Irradiated sporozoites prime mice to produce high antibody titres upon viable *Plasmodium berghei* sporozoite challenge, which act upon liver-stage development

S. CHATTERJEE[1]*, P. DRUILHE[2] *and* M. WERY[1]

[1] *Department of Parasitology, Institute of Tropical Medicine, Antwerp, Belgium*
[2] *Biomedical Parasitology, Institute Pasteur, Paris, France*

(*Received 11 August 1998; revised 28 September 1998; accepted 29 September 1998*)

SUMMARY

C57BL6 mice were protected against *Plasmodium berghei* sporozoite challenge by immunization with live 12 krad dose-irradiated sporozoites, but not by 20 krad dose-irradiated sporozoites. Immunization with 12 krad irradiated sporozoites generated low levels of antibody reactive to liver-stage parasites (titres of 1/100). Inoculation of as few as 100 live *P. berghei* sporozoites induced complete host protection accompanied by a very quick and high boost of antibody titres up to 1/4000. This sporozoite challenge-drive antibody boost was absent in mice immunized by 20 krad sporozoites and in non-protected, and non-immunized mice. Antibody was mainly liver-stage (LS) specific and due to an increase of IgG_{2a} and IgG_{2b}. The *in vitro* effect of pre- and post-challenge sera upon either sporozoite invasion or LS development was assessed in Hep-G2 cultures. Both were found to have a strong effect upon LS development even at 1/2500 dilution, and conversely a low effect upon invasion. These results suggest that sporozoites irradiated at doses that induce protection are able to prime T-cells which, upon challenge by non-irradiated sporozoites, provide help to B-lymphocytes to trigger the production of high titres of anti-LS antibodies that can inhibit LS development *in vitro*.

Key words: malaria, *Plasmodium berghei*, irradiated sporozoite, immunoglobulin.

INTRODUCTION

Protective immunity against *Plasmodium* spp. in rodents (Nussenzweig *et al*. 1967) can be generated by immunization with irradiated sporozoites. This protection is strain and species specific and is highly dependent on the schedule, number and irradiation dose of sporozoites used for immunization. Complete protection is obtained only when the irradiated sporozoites are injected i.v. into the host (Druilhe & Marchand, 1989). These data suggest that protection is dependent upon the ability of viable sporozoites to penetrate liver cells, and has led to the concept that the liver stage of the parasite is the actual target of protective immunity after immunization with irradiated sporozoites.

There is now sufficient evidence that multiple effector mechanisms including antibodies (Nudelman *et al*. 1989), helper T cells, lymphokines and cytotoxic T cells (Schofield *et al*. 1987) may all contribute to effective protection following irradiated sporozoite immunization. However, these protective mechanisms are compensatory and high levels of antibodies alone can mediate protection (Nardin & Nussenzweig, 1993). Protective antibody responses directed mostly against the circumsporozoite (CS) protein located on the sporozoite surface have been reported to prevent sporozoite attachment and/or penetration.

Our objective has been to study the antibody profile directed against liver stages (LS) developing from irradiated sporozoites, and the effects of a live sporozoite challenge in modulating this antibody profile. The aim has been to identify (a) the antibody isotypes with reactivity generated to young liver forms after immunization with irradiated sporozoites, (b) the evolution of these antibody isotypes in immunized mice after live sporozoite challenge, and (c) the inhibitory effects of pre- and post-challenge sera antibodies on sporozoite invasion and liver-stage development *in vitro*.

MATERIALS AND METHODS

Vector maintenance and sporozoite production

Anopheles stephensi was bred in our insectary, under standard conditions of 25 °C temperature and 85% relative air humidity. Sporogonic stages of *Plasmodium berghei* ANKA developed in female mosquitoes that were maintained at 21 °C, after feeding on *P. berghei*-infected mice. From 21 days post-

* Corresponding author: Department of Parasitology, Institute of Tropical Medicine, Nationalestraat – 155, B-2000 Antwerp, Belgium. Tel: 00 32 3 2476355. Fax: 00 32 3 2476359. E-mail: schatterjee@itg.be

feeding, mature sporozoites were available for experiments.

