Addition of hypoxanthine to culture media allows *in vitro* cultivation of *Babesia bovis* and *B. bigemina* at reduced serum concentrations

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SUMMARY

The microaerophilous stationary phase system (MASP) was introduced in 1980 and successfully used as a standard technique for *Babesia bovis* and *B. bigemina in vitro* culture. The percentage of serum in the medium and the dependence on specific serum donors have been recognized as important constraints both for immunochemical studies and for the logistics of culture routine. In the present study the supplementation of RPMI 1640 with hypoxanthine at a concentration between 50 and 200 μM has enabled patterns of growth of *B. bovis* and *B. bigemina* to be achieved comparable to the standard technique with a simultaneous reduction of serum concentration from 40% to 5%. With hypoxanthine-supplemented medium it was possible to either replace the bovine serum from a specific donor with horse serum or use commercial adult bovine serum or foetal calf serum at 10%. When the serum replacement media Albumax II and GF21 were used, the growth of both *B. bovis* and *B. bigemina* markedly decreased after 3×72 h cycles. However, when these species were cultivated in culture flasks previously coated with cells from a murine peritoneal lavage, continuous parasite growth was achieved.

Key words: *Babesia bovis*, *Babesia bigemina*, *in vitro* culture, hypoxanthine, feeder cell layers, serum.

INTRODUCTION

The successful *in vitro* cultivation of *Babesia bovis* and *B. bigemina* (Levy & Ristic, 1980; Vega *et al.* 1985) has provided an effective alternative system to *in vivo* infections for a wide range of applied studies. Furthermore, the potential of the *in vitro* culture systems for mass production of parasites or parasite-derived molecules makes them an important logistic resource for the development of diagnostic assays and for vaccine production. Serum is considered to be a crucial factor for the continuous growth of parasites in the microaerophilous stationary phase (MASP) culture technique for *B. bovis* and *B. bigemina* (Levy & Ristic, 1980). Culture medium (M199) containing bovine adult serum at 40% inclusion optimizes parasite growth and is routinely used in this system. Nevertheless, variable abilities to support parasite growth in culture have been observed amongst sera from different individual adult cattle, which may account for significant variability in parasite multiplication (Neves, 1991). Also, the presence of bovine proteins in relatively large amounts in the culture medium commonly poses a problem for the isolation and purification of parasite proteins (James, Levy & Ristic, 1981). Several attempts to circumvent this problem by replacement of bovine serum in cultures have been reported (Levy & Ristic, 1983; Mishra *et al.* 1991; Mishra, Clabaugh & Kakoma, 1992). The substitution of serum by a mixture of more defined components considered fundamental for *in vitro* multiplication constitutes the rationale for 'serum-free' culture systems (Heath, 1993). The immediate advantages of these systems would be a higher potential for standardization, the facilitation of the isolation and purification of target molecules from cultures and the solution of safety problems posed by manipulation of fresh serum. Successful cultivation in serum-free conditions has been achieved with *P. falciparum* (Schrével, Grellier & Rigomer, 1992; Asahi & Kanazawa, 1994; Asahi *et al.* 1996, Cranmer *et al.* 1997) and with *B. divergens* (Grande *et al.* 1997).

Here, we report a series of experiments that indicate an important role for hypoxanthine in enabling the reduction of the serum content in *B. bovis* and *B. bigemina* cultures. The relationship between hypoxanthine and the possibility of replacing bovine serum either by semi-defined media or serum from alternative species is also described. Finally, we report the continuous growth of *B. bovis*

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and *B. bigemina* in the serum replacement media Daigo's GF and Albumax II with feeder cells.

MATERIALS AND METHODS

Materials

M199, RPMI, Hanks salts, *N*-[2-hydroxyethyl]-piperazine-*N*′[-2-ethanesulphonic acid] (HEPES), L-glutamine, normal bovine serum, horse serum, foetal calf serum, Albumax II and penicillin/streptomycin were all obtained from Gibco Ltd, UK. Bovine lipoprotein and hypoxanthine were supplied by Sigma, UK and Daigo's GF by Wako Pure Chemical Industries.

