Continuous *in vitro* culture of *Babesia divergens* in a serum-free medium

N. GRANDE[1], E. PRECIGOUT[1], M. L. ANCELIN[2], K. MOUBRI[1], B. CARCY[1], J. L. LEMESRE[3], H. VIAL[2] *and* A. GORENFLOT[1]*

[1] *Laboratoire de Biologie Cellulaire et Moléculaire, UPRES No. 699, UFR des Sciences Pharmaceutiques et Biologiques, 15 Avenue Charles Flahault, F-34060 Montpellier Cedex 02, France*
[2] *UMR 5539 CNRS, Université des Sciences et Techniques du Languedoc, Dynamique Moléculaire des Interactions Membranaires, CC 107, Place Eugène Bataillon, F-34095 Montpellier Cedex 05, France*
[3] *Laboratoire des Grandes Endémies Tropicales, Unité de Biologie Parasitaire, ORSTOM, 911 Avenue Agropolis, BP 5045 F-34032 Montpellier Cedex, France*

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SUMMARY

Babesia divergens was cultivated in RPMI 1640 (25 mM HEPES) supplemented with 10% human serum (RPMI-10% HS) with a high percentage of parasitized erythrocytes (PPE) ($\geqslant$ 40%). Standardization of *in vitro* tests, purification of exoantigens, biochemical studies and the safety of the culture handler motivated the development of a serum-free defined medium. Removal of serum greatly reduced the PPE but, after a period of adaptation, the culture was continuous and the parasite was able to develop a 3% routine PPE. Addition of vitamins or reduced glutathione in basal medium (RPMI) did not improve the PPE. The supplementation of basal medium with lipidic carrier (Albumax I or bovine serum albumin–Cohn's fraction V) promoted the growth of *B. divergens* with high PPE ($>$ 30%) close to those obtained in RPMI–10% HS. Neither protein nor lipid fractions alone were able to restore the growth of *B. divergens*. Nevertheless, the whole lipid fraction from serum or Albumax I added to delipidated albumin partially restored the growth (7% PPE), indicating that the presentation of specific lipids by a carrier is crucial for the parasite. All the data indicate that Albumax I can replace human serum offering the advantages of safety, standardization for chemosensitivity tests, and exoantigen purification.

Key words: *Babesia divergens*, serum-free culture, bovine serum albumin–Cohn's fraction V (BSA–FV), fatty acids, serum lipids, Albumax I.

INTRODUCTION

Babesiosis is a tick-borne haemoprotozoan disease affecting a wide variety of wild and domestic animals, including cattle, horses, dogs and rodents. Humans are rarely affected but the disease is increasingly recognized in the United States and in Europe, where the babesiosis agents are *Babesia microti* and *Babesia divergens* respectively (Gorenflot *et al*. 1990). *B. divergens*, the main agent of bovine babesiosis in Europe, is responsible for major economic losses (Kuttler, 1988). The vector tick (*Ixodes ricinus*) inoculates the infective sporozoites into the bloodstream of the host where they invade erythrocytes and differentiate into 2–4 merozoites that initiate an erythrocytic cycle.

Attempts to elaborate an efficient *B. divergens* long-term *in vitro* culture have encountered difficulties for a long time. Several such attempts to cultivate *B. divergens* in a microaerophilous stationary phase system (MASP containing RPMI 1640 + 40% bovine serum) have achieved approximately 10% parasitized erythrocytes (Varynen & Tuomi, 1982; Konrad *et al*. 1984, 1985; Pudney, 1984). Using the cultivation method developed by Trager & Jensen in 1976, for *in vitro* culture of another haemoprotozoan, *Plasmodium falciparum*, a continuous culture of *B. divergens* reaching an average percentage of parasitized erythrocytes (PPE) of 40–50% was obtained (Gorenflot *et al*. 1991). This cultivation protocol is based on a basal synthetic medium (RPMI 1640 buffered with HEPES) supplemented with 10% human serum (RPMI–10% HS). Although we know that serum is essential for the *in vitro* culture of haemoparasites, the identification and role of specific components have not been precisely defined. The critical factors may include serum proteins, lipids, sugars, vitamins, growth factors and hormones that synthetic media fail to provide. To develop a serum-free medium which would supply essential nutrients and consequently allow optimum growth of the parasite and easier characterization/purification of the parasitic exoantigens, we have carried out a multi-step supplementation of *B. divergens in vitro*. The first step consisted of the development of a synthetic medium

