Evaluation of an *in vitro* assay aimed at measuring protective antibodies against sporozoites

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We have evaluated the in vitro *biological activities of antibodies directed against sporozoites and compared them with their capacity to protect against challenge with both human and rodent malaria. The anti-*Plasmodium falciparum *antibodies evaluated with the test included monoclonal antibodies (MAbs) NFS1 and NFS2 as well as polyclonal antibodies contained in human hyperimmune sera directed against sporozoites of* P. falciparum. *The inhibitory effect of these antibodies was dependent on their concentration. However, total inhibition was not observed except occasionally with highly concentrated MAbs (10–100 μg/ml). Strong but also incomplete inhibition was observed with sera from humans living in hyperendemic areas. In the* P. yoelii *rodent system, we tested sera from mice immunized with subunit vaccines. None of these mice were protected* in vivo *against challenge with 40–200 sporozoites.* In vitro *only a sub-total inhibition was achieved (maximum 91% at 1:10 serum dilution). In contrast, we tested sera from mice that received NYS1, an IgG3 MAb, in passive transfer and were protected against challenge with 5000 sporozoites. At 1:10 dilution, 100% inhibition was achieved* in vitro *while IFA titres from these mice were similar to those of vaccinated mice. These data show a close correlation between* in vivo *and* in vitro *findings and thus suggest that the inhibition of liver-stage development assay (ILSDA) appears appropriate to evaluate the potential of antibodies.*

Introduction

The feasibility of successfully developing a vaccine against sporozoite challenge has rested largely on successes in protecting mice and some humans with irradiated sporozoites (*1–4*). Several studies have indicated that antibodies (*5*), $CD8^+$ T cells (*6–9*), and cytokines (*7, 10–12*) released by immuno-competent cells can interfere with the pre-erythrocytic phase of parasite development. However, information from malaria animal models have demonstrated that antibodies alone, either monoclonal (MAb) or polyclonal, can be fully or partially effective in preventing infection in sporozoite challenged animals (*5, 13*). In human falciparum malaria, our former *in vitro* studies (*14*) also showed that both MAb directed to circumsporozoite protein repeats, and antibodies raised in animals to recombinant molecules containing these repeats could reduce the number of sporozoites developing into liver stages. The inhibitory effect was found to be dependent on antibody concentration and in this preliminary study, total inhibition was reached at the highest concentration tested.

On the other hand, several recent subunit vaccine trials designed to elicit antibodies against the CS protein have produced disappointing results (*15, 16*). One explanation is that the vaccine formulations tested in human volunteers thus far have been marginally immunogenic eliciting an antibody response too weak to be clinically effective. It was thus thought that if proper immunization conditions and high levels of antibodies could be reached, protection could be achieved. We carried out complementary investigations using the *in vitro* hepatocyte culture model in order to evaluate, first the effect of very high antibody concentrations, and secondly the *in vivo* correlativity of this *in vitro* test in man and in mice.

Materials and methods

Preparation of primary cultures of hepatocytes

Mouse hepatocytes were obtained by *in situ* perfusion, and rat or human hepatocytes from liver biopsies by microperfusion. As previously described (*17*), we used Ca^{++}-free HEPES buffer, following by a Ca^{++} collagenase solution (Sigma) to dissociate the hepatocytes. The perfused fragment or the liver was minced and the free cells were washed three times in HEPES buffer and suspended in medium MEM supplemented with 0.2% serum albumin bovin, 10% fetal calf serum, penicillin–streptomycin, insulin, glutamine, and non-essential aminoacids. The cells were seeded in eight-chamber Lab-tek plastic slides (Miles), at 10^5 cells per

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chamber, and were incubated at 37°C in an atmosphere of 5% CO_2, 95% O_2. After 24 hours of incubation, hydrocortisone 7×10^{-5} mol/l was added to the cultures.

Parasites

(*a*) Sporozoites of *P. falciparum* were obtained either from infected *Anopheles stephensi* or *A. freeborni* mosquitos after membrane feeding on gametocytes from cultures of the NF54 strain, presumed from Africa, or the 7G8 clone from a Brazilian isolate.

(*b*) Sporozoites of *P. yoelii* (17XNL) were extracted from infected *A. stephensi*, 14 days after the mosquitos received an infective mouse blood meal.