Irradiation and processing of sporozoites

Irradiated sporozoites were obtained by dissection of *P. berghei* ANKA-infected mosquitoes irradiated the previous day. The dose given to the infected mosquitoes was delivered by ^{60}Co irradiation by means of a teletherapy machine (Theratron 780, courtesy Mr Schaeken, Middelheim Hospital, Antwerp, Belgium) as described previously (Chatterjee *et al*. 1996). Mosquitoes were dissected in cold GLSH medium (glucose, lactalbumin, fetal calf serum, haemoglobin) (Le Ray, 1975), and the salivary glands were transferred to a glass homogenizer on ice and gently disrupted to release sporozoites. The number of sporozoites in the suspension was counted in a haemocytometer.

Mice and immunization schedule

Female 6-week-old C57BL6 mice (IFFA Credo, Brussel) were used for immunizations. Groups of 5 mice were immunized every 2 weeks by intravenous injections of 30000 irradiated *P. berghei* sporozoites into the tail vein. In all, 3 immunizations were administered. An irradiation dose of 12 krad was selected for all experiments except 1 in which 20 krad was used. After every immunization, blood smears were collected from individual mice and stained with Giemsa to check for the absence of parasitaemia. At 2 weeks after the last immunization, mice were challenged with 100 live, non-irradiated *P. berghei* sporozoites. A group of unimmunized mice was included as a control to confirm the viability of the sporozoites. From day 4 post-challenge, thin blood smears were made from tail blood, fixed with methanol, and Giemsa stained. The number of parasites per 10000 erythrocytes was counted and the mean level of parasitaemia was calculated.

Just before challenge (day 0), and then on days 3, 10, 17, 24 and 31, immunized mice were bled and the serum was stored at −20 °C until required for testing. The control unimmunized mice were bled on days 0, 3 and 10 post-challenge.

Delipidation of serum samples

Two volumes of individual test serum were stirred together with 1 volume of Freon (Sigma 1.1.2 trichlorotrifluoroethane) for 30 min at 4 °C. The suspension was then centrifuged at 10000 ***g*** for 30 min at 4 °C, whereby the lipids and Freon separated out from the serum into the lower layer. The delipidated serum in the upper layer was stored at −20 °C until required for testing in the indirect fluorescent antibody test (IFAT), the inhibition of sporozoite invasion assay (ISI) or in the inhibition of liver-stage development assay (ILSDA).

Indirect fluorescent antibody test (IFAT) for titration of anti-plasmodial Ig isotypes

For titration of anti-liver stage Ig isotypes, serial dilutions of sera in phosphate-buffered saline (PBS; dilution range, 1/100 to 1/4000) were applied to antigen slides. The antigen used was *P. berghei* liver-stage parasites at 24 h of hepatic-stage development. The human hepatoma cell line Hep G2, which supports infection with *P. berghei* sporozoites and the complete development of the hepatic cycle (Hollingdale, Leland & Schwartz, 1983), is maintained in continuous culture in our laboratory. For parasite culture, 0·81 cm² sterile chamber slides (Lab-Tek, Nunc; 8 chambers/slide) were used as described previously (Chatterjee *et al*. 1996). Monolayers of Hep G2 cells in every chamber were incubated with 10000 *P. berghei* sporozoites in 50 μl of MEM Rega 3 medium for 2 h, a period that allows the sporozoites to penetrate into the host cells (Zavala *et al*. 1985). Non-invasive sporozoites were then removed by replacement of the medium in the chamber slides several times. Liver-stage parasites were allowed to develop in 200 μl of complete MEM Rega 3 medium for up to 24 h, at which time the cells were fixed with cold methanol, washed with PBS and incubated with 50 μl of the appropriate dilutions of test sera. After 30 min of incubation the slides were washed, and 50 μl of fluorescein-conjugated, affinity-purified, rabbit anti-mouse IgM, IgG, IgG_1, IgG_{2a}, IgG_{2b} or IgG_3 monoclonal antibody (Rockland, PO Box 316, Gilbertsville, PA 19525) diluted 1/10000 in Evan's blue solution was added and the slides were incubated for a further 30 min. After a final wash, mountant was added and the slides were examined under a fluorescence microscope.

