

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

The leishmaniasis 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 4.0×10^6 to almost 4.2×10^7 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 μM) enhanced the growth of *L. major*. Of these, only xanthine

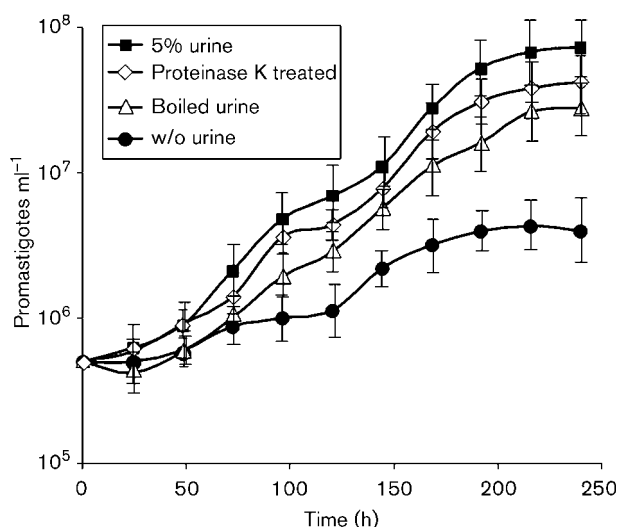


Fig. 1. Growth curves of *Leishmania major* in RPMI 1640. Addition of 5 % human urine to the medium significantly enhances growth rate and prolongs the stationary phase. Enhancement is not abrogated by boiling or proteinase K treatment. Experiments were repeated three times.

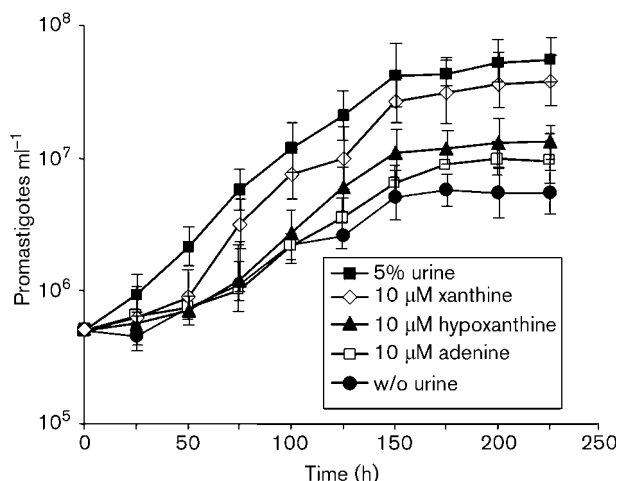


Fig. 2. Growth curves of *Leishmania major* in RPMI 1640 with added purines (10 μM). Xanthine enhances growth almost as much as 5 % human urine. The experiment was repeated four times.

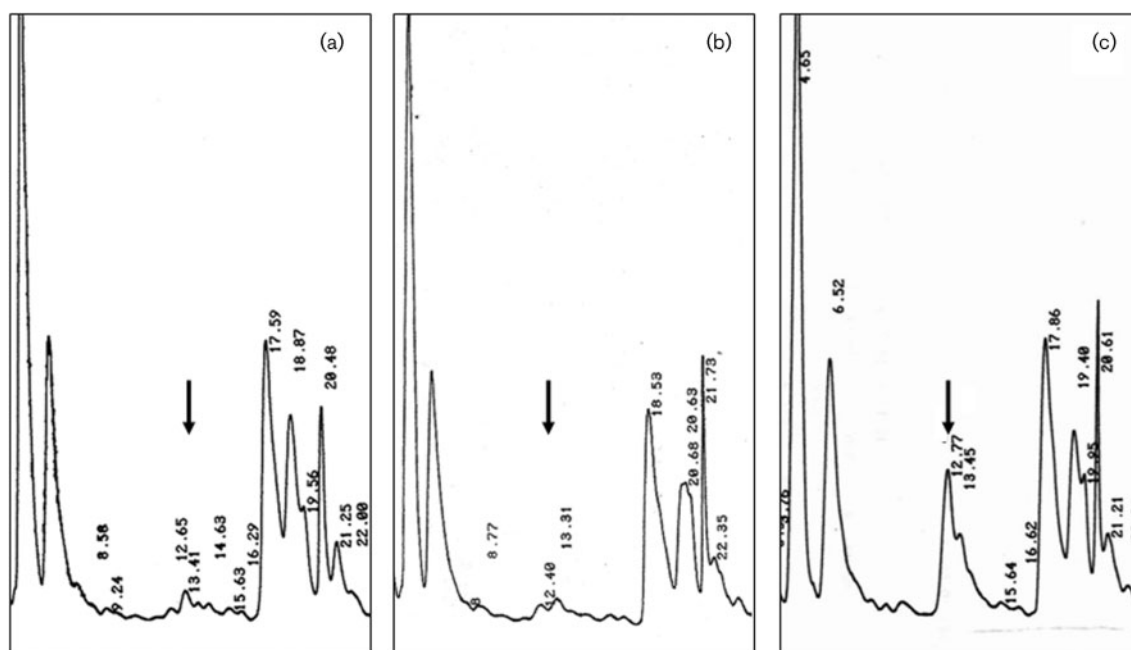


Fig. 3. HPLC analysis of human urine. (a) Chromatogram of normal urine. (b) Chromatogram of urine depleted of xanthine after passing through an oligonucleotide purification cartridge. (c) Chromatogram of xanthine-depleted urine to which 10 mM synthetic xanthine was added. Arrows point to peaks corresponding to xanthine.

