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*

By S. M. SHAMSUZZAMAN*

Department of Parasitology, Faculty of Medicine, Kochi Medical School, Nankoku, Kochi, Japan, and Department of Microbiology, Dhaka Medical College, Dhaka, Bangladesh

M. FURUYA

Institute for Laboratory Animals, Kochi Medical School, Nankoku, Kochi, Japan

M. KORENAGA, K. IMAMURA AND Y. HASHIGUCHI

Department of Parasitology, Faculty of Medicine, Kochi Medical School, Nankoku, Kochi, Japan

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The effect of supplementing *in-vitro* cultures of *Leishmania donovani* with urine was investigated. The parasites were isolated from Bangladeshi patients with visceral leishmaniasis. The urine samples used were collected from healthy human donors, patients with nephrotic syndrome, diabetic nephritis (DN) or diabetes mellitus, a dog and a cow. Promastigotes from blood-agar cultures were inoculated into RPMI-1640 basal medium with 10% heat-inactivated foetal calf serum (FCS) and/or 1%–20% urine. The parasites were then counted in a haemocytometer, on days 2, 4, 5, 6, 7, 8, 10, 12 and 14 post-inoculation. From day 4, the numbers of parasites/ml in cultures containing 5% healthy-human urine but no FCS were at least as high as those in cultures containing 10% FCS but no urine ($P = 0.191$). The wet weights of parasites harvested from mass cultures of the parasites in RPMI-1640 plus 5% healthy-human urine and in RPMI-1640 plus 10% FCS were practically the same. Multiplication of the parasites in the presence of 5% urine from a DN patient was significantly greater ($P < 0.01$) than that seen with other urine samples at the same concentration or with 10% FCS. The multiplication seen with 8% canine urine was almost the same as with 5% healthy-human urine. Parasites could be maintained in RPMI-1640 plus 5% healthy-human urine for at least 40 days, sub-culturing every 4 days. Urine may be a better and much cheaper stimulant of *Leishmania* multiplication *in vitro* than FCS.

For almost 70 years, foetal calf serum (FCS) has been considered a useful if not essential component of the media used for *in-vitro* cultivation of various animal cells, including many parasites. However, FCS is not only costly everywhere but also either impossible to obtain or difficult to obtain as a reliable supply

in many countries of the developing world (Gholamhossienian and Vassef, 1988; Armstrong and Patterson, 1994). It has been estimated that using 10% FCS in combination with some basic, powder-based media accounts for 75%–90% of the total material costs of cultivation (Keay, 1977). The cost is particularly prohibitive when large numbers of cells have to be produced *in vitro*. A cheap, easily available, serum-free but good medium has been the dream of those involved in cell

* Address for correspondence: Department of Parasitology, Kochi Medical School, Nankoku City, Kochi-783-8505, Japan. E-mail: shamsuzz@kochi-ms.ac.jp; fax: + 81 888 80 2415.

TABLE
 Characteristics of the urine samples from the patients with diabetic nephritis (DN) or nephrotic syndrome (NS)

	Urine from DN patient	Urine from NS patients
Specific gravity	1.010	1.012
pH	6.5	6.0
Sugar (mg/dl)	200	0
Protein (mg/dl)	600	200
Occult-blood score	1 +	3 +
Urobilinogen (mg/dl)	0.2	0.2
NO. PER HIGH-POWER FIELD		
Red blood cells	1-4	> 100
White blood cells	1-4	5-9
Casts per low-power field	0-1	0-1

culture since the beginning of the in-vitro cultivation of animal cells (Barnes and Sato, 1980). Although serum-free media have been devised for several *Leishmania* species, they tend to be complex and not particularly cheap (Gholamhossienian and Vassef, 1988; Gupta and Saran, 1991). Human urine has proved to be a potential stimulant for many urinary pathogens (Brooks and Keevil, 1997) as well as *Leishmania* promastigotes (Howard *et al.*, 1991; Armstrong and Patterson, 1994). It seemed possible that it could be used, instead of FCS, as a supplement to a fairly basic medium, to give a cheap, easily available and easily preparable serum-free media for *Leishmania* cultivation. The aim of the present study was to investigate this possibility.