* Corresponding author: Laboratoire de Biologie Cellulaire et Moléculaire, UPRES No. 699, UFR des Sciences Pharmaceutiques et Biologiques, 15 Avenue Charles Flahault, F-34060 Montpellier Cedex 02, France. Tel: +33 04 67 54 64 81. Fax: +33 04 67 54 66 21. E-mail: agorenf@pharma.univ-montpl.fr.

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supplemented only with high-density lipoproteins (Valentin *et al*. 1991); the parasitic growth lasted a few cycles in this semi-defined medium.

In this work, using the *B. divergens* Rouen 1987 isolate which has been adapted to grow in RPMI 1640 without serum (RPMI), we first tested the role of vitamins and anti-oxidants on *B. divergens in vitro* growth. Considering that lipids may play an important role, as was previously demonstrated in *P. falciparum* (Vial & Ancelin, 1992), we tested various lipid-rich supplementations and demonstrated that lipidic carriers such as bovine serum albumin (Cohn's fraction V) or Albumax I can be used in place of serum.

MATERIALS AND METHODS

Materials

RPMI 1640 buffered with 25 mM 4-2-hydroxyethyl-1-piperazine-ethanesulfonic acid (HEPES), Albumax I and the vitamin solution were obtained from Gibco-BRL (France). The 1 × concentration of vitamin solution consists of 0·2 mg/l biotin, 0·25 mg/l calcium pantothenate, 3 mg/l choline chloride, 1 mg/l folic acid, 2 mg/l para-aminobenzoic acid, 35 mg/l *i*-inositol, 1 mg/l nicotinamide, 1 mg/l pyridoxine hydrochloride, 0·2 mg/l riboflavin, 1 mg/l thiamine hydrochloride and 0·005 mg/l B12 vitamin. Fatty acid-free bovine serum albumin (BSA) from BSA–Cohn's Fraction V (Ref. A6003), BSA–Cohn's Fraction V (Ref. A4503), palmitic acid, stearic acid, oleic acid, linoleic acid and activated charcoal were purchased from Sigma (France). Reduced glutathione and BSA–linoleic acid (Ref. 1243 241) were obtained from Boehringer Mannheim (Germany). Human blood (blood group O) and human serum were obtained from the Centre Régional de Transfusion Sanguine of Montpellier (France).

In vitro *culture of* B. divergens

The *B. divergens* Rouen 1987 isolate was obtained from a patient who recovered from acute babesiosis. The *in vitro* culture technique used has been previously described (Gorenflot *et al*. 1991). The parasites were grown in 25 cm^2 culture flasks, in O human erythrocytes suspended at 5 % haematocrit in basal medium consisting of 5 ml of RPMI 1640 buffered with 25 mM HEPES, 25 mM $NaHCO_3$, pH 7·3 and supplemented with 10 % human serum. The medium was changed daily until the PPE reached 30–50 %. Then, the infected erythrocytes were diluted, to obtain a PPE of 1 %, with normal human erythrocytes which were twice washed in basal culture medium. *In vitro* cultures were maintained at 37 °C after flushing with a 91 % N_2, 6 % O_2 and 3 % CO_2 gas mixture.

When the cultures were grown in RPMI, the erythrocytes were extensively washed: 1 volume of erythrocytes was centrifuged (1200 ***g***, 10 min) 12 times in 50 volumes of RPMI.

Periodically, aliquots were taken and the percentage of parasitized cells was estimated on erythrocyte smears stained with Diff-Quick (Dade S.A., France) by counting 2000 erythrocytes.

