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Development of Exoerythrocytic Forms of *Plasmodium gallinaceum* in Chick Embryo Culture

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ABSTRACT : Six inoculation methods were attempted on the culture of *Plasmodium gallinaceum* in developing chick embryos. Materials used as inocula were : (A) the parasitized blood from embryos in blood-induced infection; (B) the infected organs and (C) the parasitized blood, both taken from embryos inoculated with chick brain containing the exoerythrocytic forms and possibly few, if any, erythrocytic forms by the suprachorioallantoic implantation. Each material was inoculated into embryos by the intravascular injection or the suprachorioallantoic implantation. The inouclated embryos were examined for growth of the parasite, especially of the exoerythrocytic forms, on the 7th or 10th day of incubation. (1) Numerous exoerythrocytic forms developed most frequently in embryos inoculated with the embryonic organs (material B) containing the exoerythrocytic forms and possibly the erythrocytic forms as well by the suprachorioallantoic implantation. On the other hand, marked parasitemia commonly resulted from the intravascular injection of the parasitized blood (material A) from embryos in blood-induced infection. (2) The exoerythrocytic forms inoculated on the chorioallantoic membrane seemed to have been introduced via chorioallantoic vessels into various organs, where they would first develop as the exoerythrocytic forms and then produce the erytrocytic forms. (3) It was suggested that the parasitized blood (material C), in addition to the erythrocytic forms, contained some exoerythrocytic forms in it.

Many attempts to grow the exoerythrocytic forms of avian malarial parasites have been performed in chick embryo and tissue cultures. According to the reports so far published, the species of avian *Plasmodium* most frequently used in these fields of study are *Plasmodium gallinaceum*, *Plasmodium fallax*, and *Plasmodium lophurae* (Davis *et al.*, 1966 ; Huff, 1969 ; Beaudoin *et al.*, 1970). Procedures for production of the exoerythrocytic forms of *P. fallax* and *P. lophurae* in tissue culture have already been established (Beaudoin *et al.*, 1974).

Development of the exoerythrocytic forms of *P. gallinaceum* in chick embryo culture has

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been reported by many researchers. Shortt *et al.* (1940) deposited on the chorioallantoic membrane of developing chick embryos the salivary glands of *Aedes aegypti* previously infected with *P. gallinaceum*. Heavy infection of the brain, the liver, the spleen, and bone marrow of the embryos with the exoerythrocytic forms resulted, but smears from the heart blood merely showed a low grade of parasitemia. Haas and Ewing (1945) observed exoerythrocytic forms in the brain in sporozoite-induced embryonic infection. Zuckerman (1946) described more precisely the chick embryo culture of *P. gallinaceum*. The chick brain containing numerous exoerythrocytic forms was inoculated on the chorioallantoic membrane. About 60% of the embryos survived and showed heavy infection, especially of the brain, the liver, and the spleen with the exoerythrocytic forms. Serial subinoculation of the exoerythrocytic forms from embryo to embryo was carried out successfully. Further, the same author (1946), in addition to the suprachorioallantoic implantation, employed the intramuscular injection of infected chick blood. As the results, marked parasitemia occurred in the embryos, of which some were found to contain a few exoerythrocytic forms in their tissues. Throughout the embryonic infections with *P. gallinaceum* mentioned above, it was proved that the organs in which the exoerythrocytic forms could be most commonly detected were the brain, the liver, and the spleen.

In the present study, the authors attempted to grow *P. gallinaceum* in developing chick embryos employing several ways of inoculation. The purpose was to compare the growth patterns of the parasite, especially of the exoerythrocytic forms, which would be produced in the different infection systems.

MATERIALS AND METHODS

Plasmodium gallinaceum used in this study was given by Mr. G. T. Shute, WHO Technical Officer, Malaria Eradication Programme, Manila, Philippines, in 1971 and has been maintained by serial blood passage using White Leghorns in this laboratory. *Aedes aegypti* used for producing the sporozoite-induced infection in chicks had also been bred in this laboratory. Fertilized hen's eggs were obtained commercially and incubated at 38°C in adequate moisture.

Infections of chick embryos with P. gallinaceum: The technics described by Zuckerman (1946), and Pipkin and Jensen (1958) were employed with slight modifications in the inoculation of infected materials into chick embryos. The following six methods of inoculation were adopted:

Inoculation I. Intravascular injection of parasitized blood taken from embryos in blood-induced infection

Inoculation II. Suprachorioallantoic implantation of the same material as in *Inoculation* I

Inoculation III. Suprachorioallantoic implantation of infected embryonic organs containing the exoerythrocytic forms and possibly the erythrocytic forms as well

Inoculation IV. Intravascular injection of the same material as in *Inoculation* III

Inoculation V. Intravascular injection of parasitized blood taken from the embryos which were used in *Inoculation* III

