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The life cycle of *Plasmodium vinckei lentum* subsp. nov.* in the laboratory; comments on the nomenclature of the murine malaria parasites

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In 1966 Adam, Landau and Chabaud discovered two malaria parasites in the forest rodent *Thamnomys rutilans*† captured in the Congo (Brazzaville). From their morphology in the blood the parasites were identified as *Plasmodium berghei* Vincke and Lips, 1948, and *P. vinckei* Rodhain, 1952, but, since their complete life-cycles were not then known, it was not possible to determine their subspecific status and relationships to similar parasites from other parts of Africa.

In later work, the *berghei*-like parasite was found to be easily transmitted cyclically in the laboratory, and the sporogony and tissue schizogony of this subspecies, *P. berghei killicki*, have been described elsewhere (Landau, Michel and Adam, 1968). In contrast, stages of the life-cycle of the *vinckei*-like parasite were less easily obtained, and attempts were made using many new isolates before the best methods of cyclically passaging this subspecies in the laboratory were determined.

In the present paper, a description is given of the complete life cycle of *P. vinckei lentum* subsp. nov., from Brazzaville, and the nomenclature of the murine malaria parasites is discussed.

MATERIAL AND METHODS

In general, the techniques used were those previously employed in the study of the life-cycles of malaria parasites of rodents from the Central African Republic (Landau and Killick-Kendrick, 1966). It is well established that many factors can interfere with the development of a malaria parasite, e.g. the dietary content of para-amino-benzoic acid. We had the possibility of working in two different laboratories—at the Museum in Paris, and at the London School of Hygiene and Tropical Medicine. As the numerical results obtained were comparable in both places, we consider that these factors can be disregarded when one makes comparisons between strains handled under similar experimental conditions.

*The type material of *P.v. lentum* is deposited at the Muséum National d'Histoire Naturelle, Laboratoire de Zoologie, Vers, 57, Rue Cuvier, Paris 5e, (Slide No. P I 215 [194 ZZ]).

†In the original report, the specific identity of some of the infected rodents was in doubt; all have since been identified as *Thamnomys rutilans*.

Strains

Of the many isolates studied, two strains (194 ZZ and 201 ZZ) were used for the present description. These were isolated in September, 1966, from two of many infected *T. rutilans* caught alive near the village of N'Ganga Lingolo, 15 km. from Brazzaville. The histories of the two strains are as follows:

Thamnomys—23/3/67→Mouse—14/4/67→Mouse—24/4/67→
201 ZZ 216 ZZ 271 ZZ

Mouse 347 ZZ
A. stephensi—10/5/67→Mouse 348 ZZ
318 ZZ Mouse 349 ZZ

Thamnomys—28/10/68→Mouse—9/11/68→Mouse—15/11/68
194 ZZ 623 CC 688 CC

→Mouse—29/11/68→*Hybomys*—4/12/68→*A. stephensi*—
739 CC 860 CC 882 CC

17/12/68→Mouse 22 VC

Mosquitoes

Anopheles stephensi originating from the colony at the Malaria Reference Laboratory at Epsom, were bred at the London School of Hygiene and Tropical Medicine and the Muséum National d'Histoire Naturelle, Paris. After infective feeds, the mosquitoes were incubated at 23–25°C.

Rodents

Mice were from the same strain (T.O.) as those used in the study of the tissue stages of *P. v. chabaudi*, (Landau and Killick-Kendrick, 1966), and also from an outbred colony at the Zoo de Vincennes. Mice inoculated with blood to determine the minimum time at which merozoites were released from tissue schizonts were splenectomized to increase their suceptibility.

Hybomys univittatus Peters, 1876, were obtained from a colony established by Dr. F. Petter at the Muséum National d'Histoire Naturelle (Petter and Genest, 1967).

RESULTS

Sporogony of P. v. lentum subsp. nov.

