A strain of *Plasmodium vivax* characterized by prolonged incubation: morphological and biological characteristics

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Numerous strains of P. vivax, *distinguishable chiefly by their biological characteristics, are known to exist. Two main varieties are recognized: the so-called temperate and tropical strains. The most extreme example of the former—designated by Nikolaev as* P. vivax hibernans—*constantly exhibited an extremely long incubation period. The strain is no longer in existence and no type material has been preserved. In its place, a North Korean strain with a generally long incubation period has been studied and compared with the well-known tropical Madagascar strain, which frequently but not constantly has a short incubation period. The data presented here concern the behaviour of various strains from the USSR and the morphological characteristics of the North Korean and Madagascar strains. Splenectomized chimpanzees were used as the host of these parasites, particularly in regard to exoerythrocytic schizogony. Attempts were also made, by late biopsies of the liver of the apes, to elucidate the prolonged latency of the North Korean strain. Although there was no evidence of specifically dormant forms, it is probable that certain sporozoites fail to develop in the normal time and that they are reactivated by an unknown factor a year or more after inoculation.*

Various strains of *Plasmodium vivax* have been described that have proved to be antigenically distinct in that infection with a certain strain may confer no cross-immunity to another. Whereas minor differences have been reported in their relative infectivity to different species of mosquito or response to drugs, the major biological difference between the strains concerns the relapse patterns and variations in the length of the incubation period. A prolonged delay of 9 months or longer was described by James (13), Mühlens (17), Korteweg (15), Winckel (40), Swellengrebel (33), Swellengrebel & de Buck (34), Shute (31), Nikolaev (19), Sergiev & Tiburskaja (27), and Tiburskaja (35), with particular reference to the Dutch, Madagascar, and USSR strains. A similar phenomenon, but in a less pronounced form, was noted in American strains (14), and the characteristics of the " temperate zone " St Elizabeth *P. vivax* were described in detail by Coatney & Cooper (4). Strains of *P. vivax* have been studied extensively in the USSR by Nikolaev (18–26) and more recently by Tiburskaja (35–38), Sergiev et al. (29), and Sergiev & Tiburskaja (27, 28).

Usually strains with prolonged latency show the phenomenon to a greater or lesser degree. This latency has been attributed either to " senility " of

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the sporozoite towards the end of the season (34) or to the minute numbers of sporozoites in the infective bite (G. Shute et al., unpublished observations, 1968–73). Other factors were summarized by Shute (31), who discussed the effect of certain drugs, the presence of a second species of malaria parasite (*P. falciparum*), and some degree of immunity in the host. Sergiev & Tiburskaja (28), considering data based on numbers of mosquitos not of sporozoites, concluded that the dose of sporozoites was not concerned and that the length of incubation depended on inherent properties of the strains.

The classification of strains proposed by WHO (39) was largely based on an analysis of data from North America, the United Kingdom, and the USSR. The following types were recognized:

Type I. The incubation period is short (12–20 days), relapses are frequent, and there are no prolonged periods of latency.

Type II. The incubation period is short (12–20 days) and there is a prolonged period (7–13 months) of latency between the primary attack and the first relapse or series of relapses at short intervals.

Type III. The incubation period is prolonged (6 months or more). The delayed primary attack may be succeeded by a series of relapses at short intervals and then by a second prolonged period of latency and further relapses.

Deviations from these patterns are common. Some strains may cause infections having the features of both Type II and Type III.

In 1967, with a view to studying further the problem of relapsing vivax malaria, the possibility of obtaining a strain of Nikolaev's parasite through the Marcinovskij Institute, Moscow, was explored. However, this strain was no longer in existence and the next most suitable strain appeared to be a North Korean *P. vivax* strain that had been isolated in 1953 and maintained in a Moscow psychoneurological hospital in patients undergoing malaria therapy. Plans were made to carry out a joint experiment with sporozoites of that strain, using splenectomized chimpanzees as the mammalian host. The main objective of the investigation was to see if the strain differed in the sporogonic and exoerythrocytic stages from the Madagascar strain, and in particular to observe the phenomena of latency and relapses in relation to the tissue phase of the parasite in the liver of the experimental animals. It is necessary, however, to bear in mind that the splenectomized chimpanzee is an abnormal host in which the parasite persists in low numbers in the blood for long periods (see pp. 30–31).

EXPERIMENTAL PROCEDURES

Of 7 patients undergoing malaria therapy in Moscow in May 1968, only 3 showed gametocytes in reasonably large numbers; it was on these patients that mosquitos were infected. About 2 000 *Anopheles atroparvus* had been taken by air from London to Moscow for the purpose. A few days after the infective feeds, the mosquitos were brought back to London and kept at a temperature of 28°C and a relative humidity of about 80%. On 7 June 1968—2 days after sporozoites appeared in the salivary glands—the mosquitos were dissected and the suspension (containing approximately 162 000 sporozoites) in medium 199 (Difco) was inoculated by the intravenous route into a young female chimpanzee (Bonnie) weighing 4 kg. This animal had been under observation for 3 months; it had shown a fleeting infection with *P. malariae* in March 1968; its spleen had been removed on 6 May, and it received a 10-day course of quinine (60 mg daily) by intramuscular injection later that month. Three days after the introduction of sporozoites, the chimpanzee developed diarrhoea and pulmonary symptoms; it was accordingly treated for 2 days with oxytetracycline (140 mg each day), and these conditions disappeared. A biopsy of liver was taken on 15 June at open operation, 8 days after the inoculation of sporozoites, and the material was submitted to the routine Giemsa–colophonium technique after fixation in Carnoy's fluid.

Daily blood films were taken from 14 June, but no parasites appeared in the animal's blood until 27 June—i.e., after a prepatent period of 20 days—when scanty trophozoites and schizonts were observed. These increased in number, and by the fifth day of the infection (3 July 1968) gametocytes appeared. No exoerythrocytic schizonts were found in 200 large sections of the liver biopsy material. We tentatively assumed that this result was due to one of the following factors:

(1) The North Korean strain was behaving rather as expected in producing sporozoites of low viability. Exoerythrocytic schizonts would be correspondingly rare; several cycles of schizogony in the blood would be required before parasites became visible in the blood, and the prepatent period would be delayed.

