which range from about 10^h02^m at the equator to about 11^h03^m at 57° latitude. By dividing the analysis interval in half and subjecting each half to the above spectral analysis, we can evaluate the secular change in the rotation period. We find that the period is stable to within ± 43 seconds over the analysis interval, or ± 1 second per rotation at the 5 percent confidence limit.

Although it is difficult to judge definitively because of the limited volume of data at hand, it appears that the Saturn events are not organized in rotation phase as tightly as the Jupiter radio events that we have been observing for many years. This was predicted by Acuña and Ness (*4*) on the basis of the Pioneer 11 in situ field measurements, which indicate a rotationally axisymmetric magnetic field for Saturn. It is thus unlike both Jupiter and Earth, which have magnetic dipoles tipped at relatively large angles to their rotation axes. Therefore, any modulation which appears in the SKR is probably due to near-surface anomalies in the field which are undetectable from the Pioneer 11 observations.

In spite of the fact that the ~1-MHz Saturn radio events reported by Brown (*1*) would be more than 20 dB above our present detection threshold, we have failed to record any Saturn emission in the vicinity of 1 MHz. Reexamination of the Interplanetary Monitoring Platform (IMP-6) data used by Brown shows that for much of the period covered by his figures 1 and 2, TKR was clearly evident below 600 kHz. Moreover, the dynamic spectra for this period are strongly reminiscent of Jovian hectometer-wave emission, which was observed often by IMP-6. In a recent study, Fainberg (*13*) has shown that, when two or more radio sources are emitting simultaneously, the IMP-6 direction-finding analysis "points" to the intensity-weighted mean angle between the sources. It is possible that Brown may have observed a signal coincident with the Saturn direction which was formed by a combination of signals from Jupiter and Earth (or perhaps the sun). Another possibility exists if Saturn's radio emission is tightly beamed. During the period of Brown's observations the sub-IMP-6 point on Saturn was at $< -20°$ latitude, as compared with +9° latitude for the Voyager observations reported here. This possibility can be tested after the V-2 encounter in August 1981, when the outbound trajectory reaches large negative latitudes.

Closest approach to Saturn for V-1 is 12 November 1980 and for V-2 is 27 August 1981. During the next year and a half we expect to be able to refine the rotation period. We also plan to search for an LH polarized component and investigate the nature of the rotation modulation of SKR.

Note added in proof: With data analyzed through mid-July 1980 (totaling 202 hours of SKR activity), the best estimate of the Saturnian rotation period is $10^h39.4^m \pm 0.15^m$.

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Human Cutaneous Leishmania in a Mouse Macrophage Line: Propagation and Isolation of Intracellular Parasites

Abstract. *A mouse macrophage line, J774G8, supports continuous and prolific intracellular growth of* Leishmania mexicana amazonensis, *the etiological agent of a South American cutaneous leishmaniasis. The intracellular parasites from these infected cultures can be isolated with high recovery rate and purity by simple Percoll gradient centrifugation.*

Human leishmaniases are parasitic diseases caused by trypanosomatid protozoa of the genus *Leishmania*. Characteristic of all leishmanial infections is the intracellular parasitism of macrophages by amastigotes, the mammalian stage of these parasites responsible for all the symptoms and pathology. The vector stage of leishmania, the promastigote, can be cultured readily in artificial media and has been used frequently for investigation. Unfortunately, very little is known about the intracellular amastigotes because of the difficulties of procuring them in sufficient quantity and purity for investigation. It is now especially urgent to solve this problem in order to deal with the resurgence of the leishmaniases around the world. I report a culture system in which a macrophage cell line is used for continuous propagation of leishmanial amastigotes and a method for their isolation from the cultured material. Until now, these intracellular parasites have not been cultured in large enough numbers for isolation, although similar cell lines were found to support their growth (*1*).

The permanent cell line used for this study was J774G8, originally derived from macrophages of the oil-induced peritoneal exudate of BALB/c mice (*2*). Parasites used were promastigotes of *Leishmania mexicana amazonensis*, which were cultured in a medium previously described (*3*). A macrophage suspension was prepared at a cell density of 10^6 per milliliter in medium RPMI 1640 plus 20 percent heat-inactivated fetal bovine serum (HIFBS) and Hepes buffer. This suspension was added to parasites pelleted by centrifugation at a parasite to macrophage ratio of 10 to 1 and then mixed thoroughly. The mixture was placed in tightly capped tissue culture flasks and incubated at 35°C. During the first 3 days, promastigotes gained entry into the macrophages, transformed into amastigotes, and induced the formation of huge vacuoles characteristic of this leishmania species (Fig. 1A). On day 3 and every 3 days thereafter, cells were removed from the culture flask by vigorous rinsing with a Pasteur pipette, the medium was renewed, and the intracellular parasites were counted. For change of medium, cells were centrifuged at 200*g* for 3 minutes, suspended in fresh medium, and returned to the original flask. For counting the intracellular parasites, a thin wet mount was made by covering 10 μl of the culture fluid with a 22-mm^2 cover slip on a slide. The percentage of infected cells and the average numbers of amastigotes per cell were determined by examining at least 100 macrophages by phase-contrast microscopy with an oil immersion lens. The total number of macrophages was determined from the volume of the culture and mac-

