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STUDIES ON THE INFECTIVITY OF *PLASMODIUM BERGHEI* SPOROZOITES IN EXPERIMENTAL HOSTS

by

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Summary — Sporozoites of *P. berghei* are released in A. stephensi between the days 21 and 28 after the infective blood meal, from two oocysts populations displaying a different growth rate. Sporozoites are, as a consequence, more numerous in the salivary glands at day 28 than at day 21, and their infectivity remains practically unchanged up to the 30th day.

C57 Black mice are at least 7 times more receptive to sporozoites than TB, OF1 and CBA mice. Their susceptibility is about 80 percent of that of the *Thamnomys*. Other species of wild rodents like *Pelomys* and *Aethomys* are as susceptible as *Thamnomys*.

Inoculation in a mesenteric vein increases about four times the concentration of schizonts in the liver as compared with the inoculation in a vein of the tail.

A decrease in the number of EE schizonts produced by a standardized inoculum of sporozoites was observed after injection of cyclophosphamide, in the presence of acute parasitemias with *P. berghei* or *T. evansi* or after immunization with sporozoites. On the other hand chronic infections with *T. gambiense* had no influence.

Changes in the liver conditions like the presence of numerous eggs of *Hepaticola*, fatty degenerescence or previous portal vein ligature were found to have no influence on the number and growth of schizonts.

KEYWORDS : *Plasmodium berghei*; Sporozoites; Infectivity; Experimental Hosts

1. Introduction

The study of the host-parasite relationship is of particular interest in the epidemiology of malaria. It is only after 5 to 10 years of uninterrupted contact with the parasite that the protective immunity becomes effective in endemic areas. This long delay in the mounting of protective immunity in natural infections might be due to the occurence of antigenic variation of the erythrocytic stages as has been demonstrated in animal models by Brown, Brown and Hills, 1968, Voller and Rossan, 1969, Wéry and Timperman, 1979.

This also means that the sporozoites continue to enter the host organism and hepatic cells with little interference from the host immune mechanism. It seems that antisporozoite antibodies are present at high levels in adults only (Nardin *et al.*, 1979). Indeed, Orjih and Nussenzweig (1979) demonstrated the inhibitory effect of an acute blood stage infection, common in children, on the synthesis of antisporozoite antibodies.

The search for factors that would prevent the sporozoite penetration into the livercells is therefore important, but needs the design of a precise experimental model for tracing the fate of the sporozoites in the vertebrate host.

In natural conditions, the sporozoites are transferred from the salivary glands of the mosquito straight into the blood or subcutaneous tissues of the vertebrate host where they somehow manage to find their way towards the parenchymal cells of the liver. Little is known however about the efficiency of the transfer (Vanderberg, 1977).

In epidemiological studies (Pull. 1976), the actual inoculation rate (h2) is calculated from the values of m (the anopheline density), a (the frequency of human blood meals), s (the sporozoite index) and b (the proportion of infected anopheles which succeed in establishing an infection).

In contrast to m, a and s, it is not possible to calculate b from entomological surveys and h2 must as a consequence be drawn from parasitological surveys, i.e. the observation of the number of new infections per unit of time produced in a cohort of newborn children. The combined approaches allow an estimation of b depending on the number of viable sporozoites present in a single infected bite.

In an experimental model, however, standardization of the parameters is possible as the infectivity of a suspension of sporozoites can be estimated either by the minimal number necessary to infect all the recipients or by histological examination of liver sections.

In the present work, the possibilities of standardization of the parasite- or host-dependent parameters were investigated. Rodent malaria parasites were chosen for that purpose. *P. berghei* sporozoites were studied in the anopheline host, harvested, injected in laboratory rodents and followed up to fully grown exoerythrocytic schizonts.

The following parameters were given special interest: (i) parasite-linked factors, like number and viability of the sporozoites matured in the body of the mosquito; (ii) host-dependent factors, like susceptibility of different species or strains of rodents (Most *et al.*, 1966; Vanderberg *et al.*, 1968; Vincke and Bafort, 1968; Wéry and Killick-Kendrick, 1967; Wéry, 1968), the immune status, concurrent infections, physiological conditions of the parenchymal cells, route of inoculation (Wéry and Killick-Kendrick, 1967).

The combined effects of all these factors will result in a relation between the number of sporozoites injected and the total number of schizonts developped in the liver.

2. **Material and methods**

2.1 *Investigations on the sporogonic cycle in* A. stephensi

Methods for maintenance of the *Plasmodium berghei - Anopheles stephensi* system have been previously described by Vincke, Bafort and Scheepers Biva (1966). Batches of more than 500 mosquitoes of the same age were infected on the same day on a homogeneous group of gametocyte carriers presenting a similar percentage of infected red blood cells sorted out amongst mice inoculated four days earlier.

From the tenth day onwards after the infective blood meal, samples of twenty five mosquitoes were removed from the cages every second day. Thoraces and abdomens were separated and crushed in

GLSH (*) with 10 percent foetal calf serum (Le Ray, 1975; Ngimbi *et al.*, 1979). Sporozoites were counted in suspension after filtration through a 14 μ pore size membrane.

2.2. *Preparation of sporozoite antigens*

Sporozoites were obtained from salivary glands dissected and crushed in GLSH with 10 percent foetal calf serum. After filtration through a 14 μ pore size membrane, drops of the parasite suspension were distributed on siliconed slides and air dried for 30 min. Slides were fixed with a 1 percent glutaraldehyde solution in PBS for 30 minutes (modified from Nardin *et al.*, 1979). Dried slides were stored at $-$ 25 °C.