(2) The use of the antibiotic had inhibited the development of the parasite in the liver.

(3) Some deficiency in the experimental technique had occurred.

Fortunately, we were able to put these theories to the test, because the vivax infection in Bonnie was accompanied by gametocytes (appearing on the fifth day of the infection), and new batches of mosquitos were fed at appropriate times (3, 4, and 5 July 1968). The salivary glands were dissected and suspensions of sporozoites were reinoculated into the same animal and into other animals as follows:

(1) Ten million sporozoites from 104 *A. atroparvus* and 96 *A. stephensi* were inoculated into Bonnie by the intravenous route on 15 July 1968, and 500 *A. stephensi* were allowed to bite her on the same day. (Garnham & Bray had previously shown that the liver of immune animals was entirely suitable for the normal development of further homologous parasites (11); it was accordingly felt that this second inoculation would not interfere with the main purpose of the experiment—i.e., observations on late relapses.)

(2) Twenty-four million sporozoites from the same batch of *A. atroparvus* (300) were inoculated on the same day (15 July) into Sally—an older chimpanzee (a female approximately 5 years of age)—and 500 infected *A. stephensi* were allowed to bite her. She had been splenectomized in 1964 and was the subject of long-term experiments involving sporozoite infection with *P. malariae* (16).

A natural infection with *P. schwetzi* had been detected in the blood on 12 February 1965 and parasites of this species were last seen on 22 June 1966. Although this animal was far from " clean " it was the only other chimpanzee available and had never been exposed to *P. vivax*; we therefore felt that we should be able to interpret correctly the later parasitological manifestations. Subsequently the occurrence of a normal infection of *P. vivax* in Sally indicated the absence of any cross-immunity between these closely allied species.

Bonnie showed a parasitaemia of *P. vivax* that fluctuated during the course of the next 12 months and presumably originated from the two previous inoculations, although no upsurge of parasites had occurred after the second inoculation of sporozoites. There were seven irregularly spaced recrudescences (with 1 000–10 000 parasites per mm³) during the year (2).

Bonnie was not subjected to an immediate second biopsy of the liver as she did not respond well to anaesthesia; we did not want to risk her life during the early period of the experiment. Instead, she was kept for long-term observations on relapses, and a liver biopsy was performed 8 months after the second inoculation.

Sally, however, had a piece of liver removed after laparotomy on 23 July, 8 days after the inoculation of sporozoites. The blood was invaded by tiny rings of *P. vivax* on that day and the liver showed exoerythrocytic schizonts (see below)—thus demonstrating the normal degree of viability of sporozoites of this strain when not exposed to oxytetracycline. We later showed the inhibitory effect of this antibiotic on the tissue stages of *P. cynomolgi* in the liver of rhesus monkeys (10). The presence of gametocytes of *P. vivax* in the blood of Sally enabled us to infect still further batches of mosquitos.

Sporozoites were harvested as before and were inoculated into animals by the intravenous route on 13 August 1968 as follows:

3 million sporozoites into one *Aotus trivirgatus*;

1.6 million sporozoites into one *Cercopithecus neglectus* (chronically infected with *Hepatocystis cercopitheci*, which had last been seen in the blood in August 1967);

1.6 million sporozoites into one unsplenectomized *Saimiri sciureus*;

1.6 million sporozoites into another *S. sciureus*, which was splenectomized at the same time as a liver biopsy was taken.

These four primates were subjected to laparotomy 8 days after the inoculation of sporozoites, and portions of liver were removed. No parasites were observed in the blood of the last three animals, but *P. vivax* appeared in the blood of the *Aotus* on 15 November 1968, 95 days after infection. Exoerythrocytic schizonts were found in sections of the liver of the unsplenectomized *Saimiri* monkey (see below), but were not detected in the other animals.

Finally, 5.5 million sporozoites (from a later batch of infected mosquitos) were inoculated by the intravenous route into a marmoset (*Saguinus oedipus*), but no parasites were found in its blood or liver.

SPOROGONY OF THE NORTH KOREAN STRAIN
OF *P. VIVAX*

Exflagellating microgametocytes

The maximum number of microgametes seen in the North Korean strain was eight, and at 22°C they

became free from the surface of the gametocytes in 15 min.

Ookinetes

By 18 h at 27°C the male and female nuclei had merged. Some laggards were still in the spherical form, about 7 μm in diameter, but were on the point of " unrolling " (Fig. 2 A). The maximum length of the ookinete at 18 h was 17 μm and the breadth 3.5 μm (Fig. 2 B). The cytoplasm stained a deep blue colour, and in addition to the pigment three prominent structures were seen in the parasite, as follows:

(1) A nucleus consisting of dense chromatin (often arranged in a few particles) within a pale zone bounded by the nuclear membrane; it was 4 μm long. Later in growth the pale halo became less conspicuous.

(2) An anterior cap, 2 μm or more in length, orange in colour, in which the pigment later became concentrated.

(3) One, or occasionally two, circular vacuoles.

The pigment when scattered throughout the cytoplasm was fine and very dark or black in colour; it was sometimes condensed at the posterior extremity and seemed about to be extruded. This extremity was always curved and looser in appearance than the rest of the ookinete; it resembled a fish's tail or hawk's head.

Oocysts

In the three-day oocysts the pigment granules were numerous and were scattered throughout the cyst. The colour of the pigment was dark brown to nearly black; the granules were uneven in size and were in the form of large dots and very short rods. On the fourth day and onwards, with the size of the cysts increasing daily, the pigment granules tended to become arranged in rows or in some kind of pattern (Fig. 1). By the seventh day, with advanced sporogony, much of the pigment had become obscure, but in some oocysts a few grains were still to be seen. Table 1 gives the size of oocysts of this strain, compared with those of the Madagascar strain in similar conditions.