In spite of an early success in transmitting the parasite through *A. stephensi*, using mice as gametocyte carriers, later work showed the mouse to be a poor laboratory host for the production of gametocytes. Parasitaemia was often lighter than with *P. v. chabaudi*, and a few mice appeared to be completely refractory to infection. Only few gametocytes were produced, and these were usually not infective to mosquitoes. In the course of many experiments using mice, only one batch (318 ZZ) of mosquitoes, fed on the 10th day of infection, became well infected, with numerous oocysts on the midgut and infective sporozoites in the salivary glands.

In other work in progress at the same time, in which the susceptibility of different rodents to malaria parasites were being investigated, splenectomized *H. univittatus* were found to develop higher infections of *P. v. lentum* than white mice, accompanied by the production of many more gametocytes. The infection rate in *A. stephensi* (batch 882 CC) fed on *Hybomys* 860 CC on the fifth day of infection was 90 per cent., with the number of oocysts reaching a maximum of 50.

Throughout the duration of sporogony at 23–25°C. the oocysts grew very evenly, and on any given day there was little variation in their mean diameters. On day 4 they measured 9μ; on day 5, 12·5μ; on day 6, 19·5μ; on day 7, 21·5μ; on day 8, 33·5μ; on day 10, 39μ; and on day 11, 44μ. The first oocysts containing sporozoites were seen on day 8; the mean diameter of 11 mature oocysts on day 11 was 47μ (maximum 56μ).

Sporozoites were first seen in the salivary glands of mosquitoes on day 10, two days after the earliest maturation of oocysts. The mean length of the midlines of 25 sporozoites in stained preparations was 21·2μ (18–25μ).

Primary Exoerythrocytic Schizogony of P. vinckei lentum *subsp. nov.*

Sporozoites of strain 201 ZZ from the salivary glands of 30–40 mosquitoes (cage 318 ZZ) were inoculated intravenously into each of three white mice. Liver was taken at autopsies 28, 50 and 53 hours later; liver from the mouse killed at 53 hours was also taken at biopsy at 42 hours. At 53 hours there were 2–5 primary exoerythrocytic schizonts in each section.

TABLE I
Showing the sizes of primary exoerythrocytic schizonts of *P.v. lentum*

Age of schizonts (hours)	No. measured	Mean diameters	Range of diameters
28	2	7μ	
42	7	12·5 × 8·9μ (11μ)	15—11·25μ × 11·25—6·25μ
50	22	22·1 × 13·8μ (18μ)	31·25—16·25μ × 18·75—10μ
53	11	24 × 16·5μ (20μ)	27·50—20μ × 25—12·50μ
62½	15	39·9 × 33·5μ (36·7μ)	46·50—32·55μ × 40·30—26·35μ

One mouse was inoculated intravenously with sporozoites of strain 194 ZZ obtained from the salivary glands of 40 mosquitoes (882 CC). Liver was taken at 62½ hours by biopsy, and at 73 hours at autopsy. About one schizont was found in every eight sections. Blood was first found infective at 72 hours, but since no subinoculations were made between 65 and 72 hours, the minimum time of rupture of mature schizonts may be less. The first ring forms seen in thin smears of peripheral blood were found at 72½ hours.

This prepatent period is longer than that recorded for other subspecies of murine *Plasmodium*, and is related to a relatively slow growth of the hepatic stages. Even at 62½ hours no tissue schizonts were found with merozoites, and at this time the tissue forms are indistinguishable from those of *P. v. chabaudi* at 50 hours.

The sizes of 28, 42, 50, 53 and 62½ hour-old schizonts are given in Table I.

From 28–53 hours the tissue forms appeared as dark blue masses, round or oval, regular in outline, with a heavily staining cytoplasm forming a number of basophilic aggregations. The nuclei were small and evenly distributed. There were no vacuoles within the schizonts, and, unlike *P. b. killicki* from the same locality, the nucleus of the host cell was unaffected by the presence of the parasite.

