from the site of denervation. The increase of unmyelinated axons is approximately 22 percent (15 and 29 percent) one segment from the lesion and approximately 13 percent (8 to 18 percent) in roots two and three segments from the lesion. Since the sprouting can be quantified, it will be possible to determine the effects of age on the amount of sprouting and to see if different types of spinal denervation produce different amounts of sprouting. Such studies may lead to a greater understanding of the various determinants of sprouting of dorsal root axons in response to spinal injury.

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5. The neural components of the dorsal root are myelinated and unmyelinated axons. Sprouting is an increase in the number of myelinated or unmyelinated axons, or both, in a test pathway, in this case the dorsal root. The studies (*2, 3*) demonstrated sprouting in the following way. An area of spinal cord into which a test root projected was partially denervated—for example, by hemisection or by cutting other dorsal roots. Enough time was allowed for products of degeneration to disappear and sprouting to occur. Then the test root and its mate on the intact side of the cord were cut. When axonal degeneration was maximal, the animal was killed, the spinal cord was sectioned histologically, and a silver stain was applied. The usual finding was that more silver was deposited on the sectioned side of the cord, and the conclusion was that the increased staining on the denervated side represented an increased number of axons. Some alternate explanations are that silver staining is capricious, that shrinkage of neural tissue leads to an illusory enhancement of the staining, or that axons on the damaged side change—by an increase in neurofilaments, for example—so that more silver is deposited per nerve fiber. Thus, a technique that allows all axons to be counted is to be preferred over the use of silver stains or other light microscopic techniques that do not permit the unmyelinated axons to be resolved. At present, therefore, electron microscopic examination of the tissue is necessary.
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In vitro Cultivation of the Exoerythrocytic Stage of *Plasmodium berghei* from Sporozoites

Abstract. *When inoculated with sporozoites of* Plasmodium berghei, *the human embryonic lung cell line WI38 supports the complete asexual developmental cycle of the exoerythrocytic stage. Cultured parasites were sensitive to primaquine, were shown to resemble parasites in living hosts by immunofluorescent antibody tests, and on subinoculation into mice induced a red blood cell infection, the gametocytes of which produced sporozoites in anopheline mosquitoes.*

Infection of the mammalian host with malarial parasites begins with the bite of an infected *Anopheles* mosquito and injection of sporozoites, which invade liver parenchymal cells and undergo schizogony, a cycle of asexual multiplication. These exoerythrocytic schizonts rupture to release merozoites, which may invade erythrocytes and initiate repeated cycles of asexual development with which are associated the symptoms of malarial infection. Some merozoites enter erythrocytes and differentiate into sexual forms called gametocytes. When a mosquito again bites, gametocytes are ingested, undergo sexual development, and ultimately produce sporozoites. In the rodent malarial parasite *Plasmodium berghei* only a single exoerythrocytic cycle is believed to occur and does not cause recognizable disease. However, blood infections with this parasite are usually lethal (*1*).

Although the exoerythrocytic stages of avian malarial parasites have been in continuous tissue culture since 1966, in vitro cultivation of the exoerythrocytic stages of a mammalian malarial parasite was not accomplished until recently. In 1979 Strome *et al.* (*2*) reported that sporozoites of *P. berghei* successfully entered rat embryonic liver and brain cells and developed into multinucleated schizonts. However, only 10 percent of the cultures became infected, the number of exoerythrocytic parasites was low, and complete cytoplasmic division did not occur. We now report the consistent development of large numbers of exoerythrocytic forms of *P. berghei* and the completion of schizogony in these parasites, with release of merozoites. Thus the complete asexual cycle of the exoerythrocytic stage of a mammalian malarial parasite is now available in vitro.

Human embryonic lung cells (line WI38) were obtained from the American Type Culture Collection and grown in Falcon 25-cm^2 flasks in NCTC-135 medium supplemented with 10 percent heat-inactivated fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 μg/ml) at 37°C in air containing 5 percent CO_2. When confluent, the cells were removed by trypsinization (0.25 percent trypsin in Hanks balanced salt solution) and subcultured onto 1-cm^2 round cover slips in vials with 0.5 ml of medium. They were grown for 2 to 3 days under the above conditions until nearly confluent. It was found that allowing up to 3 days of growth before the sporozoites are added greatly increases the susceptibility of cells to infection.