Standard B. bovis *culture establishment and maintenance*

The microaerophilous stationary phase (MASP) *B. bovis* culture technique of Levy & Ristic (1980) was used. An Israeli clone of *B. bovis* (C61411, from Israel vaccine strain) was received from the Department of Infectious Diseases and Immunology, Veterinary Faculty, Utrecht University as a culture suspension (4·68 % parasitaemia) and was used directly to initiate the cultures.

The parasites were cultured in 50 ml/25 cm^2 Nunclon flasks in 5 % bovine red blood cell (RBC) suspension in medium at 37 °C, and gassed with a mixture of 2 % O_2, 5 % CO_2 and 93 % N_2. The volumes of culture were 5 ml when incubated vertically, 12–15 ml when incubated horizontally and 1·2 ml in 24-well plates. In all cases the maximum depth of culture was 6–7 mm.

The standard culture medium was M199 with Hanks salts supplemented with 25 mM HEPES, 2 mM L-glutamine, 40 % normal bovine serum from a known growth-supportive donor, penicillin 100 U/ml and streptomycin 100 μg/ml. The serum was prepared by clotting and centrifugation (1700 ***g***, 4 °C, 30 min) and kept at 4 °C (short-term storage) or −20 °C. Medium was freshly prepared every week and stored at 4 °C.

Medium changes were carried out every 24 h. Approximately 4/5ths of the overlying medium was removed gently without disturbing the layer of settled RBC and replaced with fresh medium. Subcultures were prepared every 48 h, by dilution of old cultures 1 : 2, 1 : 5 or 1 : 10 in 5 % fresh uninfected RBC suspension to obtain a parasitaemia of approximately 1 % after subculture. For the 5 % uninfected RBC suspension, blood was aseptically collected from 1 of 2 known donor adult cows and defibrinated with glass beads. The defibrinated blood was centrifuged at 1000 ***g*** for 10 min. The plasma, buffy coat and 20 % top RBC were discarded. The remaining pellet was diluted in medium and stored at 4 °C for up to 1 week.

Standard B. bigemina *culture establishment and maintenance*

For *in vitro* cultivation of *B. bigemina*, the method described by Vega *et al.* (1985) was used. An *in vitro* adapted line of a Mexican isolate of *B. bigemina* was obtained from Imperial College, London. All the procedures were similar to those described above for *B. bovis* cultures, except that 10 % RBC suspension was used and in all experiments cultures were incubated in an atmosphere of 5 % CO_2 in air, 95 % relative humidity (RH), over 72 h cycles. In all experiments, culture parasitaemias were assessed by counting 10000 erythrocytes on Giemsa's stained thin blood films and a mean percentage of parasitized erythrocytes was calculated.

Feeder cell layers

Feeder cells consisting of peritoneal macrophages and fibrocytes were obtained under aseptic conditions from Biozzi mice by injection into the peritoneal cavity of 7 ml of cold RPMI 1640. After a quick massage, 3–5 ml were aspirated and transferred to a sterile 15 ml conical centrifuge tube kept on ice. The tubes were centrifuged at 400 ***g*** for 10 min and the cells washed twice in the same medium. Finally, the cells were gently resuspended in 5 ml of RPMI 1640 containing 10 % foetal calf serum (FCS) and incubated in 50 ml/25 cm^2 flasks at 37 °C with 5 % CO_2 in air. When a relatively dense layer of feeder cells was established, the supernatant was completely removed and *Babesia* cultures were transferred to these flasks immediately after subcultivation and kept for the entire cycle of 48 h (*B. bovis*) or 72 h (*B. bigemina*).