2.3. *Procedure of immunization*

Wistar rats were immunized by weekly exposure to the bites of about 100 infected mosquitoes, during three weeks (Verhave, 1975). A homologous challenge was given on the fourth week. Liver specimen were obtained at autopsy or through liver biopsy 45 hours after the challenge inoculation. Chloroquine treatment (25 mg per os per kg on days 1, 2 and 3) was given after each sporozoite inoculation to avoid development of blood forms. Serum samples obtained 45 hours after the last inoculation were tested for antibodies using the indirect immunofluorescence test (IFAT) (Nardin, Gwadz and Nussenzweig, 1979). C57 black mice were immunized using the same scheme during four weeks and the challenge was given on the fifth week.

2.4. *Route of inoculation*

Inoculation into the caudal vein versus inoculation and into a branch of the portal vein were compared. For intraportal injection, the abdominal cavity of the anaesthetized mouse was opened and the caecum exteriorized. Two loose silk sutures were applied on the caecal vein and 0.3 ml of the suspension was injected in about 20 seconds between the loosely fitting suture knots using a tuberculin syringe mounted with a 26 G needle. Before withdrawal of the needle the caecal vein was tied off, resulting in no or minimal blood loss.

The suspension of sporozoites (250,000 parasites in 0.3 ml per inoculum) in GLSH with 10 percent foetal calf serum was maintained in an ice bath until the inoculation.

In a second experiment we compared the intraportal and intracaudal routes using suspensions of sporozoites from the ice bath or brought to 37 °C before the injection.

(*) A medium containing glucose, lactalbumin, foetal calf serum and hemoglobine.

2.5. *The counting of exo-erythrocytic forms* (E. E. F.)

This was performed by examining 50 serial sections (4 μ thick) of standardized size, cut from the left lobe. However, the examination of 25 sections selected on the base of a 10 percent sample in a series of sections was found to give equally reliable results. The Giemsa colophonium technique was used for staining (Bray and Garnham, 1962). The distribution of the schizonts in the left lobe was found to be uniform (Table 1).

TABLE 1
Distribution of exo-erythrocytic forms (EEF) in three different samples of the left liver lobe *

Animal nr	EEF per cm² **			
	Sample 1	Sample 2	Sample 3	Average
1	4.82	3.68	4.84	4.35
2	0.80	1.45	0.98	1.05
3	8.92	9.25	9.56	9.25
4	5.23	4.91	5.62	5.24
5	6.69	8.16	11.07	8.87

* 45 hours after intravenous inoculation of 219,000 sporozoites.
** 25 sections examined in each sample

2.6. *Vertebrate hosts*

The animals used in the different experiments are listed below with their origin :

OF1 mice	IFA Credo, Oncins, France
CBA mice	Inserm, Lille, France
TB mice	UCL Woluwe, Belgium
C57 Black 6J mice	KUL, Leuven, Belgium
OFA rats	IFA Credo, Oncins, France
Wistar rats	KUL, Leuven, Belgium
Thamnomys sp.	trapped in Shaba (Zaire)
Pelomys sp.	trapped in Shaba (Zaire)
Aethomys sp.	trapped in Shaba (Zaire)

Laboratory young adult females were used whenever possible. Wild rodents were kept in the laboratory for 6 months before the inoculation of sporozoites. In some animals, total ligation of the portal vein was performed according to Cheever and Warren (1963).

2.7. *Sporozoites viability tests*

Sporozoite suspensions were obtained from dissected salivary glands crushed in GLSH medium by use of a teflon grinder and kept in ice until the time of inoculation.

Schizonts were counted in sections of livers taken 45 hours after inoculation. The surface of the sections was standardized.

2.8. *Immunofluorescence tests*

Antibodies were titrated in sera of rats and mice using the indirect immunofluorescence technique. Antirat and antimouse conjugates from Nordic Pharmaceuticals were used at a dilution of 1/40.

Sporozoite antigens were fixed with glutaraldehyde (Nardin and Nussenzweig, 1979), while blood forms antigens were made of unfixed infected blood smears.

3. **Results**

3.1. *Growth of the oocysts and production of sporozoites*

The number of sporozoites harvested from abdomen and thoraces at different moments during the infection in mosquitoes is expressed as the mean number of free sporozoites per mosquito (Table 2). The differences observed between the four experiments can be attributed to a difference in the initial level of infection caused by different batches of donor mice. However, mosquitoes may also reach different levels of infection if fed concurrently on the same gametocyte carrier (Rutledge *et al.*, 1970). The number of sporozoites in the abdomen reached a maximum between day 21 and day 30 after which it decreased steadily indicating a migration towards the salivary glands in the absence of new waves of maturing oocysts. These sporozoites originate from two distinct oocysts populations, as shown in Fig. 1 (experiments 2 and 3). At a temperature of 20 °C, a first group of oocysts reaches maturity around the twenty second day and a second group becomes mature between the days twenty seven and twenty nine.

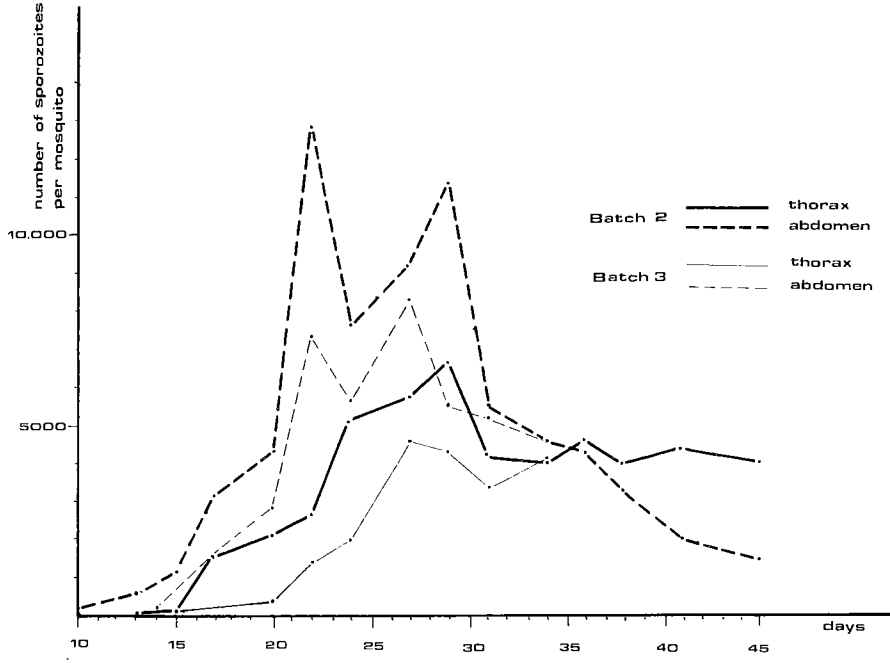

Figure 1.
Evolution of the number of sporozoites harvested from the abdomen and the thorax of *A. stephensi* (batches 2 and 3).

