Observations on the In Vitro Cultivation of *Leishmania braziliensis*

The general difficulty of culturing some parasites of the *Leishmania braziliensis* complex, compared with the extreme ease of cultivation in virtually any blood agar medium of members of the *L. mexicana* complex, constitutes one of the characters for separating the two complexes. However, the inability to cultivate certain members of the *L. braziliensis* complex has been a serious obstacle to comparative physiological, immunological, and epidemiological studies.

The medium routinely used by the Department of Parasitology of the Instituto Evandro Chagas, Belém, in attempted isolations of *L. b. braziliensis* from man and wild animals has been NNN medium. This is prepared as follows: 28 g Oxoid Nutrient Agar (Code CM3, available from Oxo Company, Southwark Bridge Rd., London SE1) per 1,000 ml distilled water, and 5% defibrinated rabbit blood added. When cool, an overlay of 0.5 ml sterile physiological saline (0.9% w/v) containing 500 I.U. of penicillin and 500 μg of streptomycin per ml is added. Attempts were also made to cultivate *L. b. braziliensis* in modifications of the same medium; 10% and 20% defibrinated rabbit blood, or different overlays, such as condensation fluid, Locke's solution, and 199 tissue culture medium, were used. In no case, however, was it found possible to cultivate the parasite successfully. We consider cultivation to be successful when it is possible to maintain the strain by subpassaging. Often there may be good initial growth in certain media, but subsequently it proves impossible to maintain the strain.

A very similar blood agar medium has been routinely used by the U.S. Army Medical Research Unit, Panama (USAMRU) for isolation attempts and maintenance of stock strains of *Leishmania* (Walton et al., 1972, Am J Trop Med Hyg **21**: 296–299). This is prepared from 40 g Difco Blood Agar Base (Code B45, Difco Laboratories, Detroit, Mich.) per 1,000 ml distilled water with 15% defibrinated rabbit blood. The blood agar slants are left at room temperature for several hours to allow formation of condensation fluid, and then stored at 4 C until used. Antibiotics are not routinely used, but penicillin/streptomycin are added on occasion for culturing contaminated lesions, with no apparent adverse effect. In this medium *Leishmania* isolated from patients and sandflies in Panama grew rapidly and luxuriantly. These strains came from the same general area as the *L. b. panamensis* studied by Lainson and Shaw, and over 20 isolates inoculated into hamsters demonstrated the small, slowly developing, nonmetastasising lesion characteristic of *L. braziliensis*; there is no doubt these strains represent *L. b. panamensis*. In 1974 another isolate from a case of espundia acquired in Peru, an undoubted member of the *L. braziliensis* complex (Walton et al., 1977, Am J Trop Med Hyg: in press), was also found to grow rapidly and abundantly in this medium.

In 1975 attempted isolations were made from a patient (M 2903) suffering from cutaneous leishmaniasis that had been contracted in the Serra dos Carajás of Pará. The epidemiology of cutaneous leishmaniasis in this area has been studied in detail (Lainson et al., 1973, Trans R Soc Trop Med Hyg **67**: 184–196) and all strains isolated from man have proved to be *L. b. braziliensis*. Material was aspirated from the edge of the lesion and inoculated in alternating tubes of Belém NNN (Oxoid CM3 base) and USAMRU medium (Difco C45 base). From this series, strain M 2903 grew rapidly and well in the USAMRU medium and became established by subpassage every 7 to 14 days, while the Belém NNN medium did not become positive. The promastigotes that grew in the USAMRU medium were infective for hamsters, producing the same type of small, slow-growing lesion that is typical of *L. b. braziliensis* and which was produced by the inoculation of the original biopsy material. The growth of this strain of *L. braziliensis* from Brazil on USAMRU medium confirms the prior observation on the cultivability of *L. braziliensis* strains from Panama and Peru.

This marked difference of the growth of a single strain in two variants of basically similar media is apparently due to differences in the nutrient agar. It might be suggested that the differences observed in our two laboratories were due to a rabbit blood factor, but this

possibility has been eliminated. Both media were prepared with blood from Belém rabbits and isolates of M 2903 were made from hamsters. The strain again grew well in the USAMRU-Difco medium but not in the Belém-Oxoid medium.

In the past, instances of lack of agreement between laboratories concerning characteristics of certain strains have marred the study of *Leishmania*. Such discrepancies emphasize the importance of detailed descriptions of the media used, including the proprietary components, when reporting culture characteristics of *Leishmania*.

However, it must be pointed out that this medium does not support the growth of all strains of *L. braziliensis* equally well, but that some remain refractory to in vitro culture. Strain M 1287 (Shaw and Lainson, 1975, Trans R Soc Trop Med Hyg **69**: 323–335), originally isolated from a nasal biopsy of a patient from Pará with mucocutaneous leishmaniasis of some 18 years duration, was maintained by serial hamster-to-hamster passage. During a period of over a year, repeated attempts to grow the parasite in Panama from needle aspirates and in Brazil from pieces of infected hamster tissue in different batches of USAMRU medium were unsuccessful. Sometimes cultures showed no growth, but usually they yielded a few languidly motile promastigotes which would not grow upon subpassage. Inoculation of triturated tissue yielded more organisms, but they also died upon subpassage. Tubes of medium used from the same batches supported copious growth of *L. b. panamensis* and strain M 2903. Similarly, a small percentage of patients from Panama has been encountered in which the parasite could not be established in culture (Walton et al., 1968, Am J Trop Med Hyg **17**: 814–818). It is of interest that all of these cases refractory to culture originated from Fort Sherman on the Caribbean coast of Panama, while none where encountered among the many more numerous cases originating on the Pacific slope of the isthmus.

Recent studies have shown that the genus *Leishmania* is composed of complexes of what are now regarded as infraspecific groups. It is certain that not all strains grow equally well in the same medium, some members of the *braziliensis* complex being more fastidious than members of the *mexicana* complex. The present observations indicate that the relationships of infraspecific forms are even more complex than previously recognized, and there are major differences even within the *braziliensis* group over a wide geographic distribution, as evidenced by strains from Brazil, Peru, and Panama which will grow in USAMRU (Difco base) medium and strains from Brazil and Panama which will not grow in this medium. In the future, by the use of more sophisticated media, it may well be possible to differentiate *Leishmania* on the basis of nutritional requirements of the promastigote form, in much the same way as bacteria are identified. The applicability of such an approach in the *Leishmania* has been demonstrated by Citri and Grossowicz (1955, Trans R Soc Trop Med Hyg **49**: 603), who noted that *L. tropica* grew well in a medium that they had elaborated but *L. infantum*, *L. donovani*, *L. braziliensis*, and *L. agamae* did not. Subsequently Strejan (1963, Bull Res Counc Israel Sect E **11**: 21–23), using this same medium, found that it did not support the growth of *L. mexicana*.

The successful cultivation of some strains of *L. b. braziliensis*, one of which originated from a case of espundia, on blood agar medium using Difco Blood Agar Base (Code B45) now makes it possible to study many aspects of the physiology, immunology, and epidemiology of these members of the important *braziliensis* complex.

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