Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. 81, Suppl. II :115-122, 1986. *Intern. Symp. on Malaria*

IMMUNOGENICITY AND INFECTIVITY OF MATURE AND IMMATURE *PLASMODIUM GALLINACEUM* SPOROZOITES

ANTONIANA URSINE KRETTLI*, KATIA ELIZABETH LAURENTE**
VALQUIRIA ROCHA DAHER*** & ELIANA M. MAURICIO DA ROCHA*

The infectivity and immunogenicity of two different populations of Plasmodium gallinaceum *sporozoites and their reactivity* in vitro *with normal and specific immune sera were studied in paralel experiments. Sporozoites from salivary glands (SGS) or from oocysts (OoS) contained in midguts of* Aedes fluviatilis *mosquitos gradually acquired infectivity to chicks regardless of their location. This infectivity was abolished after parasite exposure for 30 min to ultraviolete (UV) lights or to X-rays (13Krads). Inactivated OoS and SGS repeteadly inoculated i.m. or i.v. into young chicks were similarly immunogenic and elicited a strong immune response detected by the circumsporozoite precipitating (CSP) test performed with living sporozoites. Likewise, a single dose* $\geqslant 10^5$ *alive OoS or SGS inoculated i.v. into swiss mice elicited a detectable humoral anti-sporozoite antibody response measured by CSP tests. Such antibodies were clearly demonstrated only after three weeks of immunization. The titers of specific antibodies measured by CSP or by immunofluorescence against living sporozoites (IFV) were rather low (1:40) with all the immune sera whereas normal sera from chicks or mice reacted with the sporozoite when used undiluted only. The reactivity of both OoS and SGS* in vitro *was very similar whether in the presence of anti-OoS or anti-SGS sera provided they were fully infective, i.e., when recovered from 10-14 day old infected mosquitos. In this case, no detectable differences were observed in the percentage of reactive sporozoites nor in the titer of the reactions. However, the 7-8 day-old poorly infective sporozoites (SGS or OoS) were non-immunogenic and non-reactive* in vitro. *Our results are the first evidence that avian malaria sporozoites also express the CS proteins, extensively characterized in the mammalian malaria. We are now trying to characterize such proteins, in OoS and SGS at the molecular level in an attempt to elucidate further the similarities herein described between both populations and how they compare to other malaria sporozoites.*

The surface proteins of malaria sporozoites from mammalian species have been extensively studied and described to the molecular level (Nussenzweig & Nussenzweig, 1984). They are the target epitopes for protective antibodies; their size and isoelectric points have a narrow range among various species (Santoro et al., 1983) and, they contain an immunodominant epitope repeated at least twice within each molecule (Zavala et al., 1983). There are indirect evidences that the circumsporozoite (CS) proteins recognized by protective antibodies and those molecules responsible for the sporozoite penetration into the host mammalian cells are closely related since this process is inhibited *in vitro* by specific immune sera (Hollingdale et al., 1982, 1984). Very little is known about the CS proteins of avian malaria except for the work by Mulligan, Russel & Mohan (1940) with *Plasmodium gallinaceum* sporozoites. Sera from chicks immunized with these inactivated parasites displayed specific agglutinating antibodies against freshly dissected sporozoites.

We have been studying *P. gallinaceum* sporozoites in an attempt to characterize their CS proteins. Two distinct populations isolated from *Aedes fluviatilis* mosquitos (Camargo & Krettli, 1978), i.e. the salivary gland sporozoites (SGS) and the oocyst sporozoites from midguts (OoS) were studied in paralel. Both, SGS and OoS reach maturity independent on the sporozoite migration to the salivary glands (Daher & Krettli, 1980). Thus, we aimed to detect a correlation, if any, between infectivity and immunogenicity of avian plasmodia already suggested in rodent malaria. The following questions were herein addressed: a) are the CS proteins expressed by *P. gallinaceum*; b) are the immature non-infective or poorly infective sporozoites less immunogenic *in vivo* than the mature infective parasites; c) is the *in vitro* reactivity of the CS proteins variable with the sporozoites age; and, d) are the SGS and OoS able to elicit a protective anti-sporozoite immune response?

Supported by CNPq.

*Departamento de Parasitologia, Universidade Federal de Minas Gerais and Centro de Pesquisas René Rachou, Caixa Postal 1743, 30190 Belo Horizonte, MG, Brasil.