(10 μ M) 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 μ M 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

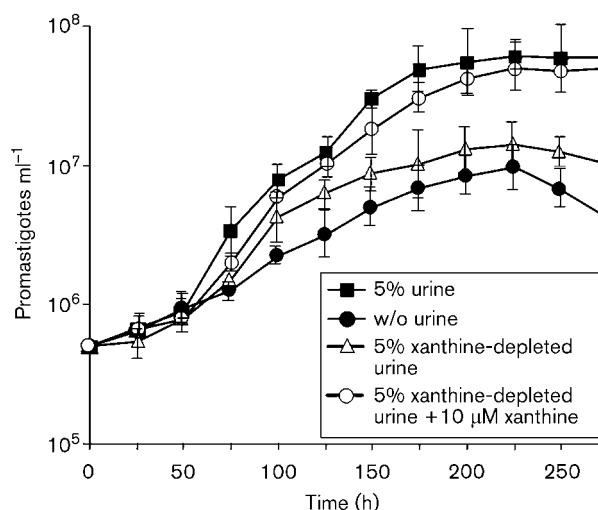


Fig. 4. Growth curves of *Leishmania major* in RPMI 1640 supplemented with 5% normal urine, xanthine-depleted urine or xanthine-depleted urine supplemented with 10 μ M xanthine (final concentration).

urine 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).

Acknowledgements

This research was supported by grant number 235/99-2 from 'The Israel Science Foundation' founded by the Israeli Academy of Sciences and Humanities.

Alon Warburg,¹ Shoshana Gelman¹ and Joseph Deutsch²

¹Department of Parasitology, The Kuvim Centre for the Study of Infectious and

Tropical Diseases, The Hadassah Medical School, Jerusalem 91120, Israel

²School of Pharmacy, The Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem 91120, Israel

Correspondence: Alon Warburg (warburg@cc.huji.ac.il)

Ali, S. A., Iqbal, J., Ahmad, B. & Masoom, M. (1998). A semisynthetic fetal calf serum-free liquid medium for in vitro cultivation of *Leishmania* promastigotes. *Am J Trop Med Hyg* **59**, 163–165.

Armstrong, T. C. & Patterson, J. L. (1994). Cultivation of *Leishmania braziliensis* in an economical serum-free medium containing human urine. *J Parasitol* **80**, 1030–1032.

Boitz, J. M. & Ullman, B. (2006). *Leishmania donovani* singly deficient in HGPRT, APRT or XPRT are viable in vitro and within mammalian macrophages. *Mol Biochem Parasitol* **148**, 24–30.

Bromberg, P. A., Gutman, A. B. & Weissmann, B. (1957a). The purine bases of human urine. I. Separation and identification. *J Biol Chem* **224**, 407–422.

Bromberg, P. A., Gutman, A. B. & Weissmann, B. (1957b). The purine bases of human urine. II. Semiquantitative estimation and isotope incorporation. *J Biol Chem* **224**, 423–434.

Desjeux, P. (2001). Worldwide increasing risk factors for leishmaniasis. *Med Microbiol Immunol* **190**, 77–79.

Deutsch, J., Rapoport, S. I. & Purdon, A. D. (1997). Relation between free fatty acid and acyl-CoA concentrations in rat brain following decapitation. *Neurochem Res* **22**, 759–765.

Howard, M. K., Pharoah, M. M., Ashall, F. & Miles, M. A. (1991). Human urine stimulates growth of *Leishmania in vitro*. *Trans R Soc Trop Med Hyg* **85**, 477–479.

Iqbal, J., Jamshid, M., Ahmed, B., Bukhari, I., Bashir, S. & Yasinzi, M. M. (2006). Some studies on human urine as promoter for the growth of *leishmania in vitro*. *Pak J Pharm Sci* **19**, 152–155.

Killick-Kendrick, R. (1999). The biology and control of phlebotomine sand flies. *Clin Dermatol* **17**, 279–289.

Marr, J. J., Berens, R. L. & Nelson, D. J. (1978). Purine metabolism in *Leishmania donovani* and *Leishmania braziliensis*. *Biochim Biophys Acta* **544**, 360–371.

Merlen, T., Sereno, D., Brajon, N., Rostand, F. & Lemesre, J. L. (1999). *Leishmania* spp: completely defined medium without serum and macromolecules (CDM/LP) for the continuous in vitro cultivation of infective promastigote forms. *Am J Trop Med Hyg* **60**, 41–50.

Schuster, F. L. & Sullivan, J. J. (2002). Cultivation of clinically significant hemoflagellates. *Clin Microbiol Rev* **15**, 374–389.

Shamsuzzaman, S. M., Furuya, M., Korenaga, M., Imamura, K. & Hashiguchi, Y. (1999). Use of urine samples from healthy humans, nephritis patients or other animals as an alternative to foetal calf serum in the culture of *Leishmania (L.) donovani in vitro*. *Ann Trop Med Parasitol* **93**, 613–620.

Singh, S., Mohapatra, D. P. & Sivakumar, R. (2000). Successful replacement of fetal calf serum with human urine for in vitro culture of *Leishmania donovani*. *J Commun Dis* **32**, 289–294.