On approaching maturity, the North Korean oocysts are rather smaller in diameter and the pigment granules after the fourth day tend to lie in double instead of single rows. The colour is similar, however, and the characteristic " Prince of Wales' feathers " pattern is found in both.
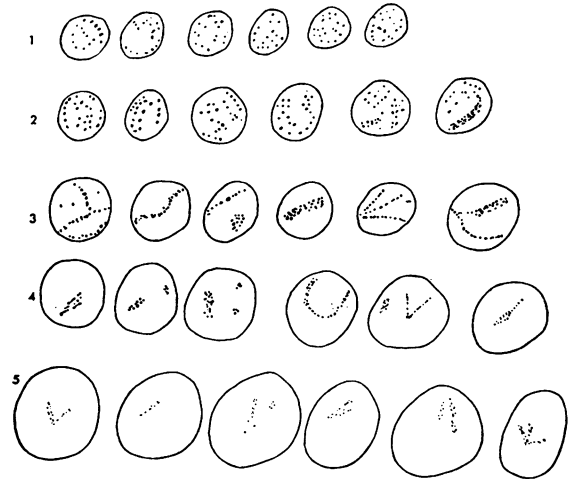

Fig. 1. Oocysts of *P. vivax,* North Korean strain, at different intervals (in days) after infection in *A. stephensi.* Whole mounts showing pigment granules.

The description of the pigment and the measurements of the oocysts are based on specimens dissected in Ringer's fluid, flattened beneath a coverslip and very lightly stained with erythrosin, dehydrated, and mounted in Euparal.

Sporozoites

Sporozoite formation was completed in the oocyst by the end of the eighth day at a temperature of 25°C, and sporozoites were found in the salivary glands on the ninth day (Fig. 2 D). Their mean length in air-dried, fixed specimens from the glands was 12 μm (range 11.0–15.5 μm); sporozoites that had been in the glands for nearly a month (at 21°C) measured 12.5 μm (Fig. 2 C); the nuclei were frequently fragmented in both.

Table 1. Mean diameters of oocysts of North Korean and Madagascar strains of *P. vivax* (25°C in *A. atroparvus*)

| Stage of sporogony (days)	North Korean strain (μm)	Madagascar strain (μm)
3	8	10
5	26	20
6	30	25
7	30	35
8	35	45

Fig. 2
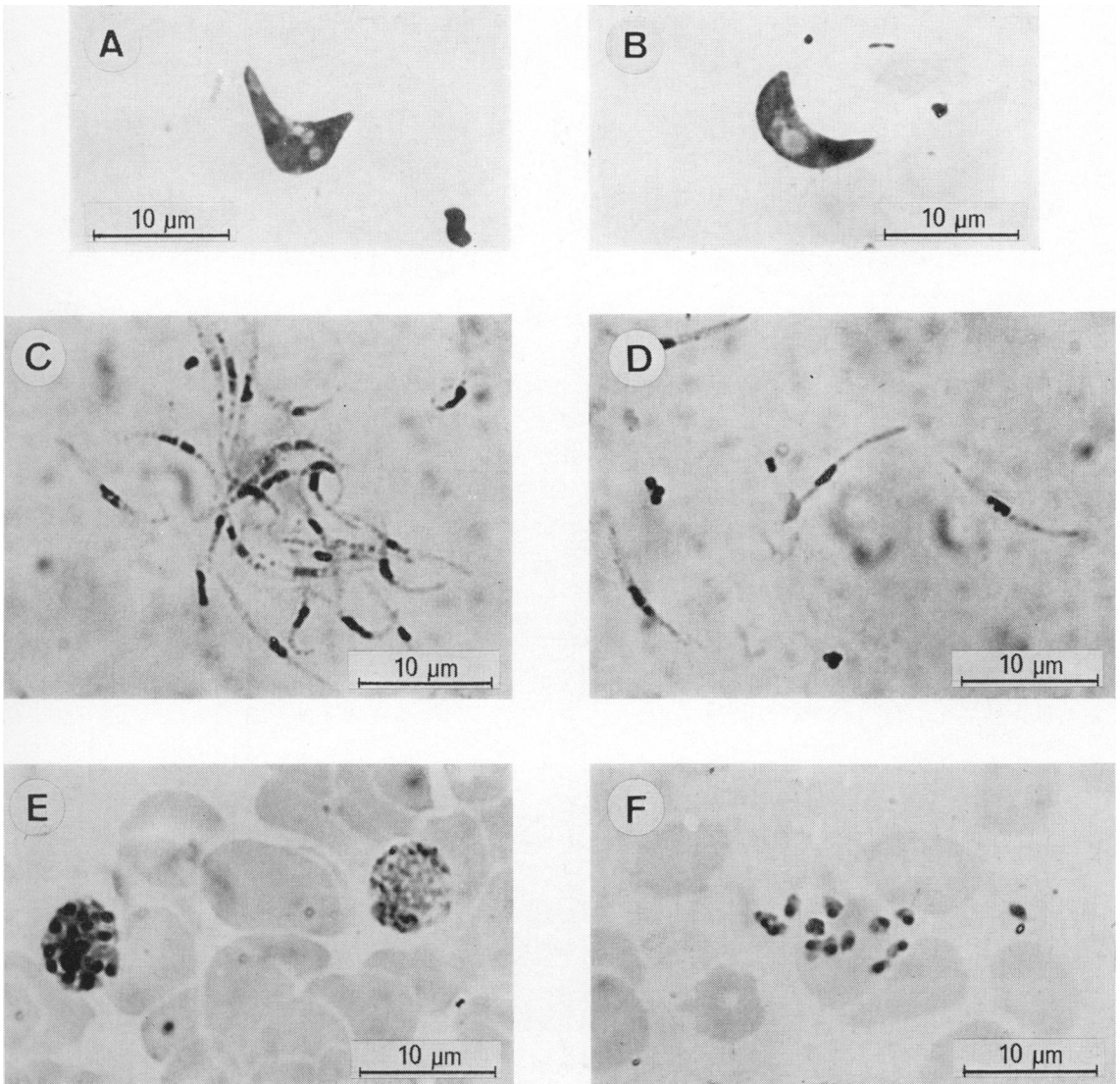
Fig. 2. Stages in the development of *P. vivax*, North Korean strain: A & B, ookinetes (18 h); C, sporozoites (25 days in salivary glands); D, sporozoites (2 days in salivary glands); E, mature schizont and macrogametocyte; F, ruptured mature schizont: 12 merozoites and pigment.