**Departamento de Bioquímica da Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil.
***Departamento de Patologia, Universidade Federal do Espírito Santo, Vitória, ES, Brasil.

We were the first to demonstrate that avian plasmodia sporozoites express the CS proteins in an age fashion rather than according to the parasite location in the mosquito. Such CS proteins, like the sporozoite infectivity, were best displayed by the mature (or older) sporozoites.

MATERIAL AND METHODS

Animals: one-day old chicks purchased from commercial stores were kept on metal cages daily cleaned provided with artificial heat, water and starter chicken food *ad libitum* and used at ages of 3, 7 and 21 days in the experiments of infectivity. One week old chicks were used for immunizations. Older chicks were used to maintain *P. gallinaceum* infections. Adult female outbred rats and mice (Swiss albino) and Balb/c mice all reared at our facilities were used for sporozoite immunization.

P. gallinaceum: the origin and passages of our parasite strain in chicks and in *A. fluviatilis* mosquitos were as previously described (Camargo et al., 1983). To produce good batches of infected mosquitos they were fed on chicks with an ascending parasitemia, ideal for production of infective gametocytes, and kept at 27°C and 75% humidity untill use being provided with a 10% glucose solution.

Sporozoites: mosquitos lightly anesthesized with ether had their thoraces sectioned from abdomens to avoid contamination of OoS with SGS. Dissections were on days 7, 8, 10 and 14 after the blood meal. Salivary glands and midguts were received in microhomogeneizers in Hank's balanced salt solution supplemented with 10% fetal calf serum (Hanks-RBS), disrupted and lightly centrifuged to avoid mosquito debris (90 g 10 min). The sporozoites kept always at 4°C were counted in a hemocytometer and diluted to desired concentrations. Before used to immunize chicks they were inactivated by X-rays (13 Krads) or ultraviolete lights (UV) for $\geqslant$ 30 min as described (Daher & Krettli, 1987). Viable sporozoites were used both, *in vitro* for circumsporozoite precipitation and immunofluorescence (IFV) tests at dilution of $\sim 10^6$ /ml, and, *in vivo* for immunization of mice and rats given in one or in multiple doses. The number of sporozoites will be specified in the results.

Circumsporozoite precipitation (CSP) tests: the test was performed as originally described (Vanderberg, Nussenzweig & Most, 1969). Freshly dissected SGS or OoS in Hanks-FCS were incubated with serum in microscope slides sealed with nail polish at 37°C 30 min. The reactions were read under phase microscopy a total of 20 sporozoites being randomly examined. Those with a threadlike precipitate and/or body deformation were scored as positive. Each serum was tested individually but most results will be expressed by group of sera (average of the percentage of sporozoite positive). Since normal sera also reacted with the sporozoites a positive test was considered only when 20% or more sporozoites were positive. Most sera were tested simultaneously against SGS or OoS. The sporozoite reactivity *in vitro* was studied using suspensions of various ages (8, 10 and 14 days after the mosquito blood meal).

Immunofluorescence (IF): the IF was performed with dead or with viable sporozoites (IFV). For IFV the parasites were incubated in plastic tubes with sera, as for the CSP, washed twice with Hanks-FCS, treated with fluoresceine conjugated anti-Ig 30 min at room temperature, washed again and examined under microscopy. For the titration of the anti-sporozoite antibodies conventional IF was performed using as antigen air-dried as well as formalin-fixed sporozoites placed on multiple well slides and kept at −20°C.

RESULTS

Infectivity of SGS and OoS to chicks: the very young SGS (seven days) but not the OoS were infective to one-week old chicks (Table I). This higher infectivity was also observed with the 8-day old SGS populations injected in chicks of various ages (Table II). However, the 14-day old OoS, like the SGS, infected 100% of chicks. The malaria prepatent period (PPP) was significantly shorter with the 14-day old SGS or OoS in relation to the 8-day old parasites. Increasing the inocula did not modify substantially the poor infectivity of the 8-day old OoS (Laurente, 1987).

Immunogenicity of the 14-day old sporozoites: sera from mice immunized with a single dose of either SGS or OoS (14 days) tested against the homologous suspentions in the CSP reaction were all positive by three weeks but negative one week after immunization. The CSP antibodies were more clearly observed in the groups which received higher inocula as shown in Fig. 1 for the OoS immunization. There was no detectable differences between the ability of SGS and OoS to elicit an specific response.