For the titration of anti-sporozoite immunoglobulins, a poly-lysine treatment of *P. berghei* sporozoites was carried out. In brief, 20 μl of poly-L-lysine (Sigma, 50 μg/ml) was coated onto multi-well slides and allowed to dry overnight at 37 °C. One million *P. berghei* sporozoites were suspended in PBS solution containing 0·001 % glutaraldehyde and centrifuged at 13000 ***g*** for 5 min. After the third wash the sporozoites in the pellet were resuspended in PBS alone, and 20 μl were applied to each of the previously poly-lysine coated multi-wells. The multi-wells were left for 1 night at 4 °C, the day after 20 μl of the respective serum was added at the appropriate dilution to each well and incubated at 37 °C in a humid chamber for 30 min. In parallel, similar serum dilutions were also added to air-dried sporozoite-coated wells. After the incubation period, wells were gently washed several times with PBS, and a second antibody (anti-mouse) conjugated to FITC was added at the appropriate dilution. Wells were further incubated at 37 °C for 30 min, washed in PBS, mounted in glycerol solution and observed under a fluorescence microscope.

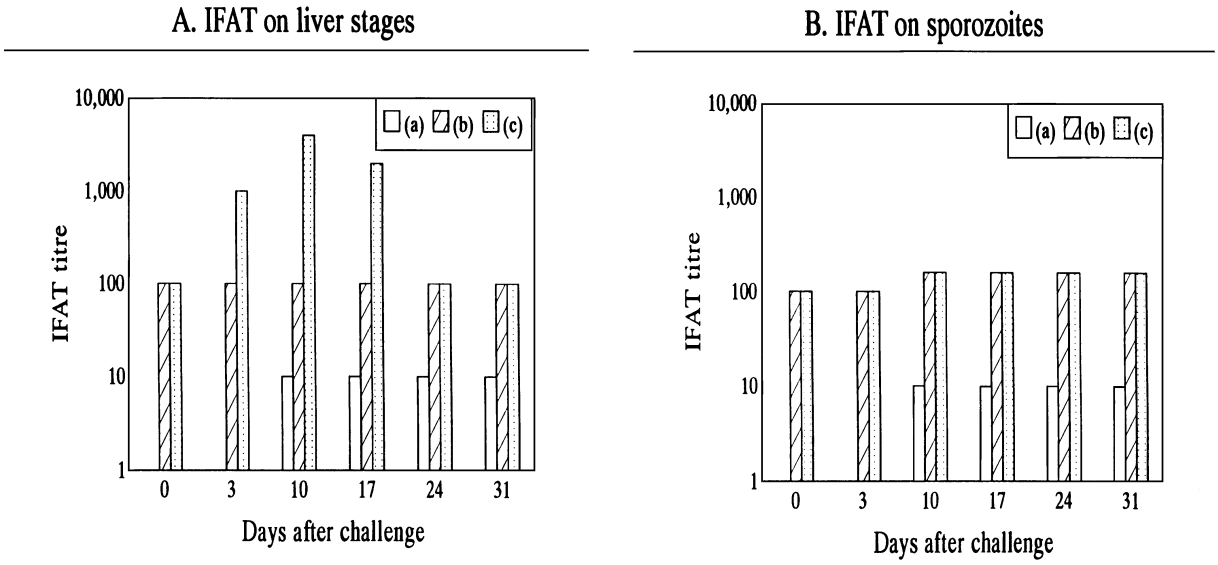

Fig. 1. Titres of whole IgG as determined on pre-challenge (day 0) and post-challenge (days 3, 10, 17, 24, 31) in (a) unimmunized controls, (b) 20 krad-dose immunized and (c) 12 krad-dose immunized C57BL6 mice. In each group, consisting of 5 mice, serum samples collected at every time-point were pooled and used for the test. In a separate experiment individual serum samples were obtained from the 3 mice in each group and checked for reactivity in IFAT. Results are shown in Table 1.