Extraction of the lipidic fraction from human serum and Albumax I

The extraction of lipids from human serum was performed according to the Bligh & Dyer (1959) procedure. First, 150 ml of human serum were mixed with 187·5 ml of chloroform and 375 ml of methanol and then 187·5 ml of chloroform were added to the mixture and blended for 1 min. Finally, 187·5 ml of double-distilled water were added whilst blending continued for a further 1 min. After decantation, the aqueous phase containing the human serum proteins was discarded. For complete separation and clarification, centrifugation (1200 ***g***, 4 min) was performed and the organic phase was evaporated under nitrogen. At the end of the evaporation step, ethanol was added to the serum lipid fraction which was then sonicated (4 × 15 sec), rapidly flushed under nitrogen and stored at −20 °C. The phospholipid content of this ethanolic fraction, determined as described by Rouser, Fleischer & Yamamoto (1979), was 194 g/l.

Lipidic and protein extraction from Albumax I was performed to test each constituent separately. The lipid extraction was done as described above except that 250 mg Albumax I were dissolved in 50 ml of distilled water and blended first with 62·5 ml of chloroform and 125 ml of methanol. After extraction and evaporation, residue was dissolved in ethanol at a phospholipid concentration of 560 mg/l as determined according to Rouser *et al*. (1979).

Extraction of protein from bovine albumin of Albumax I

The purification of bovine albumin from Albumax I was performed as described by Chen (1967). Activated charcoal was washed with distilled water, filtered through a Buchner funnel and allowed to dry at room temperature. Albumax I (1 g) was dissolved in 10 ml of distilled water. Charcoal (0·5 g) was added to the solution and the pH was lowered to 3·0 by the addition of 0·2 M HCl. The suspension was then placed on an ice bath and mixed for 1 h. Charcoal was removed by centrifugation at 20000 ***g*** for 20 min at 2 °C. The clarified solution was then brought to pH 7·0 by the addition of 0·2 M NaOH. Determination of protein was performed by the method of Lowry *et al*. (1951) using bovine serum albumin as standard. Proteins recovered by this

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procedure correspond to an 84 % purification yield and were resuspended in ethanol at a concentration of 84 g/l.

Loading of different lipidic fractions on fatty acid-free albumin

The fatty acid-free albumin was enriched with various lipid extracts consisting of (i) lipids extracted from human serum, (ii) lipids extracted from Albumax I and (iii) a mixture of the 4 essential fatty acids present in human serum.

The mixture of the essential unesterified fatty acids of serum (0·3 mM total final concentration) contained palmitic (16:0), stearic (18:0), oleic (18:1) and linoleic acid (18:2) in the molar ratio of 1·7:0·7:1·3:1·3 respectively. These free fatty acids were added in a molar ratio free fatty acids/fat-free albumin equal to 2 as observed under physiological conditions (Muller & Wollert, 1979). The lipid fractions from Albumax I and serum, expressed as phosphate content (20, 50, 100, 300 mg/l and 1 g/l), were dissolved in ethanol, then rapidly injected into the basic medium containing fat-free BSA or Albumax-extracted albumin respecting a molar ratio 2·6:1. After vortexing for 1 min, the culture medium enriched with the lipids loaded on albumin was introduced into the culture flask. The final ethanol concentration was lower than 0·3 % and did not interfere with the growth of *B. divergens*.