TABLE 2

Number of sporozoites produced during the sporogonic cycle (4 batches of mosquitoes)

Days after infective blood meal	Number of sporozoites per mosquito							
	Batch 1		Batch 2		Batch 3		Batch 4	
	abdomen	thorax	abdomen	thorax	abdomen	thorax	abdomen	thorax
10	—	—	3,000	0	—	—	—	—
13	—	—	5,600	280	—	—	—	—
14	—	—	—	—	2,200	800	—	—
15	13,340	2,520	11,280	1,240	—	—	—	—
17	—	—	31,680	16,320	—	—	—	—
20	—	—	43,320	21,420	29,680	3,640	—	—
21	58,800	41,800	—	—	—	—	87,580	62,640
22	—	—	128,800	26,520	72,800	13,520	—	—
24	—	—	76,160	51,520	56,560	19,980	—	—
27	—	—	92,560	56,680	82,800	46,200	—	—
28	—	—	—	—	—	—	62,720	36,720
29	—	—	113,880	67,080	55,180	43,200	—	—
30	40,020	18,900	—	—	—	—	—	—
31	—	—	55,640	42,120	52,080	34,320	—	—
34	—	—	46,280	40,040	45,600	41,400	—	—
35	—	—	—	—	—	—	46,800	54,880
36	—	—	43,200	46,280	—	—	—	—
38	30,720	11,000	33,600	40,200	11,400	19,800	—	—
41	—	—	20,400	44,400	—	—	—	—
45	—	—	15,680	40,200	—	—	—	—
50	3,440	20,000	—	—	—	—	—	—

Sporozoite counts in the thoraces do increase until the second population of oocysts becomes mature. After day thirty, a significant decrease is observed, followed by a long period of constant harvests, indicating that the sporozoites are stocked in the salivary glands and do not migrate anymore in significant numbers from the abdomen after the thirtiest day. A great number of abdomen sporozoites never reach the thorax.

3.2. *Effect of the age on the infectivity of salivary glands sporozoites*

The infectivity of sporozoites was tested in this experiment by injection into C57 Black mice and counting liver schizonts. Batches of 250,000 sporozoites were injected intravenously in each mouse. The schizonts counts were slightly higher when sporozoites were harvested around day 28 as compared with parasites taken on day 21 or 35 (Fig. 2). But even if the viability of sporozoites remains constant between the days 21 and 28, the harvest is more efficient on day 28, due to a considerable increase in numbers.

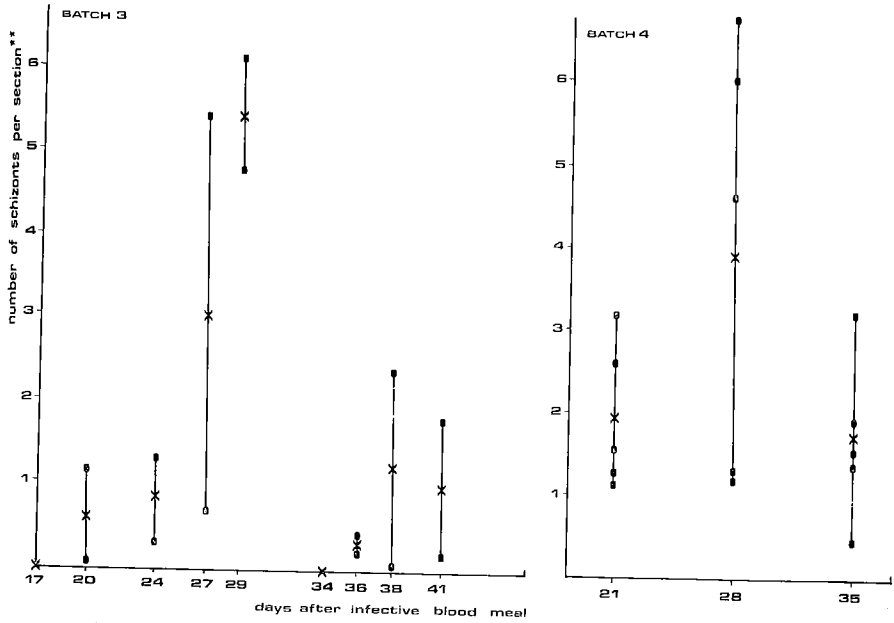

Figure 2.

Infectivity of sporozoites * from thoraces in relation to their age.

* 250,000 sporozoites injected intravenously in each C57 black mouse.

** Number of schizonts per section :

- ● individual results.
- × average.

Sporozoites obtained from crushed abdomens were found to induce always a parasitaemia in C57 Black mice when collected as early as day 13 of the infection in the mosquito. Before day 13, no infection was obtained. This shows that the mechanical disruption of maturing oocysts does produce a proportion of viable sporozoites.

3.3. *Susceptibility of different vertebrate hosts to* P. berghei *sporozoites*

In a series of experiments, differents species or strains of rodents were compared two or three at a time. These included : *Thamnomys*, *Aethomys* and *Pelomys* (trapped in the field in Shaba, Zaire); TB, OF1 and CBA white mice; C57, 65 Black mice, OFA rats (Table 3).