Inoculation VI. Suprachorioallantoic implantation of the same material as in *Inoculation* V

Parasitized blood was withdrawn from the jugular vein of a *P. gallinaceum*-infected chick into a heparinized syringe and washed 3 times in 0.5% glucose phosphate buffer saline, pH 7.2-7.4 (GBS) by centrifuging at 600 × g for 5 min. After the supernatant was discarded, a dilution containing 5×10^7 infected red blood cells per ml was prepared in GBS. Of the dilution, 0.01ml (5×10^5 infected red blood cells) was injected intravascularly into the chorioallantoic vessel of 10-12 day embryos. Marked parasitemia was frequently produced in the embryos by the 7th day of subsequent incubation. Then, serial blood passage in chick embryos was carried out by injecting 0.01 ml of the inoculum prepared similarly as described above, but from the heart blood of infected embryos. Thirty-five embryos so operated were studied for development of the parasites on the 7th day of incubation after parasite inoculation (*Inoculation* I). On the other hand, 29 embryos of 7-9 days were implanted with 0.05 ml of the same inoculum on the chorioallantoic membrane and incubated for subsequent 10 days (*Inoculation* II).

The brain containing numerous exoerythrocytic forms of *P. gallinaceum* was removed from an infected chick which had been given sporozoite-induced infection by *Aedes aegypti*, and was washed 3 times by dipping in GBS. It was then thoroughly macerated in a glass homogenizer containing a small quantity of GBS and diluted in the same solution to 3-6 times of the original volume. Of such a brain emulsion, 0.05 ml was implanted on the chorioallantoic membrane of 7-9 day embryos. Numerous exoerythrocytic forms were regularly detected in the embryonic brains, the livers, and the spleens by the 10th day of subsequent incubation, although parasitemia was usually low in these infected embryos. The embryonic brain or liver containing the exoerythrocytic forms and possibly the erythrocytic forms was used for the suprachorioallantoic subinoculation. As the spleen was too small in size to offer the sufficient amount of inoculum, it was excluded from the inoculation materials. In this way, subinoculation was carried out with 37 embryos (*Inoculation* III). Of the emulsion prepared from the infected embryonic brain or liver, 0.01 ml was injected intravascularly into 22 embryos of 10-12 days and the embryos were incubated for further 7 days (*Inoculation* IV).

Some of the infected embryos used in *Inoculation* III also developed a low grade of parasitemia. From the parasitized blood, an inoculum was prepared similarly as in *Inoculation* I. Of the inoculum, 0.01 ml was injected intravascularly into 27 embryos of 10-12 days (*Inoculation* V) and 0.05 ml was implanted on the chorioallantoic membrane of 19 embryos of 7-9 days (*Inoculation* VI).

Examination of embryos inoculated: Examination for the parasites was usually done 7 days after the intravascular injection (*Inoculation* I, IV, and V) and 10 days after the suprachorioallantoic implantation (*Inoculation* II, III, and VI). Occasionally the examination was attempted 1-2 days earlier or later to know the grade of growth of the parasites. Searching for the exoerythrocytic forms was limited to the brain, the liver, and the spleen, although other organs were known to contain such forms. Impression smears were made from these 3 organs and stained with Giemsa. Blood smears were also stained in the same manner. Parasitemia was

expressed with the parasitized red blood cell rate. Infection of the organs was graded to heavy (+++), moderate (++), or slight (+) infection, judging from the number and size of the exoerythrocytic forms found in the organs microscopically.

RESULTS

Numbers of embryos inoculated, survived, and infected with P. gallinaceum *by 6 different methods of inoculation* : The results were summarized in Table 1. In *Inoculation* I, 26 out of 35 embryos inoculated survived until the day of examination, and 25 of them were found to be infected. Blood smears taken on the 5th day of incubation after inoculation showed low parasitemia. However, on the 7th day of incubation, marked parasitemia of more than 50% occurred in 11 embryos. The highest rate of parasitemia was 92%. The exoerythrocytic forms, though very small in number, were also detected in 8 embryos with parasitemia of more than 60%. In *Inoculation* II, out of 29 embryos, 21 survived and only 3 among them produced a negligible grade of parasitemia. No exoerythrocytic forms were detected by the 7th day of incubation.

In *Inoculation* III, 33 embryos were inoculated and of 22 survivals, 14 became infected. Most of the infected embryos showed heavy (+++) or moderate (++) tissue infection with the exoerythrocytic forms accompanied by lower parasitemia than those found in *Inoculation* I. One embryo, showing no parasitemia, produced slight (+) tissue infection. The highest parasitemia recorded in *Inoculation* III was of 24%. In *Inoculation* IV, 50%(11 out of 22) of the embryos inoculated were killed by unknown cause, probably by the operative intervention, in a few days after the intravascular injection of the infected organ-emulsion. Seven embryos became infected and showed heavy (+++) or moderate (++) tissue infection with such low parasitemia as those in *Inoculation* III.