Antibodies

Antibodies to* P. falciparum *sporozoites. (*a*) *Mouse monoclonal antibodies*: purified IgG (1 mg/ml) from MAbs, NFS-1 (IgG1) and NFS-2 (IgG2a) (*18*) directed to *P. falciparum* circumsporozoite protein repeats. MAb 5-1-4 (1 mg/ml) directed to the blood stage antigen *P. falciparum* empl, but cross-reacting with the CS protein (*19*) (gifted by J. Mc Bride).

(*b*) *Human sera*: we used 10 sera from individuals aged 40 to 70 years living in three different holoendemic areas in West Africa (Cameroon, Congo, Mali), where transmission levels vary between 100 to 400 infective bites per year. IgG was prepared from sera by ion-exchange chromatography on DEAE-trisacryl column. Control sera and control IgG were from naïve French donors.

Antibodies to* P. yoelii *sporozoites. Immune mouse sera: sera from mice immunized by different protocols were used.

(i) NYS1 (IgG3) passive transfer sera (*30*): Balb/C mice were inoculated i.v. with 0.5 or 1 mg NYS1; control mice received an unrelated MAb (the *P. falciparum* anti-sporozoite MAb NFS1). Thirty minutes after antibody transfer, blood samples were collected and serum separated; all mice were immediately challenged with 5000 *P. yoelii* sporozoites.

(ii) $(KLH\text{-}QGPGAP)_4$, proteosome-$(QGPGAP)_4$ sera and PY CS.1 (*30*): Groups of mice were immunized i.m. at 3-week intervals with 100 μg $(QGPGAP)_4$ either conjugated to KLH ($KLH\text{-}(QGPGAP)_4$) or complexed to proteosomes ($Proteo\text{-}(QGPGAP)_4$), or at 2-week intervals with varying doses (500 μg) of a recombinant fusion protein (PY CS.1) including 61% of the *P. yoelii* CS protein. Two weeks after the 4th immunization with synthetic peptides or after the 5th one with PY CS.1 protein, blood samples were collected and all mice were then challenged i.v. with 200 *P. yoelii* sporozoites.

(iii) Irradiated sporozoite (Irr Spz) sera: mice were immunized i.v. with 3 doses of irradiated *P. yoelii* sporozoites (50 K, 30 K, 30 K) at 2-week intervals; blood samples were collected before challenge.

(iv) Irradiated sporozoites anti-$CD8^+$ sera (Irr Spz + anti $CD8^+$): mice previously immunized with irradiated sporozoites were depleted of $CD8^+$ T lymphocytes by giving two injections (1 mg) i.p. of the monoclonal antibody 19/178 at 24-hour intervals (*20*).

(v) Vaccinia sera: the Vaccinia CS construct encoding the full length CS protein of *P. yoelii* (Vpy) or the control Vaccinia construct (Vgalk) encoding an unrelated *Escherichia coli* galactokinase was inoculated i.p. 4 times at 2-week intervals (10^8 pfu/dose) (*20*).

Indirect fluorescent antibody test (IFAT)

Sporozoite stages. The IFAT of murine MAbs and human sera were performed as previously described (*21*) using wet preparations of *P. falciparum* sporozoites attached to poly-L-lysine (Sigma; m.w. > 500 000) films so as to measure surface reactivity. The IFAT of murine immune sera was performed with air-dried *P. yoelii* sporozoites. Depending on the antibodies being studied, the revealing serum was either FITC-labelled anti-human IgG, A, M (Pasteur Production) or anti-mouse IgG, A, M (Cappel) both at a 1/200 dilution in 0.05% Evans blue solution in phosphate-buffered saline (PBS).

Liver stages. At 48 hours after sporozoite inoculation, the infected cultures were washed three times in PBS, fixed in methanol at 4°C, rinsed in PBS, and incubated with the MAb against CS protein of *P. yoelii* or *P. falciparum*. The revealing serum was an anti-mouse IgG, A, M at a 1/200 dilution in 0.05% Evans blue solution in PBS.