The susceptibility of the recipients was estimated by calculating the minimal number of sporozoites producing an infection in 100 percent of the animals or by counting EE schizonts.

TABLE 3

Susceptibility of vertebrate hosts to sporozoites

Animal species	Number of animals	Minimal number of sporozoites to induce 100 % infection	Mean number of liver schizonts counted in a surface of 10 mm² of sections * after I. V. inoculation of 100,000 sporozoites		
			Individual results (min.)	(max.)	Average
Thamnomys	6	—	15	15	10.8
Aethomys	6	—	3	20	10.2
Pelomys	4	—	3	12	8.6
C57 black mice	25	100	3	12	8.8
OFA rats	15	—	1	3	1.7
TB mice	30	1,000	1	3	1.5
OF1 mice	35	2,500	0.2	0.4	0.3
CBA mice	10	> 10,000	0	0.1	0.04

* 50 sections examined.

The most susceptible host was found to be the natural host *Thamnomys surdaster*. *Pelomys* and *Aethomys* were almost equally susceptible. Amongst usual laboratory bred rodents, C57 Black mice were by far the most susceptible. When compared to the latter, OFA albino rats. TB mice, OF1 mice and finally CBA mice were at least 7 times less susceptible to sporozoites.

3.4. *The route of inoculation*

A comparison of intravenous (intracaudal) and intraportal inoculations showed that a three fold increment of liver schizonts was obtained using

the latter route (Fig. 3). This tends to demonstrate that when more sporozoites are given the opportunity of coming into contact with the parenchymal cells of the liver, they enter in greater numbers.

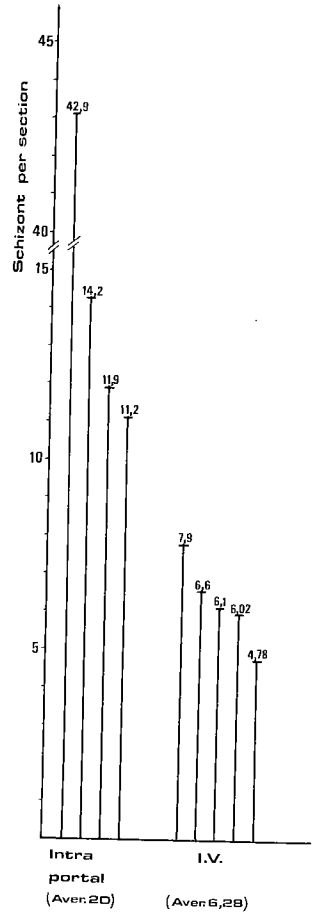

Figure 3.

Comparative results * obtained by different inoculation routes.
* 50 serial sections examined 45 hours after inoculation of 250,000 sporozoites in C57 black mice.

The temperature of the suspension of sporozoites (ice bath or 37 °C) prior to inoculation does not modify the results of the introportal inoculation (Fig. 4). Slowing down the metabolism of sporozoites by low temperature seems not to interfere with their capacity of attachment and penetration into a cell.

In the case of injection into the general circulation, preheated sporozoites are more successful. The hypothesis has been put forward that movements of sporozoites should make them capable of escaping agression by the macrophages.

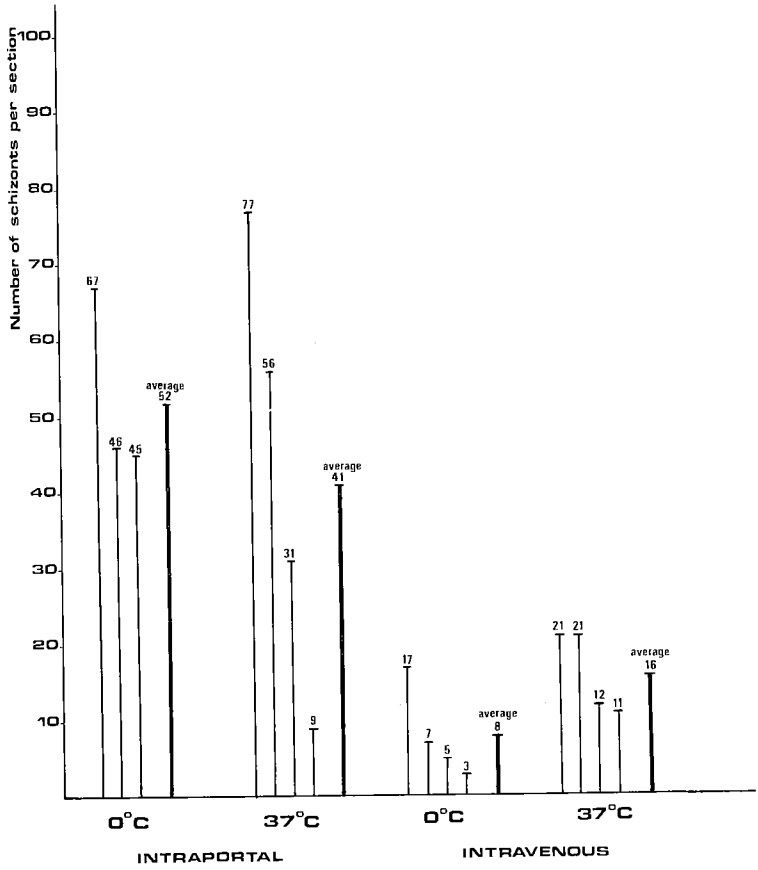

Figure 4.

Influence of the route of inoculation and the temperature of the sporozoite suspension *.
* 254,000 sporozoites per mouse (C57 black).

3.5. *Alterations of the host defence mechanisms*

3.5.1. *Non specific immunity in the recipients*

In a series of experiments, the influence of factors changing the resistance of C57 Black mice and of OFA rats was evaluated.

All animals were inoculated by intravenous (caudal) route, sacrificed after 45 hours and the histological sections of the liver examined. The results are shown in tables 4 and 5.