Fig. 3. Exoerythrocytic schizonts in the liver: A-C & E-G, chimpanzee; D, *Saimiri*. A, nearly mature, at 8 days; B, mature with merozoites forming, at 8 days; C, mature and rupturing, at 8 days; D, immature schizont, at 8 days; E, higher magnification of C, showing merozoites; F, " relapse " form at 8 months; G, " relapse " form undergoing phagocytosis.

Fig. 3
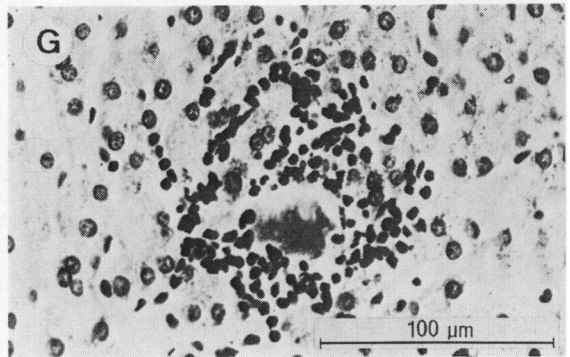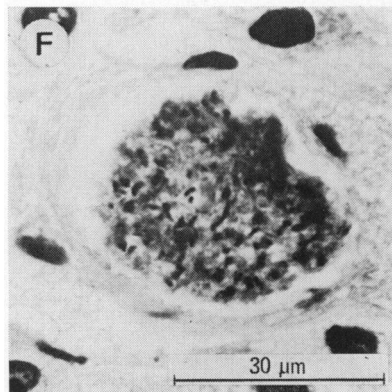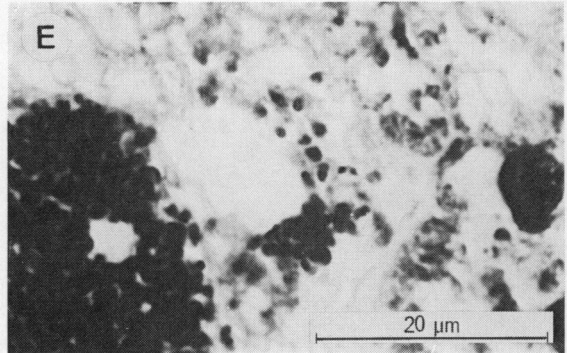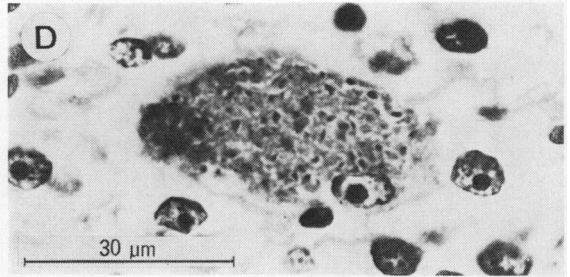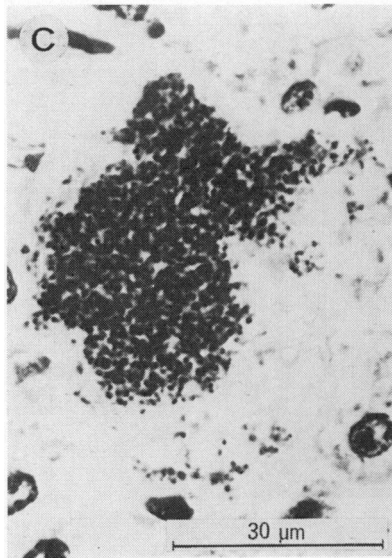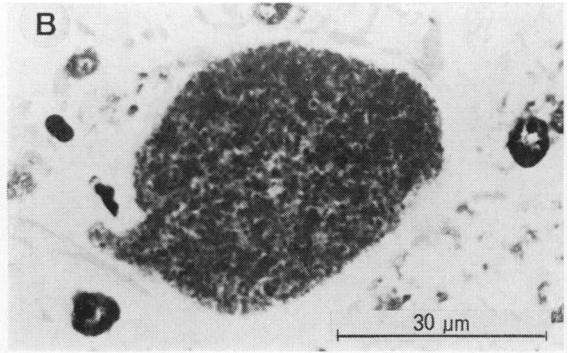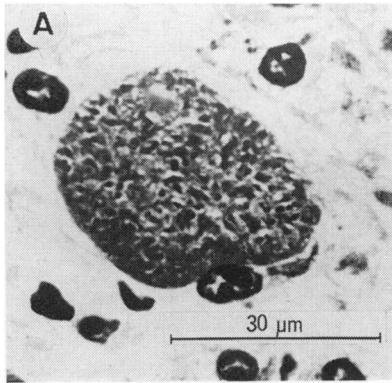
The ultrastructural characteristics of sporozoites of the North Korean strain have been described by Sinden & Garnham (32). The number of subpellicular microtubules was found to be 11+1; in the Madagascar strain there were 10+1. In other respects, the sporozoites of the two strains appeared to be identical.

SPOROZOITE-INDUCED INFECTIONS IN THE BLOOD AND LIVER

It was necessary to exercise economy in the number of chimpanzees used in the experiments, because of their expense and the undesirability of using rare animals for experimentation. Therefore our observations were based on a prolonged study of a few animals, instead of the larger numbers used as a routine in research on simian malaria.