By 62½ hours the morphology had changed. The cytoplasm stained a pale blue, and formed only few dense basophilic masses. The nuclei were very numerous, small and evenly distributed. In contrast with *P. b. yoelii* and *P. v. chabaudi*, pseudocytomeres were not seen.

At 73 hours liver was again taken from the mouse which had undergone a biopsy at 62½ hours. The schizonts still seemed as numerous as in the material collected 10 hours earlier. By 73 hours, however, fully formed merozoites were present in nearly all the schizonts, most of which had ruptured and were being invaded by macrophages. A few apparently abnormal forms were present, which had lightly staining cytoplasm and nuclei which were scanty, large and pyknotic.

DISCUSSION

The morphology of all stages of the life-cycle of two other subspecies of *P. vinckei* has been described by Landau and Killick-Kendrick (1966), and Bafort (1967, 1968). Our observations on the life-cycle of strains of *P. vinckei* from Brazzaville reveal characteristics clearly separating this parasite from *P. v. vinckei* and *P. v. chabaudi*, and we therefore consider it a new subspecies of *P. vinckei* and name it *Plasmodium vinckei lentum* subsp. nov.

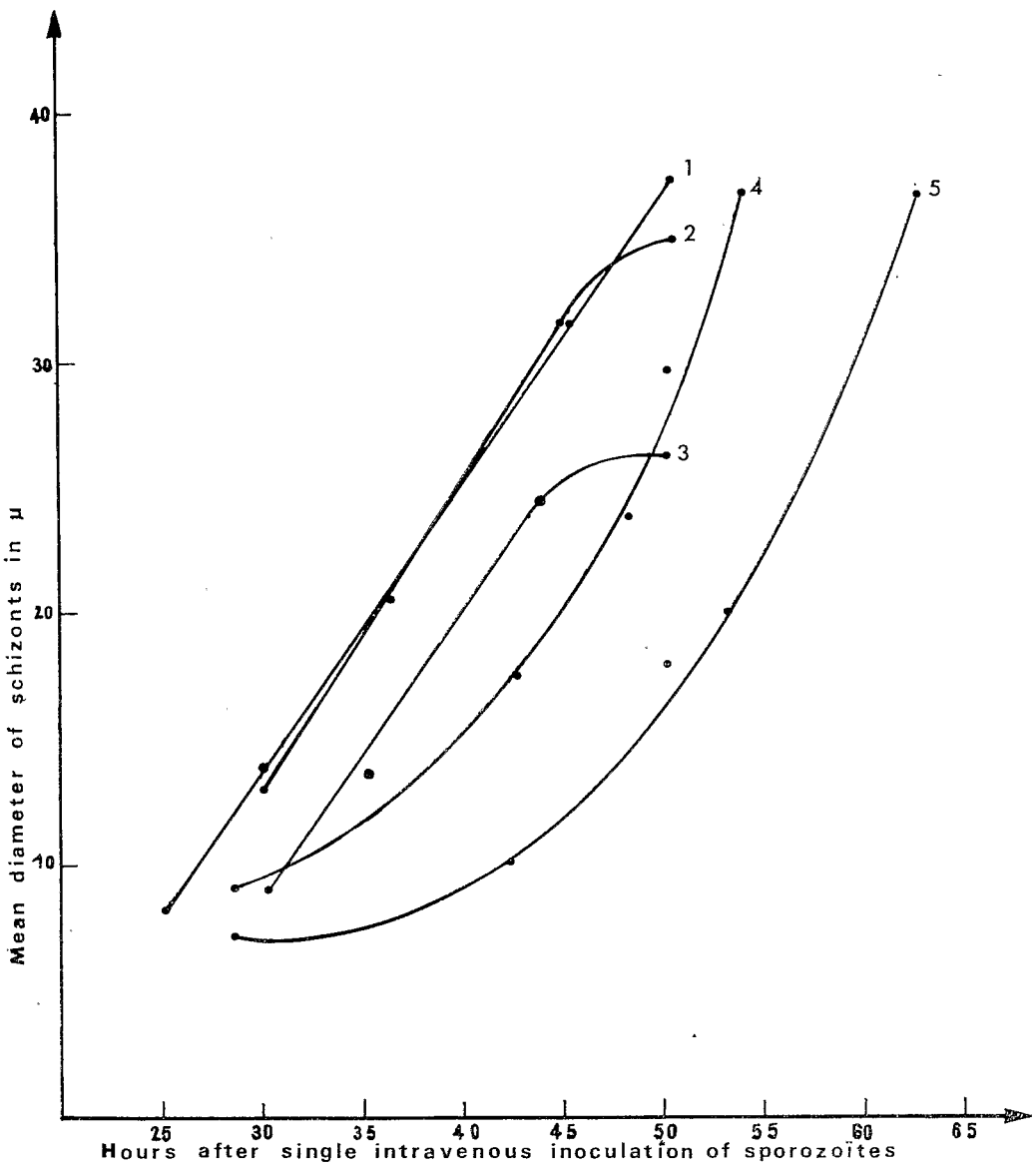
P. v. lentum differs from the other two known subspecies of *P. vinckei* in the following characteristics:

1. The mean length of the sporozoites of *P. v. lentum* in the salivary glands of *A. stephensi* is greater than 20μ, whereas that of *P. v. chabaudi* and *P. v. vinckei* is less than 15μ.

2. The mean diameter of mature oocysts is 47μ (maximum 56μ), while that of *P. v. chabaudi* is 75μ (maximum 80μ) (Landau and Killick-Kendrick, 1966). Bafort (1968) gives the range of diameters of mature oocysts of *P. v. vinckei* as 38–70μ, but gives no mean. Killick-Kendrick (*personal communication*) measured 11 mature oocysts of *P. v. vinckei* by the same methods we use, and found the mean diameter to be 40μ (maximum 43μ) at day 15 and a temperature of 21°C.

The rate of growth of the primary exoerythrocytic schizonts of *P. v. lentum* in the liver of a standard laboratory-bred host, the white mouse, is very much slower than that of *P. v. chabaudi* and *P. v. vinckei*. The difference between *P. v. lentum* and *P. v. chabaudi* is illustrated in the figure where the mean diameters of the tissue forms of different ages are plotted against the number of hours after the inoculation of sporozoites; these curves clearly show the slower growth rate of *P. v. lentum*. Similar data are not available for *P. v. vinckei* but the faster growth of *P. v. vinckei* is confirmed by the larger size of the tissue schizonts of the nominate subspecies at 48 hours, 24μ, compared to 18μ at 50 hours.

The complete life-cycles of malaria parasites of the *vinckei* and *berghei* groups from three localities in Africa (Katanga, Central African Republic and Brazzaville) have now been seen in the laboratory (Yoeli, 1965; Landau and Killick-Kendrick, 1966; Bafort, 1968; Landau, Michel and Adam, 1968). In addition, parts of the life-cycles of parasites of a fourth locality (Nigeria) have been briefly described (Killick-Kendrick *et al.*, 1968*a*). Some morphological characters are shared by the subspecies of *P. berghei* and *P. vinckei*



Graph showing the rate of growth of the primary exoerythrocytic schizonts of five subspecies of rodent plasmodia: 1. *P. b. yoelii;* 2. *P. b. killicki*; 3. *P. b. berghei*; 4. *P. v. chabaudi*; 5. *P. v. lentum*

from a given place (Landau, Chabaud, *et al.*, 1968), e.g. the long sporozoites of *P. b. killicki* and *P. v. lentum* from Brazzaville and the large oocyst and tissue forms of *P. b. yoelii* and *P. v. chabaudi* from the Central African Republic. The species are readily recognized by the morphology of the blood stages, whereas the subspecies are differentiated by morphological differences in the sporogonic and exoerythrocytic stages. The criteria upon which the species are subdivided are, we believe, clear and sufficiently constant to justify the recognition of geographical subspecies. Some of the criteria were reviewed by Garnham *et al.* (1967), others have been used since. However as Bafort (1968) has challenged both the validity of these criteria and the methods used, we shall here review briefly the principal differentiating characters of the murine malaria parasites in the light of our recent observations, and the data published by others.