RESULTS

Growth of B. bigemina *in M199 and RPMI 1640*

M199 and RPMI 1640 with 25 μM HEPES and L-glutamine containing, respectively, 40, 30, 20 and 10 % of bovine serum were used to maintain *B. bigemina* cultures in 24-well plates over 2 × 72 h cycles. The experiment was initiated from an ongoing *B. bigemina* culture in M199 40 % bovine serum. There were 6 replicates in a 24-well plate for each of the different culture conditions. Continuous parasite growth was sustained in M199 supplemented with 40–20 % serum, with percentage parasitized erythrocytes ranging from 8·4 % to 13 % in the second cycle, while the same medium with 10 % serum produced 3·5 % parasitized erythrocytes at the same time-point. All concentrations of serum in RPMI 1640 failed to support continuous growth, with the highest value of 1·7 % parasitized erythrocytes obtained at the end of the second

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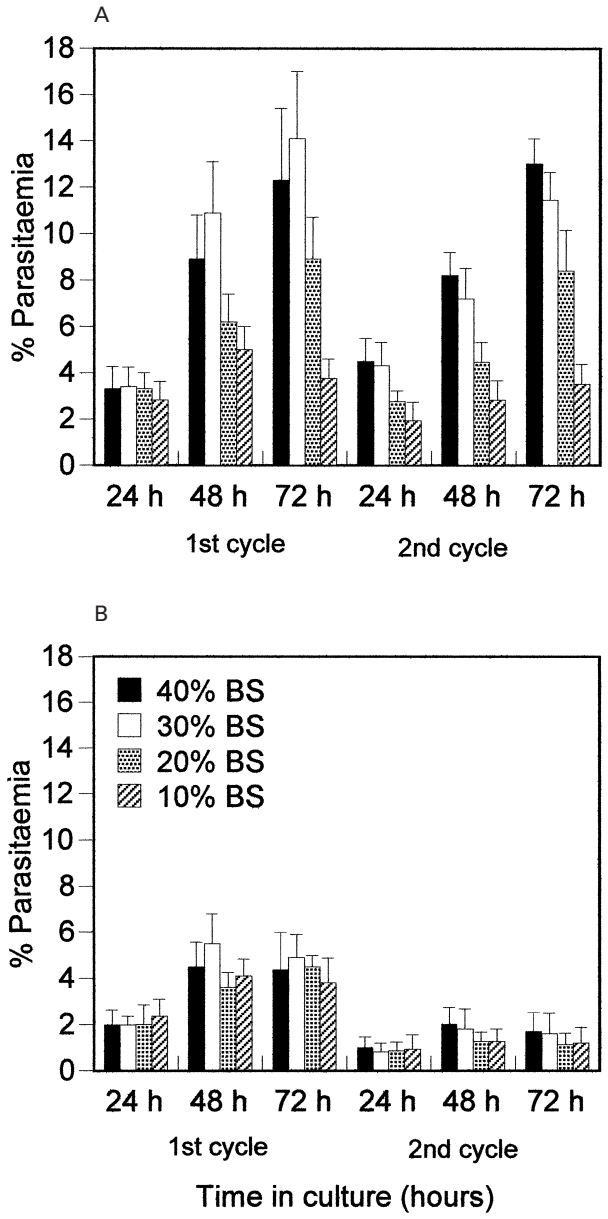

Fig. 1. Parasitaemias of *Babesia bigemina* cultured in M199 (A) or RPMI 1640 (B) over 2 cycles with different concentrations of bovine serum (BS). Bars illustrated are means from 6 replicates. Vertical lines indicate S.D.

culture cycle (Fig. 1). However, the supplementation of RPMI 1640 with hypoxanthine at 100 μM resulted in continuous *B. bigemina* growth in all the percentages of serum inclusion tested, with parasitaemia values between 7·1 and 8·2 % obtained after 3 × 72 h cycles (3 replicates for each condition investigated).

In further experiments, simultaneous titration of serum and hypoxanthine was performed. RPMI 1640 containing between 2·5 and 40 % bovine serum was used for the cultivation of *B. bigemina*, with each percentage of serum being supplemented with between 5 and 400 μM hypoxanthine. Six replicates of each combination of serum and hypoxanthine were kept in culture in 24-well plates for 2 cycles of 48 and 72 h, respectively. The highest percentage parasitized erythrocytes (11·3–13·3 %) were obtained at 10 % serum with hypoxanthine supplementation varying from 50 to 200 μM (Fig. 2). All hypoxanthine concentrations outside of this range or when a serum concentration below 5 % was used resulted in low percentage values.