Inhibition of liver-stage development assay (ILSDA)

Twenty-four hours after hepatocyte cultures had been prepared, they were inoculated with infective sporozoites (*12*). Briefly, the sporozoites were extracted aseptically from salivary glands. After removal of spent medium from a culture chamber, 25 μl of medium containing 5 to 6×10^6 sporozoites and 25 μl of a dilution of a MAb or of normal or immune mouse or human serum or 25 μl of supplemented medium were added to each chamber. Three hours after sporozoite inoculation, the medium containing

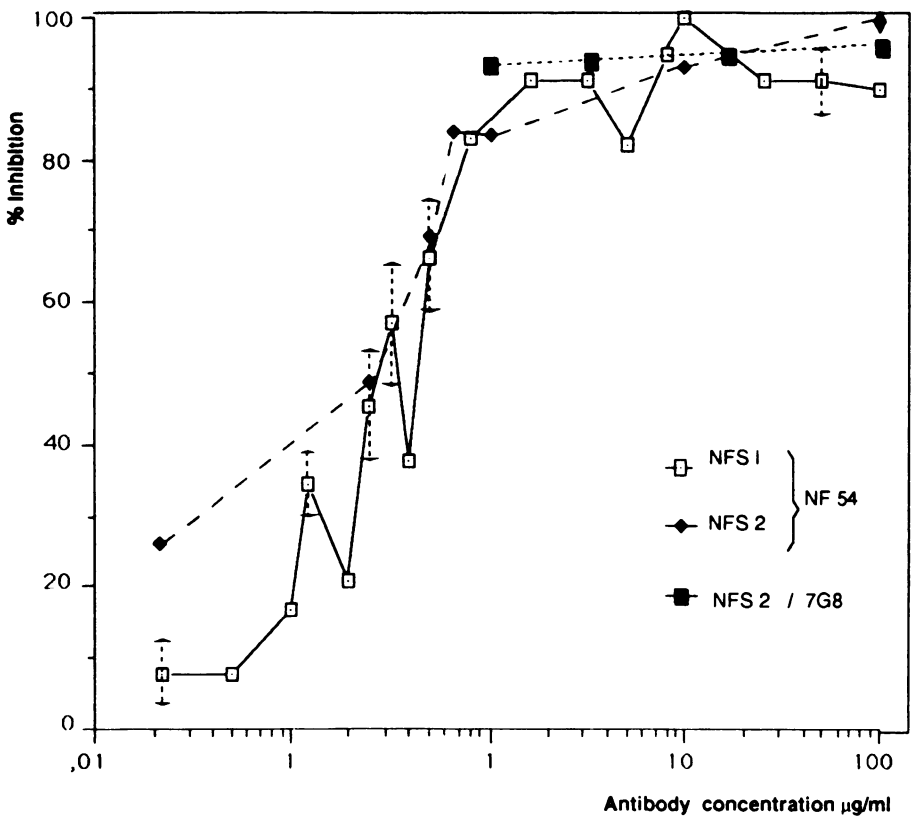
the inoculum was removed and replaced once more by fresh medium, which was thereafter changed daily. In these experiments we adopted as a standard procedure 48-hour cultivation of both *P. falciparum* and *P. yoelii* liver forms. The time was chosen to enable us to determine the number of sporozoites which had transformed into liver stages (trophozoites) in the case of *P. falciparum* and the number developing into mature liver stages in the case of *P. yoelii*. Following the IFA staining of the liver stages with relevant antibodies, the number of liver stages was counted in test and control cultures. Experiments in which cultures were contaminated or where the controls contained fewer than 20 liver schizonts were disregarded. The mean inhibition was calculated by comparing the number of liver stages in experimental cultures with the corresponding number in control cultures.

Results

P. falciparum *sporozoites*

ILSDA activity of murine MAbs. (Fig. 1). At final antibody concentrations (0.01 to 1 μg/ml), MAbs NFS1 and NFS2 inhibited the transformation of NF54 *P. falciparum* sporozoites into liver-stage trophozoites (LST) in a dose-dependent way. There was no discernible difference between the 2 MAbs. In contrast, using

Fig. 1. **Inhibition of MAbs against the *P. falciparum* CS protein (NFS1 and NFS2) on the transformation of *P. falciparum* sporozoites (NF54 and 7G8) to liver-stage trophozoites in primary cultures of human hepatocytes.** At the time of sporozoite inoculation, MAbs were added to cultures at a final concentration indicated and removed 3 hours later. Cultures were evaluated 48 hours after sporozoite inoculation. Standard deviations are shown in the figure for experiments which were performed at least 3 times in duplicate for a given concentration of the specific MAb.



a 10 to 100 times higher antibody concentration (1 to 100 μg/ml), a phenomenon of saturation of inhibition was observed (the percentage of inhibition varied between 85% and 100%). At a 100 μg/ml concentration the IFA titre of MAb NFS-1 was $1/8 \times 10^6$. Total inhibition was obtained only in 2 tests out of 26 experiments (NFS1 (10 μg/ml): 0 LST in experimental group compared to 53 in the control, NFS2 (100 μg/ml): 0 LST in experimental group versus 21 in the controls). The reproducibility of the assay was found to be satisfactory since standard deviations were low (Fig. 1) in separate experiments performed with cells from different donors.