Anopheles stephensi mosquitoes were infected by allowing them to feed on NIH/NMRI mice infected with *P. berghei* (ANKA strain). The insects' salivary glands were aseptically dissected and collected in heat-inactivated mouse serum. Five sets of salivary glands were pooled, gently disrupted by passage through an 18-gauge needle, and added to the cultures. Cultures were periodically removed and stained with Giemsa; some were also fixed with methanol at 4°C for 5 minutes, rinsed with Hanks balanced salt solution, and subjected to an indirect immunofluorescence assay (*3*). The assay was run with mouse antiserum to *P. berghei* sporozoites, prepared by injecting mice three times with 30,000 ^{60}Co-irradiated sporozoites, and reactive with air-dried sporozoites; with mouse antiserum to erythrocytic-stage parasites, prepared by giving mice repeated red blood cell (RBC) infections cured by chloroquine, and reactive with both RBC stages and sporozoites; and with a commercially prepared rabbit antiserum to mouse immunoglobulin G conjugated with fluorescein (Miles Laboratories). Exoerythrocytic parasites located by immunofluorescence assay were also examined by phase microscopy.

Over 80 percent of the cultures were infected, with an average range of 100 to 500 exoerythrocytic parasites per culture. As many as 2000 parasites were counted in a single culture, representing an infection of 5 percent of the cells. Treatment of infected cultures with primaquine diphosphate (10 μg/ml), an antimalarial drug known to affect the exoerythrocytic stage in vivo, completely destroyed the parasites in vitro. Uninucleate exoerythrocytic trophozoites 5 μm in diameter were detected by Giemsa staining and immunofluorescence as early as 16 hours after the addition of sporozoites. By 24 hours the exoerythrocytic

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parasites were circular, measured about 12 μm in diameter, contained 4 to 16 nuclei, and fluoresced strongly with mouse antiserums to sporozoites and RBC-stage parasites. By 48 hours the parasites had grown to a mean diameter of 30.5 ± 3.8 μm and were easily found by phase microscopy. These fluoresced strongly with the antiserum to erythrocytic parasites (Fig. 1A). Both the parasitophorous vacuole membrane (derived from the invagination of the cytoplasmic membrane during entry) and the parasite membrane were clearly seen in outline, and the internal cytoplasm fluoresced strongly around unstained nuclei. The fluorescence on the WI38 cell nuclear membrane appeared to be continuous with that on the parasitophorous vacuole membrane and with a clump of reactive material next to the WI38 cell nucleus on the opposite side of the parasite (Fig. 1A). Such fluorescence was also observed in exoerythrocytic parasites 24 hours after the addition of sporozoites. The parasite typically developed close to the WI38 nucleus. Nuclear membrane fluorescence was also seen with mouse antiserum to sporozoites, indicating its specific sporozoite antigenicity. The peripheral parasite membrane also fluoresced with the antiserum to sporozoites, but internal fluorescence was almost absent. It appears that, as the exoerythrocytic parasite grows in culture, the proportion of sporozoite antigens is reduced and they persist only on the surface of the parasite and on the parasitophorous vacuole membrane, whereas antigens cross-reactive with the RBC stage are subsequently synthesized and are primarily within the parasite. This agrees with the observations of Danforth *et al.* (*4*), who showed that the exoerythrocytic forms of *P. berghei*, produced in living rats by infection with sporozoites, undergo a progressive loss of reactivity with antiserum to sporozoites, and after 42 hours fluoresce most strongly with antiserum to RBC-stage parasites. Thus the in vitro development of the exoerythrocytic parasite appears similar to that observed in vivo.