In some experiments, bovine lipoprotein was incorporated in M199 in partial or total replacement of bovine serum from a known donor animal. Different concentrations of bovine high-density lipoproteins (HDL) in the range 0·25–0·75 mg protein/ml (with 0·1 mg increment) were used in combination with 20, 10 and 0 % bovine serum in the MASP culture system for *B. bigemina*. Cultures were initiated in 24-well plates by medium replacement and subcultivation, using each specific HDL-serum combination. However, none of the combinations of HDL-serum was able to support parasite multiplication for more than 1 cycle. In the combinations of 10 % and 0 % serum inclusion with any of the HDL concentrations used, a drastic decrease in percentage parasitized erythrocytes was observed after 24 h together with parasite pyknosis or degeneration (data not shown).

B. bigemina *and* B. bovis *cultivation with different serum sources*

RPMI 1640 supplemented with 100 μM hypoxanthine containing 10 % horse serum (HS), foetal calf serum (FCS) or commercial adult bovine serum (cBS), was used for *B. bovis* and *B. bigemina in vitro* cultivation. Cultures containing HS were kept for more than a year, whilst those containing FCS or cBS were maintained for 5 × 48 h cycles (*B. bovis*) and 5 × 72 h cycles (*B. bigemina*) respectively. Cultures of *B. bovis* and *B. bigemina* containing 10 % BS in RPMI 1640 supplemented with 100 μM hypoxanthine and similar cultures of *B. bigemina* with 5 % BS were maintained for more than 6 months. Serum of 3 calves which did not support *B. bigemina in vitro* cultures at 40 % level in M199 were pooled and used at a 10 % concentration in RPMI 1640 supplemented with 100 μM hypoxanthine. Three replicates were established in 24-well plates and kept over 3 × 72 h cycles. Hypoxanthine supplementation of RPMI 1640 enabled growth of *B. bigemina* and *B. bovis* parasites with all sera tested (Table 1). The typical growth patterns of *B. bovis* and *B. bigemina* in the conventional MASP culture technique are also shown in Table 1.

Use of serum replacement media and feeder cell layers

Concentrated Albumax II was added to the RPMI 1640 at a final concentration of 0·5 % (w/v) solution supplemented with 200 μM of hypoxanthine (RPMI-A). Similarly, Daigo's GF was added at 3 g/l (RPMI-GF) to RPMI 1640 supplemented with