Chimpanzee Bonnie, aged about 1 year

7.6.68 Inoculated with sporozoites.

15.6.68 Biopsy of liver; no exoerythrocytic schizonts were seen. Blood negative until:

28.6.68 Very scanty *P. vivax* in thick film.

Parasitaemia remained low until:

2.7.68 More numerous parasites with immature gametocytes; mosquitos fed on 3 successive days when gametocytes were mature.

15.7.68 Inoculated with sporozoites of above-mentioned batches; parasitaemia low from 20.7.68 until:

6.8.68 Minor recrudescence.

12.8.68 No parasites seen in thin films, but minor recrudescences occurred in September and October, and fluctuating low parasitaemia until 21 March 1969.

27.3.69 Biopsy of liver; blood negative; 5 exoerythrocytic schizonts were seen.

28.3.69 Blood negative.

29.3.69 Blood negative.

11–14.4.69 Low parasitaemia; negative from 16 April until 8 July 1969.

Dec. 1969 Blood remained negative until March 1971, when a few *P. vivax* were again found.

No biopsy was performed after the second inoculation of sporozoites (on 15 July 1968) and the animal was kept for a late examination of the liver;

on 27 March 1969 a second biopsy was made, and the following is a description of the " relapse " bodies (Fig. 3 F).

The exoerythrocytic schizonts were scanty and only five fairly large forms were detected; it is uncertain if the liver infection on the day of the biopsy was confined to parasites of approximately the same size (and possibly age), or whether smaller parasites were missed in the examination of the sections.

One schizont was pear-shaped. The contour had some deep indentations, but there was no thickening of the surface membrane. The cytoplasm stained mauve and was fairly dense. The nuclei appeared to be highly active and stained intensely; about half of them were elongated. The host cell nucleus was not seen. The dimensions (maximum) were 37×45 μm. The general appearance of this parasite did not suggest that it was in a condition of latency, but two others had the convoluted surface sometimes considered to be an indication of inactivity.

Another schizont was surrounded by adventitious cells (Fig. 3 G); the latter comprised lymphocytes, macrophages, and endothelioid cells, and outside this cellular layer were many erythrocytes. The schizont itself was not mature and its surface was indistinct, perhaps as the result of commencing cytolysis; it measured 25×37 μm, and the nuclei and cytoplasm appeared to be normal.

A further " relapse " schizont had a definitely convoluted border with an obvious limiting membrane. This schizont measured 60×52 μm and lay shrunken away from the host cell; the cytoplasm was deeply staining and studded with numerous, often elongated, nuclei. In the size and type of nuclei it resembled a primary schizont, from which, however, it differed by the border and by a circle of adventitious cells that surrounded the body. These cells were lymphocytes and mononuclear cells about three layers deep.

These observations on Bonnie showed that exoerythrocytic stages of the North Korean strain could still be found in the liver 255 days after the second inoculation of sporozoites. The elongate form of many nuclei, the presence of typical " seventh-day vacuoles ", and the absence of thickening of the surface membrane indicated that these schizonts were perhaps not in a condition of latency; certainly they were not in the form of cysts—cf. Desser et al. (6). However, phagocytic accumulation around an immature parasite and clefts or irregularities in the contour are very unusual in primary forms of *P. vivax* and are possibly of some significance.

Chimpanzee Sally, aged about 5 years

15.7.68 Infected with sporozoites (same origin as those used for Bonnie).

23.7.68 Very scanty parasitaemia (*P. vivax*). Biopsy of liver; exoerythrocytic schizonts found. Parasitaemia slowly increased until:

8.8.68 Gametocytes numerous and mosquitos fed. Parasites subsequently declined in numbers, disappearing in November 1968.

20.1.69 Parasites reappeared.

19.2.69 Parasites present.

10.3.69 Biopsy of liver; no exoerythrocytic schizonts found.

14.3.69 Parasites present.

11–16.4.69 Parasitaemia.

18.4.69 No parasites seen and blood remained negative.

21.4.69 Biopsy of liver; no exoerythrocytic schizonts found.

Although Sally was much older than Bonnie and her liver proportionately larger, exoerythrocytic schizonts were found to be fairly numerous (nearly one parasite per section) in the biopsy performed on 23 July 1968—i.e., 8 days after inoculation of sporozoites. The following is a description of these schizonts (Fig. 3 A, B, C, & E).

The primary exoerythrocytic schizonts exhibited the typical morphology of the species and some had reached maturity, as was to be expected in a biopsy performed on the eighth day. Others were less mature and still contained vacuoles. The vacuoles were usually about 3 μm in diameter and up to 5 in number. They contained an eosinophilic or orange substance, occasionally condensed into a pink staining dust.

The shape of the schizont was subspherical or ovoid, with mean dimensions of 45 × 38 μm just before maturity. On rupture of the mature form, the merozoites spread out over a wider area (Fig. 3 C). The contour was usually smooth and the surface membrane inconspicuous. One or two host cell nuclei were present and were of normal size.

Cytoplasmic clumps or strands were sometimes seen, though more often the cytoplasm was dense or granular (Fig. 3 B). The nuclei varied in shape but were often elongated, rarely even 7 μm in length; they were numerous and towards maturation tended to line up on the periphery of the schizont. The final

division resulted in the production of closely packed merozoites about 1 μm in diameter. Their structure is best seen in the newly ruptured schizont (Fig. 3 E); they are spherical bodies comprising a nucleus and a semicircle of cytoplasm.

The later biopsies were performed on the 238th and 280th days after infection, but no tissue forms were found.

Monkeys

Five monkeys (comprising 4 species) also received sporozoites derived from infections of the North Korean strain in Sally (see above), and biopsies of the livers were taken on the eighth day. In only one monkey (*Saimiri sciureus*) were exoerythrocytic schizonts detected, although 1–5 million sporozoites had been injected by the intravenous route into each animal.