TABLE 4

Influence of the non specific host defences on the sporozoites *

Number of schizonts	Controls	Milk sub cutaneous	Cyclophosphamide intra peritoneal (80 mg per kg on days − 1 and 0)	Parasitaemia *P. berghei* (day 5 after inoculation)
Individual **	11.16 14.98	14.04 12.92	6.74 4.6	1.48 0 0.28
Average	13.00	13.48	5.67	0.59

* 390,000 sporozoites per animal, intravenous.
** 50 sections examined, 45 hours after inoculation in C57 black mice.

TABLE 5

Influence of the non specific defences on the sporozoites *

Number of schizonts	Controls	Cyclo-phosphamide intra peritoneal (20 mg per kg on days − 7 to − 2)	Splenectomised	Parasitaemia (day 5 after inoculation)	
				P. berghei	*T. evansi*
	5.12	3.5	3.22	0	0
Individual **	3.02	3.23	7.80	0	0.1
	3.64	1.33	5.90	0	0
					0.1
Average	3.93	2.69	5.64	0	0.05

* 216,000 sporozoites per animal, intravenous.
** 50 serial sections examined, 45 hours after inoculation in C57 black mice.

It appears that :

(a) Attemps of alteration of the reticuloendothelial cell activity by subcutaneous injection of milk on days − 1 and 0 has no effect, the number of schizonts being comparable to that of the controls.

(b) Intraperitoneal injection of cyclophosphamide (Endoxan^{R}) (daily − 10 to − 2) before the inoculation of sporozoites caused a slight decrease in the counts of schizonts. A more important decrease was observed when a dose of 80 mg per kg of cyclophosphamide was injected on days − 1 and 0.

(c) Concomitant parasitaemia (*P. berghei*) at the time of sporozoite inoculation caused a sharp reduction of the numbers of liver schizonts.

(d) Splenectomy caused on the contrary a slight increase in the counts.

(e) Acute parasitaemias with *T. evansi* produced the same effect as parasitaemias with *P. berghei.* The explanation probably lies in the increased activity of the non specific defense mechanisms in the course of an acute infection.

It should be added that five OF1 mice (not shown in the table) chronically infected with *T. gambiense* (2 to 3 months old infections) did not displayed the same phenomenon, as the number of EE schizonts was the same as in the OF1 controls. Parasitaemias were of course very low (10^5 trypanosomes per ml).

3.5.2. *Specific immunity in the recipients*

In four weeks old rats immunized with sporozoites by means of the bite of 100 infected mosquitoes repeated three times at weekly intervals, we failed to demonstrate any liver schizont after a challenge of another hundred infective bites (Table 6).

Schizonts were seen in the non immunized control rats (nr. 6 and 7).

For the IFAT titers, two levels of fluorescence were considered : presence of a fine green layer outlining the sporozoites (+) and strong fluorescence (+++).

TABLE 6

EEF counts in the liver and titer of antibodies in Wistar rats after immunization by mosquito bites

Nr rat	Immunizing bites * week 1	week 2	week 3	Challenge week 4	EEF per section 45 hours after last inoculation	IFAT titers (serum taken at week 4) + fluor.	+++ fluor.
				INFECTIVE BITES			
1	×	×	×	×	0	1 : 10,240	1 : 2,520
2	×	×	×	×	0	1 : 10,240	—
3	×	×	×	×	0	1 : 5,120	1 : 1,280
4	×	—	—	—	0.24 (biop.)	1 : 640	1 : 80
5	×	—	—	—	0.02 (biop.)	1 : 640	1 : 80
6	—	—	—	×	1.56 (0-3)	Neg.	Neg.
7	—	—	—	×	0.21 (0-1)	Neg.	Neg.
				NON INFECTIVE BITES			
8	×	×	×	×	—	Neg.	Neg.
9	×	×	×	×	—	Neg.	Neg.

* Followed by chloroquine treatment.

One plus titers reached up to 10,000 in the immunized animals (rats nr 1 to 4). The serum of the rat nr 2 did not display a three crosses fluorescence but reached the same extinction titer as the other sera, suggesting the possibility of two types of surface antigen.

The same sera were also weakly positive against blood forms antigens (Table 7), but this is due to a transient parasitaemia appearing during sporozoite immunization in spite of the chloroquine treatment. On the other hand, sera of mice infected with blood parasites did not react with fixed sporozoites.

TABLE 7

Immunofluorescence titers obtained using antisera against sporozoites or blood forms

Sera *	Antigens **	IFAT titers (+++ fluor.)
Anti sporozoite	Sporozoite D21	1 : 2,560
	Sporozoite D28	1 : 1,280
	Sporozoite D35	1 : 2,560
	B. F.	1 : 80
Anti B. F.	Sporozoite D21, 28, 35	Neg.
	B. F.	1 : 640

* Antisporozoite antiserum of mouse nr 1 (table 6). Pool of anti-bloodforms antisera from ten mice immunized by four weekly injections of 10^6 infected red blood cells followed by chloroquine treatment given on days 4, 5 and 6.

** Sporozoites harvested from salivary glands on day 21, 28 or 35 after infective bloodmeal. Fixed with glutaraldehyde.
B. F. unfixed smears of infected blood.

Titers are much lower with sera from animals that received infected bites on one occasion only four weeks earlier (table 6, rats nr. 4 and 5). Sera of control animals (table 6, rats nr. 8 and 9), bitten by uninfected mosquitoes remained completely negative.

As shown in table 7, sporozoites harvested on days 21 to 35 of infection in the mosquitoes were compared as antigens. No difference was observed in the titers obtained.

3.5.3. *Comparison of protection afforded by specific and non specific immunity in C57 Black mice*

Immunization with sporozoites and presence of patent parasitaemia in the recipient hosts both cause an important decrease in EE forms counts. In our experiment however (table 8) stage specific immunity obtained by repeated inoculation of sporozoites is much more efficient than non specific immunity caused by the presence of blood parasites even when the challenge is injected into the portal vein. This observation shows that both specific and non specific immunity exert their effect mainly at the level of the liver.