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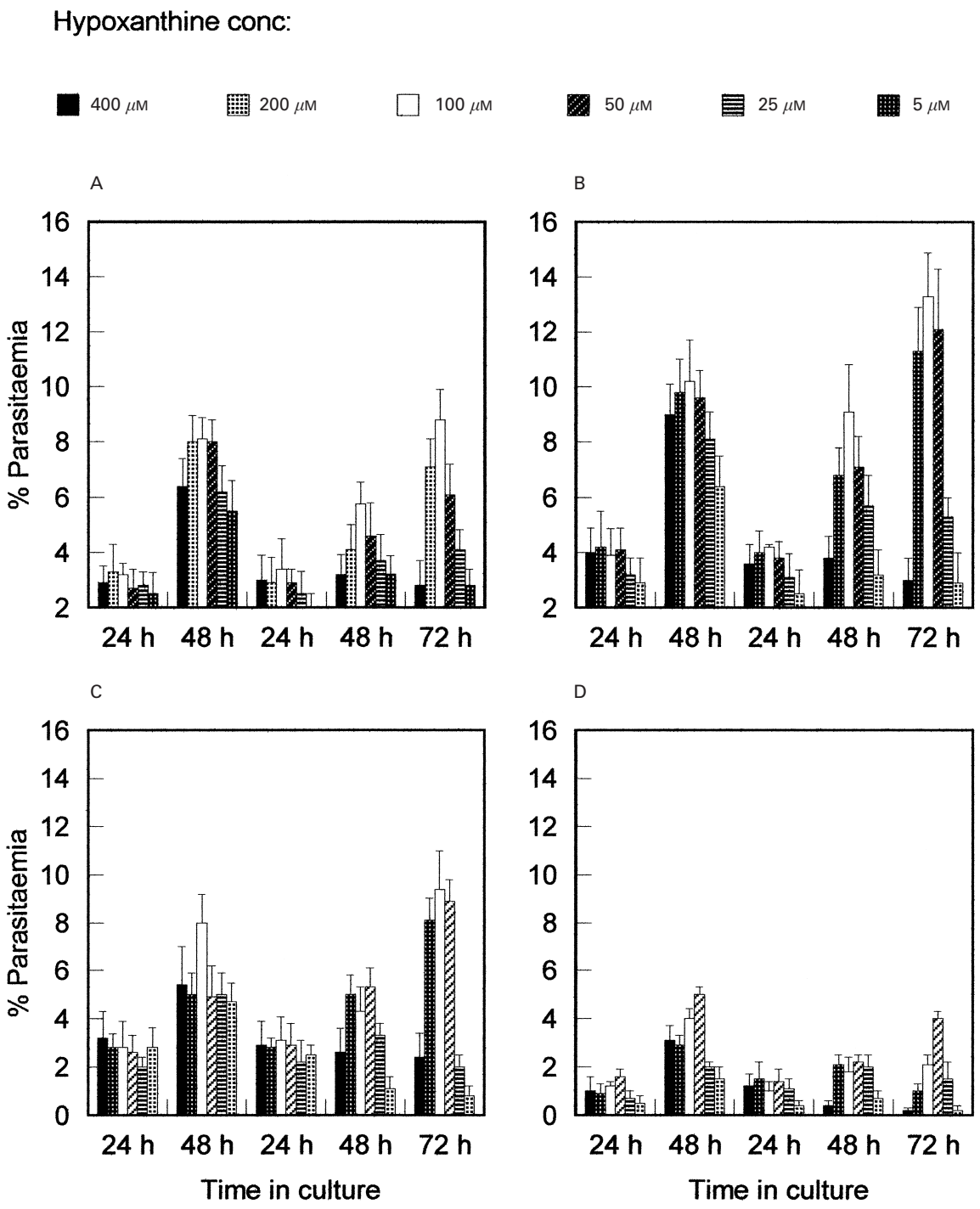

Fig. 2. Titration of hypoxanthine and bovine serum (BS) concentrations in RPMI 1640 over 2 cycles of *Babesia bigemina in vitro* culture. (A) 40% BS; (B) 10% BS; (C) 5% BS; (D) 2·5% BS. Vertical lines indicate S.D.

hypoxanthine (120 μM). These solutions were sterilized by filtration (0·2 μM porosity). RPMI-A and RPMI-GF were used in cultures of *B. bovis* and *B. bigemina* kept in 50 ml/25 cm² culture flasks (Nunc). To establish these experiments, ongoing cultures were centrifuged at 1000 ***g*** at 15 °C for 15 min and the resulting RBC pellet was diluted in a suspension of fresh RBC in RPMI-A or RPMI-GF. These two media were also used for *B. bovis* and *B. bigemina* cultures together with a feeder cell layer. Both RPMI-A and RPMI-GF supported *B. bovis* and *B. bigemina* growth over 3 cycles (Table 1), after which parasite development ceased. The culture of both parasite species in flasks containing feeder cells enabled growth (Table 1). A pool of serum samples that did not individually support *B. bigemina* development at 40% concentration in M199 was used at 10% concentration in combination with hypoxanthine-supplemented RPMI 1640. The parasite growth pattern was consistent over 3 cycles with average parasitized erythrocyte percentages at 72 h reaching 9·3% (S.D. = 1·3).

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Table 1. Growth of *Babesia bigemina* and *B. bovis* in RPMI 1640 in conventional MASP and with the addition of different sera and serum-replacement media, with and without a feeder cell layer

(Mean parasitaemias (S.D.) of 10 (10 % H.S.) or 5 cycles.)