Table 1. Numbers of embryos inoculated, survived, and infected with *P. gallinaceum* by 6 different methods of inoculation

Methods of inoculation[+]	No. of embryos			Parasitemia*	Tissue infection**	Both***
	inoculated	survived[++]	infected			
I	35	26	25	17	0	8
II	29	21	3	3	0	0
III	33	22	14	0	1	13
IV	22	11	7	0	0	7
V	27	18	15	3	2	10
VI	19	14	2	0	1	1

[+] See text.
[++] Embryos which remained alive until the day of examination.
* The numbers of embryos showing parasitemia but no tissue infection.
** The numbers of embryos showing the exoerythrocytic forms in their tissues but no parasitemia.
*** The numbers of embryos showing both parasitemia and tissue infection.

In *Inoculation* V, out of 27 embryos inoculated, 18 survived and 15 became infected. Ten embryos revealed both parasitemia and tissue infection, and 5 embryos showed either parasitemia or tissue infection. A moderate grade (10-40%) of parasitemia was recorded more frequently in *Inoculation* V than in *Inoculation* III and IV. In *Inoculation* VI, 19 embryos were inoculated, of which 14 survived and only 2 developed moderate (++) tissue infection.

Table 2. Grades of parasitemia and tissue infection in chick embryos infected with *P. gallinaceum* in Inoculation III, IV, and V

Inoculation	Embryo	Days after inoculation	Parasitemia (%)	Tissue infection		
				brain	liver	spleen
III	III- 1	10	4	+++	*	*
	- 2	9	24	+++	+++	+++
	- 4	12	< 1	++	*	*
	- 6	11	10	+++	+	*
	- 7	11	9	+++	*	*
	-11	12	< 1	++	++	++
	-16	12	2	+++	++	++
	-19	10	1	+	++	*
	-20	12	0	+	-	-
	-21	10	1	++	++	*
	-25	8	1	++	++	*
	-26	10	2	++	++	++
	-30	8	13	+++	+++	+++
	-31	10	< 1	++	++	++
IV	IV- 6	7	< 1	-	++	-
	- 9	7	< 1	+++	++	++
	-11	7	7	+++	+++	+
	-12	7	< 1	++	+++	+
	-13	7	< 1	-	+	*
	-16	8	10	+++	+++	+++
	-17	7	< 1	+	++	-
V	V- 2	8	40	++	+++	*
	- 3	5	13	+	++	++
	- 9	7	4	++	++	++
	-10	10	0	+	++	*
	-12	7	0	+	++	++
	-14	7	16	+++	+++	+++
	-17	7	8	+++	+++	+
	-18	7	18	+++	+++	+
	-19	7	1	-	++	+
	-20	6	14	+++	+++	++
	-21	7	20	*	+++	+++
	-23	7	1	-	-	-
	-24	7	4	++	++	++
	-26	7	1	-	-	-
	-27	7	2	-	-	-

* not examined

One of the 2 embryos also showed very low parasitemia but the other did no parasitemia.

Grades of parasitemia and tissue infection in embryos in Inoculation III, IV, and V: The results obtained in *Inoculation* III, IV, and V were of particular interest in that both parasitemia and tissue infection with the exoerythrocytic forms were observed more frequently in these methods of inoculation than in others (Table 2). Moreover, in these 3 embryonic infections, tissue infection was heavy (+++) or moderate (++), whereas parasitemia was usually low.

No significant difference in development of the exoerythrocytic forms were recognized among the brain, the liver, and the spleen.

Growth curves of the parasites in chick embryos inoculated by 6 different methods: The erythrocytic forms seemed to grow fast up to the maximum number in the circulating blood in case of the intravascular injection of the parasitized blood from embryos in blood-induced infection (*Inoculation* I). Numerous exoerythrocytic forms were thought to grow well in embryonic tissues, accompanied by moderate or low parasitemia, equally in *Inoculation* III, IV, and V. However, in *Inoculation* V, 3 embryos (V-23, 26, and 27) showed parasitemia but no tissue infection, on the contrary, another 2 (V-10 and 12) revealed only the exoerythrocytic forms on the day of examination. This fact might partially be due to the nature of the inoculum which was prepared from the parasitized blood, different from the infected