Sterile immunity was not obtained in this experiment after the four immunizing inocula of sporozoites, since one mature schizont was found 45 hours after intravenous challenge in one of the immunized animals (Table 8).

TABLE 8

Immune status of C57 black mice and volume of the suspension inoculated in the caudal or portal vein (number of schizonts per section)

	Controls	Animals immunized with sporozoites *	Parasitaemic animals **	Clean animals
	288,000 sp. 0.2 ml GLSH	288,000 sp. 0.2 ml GLSH	288,000 sp. 0.2 ml GLSH	576,000 sp. 0.4 ml GLSH
			INTRAPORTAL INOCULATION	
Individual results	25.30	0	6.97 (1) **	65.33
	22.49	0.93	16.33 (2) **	—
	21.62	0.12	1.00 (3) **	—
	15.45	0.07	—	—
	11.36	0.86	—	—
	37.31	—	—	—
Mean nr.	22.26	0.40	7.90	—
			INTRAVENOUS (CAUDAL) INOCULATION	
Individual results	2.43	0	1.73 (4) **	4.68
	2.94	0	0.89 (5) **	—
	1.41	0	—	—
	3.78	0	—	—
	3.07	0.04	—	—
	1.42	—	—	—
Mean nr.	2.51	0.01	1.31	—

* Four weekly immunizing inocula (bite of mosquitoes) followed by treatment with chloroquine.
** Percentage of infected R. B. C. :

	(1)	(2)	(3)	(4)	(5)
D0 (inoculation)	0.3	0.46	0.4	0.33	Neg.
D2 (sacrifice)	2.9	2.1	3.3	5.3	0.4

3.6. *Alterations of the physiological status of the liver in regard to the susceptibility to sporozoites of the parenchymal cell*

Ligation of the portal vein produces an arterialization of the liver and an increase oxygen tension (Cheever and Warren, 1963).

C57 Black mice ligated a fortnight earlier were compared with intravenous and intraportal controls. Tthe number of EE schizonts and their mean size was the same in the I. V. groups (table 9).

TABLE 9

Effect of total portal ligation on the development of EE schizonts *

	Mouse nr.	Number of schizonts per section	Diameter ** Min.	Max.	Mean	S
	4648	3.83	23.7	38.1	30.23	3.37
	4649	0.75	22.3	30.2	27.14	4.04
Ligated animals	4651	9.21	23.6	38.1	30.19	3.38
(15 days after total	4652	8.45	25	34.2	29.07	2.64
ligature of portal vein)	4653	5.04	22.3	34.2	27.54	2.82
	4655	3.80	23.6	32.8	28.74	2.32
	4656	1.86	22.3	30.2	26.3	1.72
	4657	2.67	24.3	34.2	28.95	2.68
Group average	—	4.50	—	—	27.45	—
Unoperated	4660	6.12	24.3	38.1	30.9	3.53
intravenous controls	4662	6.63	23.6	35.5	30.28	2.28
	4664	7.94	29.6	40.1	32.25	3.79
Group average	—	6.90	—	—	31.4	—
Intraportal controls	4666	14.20	27.5	36.8	31.74	2.35
	4667	42.96	26.3	38.8	32.35	2.48
	4668	11.94	24.5	36.8	31.14	3.74
	4669	11.20	18.4	27	23.17	1.98
Group average	—	20.08	—	—	29.6	—

* After IV inoculation of 300,000 sporozoites in C57 black mice.

** 20 schizonts measured (two diameters).
Minimum : schizont with smallest mean diameter.
Maximum : schizont with greatest mean diameter.
Mean : mean diameter of measured schizonts.
S : standard deviation.

Wild rodents naturally infected with *Hepaticola hepatica (Capillaria)* harbour numerous eggs of the helminth in their liver. After intravenous inoculation of sporozoites, it was observed that the numbers and the morphology of the EE schizonts were comparable to non infected rodents. Small areas of intact liver parenchyma harboured schizonts concentrated in such places (Fig. 5).

One *Aethomys* was found to present a fatty degenerescence of the liver parenchyma and yet, normal schizonts were found in the liver (Fig. 5).

4. **Discussion**

4.1. *Evolution of sporozoites in the invertebrate host*

A considerable number of sporozoites released in the abdomen by oocysts fail to migrate to the thorax and may be seen in the hemolymph and in the hemocytes of the abdomen (Sinden, 1978). Furthermore, mature oocysts often release their sporozoites directly into the tissues or in the lumen of the midgut (Beaudouin, Strome and Tubergen, 1974). All these sporozoites should be unable to migrate towards or at least to penetrate in the salivary glands (Sinden, 1978).

In our experiments, two distinct oocysts populations are clearly observed on the base of the growth rate. Delayed maturation of oocysts may be due to intracellular localization. Oocysts emprisonned in a cell of the midgut wall are often observed (Bafort, 1971; Beaudouin *et al.*, 1974; Vanderberg *et al.*, 1967) and this may result in a later release of the sporozoites.