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rophage numbers per milliliter counted in a hemacytometer. The total number of amastigotes per culture was then calculated from the following formula: total number of macrophages × percentage of infected cells × average number of amastigotes per cell. By this method, it is possible to estimate the net increase of parasites in a culture system with growing host cells (*4*).

On day 3, when the infection became stabilized at the parasite to cell ratio used, infectivity was approximately 80 percent, with an average of ten amastigotes per cell. The total amastigote numbers were thus equivalent to about 80 percent of the promastigotes used to infect the culture. These cultures (infected with the parasites for at least 3 days) were subjected to various culture environments; in this way optimal conditions were determined for continuous propagation of the intracellular amastigotes. A temperature higher than 35°C had detrimental effects on the parasites; for example, they disappeared from culture within 3 days at 37°C. Twenty percent HIFBS provided optimal growth of parasites (*5*), and supplementation of the medium with 1 percent (by volume) human red cell extract produced more consistent and higher yields of intracellular parasites. The red cell extract was obtained from a 25 percent stock solution, which was prepared by washing erythrocytes in double-distilled water (1:3 by volume), freezing and thawing three times, and centrifuging at 4°C for 2 hours at 50,000*g*. Under the culture conditions described, in the absence of J774G8 cells, the parasites survived for several days and then degenerated rapidly. The generation time of intracellular amastigotes varied considerably with the state of infection. The shortest doubling time observed was 36 hours in a lightly infected culture (50 percent infectivity and an average of five parasites per cell). The growth of the macrophages also depended on the course of infection and the preinfection conditions of the cells (*6*).

Lightly infected cells are able to undergo mitosis, indicating that macrophages and their parasites can grow together in such cultures. On the other hand, heavily infected macrophages often become multinucleated, indicative of abortive cell division. These cells are often deteriorated. Free amastigotes were seen in very heavily infected cultures, but extensive cell lysis with simultaneous release of amastigotes in large numbers was never observed under the culture conditions used (*7*). In the third week, most cultures had an infection rate of approximately 100 percent, with an average of about 100 parasites per cell and a fivefold overall increase in total number of amastigotes. A decrease in the macrophage population and slower growth of the parasites in these heavily infected cultures may account for the lower net parasite yield than might be otherwise expected. Subcultures are often desirable at this time to ensure a more uniform and low-level infection (high infectivity with few parasites per cell). For subculture, heavily infected cells suspended in phosphate-buffered saline (PBS) were passed through a 27-gauge, 0.5-inch needle three times to release intracellular amastigotes, which were used immediately or after cultivation as promastigotes to initiate infections. Such subcultures have been made repeatedly for eight passages in a period of more than 5 months, and the parasites resumed growth in each cycle. These results indicate that leishmania amastigotes can be continuously passaged in the macrophage line with good growth.

This in vitro system has been used for propagation of leishmania amastigotes in large numbers. Glass roller bottles (0.5 gallon) were seeded with infected macrophages in continuous culture. Conditions and counting were as described above, and the bottles were rotated at 1 to 2 rev/min to encourage the detachment and breakage of heavily infected cells, thereby spreading the released amastigotes for more even infection in the culture. These conditions have led to the establishment of a self-renewable host-parasite system consisting of a lightly infected cell layer around the glass wall and more heavily infected cells floating in the medium. Every week, the floating cell population along with the spent medium was removed for isolation of amastigotes, and fresh medium was added to the roller bottle containing the cell layer for regeneration of more heavily infected cells (*8*). Up to 10^9 amastigotes can be obtained from each harvest (Table 1). It is also possible to produce this quantity of parasites in stationary cultures of infected cells when large tissue culture