MATERIALS AND METHODS

Parasites

Five strains of *Leishmania* (*Leishmania*) *donovani* were isolated from patients admitted to Institute of Post Graduate Medicine and Research (IPGMR) in Dhaka, Bangladesh, by cultivation of bone-marrow aspirates (from the sternum or an iliac crest) in NNN medium at 22°C. After 15-20 passages in NNN medium, the cultures were transported to the Parasitology Department of the Kochi Medical School,

Japan, and cryopreserved for use in subsequent experiments.

Media

In Japan, the parasites were maintained on blood-agar medium (USMARU; Difco) containing 20% defibrinated rabbit blood. Proline balanced salt solution (0.4 g KCl, 0.06 g Na₂HPO₄·12H₂O, 0.06 g KH₂PO₄, 0.185 g CaCl₂·2H₂O; 0.1 g MgSO₄·7H₂O, 0.1 g MgCl₂·6H₂O, 8 g NaCl; 1.0 g L-proline and distilled water up to 1000 ml) was used as an overlay, at 2-4 ml/culture. To make the liquid medium used for the experiments, 10.2 g of powdered RPMI-1640 medium without L-glutamine was dissolved and autoclaved in a litre of water and then supplemented with 18-30 ml autoclaved 10% NaHCO₃, 10 ml 200 mM filter-sterilized L-glutamine, 100 000 units penicillin and 50 mg streptomycin. The resultant basal medium was stored at 4°C. All the foetal calf serum (Gibco, Grand Island, NY) used in the experimental cultures had been heat-inactivated (30 min at 56°C).

Urine Samples

Urine samples were collected from one healthy Bangladeshi man, Japanese patients—with nephrotic syndrome (NS), diabetes mellitus (DM) or diabetic nephritis (DN)—a dog and a cow. The concentrations of protein and sugar in each sample were estimated by the

Fig. 1. Multiplication of *L. donovani* in the RPMI-1640 basal medium with: no urine or foetal calf serum ($\square$); 5% healthy-human urine ($\circ$); or 10% foetal calf serum ($\blacksquare$).

heat-coagulation method and by use of commercial dipsticks, respectively. Only samples scored at least 2+ for protein and/or 1+ for sugar were selected for use. Each selected sample was filtered through two disposable cellulose-acetate filters, the first with 0.8- μ m pores and the second with 0.2- μ m pores, before storage at 4°C. An aliquot of each sample was sent to a specialised laboratory for detailed analysis (see Table).

Most of the samples were used without further treatment but some were used after heat-inactivation (30 min at 56°C) or after repeated freezing and thawing.

Experimental Cultures

The experimental cultures were held in sterile, plastic, disposable, screw-capped tubes (16 $\times$ 125 mm). Promastigotes from blood-agar (USMARU) cultures were centrifuged down, and resuspended in RPMI-1640 medium to give 3.5×10^7 parasites/ml. Each of the culture tubes, containing 10 ml RPMI-

1640 with no further supplementation (negative control), 10% FCS (positive control) or 1%–20% urine (see below), was then inoculated with 0.1 ml of this suspension, to give a final suspension of 3.5×10^5 parasites/ml. The capped tubes were then incubated at 25°C on a rotator. Each isolate-medium combination was replicated four times.

Parasite Counts

A haemocytometer was used to estimate the numbers of parasites/ml in samples removed from each culture on days 2, 4, 5, 6, 7, 8, 10, 12 and 14 post-inoculation. The data collected were then used to calculate mean values for the replicates and these were then plotted as growth curves.