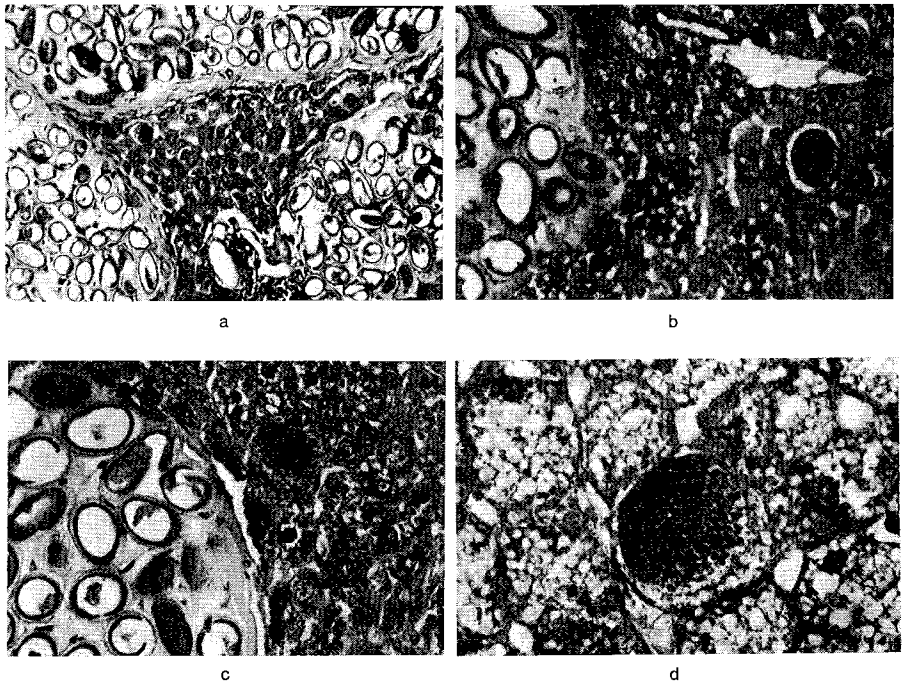

Figure 5.
45 hours preerythrocytic schizonts in the liver of rodents trapped in Shaba after inoculation of *P. berghei* sporozoites.
(a, b, c) Liver of *Aethomys* parasitized with *Hepaticola* (*Capillaria*) *hepatica* :
(a) magnification 100 ×.
(b, c) magnification 200 ×.
(d) Liver cells of Pelomys in fatty degenerescence, magnification 500 ×.

These late sporozoites show however, in our experience, the same infectivity as the others : the sporozoite content of the salivary glands increases between day 14 and day 28 and the proportion of infective parasites remain the same in the suspensions so that the total number of viable sporozoites do increase until day 28.

Later, the infectivity of the sporozoite suspensions was found to decrease slowly (Fig. 2) as Porter, Laird and Dusseau (1974) also reported.

The development of the infectivity of sporozoites is best achieved in the salivary glands, a selection being possibly made by the migration capability of the parasites. However, sporozoites of *P. berghei* have been found infective in the hemolymph of the abdomen and even in drops of blood excreted from the hindgut of the mosquito (Beaudouin *et al.*, 1974) so that Vanderberg (1975) assumed that the development of infectivity is time dependent rather than site dependent.

4.2. *Susceptibility of the vertebrate host to sporozoites*

The susceptibility of mice to sporozoite induced infections may greatly vary from one strain or species to another (Bafort, 1971, Nussenzweig *et al.*,

1966, Vanderberg *et al.*, 1968). It is known that the receptivity of the liver cell to sporozoites and of the red blood cell to merozoites are not always associated (Bafort, 1971).

We found that C57 Black 6J mice which were the most susceptible amongst currently available laboratory hosts to sporozoites, were also the most resistant to blood infection, their survival time after inoculation being the longest. The high « natural killing (NK) » activity is probably one of the factors responsible for this innate resistance (Eugui and Allison, 1979).

Hosts completely refractory to blood induced infections with *P. yoelii* as the guinea pig or the rabbit were shown to harbour pre-erythrocytic schizonts in their liver cells (Wéry, Killick-Kendrick and Bray, 1968). However the size of these schizonts was smaller than the corresponding stages in mice.

4.3. *Route of inoculation*

The influence of the route of inoculation has been previously studied (Wéry and Killick-Kendrick, 1967; Ngimbi *et al.*, 1979). In non immune recipients hosts, the proportion of successful sporozoites is remarkably constant after intravenous or intraperitoneal inoculation, considering their spreading through the whole body of the animal. This suggests a strong tropism of the parasite towards the liver.

Intraportal inoculation forces the sporozoites through the liver before the spreading occurs. Higher counts of preerythrocytic schizonts show that a majority of sporozoites establish themselves in a parenchymal cell, when first passing through the liver. It has been shown recently (Danforth *et al.*, 1980) that sporozoites actively penetrate into macrophages and that inactivated parasites fail to become interiorized. Moreover, the role of macrophages including Kupffer cells in clean animals as transporting agents of sporozoites towards the parenchymal cell was suggested by Verhave *et al.* (1980). Activated macrophages in immunized animals would remove and destroy the parasites.

It is known that sporozoites are best kept in vitro at low temperatures (0-4 °C). However, this condition could inhibit important functions such as the motility in an actively infective parasite stage (Vanderberg, 1974). The present results show however that the temperature of the sporozoites suspension does not influence the success of intraportal inoculation. This adds to the hypothesis of the sporozoite playing no active role in cell interiorization.

4.4. *Effect of specific and non specific immune defenses*

The immunosuppressive effect of cyclophosphamide (CY) on blood induced infections was shown to be both specific and non specific (Wells, Diggs and Phillips, 1977; Endardjo *et al.*, 1978).

Herman and Shiroishi (1973) observed higher initial parasitaemias with *P. gallinaceum* after sporozoite — and cryptozoite — induced infections in CY treated chickens, and concluded to a suppression of the natural (innate) immunity by the treatment.

On the contrary, a cytotoxic effect of CY on the sporozoite or the growing pre-erythrocytic parasite was demonstrated in the present work.

These contradictory results might be due to differences in the administration schemes as already observed by Spira *et al.* (1972) and Endardjo *et al.* (1978).

The increase in the number of schizonts in splenectomized animals was not significant. The spleen does not seem to play an important role in the elimination of circulating sporozoites. This has already been observed by Garnham and Bray (1956), Bray (1957), Bafort (1969, 1971) and Verhave *et al.* (1980). However, the reduction of the circulating volume in the absence of spleen might help the sporozoites to reach the liver in greater number.