Inhibition of sporozoite invasion (*ISI*)

To study the inhibitory effects of antibody isotypes on sporozoite invasion, an assay was set up as previously described (Chatterjee *et al*. 1996). In brief, sporozoites in 90 μl of GLSH medium were coincubated in chamber slides with monolayers of Hep G2 cells, together with 10 μl of the respective sera, for 2 h. The final dilution of sera in each chamber was 1/100. Later, all medium was removed from the chamber slides and the intracellular sporozoites were counted after IFAT staining using as primary antibody, 3D11 (monoclonal to the *P. berghei* CS protein repeat sequences) at 50 μg/ml. The inhibition of sporozoite invasion (ISI) was calculated using the following formula:

$$ISI = [(I_c - I_i)/I_c] \times 100,$$

where I_c represents the number of sporozoites that invaded in the presence of normal mouse serum, and I_i represents the number of sporozoites that invaded in the presence of immunized mouse test serum.

Inhibition of liver-stage development assay (*ILSDA*)

To assay for the inhibition of liver-stage development, sporozoite invasion was allowed to proceed for 2 h. Thereafter, 20 μl of test sera diluted to a final ratio of 1/100 was added to each chamber after removal of sporozoite suspension. For the day 10 serum, both untreated as well as Freon-treated samples were compared for their capacity to inhibit liver stage development *in vitro*. After incubation for 48 h, an IFAT with the monoclonal antibody 3D11 was performed to calculate the number of liver-stage parasites that had developed in the presence of the test sera. The percentage of inhibition of liver-stage development was calculated using the following formula:

$$\% \text{ liver stage inhibition} = [(L_c - L_i)/L_c] \times 100,$$

where L_c represents the liver-stage parasites developing in the presence of normal mouse serum and L_i represents the liver forms developing (at 48 h) in the presence of immunized mouse serum.

RESULTS

Protection against challenge infection in mice immunized by 12 krad, but not by 20 krad dose-irradiated sporozoites

Protection data in immunized mice, following challenge with 100 viable sporozoites, essentially confirmed earlier findings (Chatterjee *et al*. 1996). Mice immunized with sporozoites irradiated at 12 krad dose showed complete protection (i.e. none of the challenged mice developed blood parasites), whereas mice immunized with sporozoites irradiated at 20 krad dose succumbed to blood-stage infection after challenge. The day of emergence and the course of parasitaemia in the latter was identical to that of unimmunized control mice infected in parallel and was lethal in both groups of animals.

Table 1. Antibody reactivity to LS, in individual mouse sera of immune (I) and non-immune (NI) groups

((−), No fluorescence; (+/−), faint fluorescence; (+), positive fluorescence; (++), strong fluorescence; (+++), very strong fluorescence.)

Day of individual serum isolation	Reactivity at 1/100		Reactivity at 1/1000		Reactivity at 1/2000		Reactivity at 1/4000	
	(NI)	(I)	(NI)	(I)	(NI)	(I)	(NI)	(I)
Day 0								
Serum 1	+	++	+/−	+/−	−	−	−	−
Serum 2	++	++	+/−	+/−	−	−	−	−
Serum 3	++	++	+/−	+/−	−	−	−	−
Day 3								
Serum 1	+	+++	+/−	+/−	−	−	−	−
Serum 2	++	++	+/−	+	−	+/−	−	−
Serum 3	++	+++	+/−	++	−	+	−	+
Day 10								
Serum 1	+	++	−	+	−	+	−	+
Serum 2	+++	+++	+/−	++	−	++	−	++
Serum 3	+++	++	−	+	−	+	−	+
Day 17								
Serum 1	−	+	−	+	−	+	−	+/−
Serum 2	++	+	+/−	+	−	+	−	+/−
Serum 3	++	+	−	+	−	+	−	+/−
Day 24								
Serum 1	N.D.	+	N.D.	+/−	N.D.	+/−	N.D.	−
Serum 2	N.D.	+/−	N.D.	−	N.D.	−	N.D.	−
Serum 3	N.D.	+/−	N.D.	−	N.D.	−	N.D.	−
Day 31								
Serum 1	N.D.	+/−	N.D.	−	N.D.	−	N.D.	−
Serum 2	N.D.	+/−	N.D.	−	N.D.	−	N.D.	−
Serum 3	N.D.	+/−	N.D.	−	N.D.	−	N.D.	−

N.D., Not done.