	B. bigemina			*B. bovis*	
Time in culture	24 h	48 h	72 h	24 h	48 h
Without feeder cell layer					
Conventional MASP	4·2 (1·0)	9·6 (1·4)	14·0 (1·1)	3·9 (0·8)	8·1 (1·0)
100 μM hypoxanthine + 10 % HS	3·7 (0·5)	9·3 (0·7)	14·8 (0·8)	3·0 (0·6)	6·0 (0·18)
100 μM hypoxanthine + 5 % BS	3·0 (0·8)	4·2 (0·4)	8·9 (0·5)	—	—
100 μM hypoxanthine + 10 % cBS	3·5 (0·9)	7·0 (0·8)	10·0 (0·4)	3·4 (0·5)	7·0 (0·8)
100 μM hypoxanthine + 10 % FCS	3·0 (0·4)	6·0 (0·6)	8·0 (0·6)	2·4 (0·4)	5·5 (0·9)
0·5 % Albumax II + 200 μM hypoxanthine	2·2 (0·3)	4·8 (0·7)	7·6 (0·4)	2·3 (0·3)	5·2 (0·5)
3 g/l Daigo's GF + 120 μM hypoxanthine	3·5 (0·8)	5·0 (0·3)	10·0 (1·1)	2·6 (0·4)	6·4 (0·6)
With feeder cell layer					
0·5 % Albumax II + 200 μM hypoxanthine	2·9 (0·3)	5·1 (0·7)	8·2 (0·7)	2·7 (0·4)	5·0 (0·6)
3 g/l Daigo's GF + 120 μM hypoxanthine	3·3 (0·3)	5·7 (0·6)	8·6 (0·7)	2·9 (0·4)	6·9 (0·7)

DISCUSSION

The growth pattern of *B. bovis* and *B. bigemina* in the conventional MASP culture technique with 40 % normal bovine serum is characterized by low variability and the values of parasitaemia observed here appear to be within the range reported from similar systems (Levy & Ristic, 1980; Montenegro-James *et al*. 1985; Vega *et al*. 1985; Montenegro-James, 1989). When cultured in M199, *B. bigemina* growth was observed for 2×72 h cycles over a range of 40–20 % serum concentration, with proportionately lower parasitaemias with lower serum concentrations. In all combinations of serum with RPMI 1640, parasitized erythrocyte percentages were lower than with conventional MASP. These findings are in accordance with those previously reported on the standardization of the MASP technique for *B. bovis* (Levy & Ristic, 1980; Levy, Erp & Ristic, 1981) and *B. bigemina* (Vega *et al*. 1985). Nevertheless, an African strain of *B. bigemina* was recently established in culture in RPMI 1640 with a concentration of 50–60 % bovine serum (Posnett *et al*. 1998). Serum-free culture was established for *B. divergens* in medium in which serum was replaced with human high density lipoproteins (HDL) (Schrével *et al*. 1992), but this approach was not successful for *B. bovis* or *B. bigemina* when a wide range of bovine HDL concentrations were tried.

Since most of the protozoan species are unable to synthesize purines *de novo* (Bryant, 1993; Berens, Krug & Marr, 1995), they rely on the host pool of purine precursors for DNA synthesis and hence for multiplication. Studies on the incorporation of purine precursors into *B. bovis*-infected erythrocytes have demonstrated the presence of the enzymes involved in the purine salvage pathways and also the insertion into the bovine red cell membrane of a parasite-specific nucleoside/nucleobase transporter (Gero, 1989; Matias *et al*. 1990). The uptake of adenosine and hypoxanthine by *B. bovis*-infected erythrocytes was consistently greater than that of the other precursors (Conrad, 1986). Hypoxanthine supplementation was found to be crucial for parasite growth in *Babesia* cultures (*B. equi* and an unknown *Babesia* species) immediately after retrieval from liquid nitrogen or when cultures were started from live infections with very low parasitaemia (Zweygarth, Just & De Waal, 1995; Zweygarth, Van Niekerk & De Waal, 1995). It has been shown that hypoxanthine is an essential factor for the *in vitro* growth of *P. falciparum* in serum-free conditions (Asahi *et al*. 1996) and that the presence of xanthine oxidase, the enzyme that degrades hypoxanthine to uric acid, has a marked inhibitory effect on parasite growth in cultures (Berman, Human & Freese, 1991). Similarly, hypoxanthine supplementation of RPMI 1640, a culture medium that does not contain any purine precursors, has enabled *B. bigemina* growth in all tested serum conditions.

