evaluation.

Previous studies have already indicated that STARP can elicit human CTL responses [27, 28], and ongoing studies in Africa with human PBMC (L. Ralamboranto et al., in preparation) have shown that STARP peptides can induce a high frequency of T cell proliferative responses and secretion of IFN-γ, the cytokine with the most potent effect upon liver schizogony [81]. The present study documents the antigenicity of this molecule and shows that STARP-specific Ab induced by either natural exposure or artificial immunization exert a biological role at an essential phase of the pre-erythrocytic cycle, *i.e.* that of host cell invasion. The challenge now is to assess the relative importance of each STARP-specific immune effector arm in defense against parasite challenge. These results validate our search for alternative pre-erythrocytic stage molecules [24] and provide compelling evidence that STARP may prove to be a powerful component in development of a future anti-pre-erythrocytic stage malaria vaccine.

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