Long-term Maintenance in Urine-containing Medium

Each of the five *L. donovani* isolates was sub-cultured every 4 days for 40 days in RPMI-1640 medium with 5% healthy-human

Fig. 2. Peak (day-6) densities of parasites in RPMI-1640 basal medium with 5% healthy-human urine, 10% foetal calf serum or 5% heat-inactivated urine from a patient with diabetic nephritis (DN). *, $P < 0.01$; NS, not significant.

urine. The numbers of parasites/ml were checked at each sub-culture.

Mass Cultivation

The overlay from a 4-day-old culture of one of the isolates in USMARU medium was centrifuged, and the parasites resuspended in RPMI-1640 basal medium. Identical volumes of this suspension were then used to inoculate two, 200-ml, sterile, screw-capped bottles, one containing 70 ml RPMI-1640 with 5% healthy-human urine and the other containing 70 ml RPMI-1640 with 10% FCS. These were incubated at 25°C on a rotator for 4 days, when the numbers of parasites/ml were estimated and all the parasites were harvested so that the wet weight of the promastigotes in each bottle could be determined.

RESULTS

The results for each of the *L. donovani* isolates

used were virtually identical. Multiplication of the promastigotes cultured in the basal medium with 5% healthy-human urine was similar to that seen in the medium with 10% FCS (Fig. 1) and each of these two media supported much better multiplication than that seen in the basal medium with no serum or urine (Student's *t*-test; $P = 0.004$). The highest concentrations of parasites observed were in basal medium with 5% healthy-human urine, but these were not significantly higher than those seen with 10% FCS ($P = 0.191$).

Multiplication of the parasites was significantly better with healthy-human urine at 5%–8% than with the same urine at concentrations below 4% ($P = 0.004$) or above 10% ($P = 0.002$). At 5%, the urine from the DN patient only supported significantly better multiplication than 5% healthy-human urine ($P = 0.009$) or 10% FCS ($P = 0.007$) when it had been heat-inactivated (Fig. 2) and not

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Multiplication of the parasites was significantly better with healthy-human urine at 5%–8% than with the same urine at concentrations below 4% ($P = 0.004$) or above 10% ($P = 0.002$). At 5%, the urine from the DN patient only supported significantly better multiplication than 5% healthy-human urine ($P = 0.009$) or 10% FCS ($P = 0.007$) when it had been heat-inactivated (Fig. 2) and not

Fig. 3. Multiplication of *L. donovani* in the RPMI-1640 basal medium with: 5% healthy-human urine (○); 10% foetal calf serum (■); 5% urine from a patient with diabetic nephritis (●); or 5% urine from a patient with nephrotic syndrome (▲).

when it was used without heat-inactivation or after repeated freezing and thawing (data not shown). Growth curves for *L. donovani* in the media with healthy-adult, NS-patient or DN-patient urine (each at 5%) or 10% FCS are shown in Figure 3. Multiplication in the presence of the NS urine or any of several DM urine samples was significantly less than that with 5% healthy-human urine ($P=0.025$), 10% FCS ($P=0.03$) or 5% DN urine ($P=0.006$) and was unaffected by heat-inactivation of the urines (data not shown).

RPMI-1640 medium with 2% FCS and 2%–15% healthy-human urine supported significantly better multiplication of the parasites than the same basal medium with 10% FCS ($P\leq 0.01$), irrespective of the exact concentration of urine (Fig. 4); the highest numbers of parasites/ml were observed with 5% urine ($P=0.002$). At a 5% concentration, healthy-human urine supported better parasite multiplication than the bovine or canine urine

(data not shown). At its apparently optimum concentration (8%), the canine urine supported similar levels of parasite multiplication to those seen with 5% healthy-human urine ($P=0.07$; Fig. 5). The maximum concentrations of parasites seen in the trials with the bovine urine were observed when the urine was present at 10% but these were significantly lower than those seen with 8% canine urine ($P=0.006$).

Each isolate of *L. donovani* was successfully maintained in serum-free medium with 5% healthy-human serum for 40 days, with sub-culture every 4 days.