Three schizonts were found in the *Saimiri* as follows:

(1) An ovoid schizont, measuring 18 × 31 μm in its largest diameter. The nuclei were round and the cytoplasm was finely granular with a few small clumps. No surface membrane was visible. The host cell nucleus was slightly enlarged.

(2) An ovoid schizont, measuring 18 × 33 μm. There was a single deep cleft in the otherwise smooth surface. The cytoplasm resembled that of (1), but some nuclei were elongate.

(3) A larger schizont, measuring 20 × 40 μm. The nuclei were occasionally elongate and the spherical ones tended to be surrounded by a space (Fig. 3 D).

No parasites appeared in the blood of this or the other animals except in the *Aotus*, which showed a transient parasitaemia on 15 November 1968. A second biopsy of its liver was made 4 days later (i.e., 98 days after the inoculation of sporozoites) in the hope of catching an exoerythrocytic "generation", but no schizonts were seen. It may be noted that Martin Young (unpublished observations, 1973) found that the tissue stages of *P. vivax* (Panamanian strains) occurred very rarely in *Aotus*, but that they were much more easily demonstrable in the liver of *Ateles* spp.

DISCUSSION

The main objective of the first part of this study was to compare the exoerythrocytic cycle of a strain of *P. vivax* with delayed prepatency (North Korean strain) and that of a "normal" or tropical strain

(Madagascar). The work was necessarily limited to infections in splenectomized chimpanzees because the parasite never, or scarcely ever, becomes patent in intact animals. The absence of a spleen renders the course of parasitaemia entirely abnormal and possibly the parasites remain in the blood of a splenectomized chimpanzee for years, though they may be undetectable in blood films. There was no question, therefore, of observing either delayed patency or true (i.e., parasitic) relapses. The course of parasitaemia in these animals has thus taught us little.

The most we could hope for was the demonstration of " secondary " or " delayed primary " exoerythrocytic schizonts in the liver of animals inoculated with sporozoites of the North Korean strain of *P. vivax*. Fortunately this objective was realized, as was the demonstration of the primary tissue phase 8 days after the inoculation.

In the series of experiments reported above, very high doses (usually in millions) of sporozoites had to be administered in order to observe the morphological characteristics of the exoerythrocytic stages of the North Korean strain. The relationship between the actual sporozoite dosage and the duration of the incubation period in man will be dealt with in a further paper.

We are unable to say how chimpanzees would respond to the administration of low doses of sporozoites. Splenectomy is a prerequisite for the multiplication of *P. vivax* in the blood of a chimpanzee and this operation itself may well influence the course of infection, including the length of the prepatent period. Thus, studies on delayed patency of *P. vivax* require the use of man as the host.

Although no deliberate attempt has been made in this investigation to study the morphology of the erythrocytic stages of the North Korean strain, we have perforce made observations on the parasites in our daily work. Sergiev & Tiburskaja (27) and Nikolaev (22) also studied them, but found no morphological features that would distinguish this strain from the type. Field & Shute (8) thought that the variations occasionally reported in different strains of *P. vivax* were not permanent, or that they occurred only under abnormal conditions.

We also found no significant differences in the number of merozoites, length of asexual cycle, or time of maturation of the schizont. The temperature chart of patients infected with the North Korean strain of *P. vivax* showed a typical tertian periodicity after the first quotidian fever. Maturation of schizonts

and the rise of temperature occurred between 08.00 hours and noon. Such a picture is also presented by the Madagascar strain of *P. vivax*.

We hoped to find that the stage of the parasite in the mosquito host or in the liver would provide a basis for differentiation, and we compared our findings in the North Korean strain with published or unpublished work on *P. vivax vivax*.

For an adequate comparison of the sporogonic stages, it is essential that three factors should be the same for both strains: (1) species of *Anopheles;* (2) temperature of insectary; and (3) technique of preparing material, i.e., fixed or unfixed. Table 1 presents the measurements of oocysts of the respective strains. Two small differences are apparent: in the larger size of the pigment granules on the 4th and 5th days, and in the smaller size of the oocysts of the North Korean strain.

Our observations on the exoerythrocytic schizonts of the North Korean strain are based on infections in splenectomized chimpanzees. Fortunately we also have records of *P. vivax* Madagascar strain in such animals, and in most respects the observations for both strains resemble those found in man. Table 2 gives a comparison of the features of the two strains. Only minor differences in the tissue schizonts were found in the two strains, but these may be important. A careful check was made of the first appear-

Table 2. Characteristics of exoerythrocytic schizogony of *P. vivax;* Madagascar strain and North Korean strain

Characteristics	Madagascar strain in man [a]	Madagascar strain in chimpanzee [b]	N. Korean strain in chimpanzee
minimum duration to maturity:	7 days	7 days	8 days
contour:	ovoid, smooth	ovoid	subspherical, smooth
mean diameter at 7 days:	$56 \times 43 \ \mu m$ [c]	?	?
at 8 days:	?	$52 \times 44 \ \mu m$	$45 \times 38 \ \mu m$
cytoplasm:	granular	dark clumps	dense; occasional clumps
type of nuclei:	many, very elongated	often elongated	often elongated
merozoite:	$1 \ \mu m$	$1 \ \mu m$	$1 \ \mu m$ or less

[a] Shortt & Garnham (30).
[b] Bray (1).
[c] Newly measured series.

ance of parasites in the blood. The North Korean strain took a day longer than the Madagascar strain to mature in chimpanzees. The schizonts themselves were smaller in the North Korean strain.

Sections of a piece of human liver, taken 7 days after infection with the Madagascar strain, revealed schizonts (30) that were indistinguishable, except for size, from those of the same strain in chimpanzees at day 8, described by Bray (1); thus the schizonts were near maturity at 170 h, and the nuclei were round or irregular; the cytoplasm was compact and vacuoles were absent in the maturer parasites.