The simultaneous titration of serum and hypoxanthine indicates an optimal combination of the two factors at concentrations of 10 % serum and 50–200 μM hypoxanthine. Notwithstanding, growth was sustained at 5 % serum concentration in the same hypoxanthine concentration range. Combinations of 400, 25 and 5 μM hypoxanthine with all concentrations of serum were deleterious to parasite growth; this was presumably because the lower concentrations of hypoxanthine were too low to sustain growth, whilst 400 μM may have been unsuitable either because of a direct osmolarity effect, or as a consequence of the toxic effect of its metabolites. The optimal levels of hypoxanthine supplementation observed in this chequerboard experiment agree with those in use for *P. falciparum* serum-free cultures (Asahi *et al*. 1996; Cranmer *et al*. 1997).

Bovine serum has been partially replaced by human, goat and horse serum (Mishra *et al*. 1991, 1992; Yunker, Kuttler & Johnson, 1987) and totally replaced by horse and rabbit serum (40 % inclusion) (Levy & Ristic, 1983) in short-term cultures. With hypoxanthine-supplemented media, it was possible to achieve growth of *B. bovis* and *B. bigemina* over 72 h at 10 % inclusion of HS and FCS. This latter finding is inconsistent with the presence of a putative inhibitory molecule for *B. bovis* growth in FCS (Levy, Clabaugh & Ristic, 1982). In the same experiments, FCS was shown to be permissive to *B. bovis in vitro* growth after extensive dialysis against bovine adult serum, which, rather than removing a putative inhibitory molecule from the FCS, may have enabled incorporation of purine precursors from the adult bovine serum. Adult bovine serum shows different abilities to support *B. bovis* (Neves, 1991) and *B. bigemina* (Elsa Posnett, personal communication) and this variability tends to be more marked amongst young animals (Kellerman, Tsang & Kakoma, 1988). The present results indicate that commercial adult bovine serum is able to support *in vitro* multiplication of *B. bovis* and *B. bigemina*, as was a pool of previously non-supportive calf serum (*B. bigemina*) after hypoxanthine supplementation. In the serum-free culture approach for *P. falciparum* and *B. divergens*, Daigo's GF (Asahi *et al*. 1996), Albumax II (Cranmer *et al*. 1997) and Albumax I (Grande *et al*. 1997), which are all fractions of bovine serum, have replaced human serum, ensuring continuous parasite growth. When Diago's GF and Albumax II were used in combination with hypoxanthine-supplemented RPMI 1640 for *B. bovis* and *B. bigemina*, a sharp decrease in parasite development was consistently observed after 3×72 h cycles. However, when flasks containing an established feeder layer cell from murine peritoneal lavage were introduced in the culture systems of these species, continuous parasite growth was achieved with both serum replacement media. The use of feeder layer cells to stimulate parasite growth *in vitro* has been extensively reported from trypanosomes (Hirumi, Doyle & Hirumi, 1977), *P. falciparum* (Phillips *et al*. 1987; Trenholme & Phillips, 1989; Trenholme, McMonagle & Phillips, 1990) and *B. divergens* (Luis Loureiro, personal communication).

Yunker *et al*. (1987) observed some degree of strain attenuation after partial replacement of bovine serum with horse serum in short-term *B. bovis* MASP cultures. *B. divergens* strain attenuation was also demonstrated after long-term culture of this parasite (Winger, Canning & Culverhouse, 1989). The possibility of continuous growth of *B. bovis* and *B. bigemina* with 10 % HS might provide a practical means of achieving *in vitro* strain attenuation.

The combined results of these experiments indicate an important role for hypoxanthine in serum reduction and replacement in *B. bovis* and *B. bigemina* MASP cultures. The good performance of HS in these culture systems suggests that a defined fraction of this serum in combination with feeder layer cells or, alternatively, with more defined growth factors, could constitute the basis of an optimised serum-free culture system for these two parasite species.

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