Bafort emphasizes the necessity of taking account only of 'studies made in the natural host under strictly standardized circumstances'. There are three known natural vertebrate hosts of *P. b. berghei* (*Thamnomys surdaster*, *Praomys jacksoni* and *Leggada bella*) whereas the only known vertebrate host of *P. b. yoelii*, *P. b. killicki*, *P. v. chabaudi* and *P. v. lentum* is *T. rutilans;* that of *P. v. vinckei*, although assumed to be *T. surdaster*, is not known with certainty.

TABLE II

Showing the comparison between the sizes of mature oocysts and the sporozoites of six subspecies of rodent plasmodia

	Temperature of sporogony	Mean diameter of fresh mature oocysts	Mean length of sporozoites in dried preparations
P. b. berghei	21°C.	37μ (1)	11μ(2)
P. b. yoelii	22°—24°C.	75μ (1)	14μ (3)
p. b. killicki	23°—26°C.	60μ (1)	18μ (1)
P. v. vinckei	21°C.	40μ (4)	15μ (4)
P. v. chabaudi... ...	23°—26°C.	75μ (1)	13μ (3)
P. v. lentum	23°—25°C.	47μ (1)	21μ (1)

(1) Personal results
(2) Garnham (1966)
(3) Landau and Killick-Kendrick (1966)
(4) Killick-Kendrick (*personal communication*)

To attempt to confine morphological comparisons to observations in natural hosts is, in our view, impracticable, and, since the hosts differ, it means that studies cannot be made in standard conditions.

Furthermore, the invertebrate host of the parasites from Katanga is *Anopheles dureni millecampsi* (a mosquito which has never been established in the laboratory), and that of the parasites elsewhere is still undiscovered.

If observations are to be standardized, it seems best that comparisons are made between parasites in the same host and closely similar experimental conditions, and that results are expressed in the same way.

If techniques of measuring are standardized, there is little difficulty in different workers obtaining comparable data. For example, the mean diameter of the mature oocysts of *P. b. berghei* and *P. b. yoelii* recorded by Landau and Killick-Kendrick (1966) and Wéry

(1968) are closely comparable, as are the measurements of the tissue schizonts selected at random of *P. b. yoelii* and *P. v. chabaudi* reported by the same workers.

The simplest method of presenting the results of measuring oocysts, tissue schizonts or sporozoites is as mean sizes. More sophisticated statistical methods may well give a spurious security, since in a complicated life cycle involving two hosts there are many ill-defined variables. As with all populations of animals, some stages of malaria parasites are retarded and others are gigantic. The range of sizes alone as given by Bafort is therefore of little significance, and could lead to the temptation not to record consecutive observations, but to search a long time in an attempt to record the abnormal extremes.

In our experience, two valuable differentiating criteria are clearly seen during sporogony. These are the size of fresh mature oocysts, and the mean length of sporozoites measured by drawing dried stained parasites from crushed salivary glands. The differences between the sizes of oocysts and sporozoites of the six known subspecies of *P. berghei* and *P. vinckei* are shown in Table II.

The optimum temperature for the production of infective sporozoites provides valuable additional information if it is evaluated precisely by inoculating known numbers of sporozoites and counting primary exoerythrocytic schizonts (e.g. Wéry 1968).