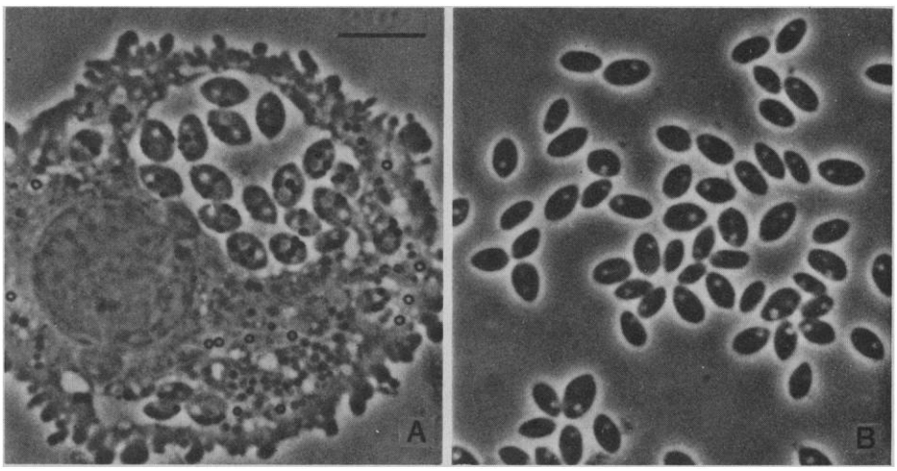

Fig. 1. (A) Representative macrophage of the J774G8 line cultured in vitro for 3 days after infection with promastigotes of *L. mexicana amazonensis*. The cells from fresh cultures were compressed under a glass cover slip on a slide to reveal intracellular amastigotes for quantitation of the infection. The upper clear area of the cell is a large parasitophorous vacuole, which contains 17 amastigotes. Production of this type of cytoplasmic vacuole is characteristic of the infection in macrophages by this and related leishmania species. The cytoplasm of the macrophages is granular and its round nucleus, on the left, is somewhat distorted due to the pressure of the cover slip. (B) Amastigotes isolated by Percoll gradient centrifugation of material from roller bottle cultures of the macrophage cell line infected with *L. mexicana amazonensis*. Phase-contrast microscopy. Scale bar, 10 μm.

Table 1. Recovery of amastigotes isolated from roller bottle cultures of J774G8 cells infected with *Leishmania mexicana amazonensis*. Floating cells along with spent medium were collected from roller bottles during weekly medium renewal and were used for isolation of amastigotes by Percoll gradient centrifugation according to procedures described in the text. Infected cells were cultured in RPMI 1640 plus 20 percent HIFBS at 35°C and at a rotation speed of 1 to 2 rev/min. Macrophages were counted in a hemacytometer. The number of purified amastigotes was estimated by the formula: total number of macrophages × percentage of infected cells × average number of amastigotes per cell.

Experiment No.	Macrophages ($\times 10^{-6}$)	Amastigotes		Recovery (%)
		Intracellular ($\times 10^{-8}$)	Purified ($\times 10^{-8}$)	
1	48	8.0	6.6	82
2	37	12.0	10.0	83
3	42	14.1	13.3	94
4	135	31.3	26.0	82

flasks are used, but more frequent medium renewals are required and infection is less stable without periodic subcultures. A similar infection can be established in this macrophage culture by use of amastigotes derived from infected animals.

Amastigotes were separated from the host cells and their cellular components in the material collected from the roller bottles. The recovery rate was more than 80 percent (Table 1), and isolated parasites were devoid of any visible host cell debris (Fig. 1B). Amastigotes were first released from heavily infected cells that were suspended in PBS plus 2 m*M* EDTA by needle passage as described above or by three cycles of vigorous mixing in a vortex mixer followed by centrifugation at 3500*g* for 5 minutes. The material was then suspended in 45 percent Percoll (Pharmacia) in PBS, layered over a cushion of 1 ml of 100 percent Percoll, and centrifuged at 3500*g* for 30 minutes. After centrifugation, amastigotes formed a sharp band at the interface between the 45 and 100 percent Percoll solutions while cells and their debris floated at the top near the meniscus. This method can also be used for isolating amastigotes from cutaneous lesions of mice infected with *L. mexicana amazonensis*. The amastigotes isolated from the in vitro culture system proved to be as infectious to laboratory animals as lesion-derived amastigotes. Parasites from both sources were injected at 10^7 per animal into the nose tips of Syrian golden hamsters (in groups of four for each parasite source). Two months later, the lesion sizes were 1.64 ± 0.3 and 1.61 ± 0.4 cm in diameter for culture- and lesion-derived amastigotes, respectively.

The in vitro system for the *L. mexicana amazonensis*-mouse macrophage line compares favorably with the in vivo animal model for this parasite in routine maintenance and production of amastigotes. The roller bottle cultures, with little attention, provide an essentially perpetual host-parasite system from which infective amastigotes can be isolated every week in quantities adequate for investigations. Flexibility is perhaps the greatest advantage of such an in vitro system, which can provide parasites on short notice and infected cells at any time for various types of assays. These cultured materials have been used to test for potential antileishmanial drugs and to study host-parasite interactions.