Supplementation of the B. divergens *culture medium*

To study the growth of *B. divergens*, various supplements were added to RPMI 1640 with 25 mM HEPES (RPMI). The different media were: RPMI–Vit (supplemented with vitamins: 0·5 ×, 1 ×, 2 ×, 3 × and 5 ×), RPMI-Glut (supplemented with reduced glutathione: 0·5, 1 and 2 g/l), RPMI–Vit–Glut (supplemented 3-fold with vitamins and 1 g/l glutathione), RPMI–Albumax (supplemented with Albumax I: 1, 5 and 10 g/l), RPMI–BSA–FV (supplemented with bovine serum albumin Cohn's fraction V: 0·4, 1, 5 and 10 g/l), RPMI–BSA (supplemented with fatty-acid free BSA: 1, 5 and 2 g/l), RPMI–Lip (supplemented with lipids extracted from human serum: 5, 10, 20, 100 and 500 mg/l), RPMI–LipAlb (supplemented with lipids extracted from Albumax I: 5, 10, 20, 100 and 500 mg/l), RPMI–BSA–Lin (supplemented with BSA loaded with linoleic acid [10 mg of linoleic acid/g of albumin]: 2 g of albumin/l, 4, 8 and 16 g/l), RPMI–BSA–4FA (supplemented with BSA loaded with a mixture of the major plasmatic fatty acids [palmitic, stearic, oleic and linoleic acid: 8 mg of fatty acids/g of albumin]: 2 g of albumin/l, 4, 8 and 16 g/l), RPMI–BSA–Lip (supplemented with BSA loaded with lipids extracted from human serum: 20, 50, 100, 300 mg/l and 1 g/l), RPMI–BSA–LipAlb (supplemented with BSA loaded with lipids extracted from Albumax I: 20, 50, 100, 300 mg/l and 1 g/l). Controls consisted of RPMI–10 % HS (supplemented with 10 % human serum) and RPMI (RPMI 1640 buffered with 25 mM HEPES).

RESULTS

In vitro *culture of* B. divergens *in RPMI*

B. divergens growth was optimal in HEPES-buffered RPMI 1640 supplemented with 10 % human serum (RPMI–10 % HS) and the PPE routinely reached 40–50 % with a multiplication index of 3 and 5 per day (Fig. 1A). The parasite material was diluted by the addition of human erythrocytes at 3 or 4 day intervals and *B. divergens* continuously maintained its normal *in vitro* asexual cycle of approximately 8 h. The *B. divergens* Rouen 1987 isolate became adapted to serum-free conditions in RPMI by gradual decrease of the serum concentration correlated with a gradual decrease of the PPE (data not shown). After 4 subcultures, *B. divergens* culture can be performed in the serum-free medium (RPMI). The PPE routinely reached 3 % with a multiplication index between 1·2 and 2 per day and the culture was continuously maintained (Fig. 1B); moreover, the extensive washing done before each subculture did not modify the growth of the parasite. The addition of 10 % serum to the isolate adapted to the absence of serum fully restored the normal growth conditions after 2 subcultures.

Supplementation of RPMI with vitamins and reduced glutathione

B. divergens was cultivated in RPMI 1640 supplemented with various concentrations of the solution of vitamins. The addition of vitamins had no effect on the average PPE of 3 % and the culture was continuous (Fig. 2). Adding 2 g/l reduced glutathione immediately had a toxic effect on parasite growth, and at 1 g/l a PPE of 1 % only was achieved (Fig. 2). At 0·5 g/l, reduced glutathione did not improve the parasitaemia and the culture could only be maintained for a few days. When cultured in RPMI medium, supplemented with both vitamins (3 ×) and reduced glutathione (1 g/l) (RPMI–Vit–Glut), the culture was continuous and a routine PPE of 2 % was obtained (Fig. 2). Since no improvement in *B. divergens* growth was observed with RPMI supplemented with vitamins and reduced glutathione in comparison with RPMI, for the following experiments, RPMI was used as basal medium.