Relapse forms of the North Korean strain were found in the chimpanzee, Bonnie, 8 months after infection. Two of the schizonts showed signs of activity, and these did not appear to be in a condition of latency; two others, however, possessed features that have been associated with dormancy and much resembled the so-called secondary exoerythrocytic schizonts of *P. cynomolgi* (9). Unfortunately, the true nature of " relapse bodies " has still to be established. Even in *P. cynomolgi* infections, carefully watched for the reappearance of erythrocytic schizogony and followed by immediate biopsies, no clear indications could be found.

The taxonomic problem of the different strains of *P. vivax* based on morphological characteristics remains largely unsolved. No strains can be ascribed with certainty to a specific locality, not even Grassi & Feletti's original *P. vivax* from Catania, Sicily. This region was exposed to infections of malaria parasites from innumerable sources around the Mediterranean and beyond, and it is doubtful if the parasites that the Italians were studying were of indigenous origin. Similarly, the Madagascar strain isolated by James (13) from a Lascar seaman possibly did not come from that island but from one of many ports at which his ship had called. Even the North Korean strain that Tiburskaja (35) isolated in 1953 and that was the source of our material, cannot be regarded with certainty as indigenous, as Korea had been exposed to foreign strains of *P. vivax* imported by American soldiers during the Korean War. However, Hankey et al. (12) noted that the malaria of this region was predominantly of the temperate type, which is characterized by a long incubation period.

Full information is available on only two of the many strains that have been described: the North Korean (temperate) and the Madagascar (tropical)

strains discussed here. The essential data are based on the biological characteristics and the features exhibited by the erythrocytic, exoerythrocytic, and sporogonic stages of the parasite. To these criteria may be added protein analysis with special reference to isoenzyme patterns.

Material obtained during this study was sent to Carter & Voller (3) for isoenzyme analysis, and we are indebted to these workers for allowing us to quote their preliminary results, which they wish to confirm on a much larger scale.

The North Korean strain showed bands of parasite activity of the two enzymes, lactate dehydrogenase (1.1.1.27) and glucosephosphate isomerase (5.3.1.9); a tropical strain of *P. vivax* (Chesson) showed a band in a position different from that of the glucosephosphate isomerase of the temperate parasite.

We recognize two subspecies of *P. vivax* in man, but the North Korean strain can be assigned to neither. These subspecies are:

P. vivax vivax (Grassi & Feletti, 1890) Nikolaev, 1949. Neotypes (consisting of exoerythrocytic schizonts, sporogonic stages, and blood forms) of the Madagascar strain (13) have been selected and deposited in the Wellcome Museum of Medical Science, London, and paratypes are in the Marcinovskij Institute of Medical Parasitology and Tropical Medicine, Moscow. These replace and augment the lost material of Grassi & Feletti. The Madagascar strain is intermediate in its relapse pattern between the Chesson strain (7) and strain XIX of *P. v. hibernans* (see below); it is, however, nearer to the former.

P. vivax hibernans Nikolaev, 1949. The type strain (No. XIX) of this subspecies was isolated from north of Moscow; no type material was deposited and the strain is now lost. Unlike *P. v. vivax*, this subspecies always showed a long incubation period (23).

In its relapse pattern and the frequency of a long incubation period, the North Korean strain resembles the Dutch and St Elizabeth strains (5). However, unlike *P. v. hibernans*, it does not always show a long incubation period. In this respect, therefore, the North Korean strain appears to lie between *P. v. vivax* and *P. v. hibernans*. Specimens of the stages of its life-cycle, described in the present paper, are deposited in the Wellcome Museum and in the Marcinovskij Institute.

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RÉSUMÉ

CARACTÉRISTIQUES BIOLOGIQUES D'UNE SOUCHE DE *PLASMODIUM VIVAX* CARACTÉRISÉE PAR UNE LONGUE PÉRIODE D'INCUBATION

Les hommes de science britanniques et soviétiques ont associé leurs efforts pour essayer de résoudre le problème des différences essentielles entre les souches de *Plasmodium vivax* de zone tempérée et de zone tropicale. L'analyse des observations de longue durée faites sur la biologie des premières a montré que l'on pouvait distinguer plusieurs types comprenant notamment *P. vivax hibernans* et une souche nord-coréenne très voisine. Aux fins de comparaison, les auteurs ont choisi, comme exemple de souches tropicales, la souche de Madagascar sur laquelle il existe une abondante documentation. Les caractéristiques biologiques essentielles de ces souches sont en rapport avec la durée de la période d'incubation et l'apparition de rechutes; pour les souches de zone tempérée, la période d'incubation est longue tandis qu'elle est courte pour les souches de zone tropicale.

La morphologie des stades sporogoniques et exoérythrocytaires des souches de Corée du Nord et de Madagascar a été étudiée chez le moustique, ainsi que chez le chimpanzé splénectomisé et chez quelques singes de petite taille. Les moustiques (*Anopheles atroparvus*) ont été infectés sur des malades soumis à paludothérapie (par la souche nord-coréenne) à Moscou, puis envoyés par avion à Londres où les sporozoïtes ont été inoculés à des chimpanzés splénectomisés à raison de 10 millions d'organismes par dose. Des biopsies du foie ont été pratiquées le 8ᵉ jour après l'inoculation et, par la suite, à des intervalles différents pendant une période de 8 mois. D'une façon générale, les chimpanzés étaient porteurs de parasites 8 jours après l'inoculation, mais le sang des singes de petite taille restait négatif ou n'accusait qu'une parasitémie retardée et de faible intensité. Des schizontes exoérythrocytaires de type normal ont été vus dans la première biopsie pratiquée 8 jours après l'inoculation des sporozoïtes. Quelques formes primaires de schizontes exoérythrocytaires ont été observées chez un singe du genre *Saimiri*.

Du point de vue morphologique, les deux souches diffèrent assez peu; des variations ont été observées dans la vitesse de la sporogonie, la taille des oocystes et la granulométrie du pigment; les schizontes exoérythrocytaires étaient plus petits dans la souche nord-coréenne et il leur fallait un jour de plus pour arriver à maturité. Cinq schizontes exoérythrocytaires ont été trouvés dans le foie d'un chimpanzé 8 mois après l'inoculation des sporozoïtes; trois de ces schizontes n'étaient pas quiescents mais, apparemment, venaient juste de sortir de l'état latent. Il est possible que les rechutes tardives observées chez l'homme s'expliquent par la persistance et le réveil de pareilles formes du parasite.