In our view the best way of comparing the sizes of tissue schizonts is as a growth curve prepared by plotting mean diameters against the time after the intravenous inoculation of sporozoites. This was first done to demonstrate differences between *P. b. berghei* and *P. b. yoelii* (Landau and Killick-Kendrick, 1966), and in the graph we included growth curves of the tissue forms of *P. b. killicki*, *P. v. chabaudi* and *P. v. lentum*. The slopes of the curves illustrate the speeds of development. The upper parts are less helpful in making comparisons; in some instances the curves bend, and at this point of growth some differential characters we once thought significant, such as size of mature schizont and the first appearance of merozoites in the peripheral blood, may not always be constant.

For example, Bafort (1968) isolated a strain of *P. b. berghei* (ANKA strain) with most precocious tissue schizonts which gave rise to parasites in the peripheral blood at an earlier time (43 hours) than do other strains of *P. b. berghei*.*

The main criteria for the recognition of the subspecies of *P. berghei* and *P. vinckei* may be summarized as follows:

(i) the mean diameter of the fresh mature oocyst.

(ii) the mean length of sporozoites in dried stained preparations.

(iii) the growth rate of the primary exoerythrocytic schizonts.

In addition, minor characters, such as the effect of the parasite on infected erythrocytes or parenchymal cells, and the optimum temperature for the production of infective sporozoites assist in recognising the subspecies.

For a greater understanding of the intraspecific divisions of the murine malaria parasites more information is needed on the continuity of their distribution in Africa. At the moment, it appears as though there are a number of unconnected foci, and in some

*Bafort's (1968) suggestion that the ANKA strain of *P. b. berghei* may be intermediate between *P. b. berghei* and *P. b. yoelii* is not supported by the morphology of the sporogonic stages which clearly place the ANKA strain with others of the Katangan subspecies. Mature oocysts of the ANKA strain measure 36μ and the mean length of 25 sporozoites is 12μ (9μ—14μ) (Killick-Kendrick, *personal communication*). The growth curves of this interesting strain may throw more light on its affinities, but on the available data the ANKA strain does not appear to differ remarkably from other strains from the same locality.

instances isolation would be expected to give rise to differences which would be manifested in the morphology.

Since it is not yet possible to determine whether or not allopatric strains could interbreed, and since the known localities are far apart from one another, it might be thought better to raise the subspecies to specific rank. If, however, taxonomy is to show affinities as well as differences, there is, as Bafort (1968) suggests, a clear case for recognising only two species, *berghei* and *vinckei*, of murine malaria parasites. By the use of the criteria suggested in the present paper, these two species are, at the moment, each clearly divided into three subspecies.

The fear that the list of subspecies might become too large and complex as new foci are discovered is unfounded. The Nigerian strains, for example, appear to be indistinguishable from those from Brazzaville (Killick-Kendrick, *et al.*, 1968*b*).

The murine malaria parasites, because of the distribution and habits of their hosts, are in classical conditions where subspeciation and speciation can be observed. The rodents do not migrate over wide areas and are normally found in discontinuous biotopes. Isolation may, however, not be total, since the anopheline hosts may allow an overlap and this might explain the fact that each different biotope does not always correspond with distinct subspecies.

SUMMARY

The life cycle of *Plasmodium vinckei lentum* subsp. nov., parasite of *Thamnomys rutilans* in Brazzaville, was studied in *Anopheles stephensi* and white mice. *P. v. lentum* is separated from the two other known subspecies of *P. vinckei* by the size of the sporozoite, and rate of growth of the primary exoerythrocytic schizont. In addition, the size of the mature oocyst clearly separates the new subspecies from *P. v. chabaudi.*

The classification and differential characters of the murine malaria parasites are discussed. They fall into two species, *P. berghei* and *P. vinckei*, each divided into three subspecies. The main criteria for division into the lower taxon are (i) the mean size of the sporozoite; (ii) the mean size of the mature oocyst and (iii) the growth curve of the primary exoerythrocytic schizont.

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