Results obtained in more limited experiments, using sporozoites from the clone 7G8 and the same MAbs, do not differ significantly and gave the same phenomenon of saturation at very high antibody concentration as seen with the NF54 strain. The percentage inhibition in this case ranged between 84% and 98% (9 LST in experimental cultures compared to 55 LST in controls, 5 in experimental group to 226 in controls).

While less than 100% of sporozoites were blocked in the ILSDA test, IFA showed 100% labelling of both NF54 and 7G8 parasites with the 2 MAbs.

Results obtained with MAb 5-1-4 directed to the blood-stage antigen *P. falciparum* emp1 show a significantly higher inhibition of 7G8 clone parasites than with the 2 other MAbs NFS1 and NFS2 (85% versus 35% and 48%, respectively, at the same concentration).

Human immune sera. In an attempt to correlate *in vitro* results from the ILSDA with *in vivo* events, and also to study the effect of polyclonal antibody to sporozoite surface antigens, we next studied human sera and the IgG derived from them.

The inhibition effect with this sera (Table 1) was correlated with the antibody concentration (IFA titre). We observed the equivalent inhibitory effect encountered with the 2 MAbs, at the final antibody concentration. Only an incomplete inhibition (88%) was obtained with the sera with the highest IFA titre for sporozoites ($1/10^4$).

At the time blood was drawn from these patients, 6/10 had blood-stage parasites detectable by microscopic examination of Giemsa-stained thick smears, thus arguing strongly against total blockade of the pre-erythrocytic phase, at least in these individuals.

P. yoelii *sporozoites*

Immune murine sera. A comparison between *in vivo* results and *in vitro* findings was made in the rodent model and sera from mice immunized with different subunit vaccines. All of the sera contained a high antibody concentration by IFA on sporozoite (range 1/2000 to 1/8000), but in most experiments the mice had marginal, if any, protective immunity. Only mice passively protected by transfer of MAb NYS-1 were protected against a sporozoite challenge (*30*).

The inhibitory effect was related to the antibody concentration for only some of these immune sera (proteosome-$(QGPGAP)_4$, PY CS.1, NYS1 passive transfer). At a 1:10 dilution (corresponding to an IFA titre of 1:800), the PY CS.1 and the KLH-construct sera inhibited 74% and the proteosome-$(QGPGAP)_4$ sera 90% (Fig. 2). However, complete inhibition of the maturation of liver stages was only observed with the NYS1 passive transfer sera (NYS1 PT) used at the same 1/10 dilution.

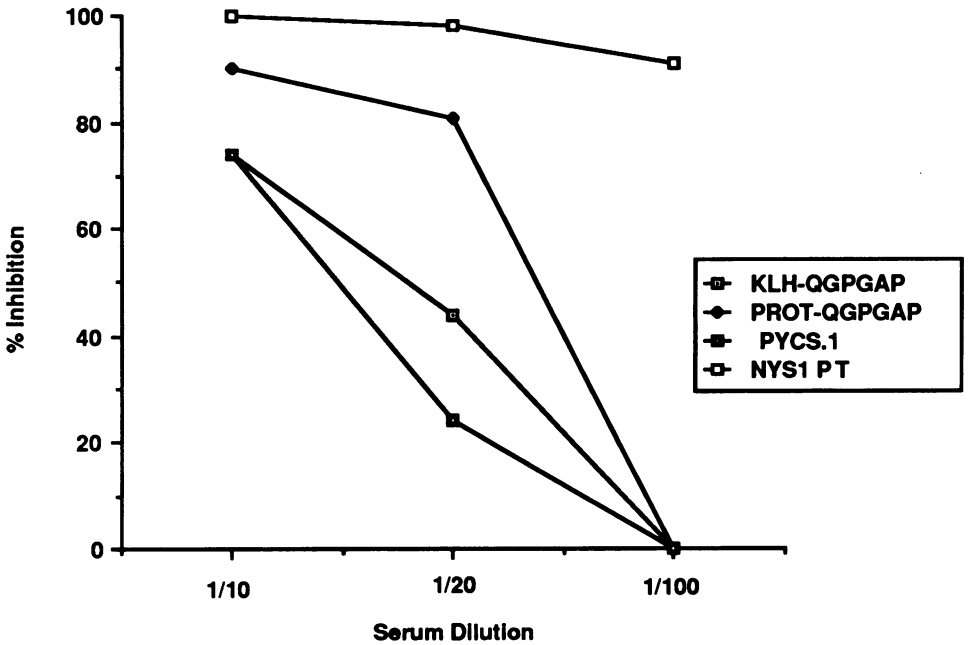
No significant inhibition could be obtained with use of sera from mice immunized with irradiated sporozoites, the Vaccinia–*P. yoelii* construct, or sera from mice immunized with the proteosome-$(QGPGAP)_4$ without adjuvant, whatever the antibody concentration tested.