En dépit de ce que voudraient les règles internationales de nomenclature zoologique, aucun matériel type du complexe *P. vivax* n'avait été déposé. Des néotypes de tous les stades de la souche de Madagascar de *P. vivax vivax* ont donc été sélectionnés et déposés au Wellcome Museum de Londres, les para-néotypes étant déposés à l'Institut Marcinovskij de Moscou. Pour ce qui est de la souche nord-coréenne, des séries analogues ont été ajoutées aux collections des deux institutions.

REFERENCES

1. Bray, R. S. *American journal of tropical medicine and hygiene*, **6**: 514 (1957).
2. Bruce-Chwatt, L. J. et al. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **64**: 2 (1970).
3. Carter, R. & Voller, A. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **67**: 14 (1973).
4. Coatney, G. R. & Cooper, W. C. *In:* Proceedings of the Fourth International Congress of Tropical Medi-

cine and Malaria, Washington, DC, 1948. Washington, DC, Department of State, 1948, vol. 1, p. 629.

5. COATNEY, G. R. ET AL. The primate malarias. Bethesda, US Department of Health, Education, and Welfare, 1971.

6. DESSER, S. S. ET AL. Canadian journal of zoology, 46: 281 (1968).

7. EHRMAN, F. C. ET AL. Science, 101: 377 (1945).

8. FIELD, J. W. & SHUTE, P. G. The microscopic diagnosis of human malaria. 2. A morphological study of the erythrocytic parasites. Kuala Lumpur, 1956 (Studies from the Institute for Medical Research, Federation of Malaya).

9. GARNHAM, P. C. C. Protozoology, 2: 55 (1967).

10. GARNHAM, P. C. C. ET AL. Journal of tropical medicine and hygiene, 74: 32 (1971).

11. GARNHAM, P. C. C. & BRAY, R. S. Revista brasileira de malariologia e doenças tropicais, 8: 151 (1956).

12. HANKEY, D. D. ET AL. American journal of tropical medicine and hygiene, 2: 958 (1953).

13. JAMES, S. P. Transactions of the Royal Society of Tropical Medicine and Hygiene, 24: 477 (1931).

14. KITCHEN, S. F. & PUTNAM, P. Journal of the National Malaria Society, 5: 57 (1946).

15. KORTEWEG, P. C. In: I. Jdo, ed., Herinneringsbuhndel voor Professor S. S. Rosenstein, Leiden, 1902, p. 272.

16. LUPASCU, G. ET AL. Transactions of the Royal Society of Tropical Medicine and Hygiene, 61: 482 (1967).

17. MÜHLENS, P. Handbuch der pathogenen Protozoen. Leipzig, Barth, 1931, p. 1474.

18. NIKOLAEV, B. P. Tropičeskaja medicina i veterinarija, 8: 380 (1931).

19. NIKOLAEV, B. P. Trudy Instituta epidemiologii i bakteriologii imeni Pastera, 2: 82 (1935).

20. NIKOLAEV, B. P. Medicinskaja parazitologija i parazitarnye bolezni, 8: 25 (1939).

21. NIKOLAEV, B. P. Medicinskaja parazitologija i parazitarnye bolezni, 8: 192 (1939).

22. NIKOLAEV, B. P. Medicinskaja parazitologija i parazitarnye bolezni, 10: 199 (1941).

23. NIKOLAEV, B. P. Trudy akademii nauk CCCP, 47: 201 (1949).

24. NIKOLAEV, B. P. Trudy voenno-medicinskoj akademii imeni S.M. Kirova, 46: 149 (1951).

25. NIKOLAEV, B. P. Trudy voenno-medicinskoj akademii imeni S.M. Kirova, 46: 168 (1951).

26. NIKOLAEV, B. P. Voprosy kraevoj patologii, 8: 110 (1957).

27. SERGIEV, P. G. & TIBURSKAJA, N. A. Medicinskaja parazitologija i parazitarnye bolezni, 13: 36 (1944).

28. SERGIEV, P. G. & TIBURSKAJA, N. A. Archives roumaines de pathologie expérimentale et de microbiologie, 26: 473 (1967).

29. SERGIEV, P. G. ET AL. Medicinskaja parazitologija i parazitarnye bolezni, 20: 312 (1951).

30. SHORTT, H. E. & GARNHAM, P. C. C. Transactions of the Royal Society of Tropical Medicine and Hygiene, 41: 785 (1948).

31. SHUTE, P. G. Transactions of the Royal Society of Tropical Medicine and Hygiene, 40: 189 (1946).

32. SINDEN, R. E. & GARNHAM, P. C. C. Transactions of the Royal Society of Tropical Medicine and Hygiene, 67: 631 (1973).

33. SWELLENGREBEL, N. H. Bulletin de la Société de pathologie exotique et de ses filiales, 22: 642 (1929).

34. SWELLENGREBEL, N. H. & DE BUCK, A. Malaria in the Netherlands. Amsterdam, Scheltema & Holkema, 1938.

35. TIBURSKAJA, N. A. Medicinskaja parazitologija i parazitarnye bolezni, 6: 643 (1962).

36. TIBURSKAJA, N. A. Medicinskaja parazitologija i parazitarnye bolezni, 33: 204 (1964).

37. TIBURSKAJA, N. A. In: [Problems of medical parasitology and prophylaxis.] Moscow, Medgiz, 1964, p. 187.

38. TIBURSKAJA, N. A. ET AL. Bulletin of the World Health Organization, 38: 447 (1968).

38. WHO Technical Report Series, No. 433, 1969 (Parasitology of malaria: report of a WHO Scientific Group).

40. WINCKEL, C. W. F. Documenta de medicina geographica et tropica, 7: 292 (1955).