Protection is associated with a strong antibody boost to LS, but not to sporozoite stages, in protected animals

Pre- and post-challenge titres of IgG antibodies to *P. berghei* sporozoite and liver stages were assessed in sera collected at various time-points from protected (12 krad dose group) and non-protected (20 krad dose group) animals from separate groups of 5 immunized mice. Antibody titres were determined on serum samples pooled for every time-point (Fig. 1 A and 1 B).

Post-immunization, pre-challenge samples showed that in both 12 and 20 krad immunized mice substantial and similar levels (titres of 1/100) of antibodies to liver-stage parasites were present. Fluorescence was uniformly distributed on the parasite inside the cell. However, as soon as the third day post-challenge, the whole IgG titres in sera from the 12 krad dose group, but not in the 20 krad dose group, rose sharply to 1/2000. There was a further rise in whole IgG titres to 4000 on day 10 post-challenge, and therefore titres decreased progressively from days 17–24 and 31 post-challenge, when the study was stopped. Levels of whole IgG in the 20 krad dose group remained unaffected at 100 throughout the test period. Finally control, unimmunized mice had no antibodies of *P. berghei* liver stages before challenge. After challenge, only low borderline positive titres of 1/10 were scored. In contrast to these observations, antibody titres to sporozoite stage were at a similar level in both 12 and 20 krad dose-immunized mice throughout the study period. Pre-challenge titres were moderate (1/100), rose only to 1/160 on challenge in both non-protected and protected mice. Since this experiment was carried out with serum that was pooled at each time-point for the respective groups, it was repeated using groups of 3 mice each. In this second experiment, serum samples from individual mice were assayed for reactivity separately (Table 1). Each of the protected 12 krad dose-immunized mice developed antibody titres of 1/4000 to liver stages on day 10, whereas on the same days 20 krad dose mouse sera reached titres of 1/100.

IgG_{2a} and IgG_{2b} are the main isotypes produced

To analyse more thoroughly the major antibody boost related to protection, the levels of 5 antibody classes were determined by IFAT using isotype-specific mAbs. As shown in Fig. 1, IFAT was carried