TABLE I

Recently emergent oocyst sporozoites (7 day-old) of *Plasmodium gallinaceum* are not infective to chicks

Sporozoite source	Sporozoite age	No. chicks infected */ No. inoculated	Mean pre-patent period
Oocyst	7	0/18	–
	8	7/7	11.3
	9	7/7	9.5
Salivary glands	7	3/3	12.0
	8	3/3	9.0
	9	3/3	8.6

*One week old chicks. Inocula = 10^4 sporozoites i.m. Adapted from Daher & Kretti, 1980. *J. Protozool.*, *27* :440.

TABLE II

Infectivity of *Plasmodium gallinaceum* sporozoites (8 and 14 days) to chicks of various ages

Sporozoite		Chicken		
Age	Source	Age (days)	No. inoculated*/ No. inoculated	% infected
8 days	Oocyst	3	10/12	83%
		7	8/25	32%
		21	0/33	0%
	Salivary glands	3	Not done	
		7	24/24	100%
		21	22/22	100%
14 days	Oocyst	7	12/12	100%
	Salivary glands	7	39/39	100%

*Dose of sporozoites varied from 250 to 5000 in paralel experiments.

TABLE III

Sera from individual rats immunized with viable sporozoites recovered from salivary glands (SGS) or from midguts (OoS) of mosquitos and tested against SGS and/or OoS gave high percentage of positive circumsporozoite (CSP) reactions

Antigen for immunization*	CSP-SGS		CSP-OoS	
	1:2	1:4	1:2	1:4
SGS	100%	80%	90%	85%
	95%	95%	90%	85%
OoS	85%	75%	60%	50%
	40%	55%	90%	100%

*Immunization schedules were different: a total of $1{,}2 \times 10^6$ SGS (3 doses of 2×10^5 *plus* one of 4.2×10^5 sporozoites) and a total of $3{,}5 \times 10^5$ OoS (one dose of 2×10^5 and one of $1{,}5 \times 10^6$ at time intervals of 2 weeks between each immunization (Rocha & Krettli, in preparation).

Chicks, rats and mice hyperimmunized with SGS or OoS also had circulating CSP-antibodies at high levels. In these cases up to 100% of positive reactions were detected (Table III). As expected, a cross-reactivity was present when sera from the hyperimmunized animals were tested against either SGS or OoS *in vitro* (Daher & Krettli, 1987). Furthermore, most hyperimmune sera gave typical threadlike precipitates characteristic of a 4+ CSP reaction.

Reactivity of SGS and OoS of various ages: sporozoites recovered at 8, 10 and 14 days incubated with a CSP positive sera gave a variable reactivity from negative to strongly positive. Thus, the poorly infective 8-day old SGS and OoS were negative by CSP tests with both, normal and immune sera whereas the older sporozoites reacted strongly with the immune sera. The sera from OoS- or SGS-immunized mice cross-reacted with the older populations no detectable differences being observed between SGS or OoS (Laurente & Krettli, 1985).

Immunogenicity of the 8-day old sporozoites: one single injection of the poorly-infective 8-day old sporozoites into mice did not elicit a detectable CSP response regardless of the origin of the antigen used for immunization (Fig. 2A). When the mice received a second dose of sporozoites, given three weeks after priming, all of them developed anti-sporozoite antibodies (Fig. 2B). Again, no detectable differences were observed between OoS and SGS immunization. Chickens hyperimmunized with the young OoS or SGS had a strongly positive sera when tested against either of the two parasites by CSP (Daher & Krettli, 1987).

Titration of anti-sporozoites antibodies: the CSP titers in the specific immune sera were usually rather low (1:40) even in the case of hyperimmunized chickens, rats or mice. The same result was observed with the IFV tests performed with alive sporozoites the end titers being around 1:40. However when the hyperimmune sera were tested by IF using the dead sporozoites as antigen we observed positive reactions up to the dilution of 1:10,000. An example of both types of serum titrations (CSP and IF) are on Table IV.