After 60 hours of cultivation early segmentation was observed. Further nuclear division had occurred and the cytoplasm appeared clumped, with deep clefts extending from the periphery. After 68 hours segmentation was complete and free merozoites were visible within the parasitophorous vacuole (Fig. 1B). Ruptured segmenters were also observed but there was no evidence of merozoite reinvasion of cultured host cells. After prolonged incubation (up to 168 hours) no additional exoerythrocytic parasites developed, suggesting that reinvasion of the WI38 cells did not occur. *Plasmodium berghei* has only one exoerythrocytic cycle in the rodent liver and then invades RBC's. However, the fact that sporozoites of a rodent malaria will, in vitro, enter cells from a naturally insusceptible host suggests that reinvasion might be induced with further modifications of the culture system.

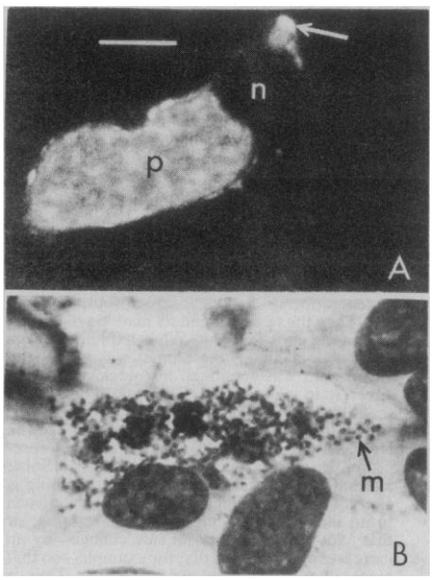

Fig. 1. (A) Exoerythrocytic-stage *P. berghei* (*p*) cultured in WI38 cell for 48 hours and reacted with mouse antiserum to erythrocytic parasites. Parasitophorous and parasite membranes and internal cytoplasm fluoresce strongly around nonfluorescing parasite nuclei. Arrow indicates clump of reactive material next to WI38 cell nucleus (*n*). (B) Mature segmenter of *P. berghei* showing merozoites (*m*) within the parasitophorous vacuole. Scale bar, 10 μm.

The infectivity of mice by developing exoerythrocytic parasites was also tested. At 24 and 48 hours after the addition of sporozoites, cells from 12 monolayers were trypsinized and resuspended in culture medium. A small portion was allowed to readhere to cover slips and the remainder was injected intraperitoneally into NIH/NMRI mice. Exoerythrocytic parasites withstood trypsinization, as shown by their subsequent development in vitro. All mice receiving WI38 cells infected with these parasites developed a patent RBC infection after 7 to 9 days, and male and female gametocytes were seen. *Anopheles stephensi* mosquitoes allowed to feed on such mice developed oocysts 15 days later, and sporozoites were found in the salivary glands 7 days thereafter. When inoculated into WI38 cells, these sporozoites produced exoerythrocytic forms. As controls for the mouse infectivity experiments, salivary gland sporozoite suspensions were held in culture medium at 37°C for 24 or 48 hours, with or without subsequent exposure to trypsin, and cell monolayers known not to be infected by *P. berghei* were inoculated with sporozoites, trypsinized, and then injected after 24 or 48 hours in culture. Control mice never developed a patent RBC infection.

Development of a vaccine against malaria is essential in view of the worldwide resurgence of the disease and its increasing resistance to chemotherapy. Experimental vaccines based on sporozoites (*5*), the RBC stage (*6*), and gametocytes (*7*) have all induced protection in animals and humans. However, the small amounts of available antigens, along with host cell or microbial contamination, have seriously hindered progress. The exoerythrocytic stage has been suggested as a potential stage for vaccine development (*8*), and its cultivation provides the opportunity to examine this experimentally. Such a procedure could be valuable in vaccine development against *Plasmodium vivax*, since its continuous culture in RBC's has thus far been unsuccessful. Isolation of *P. vivax* exoerythrocytic merozoites would be an important first step in the development of a vaccine. It also is now possible to clarify many aspects of the biology of the exoerythrocytic stage of mammalian Plasmodia by direct observation.

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