The strong inhibitory effect on the establishment of the sporozoites in the liver observed in parasitaemic mice infected with *P. berghei* or *T. evansi* confirms the results of Verhave (1975). The mechanism of this inhibition was discussed at length by the same author (Verhave, 1975; Verhave, Strickland and Jaff, 1977; Verhave *et al.*, 1980) : the production of interferon during a phase of rising parasitaemia could not be demonstrated; antibodies against bloodforms did not interfere with the infectivity of sporozoites; on the contrary, a loss of enzymes (ATP-ase and AMP-ase) by the host cell during an acute infection could account for the failure of interiorization of sporozoites or development in parenchymal cells; natural killer cells could also be involved but the major factor is probably and increased aspecific phagocytic activity.

Indeed, increased activity of macrophages triggered by specifically sensitized lymphocytes was also demonstrated in other acute parasitic infections as *Toxoplasma gondii* (Omata *et al.*, 1979), *Trypanosoma* (Clayton, 1979) and *Babesia* (Verhave *et al.*, 1980).

Moreover significant decrease of EE schizonts was demonstrated in late pregnancy. During that period, the increased oestrogen levels in the blood are known to have a macrophage activating effect (Verhave *et al.*, 1980).

The mechanism of increased numbers of macrophages in the liver (Verhave, 1975) might be the same as described by Wyler and Gallin (1977) : a chemotatic factor, presumably derived from lymphocytes during the development of parasitaemia would cause the trapping of blood monocytes in the organs of the reticulo-endothelial system.

Comparison between the results of intraportal and intravenous (caudal) inoculation suggest that in addition to the important trapping of sporozoites in the general circulation, a local trapping occurs in the liver of both immunized and parasitaemic animals.

Surprisingly in animals infected chronically with *T. gambiense* no inhibitory effect was observed on the sporozoite establishment, but in this case, the immune mechanisms are known to be progressively exhausted by the continuous challenge of antigenic variation (Greenwood *et al.*, 1973).

4.5 *Changes in the structure or the metabolism of the liver*

Important alterations of the liver parenchyma such as the presence of numerous eggs of *Capillaria hepatica*, the increased arterial blood availability by portal ligation or fatty degeneration have been found in the present work to have no influence on the fate of injected sporozoites or

on the growth of exo-erythrocytic schizonts. Landau however (1973) obtained a delayed growth of the schizonts in animals placed in a 15 °C environment showing the very high susceptibility of this parasite stage to environmental factors.

In the same line, fatty degeneration induced by ethionine, was found by Dunn *et al.* (1972) to seriously impair the development of the schizonts, which is in contrast to present results.

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Etude de l'infectivité des sporozoïtes de *P. berghei* chez des hôtes expérimentaux.

Résumé — Les sporozoïtes de *P. berghei* sont libérés chez *A. stephensi* entre le 21e et le 28e jour après le repas infectant, à partir de deux populations d'oocystes dont la vitesse de croissance est différente. Les sporozoïtes sont donc retrouvés en plus grand nombre dans les glandes salivaires le 28e jour que le 21e jour. Leur infectivité reste inchangée jusqu'au 30e jour.

Les souris C57 Black sont au moins 7 fois plus réceptives aux sporozoïtes que les souris OF1, TB et CBA. Leur réceptivité est évaluée à environ 80 pourcent de celle du *Thamnomys*. D'autres espèces de rongeurs sauvages comme le *Pelomys* et l'*Aethomys* sont comparables à ce point de vue au *Thamnomys*.

L'inoculation dans une veine mésentérique comparée à l'inoculation intraveineuse au niveau de la queue des animaux multiplie par 4 environ la concentration en schizontes exo-érythrocytaires dans le foie.

Une diminution du nombre de formes exo-érythrocytaires est observée après injection de cyclophosphamide, en présence de parasitémies aigues à *P. berghei*, ou *T. evansi* ou après immunisation par sporozoïtes. Des infections chroniques comme celle provoquée par *T. b. gambiense* n'ont, en revanche, aucune influence.

Les altérations produites au niveau de foie par la présence de nombreux œufs d'*Hepaticola*, le développement d'une dégénérescence graisseuse ou la ligature préalable de la veine porte ne change ni le nombre ni la vitesse de croissance des schizontes.

Infectiviteitsstudie met *P. berghei* sporozoïeten bij exeperimentele gastheren.

Samenvatting — *Plasmodium berghei* sporozoïeten worden door *A. stephensi* tussen de 21e en de 28e dag na de infecterende maaltijd uitgescheiden en zijn afkomstig van twee oocyste-populaties met verschillende groeisnelheid. Er worden dus meer sporozoïeten aangetroffen in de speekselklieren op de 28e dag dan op de 21e dag. Hun infecterend vermogen blijft onveranderd tot op de 30e dag.

C57 Black muizen zijn minstens zeven maal gevoeliger voor infectie met sporozoïeten dan OF1, TB en CBA muizen. Hun gevoeligheid wordt op ongeveer 80 ten honderd geraamd van deze van *Thamnomys*. Andere soorten wilde knaagdieren zoals *Pelomys* en *Aethomys* zijn, wat dit betreft, gelijkwaardig aan *Thamnomys*.

Het inspuiten in een mesenterische ader vergeleken met intraveneuze inspuiting in de staart leidt tot een viervoudiging van de concentratie exo-erythrocytaire schizonen in de lever.

Een vermindering van het aantal exo-erythrocytaire vormen wordt waargenomen na inspuiten van cyclofosfamide, bij acute parasitemische toestanden met *P. berghei* of *T. evansi* en na immunisatie met sporozoïeten. Chronische infecties daarentegen, zoals deze veroorzaakt door *T. b. gambiense*, hebben geen invloed.

Veranderingen in de lever, te wijten aan aanwezigheid van talrijke eieren van *Hepaticola*, ontwikkeling van een vettige ontaarding of voorafgaand afbinden van de vena porta verandert niet het aantal, noch de groeisnelheid van de schizonten.

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