TABLE IV

Results of serological tests with sera from Balb/c mice repeteadly immunized by i.v. route with *Plasmodium gallinaceum* sporozoites and against homologous or heterologous sporozoite used in the circumsporozoite precipitation (CSP) reaction and in conventional immunofluorescence tests (IF)

Sporozoite immunization		Mice No.	Reciprocal of serum dilution		CSP-SGS		CSP-OoS	
Origin	(Total No.)		IF-SGS	IF-OoS	1:20	1:40	1:20	1:40
SGS	(4×10^5)	1	10.240	160	65%	60%	70%	5%
	(4×10^5)	4	5.120	640	65%	40%	70%	10%
OoS	(6×10^5)	6	640	80	98%	65%	90%	85%
	(6×10^5)	7	640	160	85%	40%	90%	40%
	(1.4×10^5)	38	1.280	640	55%	15%	40%	35%
–	–	Non-immunized	Neg.	Neg.	0	5%	5%	5%

* The immunized mice received multiple doses of sporozoites at intervals of 7-15 days as foloows: no. 1-4 with 4 doses of 10^5 SGS; no. 6-7 with 2 doses of 10^5 *plus* 2 doses of 2×10^5 OoS; no. 38 with 2 doses of 5×10^4 and 2 doses of 2×10^4. All CSP were negative with serum diluted 80X.

Protective immunity induced by *P. gallinaceum* sporozoites: vaccination of chicks with inactivated OoS or SGS in three to seven consecutive doses, either i.v. or i.m., resulted in a strong protection, in some experiments, as measured by a reduced mortality. From 33 to 100% survival has been observed among groups which received five or seven doses of sporozoites. However, all immunized chicks developed a patent malaria after a similar PPP in control and test groups. Surprisingly, the parasitemia developed by most vaccinated chicks after challenge was significantly higher than in the non-immunized groups as illustrated in one experiment in Fig. 3. The route of immunization or that of sporozoite challenge did not seem to influence these results. Furthermore, no detectable differences were observed among groups vaccinated with either OoS or SGS.

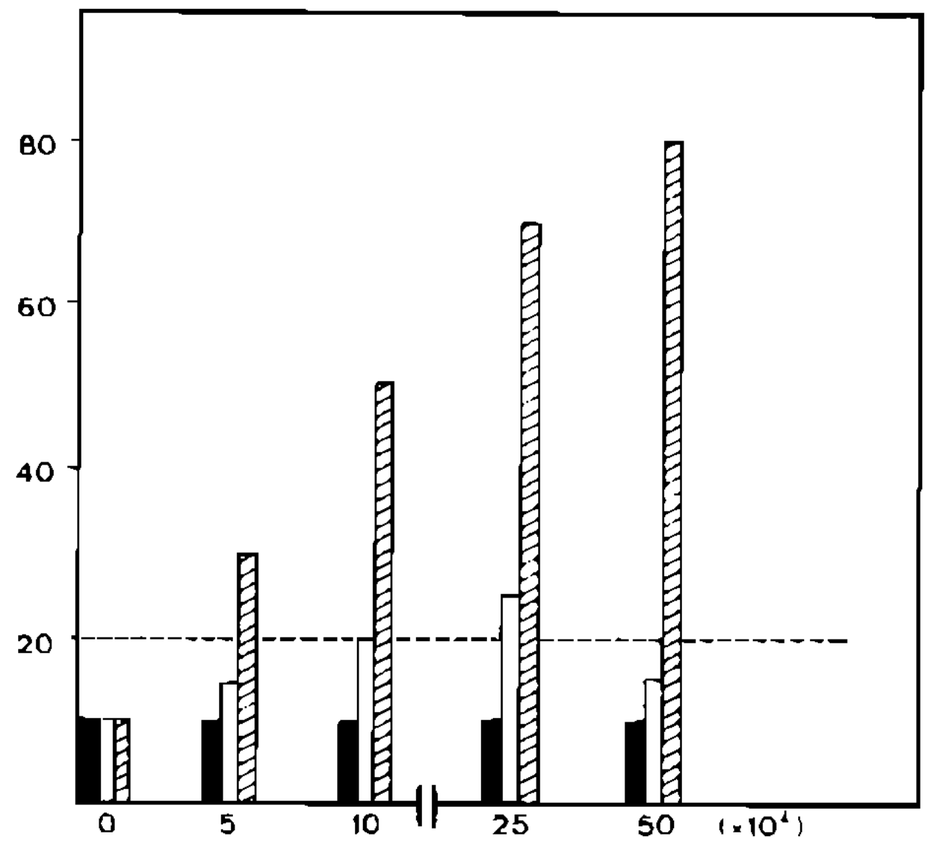

Fig. 1: Percentage of positive circumsporozoite precipitation (CSP) reactions in sera from mice immunized with a single injection of *Plasmodium gallinaceum* mature oocyst sporozoites (14-day old OoS) isolated from midguts of *Aedes fluviatilis* mosquitos. Sera were obtained at one ■, two □ or three ▨ weeks after mice intravenous immunization and tested against the homologous 14-days old OoS.