Furthermore, three of the sera tested (Vaccinia–*P. yoelii*, irradiated-sporozoite sera, and

Table 1: **Inhibitory effect of human hyperimmune sera (final dilution 1/10) on the transformation of NF54 *P. falciparum* sporozoites to liver-stage trophozoites (48 hours) in primary cultures of human hepatocytes**

Human hyperimmune sera	IFA titre	No. tested/No. of controls	Mean % inhibition
1	1.6×10^3	38/68	44
2 (IgG)	6.2×10^3	10/23	56
3	6.2×10^3	39/68	42
4	1.3×10^4	7/53, 31/226, 14/53	82
5	1.3×10^4	10/31, 6/21	70
6	1.3×10^4	5/20	75
7 (IgG)	2.5×10^4	14/79	82
8	2.8×10^4	7/27, 6/21	82
9	5×10^4	4/33	88
10	5×10^4	6/33	82
11	10^5	6/33	82

Fig. 2. **Inhibition of immune mouse sera on the development of *P. yoelii* liver-stage parasites in mouse hepatocytes.** Cultures were stopped at 48 hours after sporozoite inoculation. Only sera from mice that received MAb NYS1 in passive transfer (NYS1 PT) gave consistently higher inhibition at a 1:100 dilution.



irradiated sporozoite + anti-CD8$^+$ sera) produced at higher antibody concentration an enhancement of the number of liver stages recovered, compared with the relevant controls (Fig. 3). This surprising result was confirmed by several *in vitro* assays and was found to be consistent, particularly with irradiated sporozoite sera.

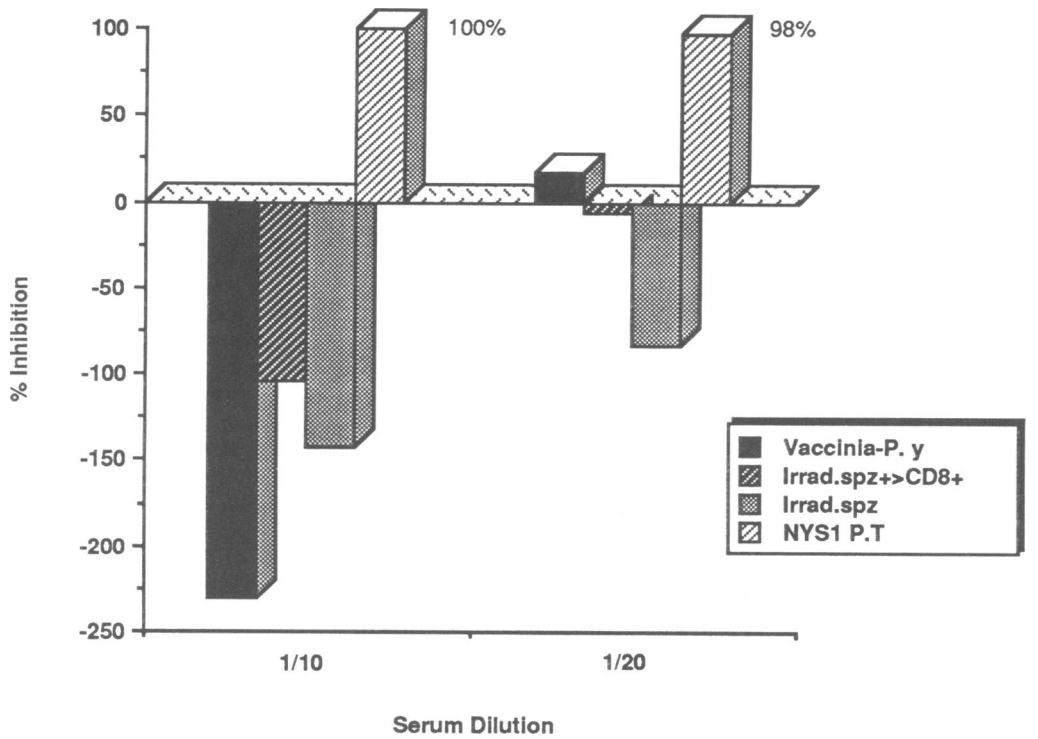
Using rat instead of mouse hepatocytes, an enhancement with Vaccinia construct sera and irradiated sporozoite sera was also obtained. In contrast, inhibition of parasite development with the MAb passive transfer sera was never total in rat cells (data not shown). This difference in the level of inhibition reached with MAb-transferred mouse sera was consistent among 5 separate experiments with mouse hepatocytes and 3 with rat hepatocytes.