DISCUSSION

Foetal calf serum is expensive everywhere. In the developing world it is difficult to transport and often impossible to obtain as a reliable supply. Could urine be a free, suitable and readily available alternative? In the present

Fig. 4. Multiplication of *L. donovani* in the RPMI-1640 basal medium containing 2% foetal calf serum (FCS) and further supplemented with 0% (▤), 2% (□), 5% (○), 10% (△) or 15% (▲) healthy-human urine. Development in RPMI-1440 basal medium containing 10% FCS is shown for comparison (■).

study, cultivation of *L. donovani* promastigotes in basal medium was at least as good with 5%–8% urine from a healthy human donor as it was with 10% FCS. Higher concentrations of urine were not as good, probably because of a fall in pH and inhibitory concentrations of human metabolites. Even with 2% FCS in the medium, concentrations of human urine around the 5% level appeared optimal. With 10% FCS in the medium, Howard *et al.* (1991) found that lower concentrations of normal human urine (1%–2%) supported the best multiplication of various *Leishmania* spp. *in vitro*.

Although Armstrong and Patterson (1994) successfully cultured *L. (Viannia) brasiliensis* in serum-free M199 medium with 5% human urine, they added several chemicals (HEPES, haemin, hypoxanthine and NaHCO_3) as supplements and compared the multiplication they obtained using their final medium with that seen in the presence of 5% FCS. In

contrast, in the present study, the basal medium was RPMI-1640 with just two extra supplements apart from antibiotics (L-glutamine and NaHCO_3) and the medium used as a positive control was enriched with 10% FCS. After the successful use of urine by Howard *et al.* (1991), several other researchers used urine along with FCS in mass cultivation (Belli *et al.*, 1994; El-Masum and Evans, 1995). In the present study, the wet weight of parasites produced by mass cultivation in medium with 5% healthy-human urine but no FCS was found to be the same as that produced in medium containing 10% FCS. (The fact that more parasites but no extra weight of parasites was produced in the urine-containing medium indicates that parasite morphology may be affected by the urine.)

Perhaps the most interesting aspect of the present study was the comparison of the urine from a healthy donor with samples from a dog, a cow and patients who were likely to be

Fig. 5. Multiplication of *L. donovani* in the RPMI-1640 basal medium supplemented with: 10% foetal calf serum (■); 8% canine urine (△); or 10% bovine urine (▲).

producing abnormal urine. Sera (including FCS) are complex fluids that are rich in protein and many other nutrients. Urine is also a complex fluid and it contains many essential and non-essential amino-acids, minerals, vitamins and various metabolites (Brooks and Keevil, 1997). Nephritis patients, who have damaged kidneys, produce urine with unusually high levels of protein and other serum constituents. It seemed plausible, therefore, that urine from such patients would make a particularly good replacement for FCS. The best multiplication observed during the present study was produced using the basal medium supplemented with the heat-inactivated and protein-rich urine of a diabetic nephritis patient (see Table). This multiplication was significantly better than that seen with healthy-human urine or 10% FCS. The urine from a patient with nephrotic syndrome did not have such a high protein concentration as the DN urine (see Table) and, like the

samples from patients with diabetes mellitus, appeared less useful than the normal urine from a healthy donor or 10% FCS. Heat-inactivation of the DN urine may have benefitted the parasites by reducing the activity of inhibitors normally present in serum and possibly excreted in the urine of patients with badly damaged kidneys.

As 8% canine urine stimulated the multiplication of parasites almost as well as of 10% FCS or 5% healthy-human urine, it appears that normal urine samples from humans or dogs may at least as useful as FCS in the cultivation of *L. donovani* promastigotes. The heat-inactivated urine of some nephritis patients may be even better. Although primary isolation of parasites into serum-free media supplemented with urine still has to be explored, many more researchers in developing countries will be able to carry out extensive research on leishmaniasis, using urine instead of FCS.

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