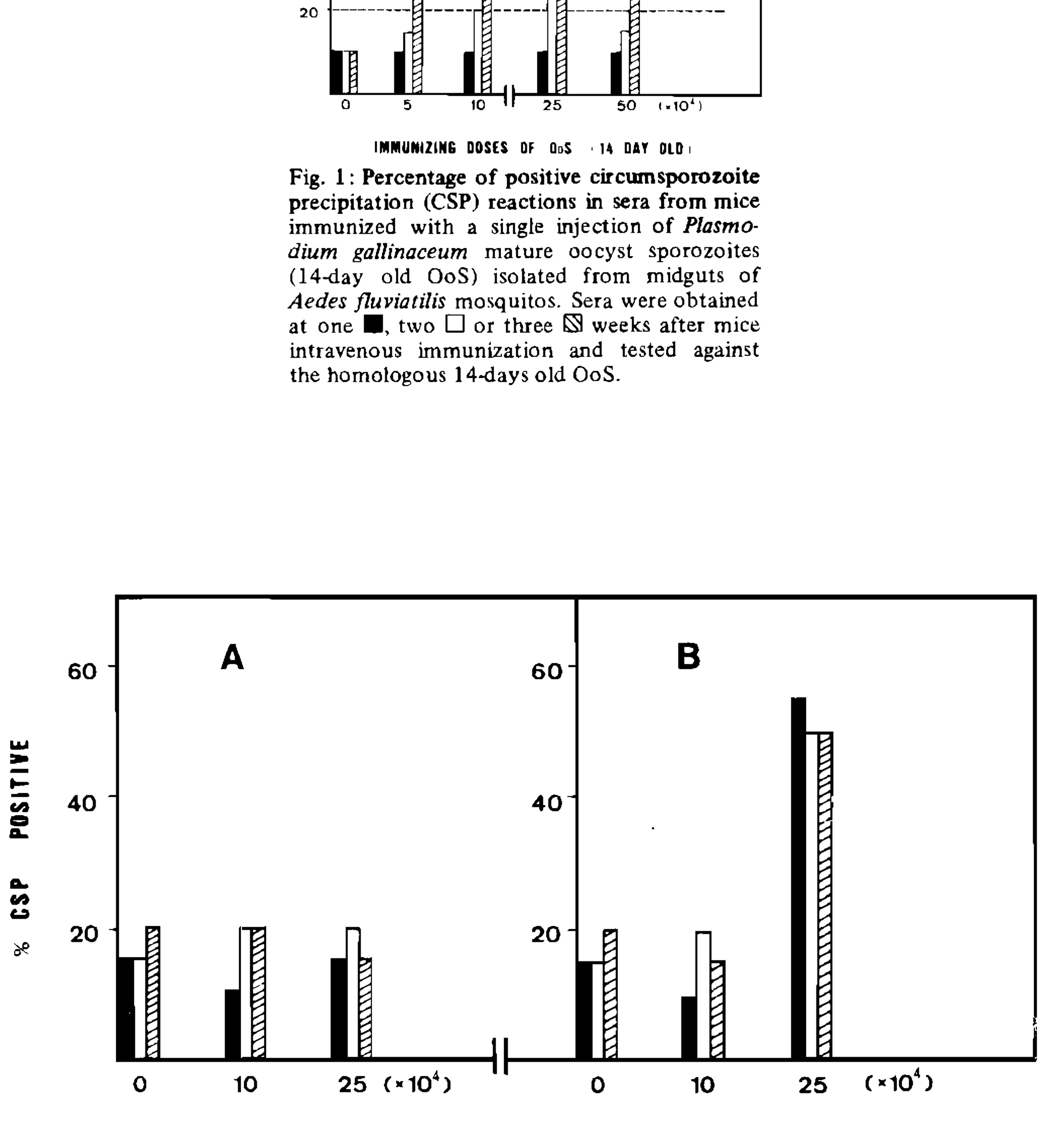

Fig. 2: Percentage of positive circumsporozoite precipitation (CSP) reactions in sera from mice immunized with one (A) or two (B) consecutive doses of immature salivary gland sporozoites (8-day old SGS) given with one week interval. Sera were obtained at one ■, two □ or three ▨ weeks after single or twice immunization and tested against 14-day old SGS.