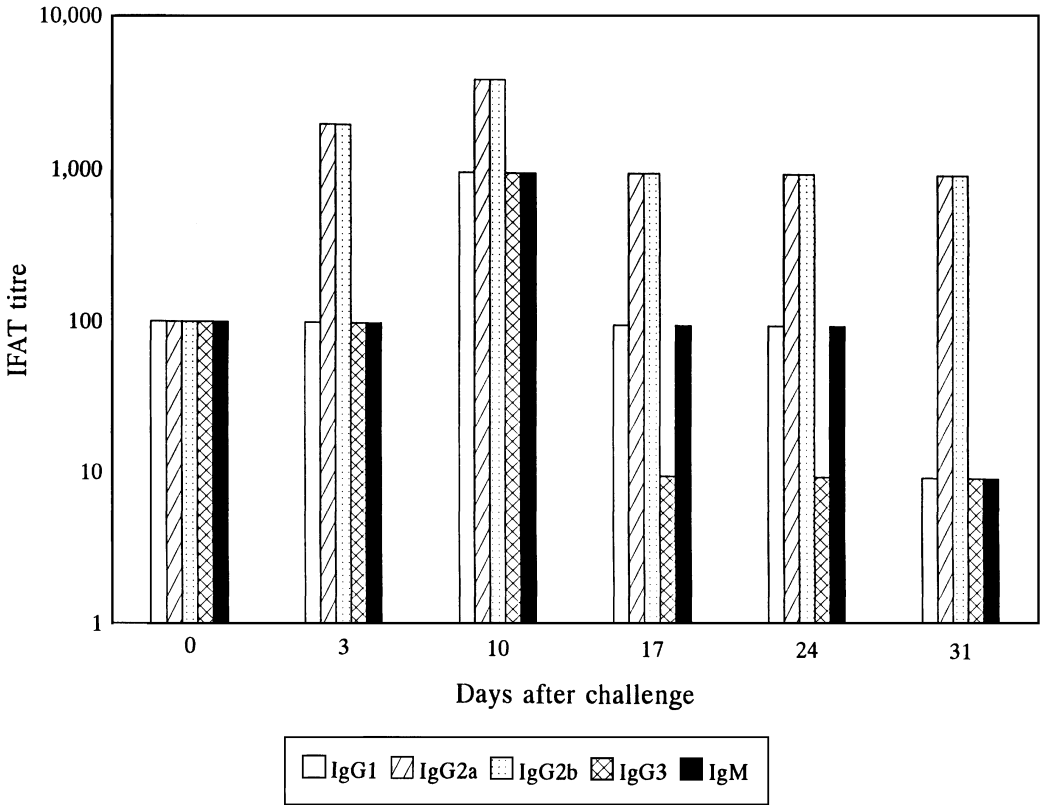

Fig. 2. Titres of IgG subclasses and IgM to liver stages as determined before and after challenge in 12 krad-irradiated sporozoite immunized C57BL6 mice. As in Fig. 1 serum samples collected from mice of the same group were pooled together at each time-point.

Table 2. The ISI and ILSD activity *in vitro* of sera, generated in C57BL6 mice immunized with irradiated sporozoites and subsequently challenged with live sporozoites

(Results show the percentage inhibition as well as the number of parasites counted in duplicate wells. In control wells using normal mouse serum a total of 239 liver schizonts were calculated at 48 h of maturation (2·4% conversion into normal liver forms from sporozoites).)

Sporozoite irradiation dose and day of sera collection:	Inhibition of sporozoite invasion at: 1/100 sera dilution	Inhibition of liver-stage dev. at: 1/100 sera dilution	1/500 sera dilution	1/2500 sera dilution	1/12500 sera dilution
12 krad					
Day 0	15% (222, 184)	80% (50, 63)	76·6% (50, 62)	40·2% (112, 174)	15·5% (191, 213)
Day 10	11% (205, 219)	81% (53, 55)	84·1% (22, 54)	71·6% (59, 77)	33·1% (112, 208)
20 krad					
Day 0	17% (220, 176)	78% (47, 57)	17·7% (175, 219)	17·3% (187, 209)	N.D.
Day 10	9% (194, 240)	55% (99, 115)	8·2% (207, 231)	5·2% (211, 241)	N.D.

out on serum samples that were pooled from the 5 animals in each group at every time-point (Fig. 2). Post-immunization, pre-challenge samples showed a homogeneous distribution of responses among the 4 IgG subclasses and IgM. In contrast the strong antibody boost occurring early on day 3 post-challenge was principally of IgG_{2a} and IgG_{2b}, and was followed on day 10 by a further more moderate

rise in IgG_1, IgG_3 and IgM. This experiment was repeated several times to establish the consistency of the results.

Antibodies elicited by immunization and by challenge strongly inhibit LS development

The potential biological effect of the antibodies produced was estimated by assessing the effect of sera collected at various time-points upon either sporozoite invasion or intra-hepatic liver-stage development in the Hep G2 cells system *in vitro*. In agreement with our previous studies (Chatterjee *et al*. 1996), using post-immunization samples (day 0), only a very low, borderline significant inhibitory effect was seen upon sporozoite invasion, whereas a strong inhibition was obtained when adding the sera after invasion, i.e. upon liver-stage development. Moreover, the same proved true for post-challenge samples in which, despite the rise in specific antibodies, invasion inhibition increased only slightly whilst inhibition of liver-stage development reached high values.