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4. It is important to note that the counting method is made possible by certain properties of the parasite species and the macrophage line used. The fact that J774G8 cells under the present culture conditions are loosely adherent and can be washed from the culture flasks by vigorous aspiration facilitates their counting. The relatively large sized amastigotes of the leishmania species and the huge parasitophorous vacuoles produced by them in the macrophages render their visualization and enumeration rather easy by phase-contrast microscopy of fresh material with an oil immersion lens. Previously, infected cultures were fixed and stained before observations, and it was very difficult to estimate the total cell numbers.
5. Fetal bovine serum from commercial sources must be screened for its ability to support the growth of the macrophage and the parasite before use.
6. Under the culture conditions described, macrophages double their numbers twice during the first 2 weeks, after which the cell population frequently decreases in heavily infected cultures. When macrophages used for infection experiments were previously cultured in MEM plus 10 percent HIFBS, they normally did not grow during the first 2 weeks.
7. Large numbers of free amastigotes may appear in heavily infected cultures when the medium is not changed for more than 3 days. Such cultures usually deteriorate. Heavily infected cells do not burst, but trap large numbers of amastigotes until they deteriorate. They are thus not made available to infect other macrophages in the same culture. This would explain the fact that uniform infection cannot be maintained in the stationary culture unless amastigotes are purposely released to initiate a new infection. There is no evidence for extensive destruction of macrophages by leishmania in natural infections. Such an event may not be advantageous for intracellular parasites that use these cells exclusively as their host cells.
8. Repeated collections of the floating cell population may sometimes thin out the cell layer and necessitate the addition of infected cells.
9. I thank W. Trager for suggestions in reviewing this report; J. Michl and R. S. Bray for supplying the macrophage cell line and the parasite, respectively; and J. Stanorski and C. Klein for technical help. Supported by the U.N. Development Programme-World Bank-World Health Organization Special Program for Research and Training in Tropical Diseases contract 780040, by research grant AI-15183 from the National Institute of Allergy and Infectious Diseases, and by an Irma T. Hirschl career scientist award.

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Cerebral Arteriolar Damage by Arachidonic Acid and Prostaglandin G_2

Abstract. *Application of arachidonic acid or prostaglandin G_2 to the brain surface of anesthetized cats induced cerebral arteriolar damage. Scavengers of free oxygen radicals inhibited this damage. Prostaglandin H_2, prostaglandin E_2, and 11,14,17-eicosatrienoic acid did not produce arteriolar damage. It appears that increased prostaglandin synthesis produces cerebral vascular damage by generating free oxygen radicals.*

Severe arterial hypertension causes arteriolar necrosis by mechanisms that are not well understood (*1*). In cats, acute hypertension, induced by experimental brain injury or by intravenous administration of vasoconstrictor agents causes discrete destructive lesions in the endothelial lining of brain pial arterioles (*2–4*). After the hypertensive episode, these arterioles display sustained vasodilation and reduced responsiveness to change in the partial pressure of CO_2 in arterial blood (P_aCO_2) and to changes in arterial blood pressure. All of these abnormalities are minimized or completely inhibited by treatment with cyclooxygenase inhibitors or by topical application of free radical scavengers to the brain surface (*3, 4*). These findings suggest that the cerebral arteriolar abnormalities in acute hypertension are caused by free oxygen radicals generated as a result of increased prostaglandin synthesis. It is known that intermediate steps in the biosynthesis of prostaglandins from their precursor, arachidonic acid, produce free oxygen radicals (*5–9*). These highly reactive forms of oxygen are injurious to tissues and may cause lysis of red cells (*10*), destruction of endothelial cells (*11*), and peroxidation of lysosomal (*12*) and mitochondrial membranes (*13*).

If this hypothesis for the pathogenesis of the cerebral arteriolar damage in acute hypertension is correct, the cerebral arteriolar abnormalities seen in hypertension would be produced by application of exogenous prostaglandins, and this effect of the prostaglandins would be inhibited by administration of free radical scavengers. We tested this hypothesis in cats anesthetized with pentobarbital (30 mg/kg, given intravenously) and ventilated artificially with a positive-pressure respirator, after skeletal muscles were paralyzed with decamethonium bromide (0.5 mg/kg, intravenously). Arterial blood pressure and CO_2 concentration in expired air were monitored continuously. The pial microcirculation of the parietal cortex was

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