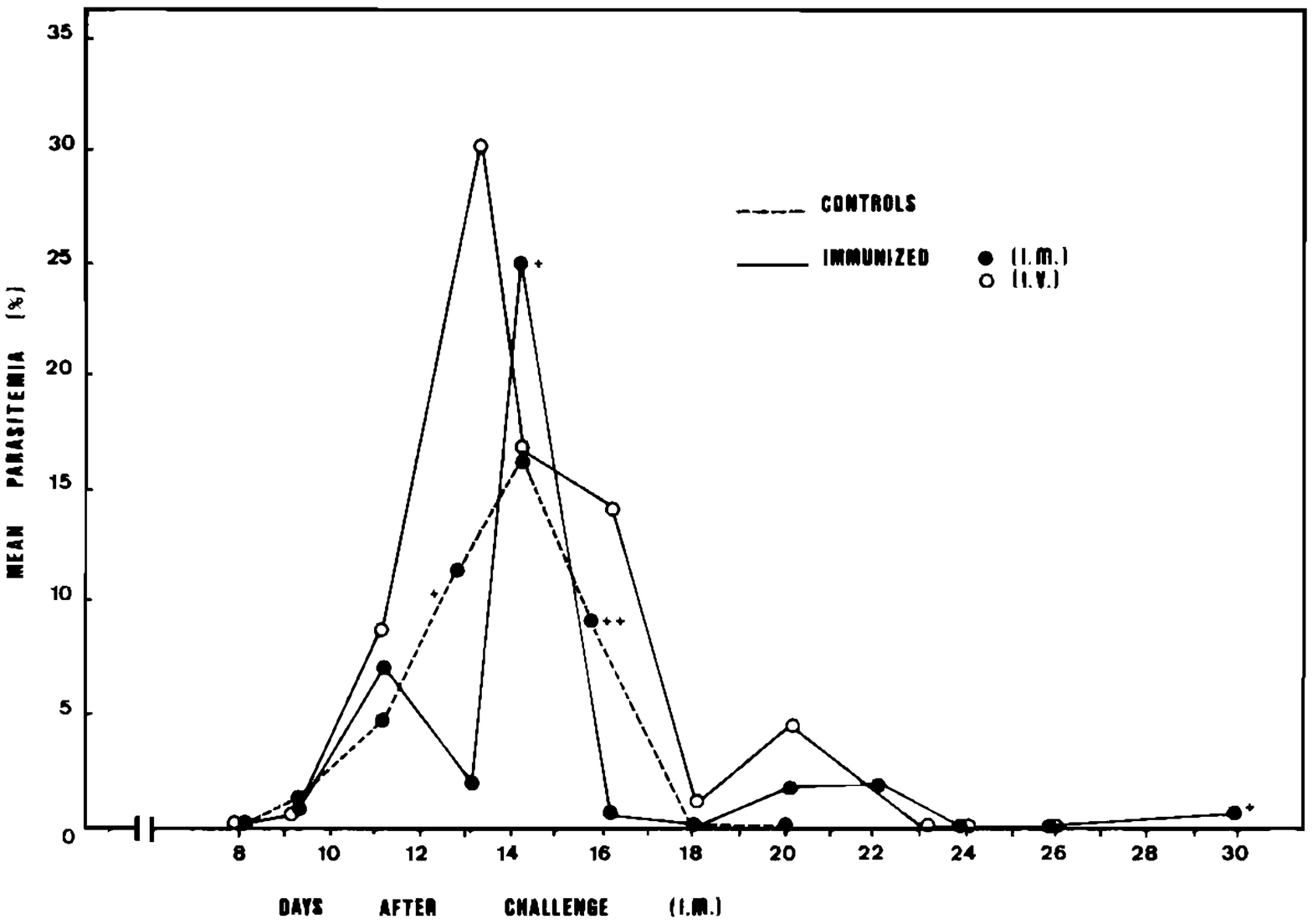

Fig. 3: ***Plasmodium gallinaceum*** **mean parasitemia in groups of three chicks each challenged with salivary gland sporozoites (10^4 intramuscularly). Normal and** sporozoite immune groups were of same age. Immunization was by intravenous (i.v.) or intramuscular (i.m.) routes with a total of $6x10^4$ oocyst sporozoites (OoS) from midguts of *Aedes fluviatilis*, inactivated by UV lights and given in three consecutive weekly doses. In this experiment mortality was equal although somewhat earlier in normal than in vaccinated groups.