Further studies were aimed at analysing the differences between protected and non-protected animals and the dose effect over a wide range of dilutions. Results (Table 2), show a clear-cut difference between 12 and 20 krad dose-immunized mice. In the sera of protected mice an inhibition of liver-stage development was still detectable up to a titre of 1/2500 whereas in sera from non-protected mice it was detectable only at a titre of 1/100. Moreover in protected mice a dose-dependent effect was seen in both day 0 and day 10 samples, although this was more pronounced at day 10, in agreement with the immunological events described above. Since it has been observed that lipidic components in sera can be rapidly oxidized and exert a toxic effect upon cultured cells, the assays were repeated following delipidation of sera by Freon treatment. Delipidated sera (day 10 samples) yielded identical inhibitory effects as whole sera (not shown). In a separate experiment 3D11 (monoclonal antibody to CS protein repeats) was added at a concentration of 50 μg/ml to Hep G2 monolayers. While a high inhibition of sporozoite invasion activity was noted in the presence of 3D11 (93%), the corresponding inhibition of liver-stage development was low (5%).

DISCUSSION

Antibody boost observed after live sporozoite challenge of immunized mice

Our study shows a phenomenon which, to our knowledge, has not been described to date: a major antibody boost after challenge. It is an intense phenomenon since titres increase by 40 times. Pre-challenge sera from irradiated sporozoite immunized C57BL6 mice showed reactivity to young 24 h hepatic schizonts up to titres of 100, whereas after challenge the IgG titres to the same rose to 4000. This antibody boost was triggered by a minimal amount of protein derived from as few as 100 challenge sporozoites. This is in sharp contrast to the much larger number of sporozoites that are usually used for experimental immunization purposes. The antibody boost was induced only by live parasites and was very fast appearing on day 3 post-challenge, hence a secondary type of response, suggesting that irradiated sporozoites mainly prime T-helper cells. The antibody response involves IgG, particularly the IgG_{2a} and IgG_{2b} antibody isotypes, and is a transient phenomenon since antibody titres decreased rapidly in the following month (which may explain why it has not been described previously). Finally, this boost is directed to antigens which are expressed only at the liver not at the sporozoite stage, stressing again the importance of liver-stage antigens. However, it is not clear whether or not these boosted anti-liver stage antibodies contribute to protection *in vivo*.

Role of antibodies in protection

In the rodent model, the inhibition of sporozoite invasion (ISI) (Hollingdale *et al*. 1982) and the inhibition of liver-stage development (ILSD) (Ferreira *et al*. 1986) are the 2 *in vitro* assays known to correlate with protection by antibodies from sporozoite challenge.

Our results show that even at a dilution of 1/2500, 12 krad irradiated sporozoites induce antibodies that block liver-stage development *in vitro*, whereas those from 20 krad irradiated sporozoites do not. In a related study done by Krzych *et al*. (1995) recall T cell responses in irradiated sporozoite-immunized volunteers to liver-stage and blood-stage antigens were observed, but not to sporozoite antigens. This result suggested that liver-stage antigens are expressed by irradiated sporozoites and are thus potentially boostable by live sporozoite challenge. The difference in recall responses also indicates that liver-stage antigens are processed differently from sporozoite antigens, and our findings further confirm this since in our experiments live sporozoite challenge did not boost anti-sporozoite antibodies.

Whether this antibody boost accompanies other types of immune responses which are responsible for protection, and hence is only a marker of protective immunity, or constitutes an effector arm of defence itself, cannot be yet determined. *In vitro* experiments suggest that antibodies may play a role in protection since antibodies from protected animals show more inhibition of live-stage development even at high sera dilutions, than those from non-protected animals. Passive transfer of monoclonal antibodies to *P. berghei* circumsporozoite (CS) protein has been reported to confer partial protection against spo-

rozoite challenge (Yoshida *et al*. 1980). However, sera from mice immunized with irradiated sporozoites have not been reported to date to passively protect naïve mice. Taken together our results suggest that in the so called irradiated sporozoite vaccine, it may well be the antigens expressed when the parasite lies within the hepatocytes that are triggering specific immune responses which are boosted following live sporozoite challenge and may play a role in the induction of protection.

