Xanthine in urine stimulates growth of *Leishmania* promastigotes *in vitro*

The leishmaniases are parasitic diseases that affect large populations in vast areas of the world (Desjeux, 2001). The causative agents of these diseases, protozoan parasites belonging to the genus *Leishmania* (Kinetoplastida: Trypanosomatidae), are transmitted by phlebotomine sand flies (Killick-Kendrick, 1999). In culture media (at 26–28 °C, pH ~7.2), *Leishmania* parasites develop as motile promastigotes similar to those found in the sand fly midgut. A number of reports have shown that the addition of 1–5% human urine stimulates growth, leading to more rapid multiplication and a higher concentration of parasites at the stationary phase (Ali et al., 1998; Armstrong & Patterson, 1994; Howard et al., 1991; Iqbal et al., 2006; Shamsuzzaman et al., 1999; Singh et al., 2000). Preliminary studies have indicated that the factor responsible for this enhancement is a small molecule which is not destroyed by autoclaving (Ali et al., 1998). However, despite the substantial advantages of using defined media for the culture of human pathogens (Schuster & Sullivan, 2002), the factor(s) in urine that is responsible for promoting growth of *Leishmania* has not been identified.

Preliminary experiments confirmed that the addition of 5% (v/v) human urine to *Leishmania major* promastigotes cultured in RPMI 1640 enhanced the rate of multiplication about 10-fold (two-tailed t-test, \(P < 0.005\)). The final concentration at stationary phase increased from \(4.0 \times 10^6\) to almost \(4.2 \times 10^7\) parasites ml\(^{-1}\). This exacerbative effect was not abolished by boiling the urine or treating it with proteinase K (Fig. 1).

Since *Leishmania* parasites are auxotrophic for purines and human urine contains low concentrations of these molecules (Boitz & Ullman, 2006; Bromberg et al., 1957a, b), we tested whether adenine, hypoxanthine and xanthine (10 \(\mu\)M) enhanced the growth of *L. major*. Of these, only xanthine

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**Fig. 1.** Growth curves of *Leishmania major* in RPMI 1640. Addition of 5% human urine to the medium significantly enhances growth rate and prolongs the stationary phase. Enhancement is not abrogated by boiling or proteinase K treatment. Experiments were repeated three times.

**Fig. 2.** Growth curves of *Leishmania major* in RPMI 1640 with added purines (10 \(\mu\)M). Xanthine enhances growth almost as much as 5% human urine. The experiment was repeated four times.
(10 μM) significantly and repeatedly enhanced the growth of promastigotes in vitro (two-tailed t-test, \(P < 0.005\)) (Fig. 2).

In order to ascertain whether xanthine is the major component in urine that enhances leishmanial growth in vitro, fresh urine was depleted of xanthine by passing it through an activated oligonucleotide purification cartridge (OPC) (Applied Biosystems) according to a variation of the manufacturer’s protocol as described previously (Deutsch et al., 1997). Depletion was demonstrated by HPLC analysis (Fig. 3). The peak at RT 13.41 min corresponding to xanthine (Fig. 3a) was markedly smaller after passing the urine through an OPC (Fig. 3b; RT 13.31). The observed deviations in RT were minor and reasonable for inter-days deviations (measuring samples on different days). Addition of exogenous xanthine restored a peak at almost the same RT (Fig. 3c, with minor changes due to the different extent of ionization of the synthetic xanthine).

Finally, to make sure that xanthine is the main component that enhances multiplication of *Leishmania in vitro*, the three urine types shown in Fig. 3 were used to supplement RPMI 1640 culture medium at 5% (v/v). Results depicted in Fig. 4 confirm that depletion of xanthine from human urine abrogates its capacity to enhance growth of *L. major* promastigotes (two-tailed t-test, \(P < 0.005\)). Moreover, addition of 10 μM xanthine to the urine restored this capacity (Fig. 4).

Unlike mammalian cells that synthesize purines de novo, all protozoan parasites studied thus far are incapable of synthesizing the purine ring, rendering them auxotrophic for purines (Marr et al., 1978). Xanthine is excreted in human urine. Fig. 3. HPLC analysis of human urine. (a) Chromatogram of normal urine. (b) Chromatogram of urine depleted of xanthine after passing through an oligonucleotide purification cartridge. (c) Chromatogram of xanthine-depleted urine to which 10 mM synthetic xanthine was added. Arrows point to peaks corresponding to xanthine.

Fig. 4. Growth curves of *Leishmania major* in RPMI 1640 supplemented with 5% normal urine, xanthine-depleted urine or xanthine-depleted urine supplemented with 10 μM xanthine (final concentration).
xanthine at approximately 10 mg per 24 h, and its concentration in normal urine is around 0.2–0.5 mM. Our data show that like 5% (v/v) human urine, 10 μM xanthine significantly enhances the growth of L. major promastigotes in culture (Fig. 2). Furthermore, the depletion of xanthine from urine abrogated its capacity to exacerbate growth of Leishmania promastigotes (Fig. 4). Although passage of the urine through the OPC may have removed other purines as well, we showed that the exacerbative effect can be restored almost entirely by adding commercial xanthine at 10 μM (Fig. 4). The results strongly suggest that xanthine is the one most active molecule in human urine that enhances the growth of L. major promastigotes in culture, and its routine addition at 10 μM should improve the culture of Leishmania in vitro (Merlen et al., 1999; Schuster & Sullivan, 2002).

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