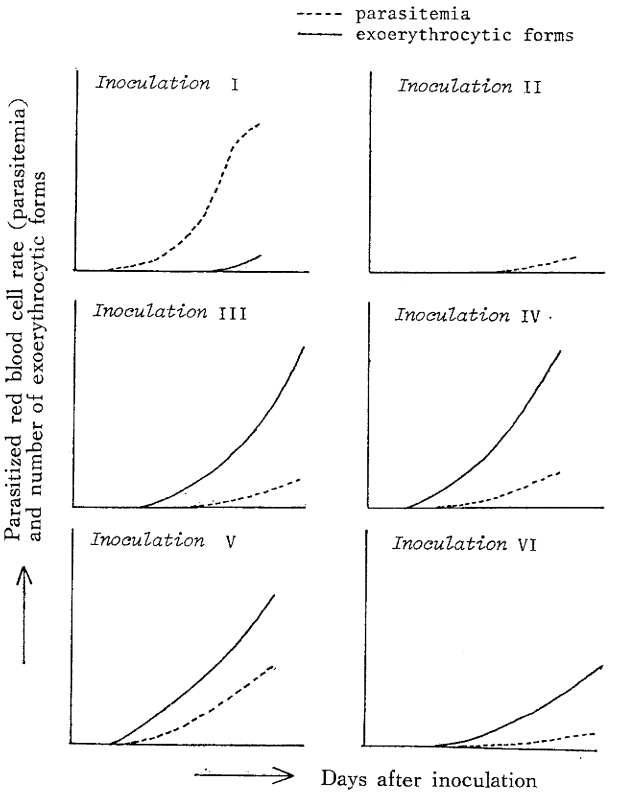

Fig. 1. Estimated growth curves of *P. gallinaceum* (parasitemia and exoerythrocytic forms) in chick embryo culture.

organs in *Inoculation* III and IV.

From the above data, the growth curves of *P. gallinaceum* in chick embryo culture were estimated and shown in Fig. 1.

DISCUSSION

Numerous exoerythrocytic forms, usually with moderate or low parasitemia, were detected in the infected embryos commonly in *Inoculation* III, IV, and V. However, the survival rate of embryos and the frequency of the exoerythrocytic form formation in the infected, were lower in *Inoculation* IV and V, respectively, than in *Inoculation* III. Therefore, it is concluded that the best method of those used for production of the exoerythrocytic forms is the suprachorioallantoic implantation of the brain or liver containing the exoerythrocytic forms and possibly the erythrocytic forms. The erythrocytic forms detected in the embryos infected by this method seemed to be derived from the exoerythrocytic forms in the inoculum. That is, the exoerythorcytic forms inoculated by the suprachorioallantoic implantation seemed to have been introduced via chorioallantoic vessels into various organs, where they first developed as the exoerythrocytic forms and then produced the erythrocytic forms. Because, the erythrocytic forms, when implanted on the chorioallantoic membrane, frequently failed to develop as observed in *Inoculation* II and VI. As Zuckerman (1946) stated before, only the exoerythrocytic forms might have the ability to infect embryos when inoculated on the chorioallantoic membrane. However, it is also reasonable that some of the erythrocytic forms in the inoculum, when placed on the chorioallantoic membrane, were introduced into the blood stream through the possibly injured site of the chorioallantoic vessels and eventually could grow in the circulating blood.

Significant difference between *Inoculation* I and V was observed in development of the exoerythrocytic forms. That is, heavy or moderate tissue infection with the exoerythrocytic forms frequently occurred in *Inoculation* V, whereas, in *Inoculation* I, very small numbers of the exoerythrocytic forms were produced in only 8 out of 25 infected embryos. These data may suggest that the parasitized blood used as inoculum in *Inoculation* V also contained some exoerythrocytic forms in it; or that the erythrocytic forms in the inoculum had a tendency to give rise to the exoerythrocytic forms, different from those with little tendency in *Inoculation* I.

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鶏マラリア *Plasmodium gallinaceum* の発育鶏卵培養に於ける赤外型の出現について

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Plasmodium gallinaceum の鶏卵培養を6つの接種方法で試みた。接種材料は次の通りである。(A) 卵血管内接種によって継代維持された赤血球型を含む血液．赤外型を含むひなどりの脳（厳密には赤血球型をも含む）の漿尿膜上接種によって得られた (B) 赤外型感染臓器（ときに赤血球型をも含む）及び(C)感染血液．各接種材料をそれぞれ卵血管内及び漿尿膜上に接種した．原則として卵血管内接種法では10—12日卵を用い7日後に開卵し，漿尿膜上接種法では7－9日卵を用い10日後に開卵し，とくに赤外型の増殖の態様について検討した．結果：(1) 赤外型の増殖は赤外型を含む臓器（ときに赤血球型をも含む）の漿尿膜上接種法で著明であった．(2) 漿尿膜上に接種された赤外型は漿尿膜血管を介して各種臓器に運ばれそこでまず赤外型として増殖し，次いで赤血球型を生ずるものと考えられた．(3) 赤外型を含む臓器（ときに赤血球型をも含む）の漿尿膜上接種によって感染した発育鶏卵はときに軽度の赤血球原虫感染を呈したが，その血液は更に赤外型をも含む可能性が示唆された．

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