The authors are grateful for the excellent technical assistance of Anne Correwyn and Chantal Van Overmeir. Irradiation facilities were obtained with the help of Bob Schaeken of the Middelheim Hospital. Antwerp. This study was funded by the EC project entitled 'Selection of *P. falciparum* genes for mpes vaccine development', contract number: IC18-CT95-0016 (DG12-SNRD).

REFERENCES

CHATTERJEE, S., FRANÇOIS, G., DRUILHE, P., TIMPERMAN, G. & WERY, M. (1996). Immunity to *Plasmodium berghei* exoerythrocytic forms derived from irradiated sporozoites. *Parasitology Research* **82**, 297–303.

DRUILHE, P. & MARCHAND, C. (1989). From sporozoite to liver stage: the saga of the irradiated sporozoite vaccine. In *New Strategies in Parasitology* (ed. McAdam, K. P. W. J.), pp. 39–48, Churchill Livingstone, Edinburgh.

FERREIRA, A., SCHOFIELD, L., ENEA, V., SHELLEKENS, H., VAN DER MEIDE, P., COLLINS, W. E., NUSSENZWEIG, R. S. & NUSSENZWEIG, V. (1986). Inhibition of development of exoerythrocytic forms of malaria parasites by gamma interferon. *Science* **232**, 881.

HOLLINGDALE, M. R., ZAVALA, F., NUSSENZWEIG, R. S. & NUSSENZWEIG, V. (1982). Antibodies to the protective antigen of *Plasmodium berghei* sporozoites prevent entry into cultured cells. *Journal of Immunology* **128**, 1929–1930.

HOLLINGDALE, M. R., LELAND, P. & SCHWARTZ, A. L. (1983). *In vitro* cultivation of the exoerythrocytic stage of *Plasmodium berghei* in a hepatoma cell line. *American Journal of Tropical Medicine and Hygiene* **32**, 682–684.

KRZYCH, U., LYON, J. A., JAREED, T., SCHNEIDER, I., HOLLINGDALE, M., GORDON, D. M. & BALLOU, W. R. (1995). T lymphocytes from volunteers immunized with irradiated *Plasmodium falciparum* sporozoites recognise liver and blood stage malaria antigens. *Journal of Immunology* **155**, 4072–4077.

LE RAY, D. (1975). Structures antigéniques de *Trypanosoma brucei* (*Protozoa*, *Kinetoplastida*). Analyse immuno electro-phorétique et étude comparative. *Annals of the Belgian Society of Tropical Medicine* **55**, 129–311.

NARDIN, E. H. & NUSSENZWEIG, R. S. (1993). T cell responses to pre-erythrocytic stages of malaria. *Annual Review of Immunology* **11**, 687–727.

NUSSENZWEIG, R. S., VANDERBERG, J., MOST, H. & ORTON, C. (1967). Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei*. *Nature*, *London* **216**, 160–162.

NUDELMAN, S., RENIA, L., CHAROENVIT, Y., YUAN, L., MILTGEN, F., BEAUDOIN, R. L. & MAZIER, D. (1989). Dual action of antisporozoite antibodies *in vitro*. *Journal of Immunology* **143**, 996–1000.

SCHOFIELD, L., VILLAQUIRAN, J., FERREIRA, A., SHELLEKENS, H., NUSSENZWEIG, R. S. & NUSSENZWEIG, V. (1987). Gamma interferon, CD8+ T cells and antibodies required for immunity to malaria sporozoites. *Nature*, *London* **330**, 664–666.

YOSHIDA, N., NUSSENZWEIG, R. S., POTOCNJACK, P., NUSSENZWEIG, V. & AIKAWA, M. (1980). Hybridoma produces protective antibodies directed against the sporozoite stage of malaria sporozoites. *Science* **207**, 71.

ZAVALA, F., HOLLINGDALE, M. R., SCHWARTZ, A. L., NUSSENZWEIG, R. S. & NUSSENZWEIG, V. (1985). Immunoradiometric assay to measure the *in vitro* penetration of sporozoites of malaria parasites into hepatoma cells. *Journal of Immunology* **134**, 1202–1205.