Discussion

In the attempt to evaluate the biological effects of antibodies directed against sporozoites, we used an *in vitro* test ILSDA which takes into account not only the penetration but also the development of the parasite in its normal host cell, the hepatocyte. This *in vitro* test is particularly appropriate for the *P. falciparum* system, since, until now, only primary cultures of human hepatocytes have been found able to support the development of this human parasite. The reproducibility of the *in vitro* test appears to be satisfactory and the results from it appear to correlate with resistance to sporozoite-induced malaria *in vivo*.

In agreement with our prior findings (*14*), the results obtained in this study demonstrate a consistent antibody-induced dose-dependent inhibitory effect in the human model with monoclonal antibodies or polyclonal sera. A saturation effect has been observed at high antibody concentrations although the mechanism by which the few remaining infective sporozoites escape the action of antibody is unknown. The significant point is that a similar phenomenon is also observed *in vivo* (*22*). The mechanism could possibly be related to the inability of antibody to block all the interactions between parasites and hepatocyte receptors, or alternatively it may even result from the inordinately fast turnover and shedding of

Fig. 3. **Immune mouse sera inhibit or enhance the development (48 hours) of *P. yoelii* parasites in cultures of mouse hepatocytes.**



CS protein by these few sporozoites (*23*) which perhaps can bind and thereby inactivate antibodies that would otherwise block sporozoite entry.

The correlation of the ILSDA test with *in vivo* protective immunity is supported by several observations. In the *P. falciparum* human hepatocyte model, sera from humans exposed daily to inocula of several hundred sporozoites over a period of 40 to 70 years do not appear to contain blocking antibodies that fully protect despite the very high titres generated. Comparing these results to the infection rate in the individuals donating the serum, 60% of them were found to harbour blood parasites at any given time and extended studies indicate that this figure is close to 100% if the subjects are monitored daily by blood examination (*24–26*). Thus, less than complete inhibition by ILSDA seems to indicate less than complete immunity in man.

Observations in the rodent model support this interpretation and are particularly helpful because of the high rate of penetration of *P. yoelii* sporozoites in *in vitro* conditions, which far exceeds that for *P. falciparum*, and thus increases the reliability of the results. In rodent experiments, only the NYS1 passive transfer sera produced complete inhibition of liver-stage maturation *in vitro*. With the other immune sera, whatever the antibody concentration used, we observed significant inhibition which was occasionally high, but never complete and with some of these sera no inhibition was seen at all. *In vivo*, the NYS1 passive transfer protected mice against a 5000 sporozoite challenge inoculum (*30*). All the mice immunized with the subunit vaccines used in this study resulted in a blood parasitaemia after challenge with 40–200 sporozoites (*20, 30*). Moreover, the failure to inhibit parasite maturation with sera from mice immunized

with irradiated *P. yoelii* sporozoites is particularly relevant since passive transfer of these same sera likewise failed to protect *in vivo* (Y. Charoenvit, unpublished data).

Reproducible differences were obtained using mouse and rat hepatocytes, total inhibition by antibodies being consistently achieved in one model and not in the other. This observation raises the problem of models for malaria research even in *in vitro* conditions, and thus stresses the importance of using, as much as possible, the natural host cell, i.e., the human hepatocytes for *P. falciparum*.

In conclusion, our data indicate that ILSDA may provide a meaningful test in the evaluation of the antibody component of the immune response. The antibody concentrations 10–100 μg/ml required to provide significant inhibition in the ILSDA represent a formidable goal for vaccine developers.

Except when using MAbs, the effect of antibodies alone appears incomplete and insufficient, but it can be hoped that antibodies may constitute a first barrier which will be completed by the effects of cytokines (*7, 10–12*) and cytotoxic T cells (*7, 8, 27–29*) at the liver-stage level.

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