DISCUSSION

The differences between the infectivity of SGS and OoS of *P. gallinaceum* shown in our previous work (Daher & Krettli, 1980) were now further confirmed using chicks of various ages and different inocula. Thus the infectivity of OoS is not a result of their contamination with SGS and the acquisition of molecules responsible for the sporozoite infectivity is a gradual process independent on parasite migration towards the salivary glands. The apparently greater infectivity of the younger SGS (7-8 days) may be atributed to them being indeed older (they need time to migrate) than the OoS recovered at the time. Since the recovery of OoS is less tedious, easier and faster we have suggested the use of this target cells instead of the SGS for the characterization of *P. gallinaceum* circumsporozoite proteins (Daher & Kretti, 1987).

Hyperimmune sera of chicks, mice and rats injected with SGS or OoS all displayed strong and similar levels of anti-sporozoite antibodies detected by CSP and by IF. However, the use of a single immunizing dose of either SGS or OoS allowed the demonstration of a lower antigenicity of the younger poorly-infective (7-8 days) sporozoites. These mice sera were unable to give a positive CSP when tested against sporozoites of various ages but after a second booster they became CSP positive.

The *in vitro* reactivity of *P. gallinaceum* sporozoites was also correlated to their age, but not their origin, only the younger poorly-infective ones being non-reactive. The CSP reactions given by 10- and 14-day old sporozoites were intense and alike. Again, such differences were only observed in the presence of sera from a single immunization.

The development of stage specific surface antigens by OoS and SGS of rodent and simian malaria, studied with alive sporozoites by CSP and IFV tests, show a different picture (Nardin, Gwadz & Nussenzweig, 1979). The OoS were found to give mostly negative or weakly reactions *in vitro*. The 11-day old OoS of the simian *P. knowlesi* used air-dried or fixed in conventional IF gave also poorly reactions whereas the 20-day old SGS reacted strongly at dilutions up to 1:1024. Such differences have been characterized at the molecular level in a detailed study on the biosynthesis of the protective antigen by *P. berghei* sporozoites in various developmental stages. It was shown that this synthesis is strictly associated with the mature SGS. In contrast to SGS, in OoS the main antigen and its precursors were found in only minute amounts (Yoshida et al., 1981). It seems therefore that maturation of the *P. gallinaceum* sporozoites is more rapid than that of mammalian malaria OoS. Differences in temperature would not explain those data since only *P. berghei* sporogony needs low temperatures.

The CSP reactions with *P. gallinaceum* SGS or OoS were typical and often were of a 4+ described with *P. berghei* (Vanderberg, Nussenzweig & Most, 1969) i.e. most sporozoites had a long threadlike precipitate when incubated with hyperimmune sera. Similarly to the mammalian systems the use of diluted sera was a limiting factor. Thus, titration of *P. gallinaceum* CSP antibodies showed positive tests only at dilutions $\leqslant$ 1:40. Conversely, positivy of those anti-sporozoites antibodies measured by IF against air-dried or formaline fixed sporozoites persisted at high dilutions ($\sim$1:10.000). Such differences paralel previous finds on simian malaria (Nardin, Gwadz & Nussenzweig, 1979).

Vaccination of chicks with *P. gallinaceum* inactivated SGS or OoS were attempted by i.v. or i.m. routes. Our rationale was that avian sporozoites may develop in macrophages or in endothelial cells at the site of inoculation. Indeed, provided we used multiple doses of antigens a strong protection measured by increased chicken survival was acchieved regardless of the route of immunization, the origin or age of the sporozoites. However, there was a great variation in the percentage of surviving chicks. This was partly attributed to non related virus or coccidia infections among our animals. Antiviral vaccines and coccidiostatic drugs could not be used since they were shown to interfere with the malaria infections (Daher, 1979).

A curious but yet not elucidated phenomena was the increased malaria parasitemia observed in most vaccinated groups. In previous work with *P. gallinaceum* sporozoites vaccination (Mulligan, Russel & Mohan, 1941) the authors described an increased chick survival but did not follow the parasitemia of the immunized chicks. This result deserves further classification. We are now trying to produce monoclonal antibodies against *P. gallinaceum* sporozoites in an attempt to characterize further the protective antigens expressed by OoS and SGS.

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