Supplementation of RPMI with Albumax I or BSA–Cohn's Fraction V

Albumax I at the concentration of 1 g/l gave a PPE of 14 % (data not shown) but with the 5 g/l

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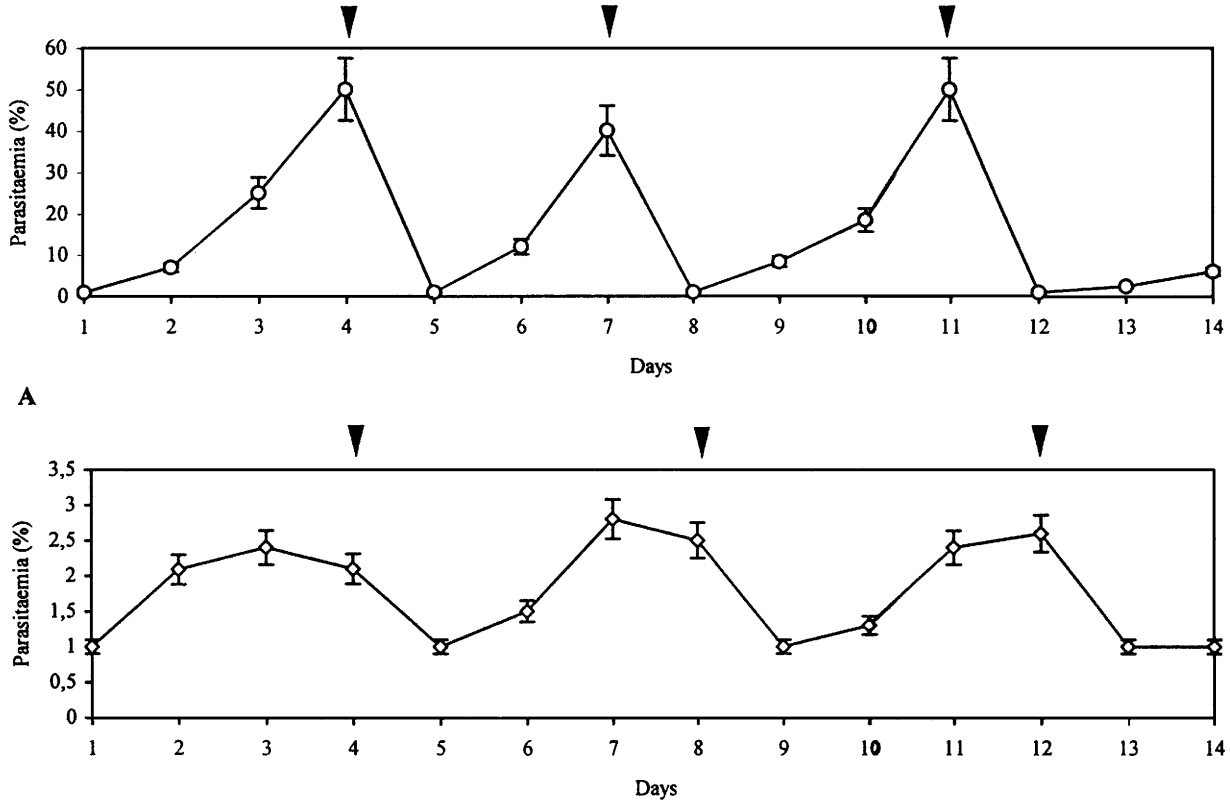

Fig. 1. *In vitro* growth of *Babesia divergens* Rouen 1987 isolate in RPMI 1640 (25 mM HEPES) with 10% human serum (A) or without serum (B). Subcultures are indicated by arrows. Each bar represents the mean ± S.D.

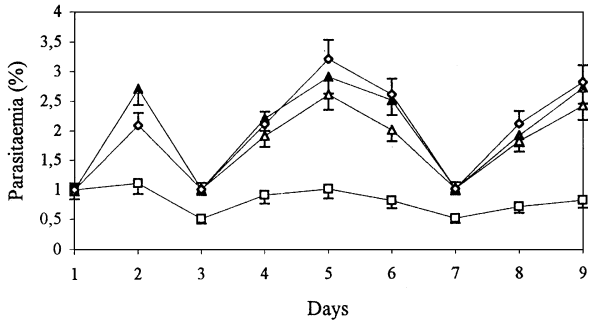

Fig. 2. Effect of vitamins and glutathione on the *in vitro* growth of *Babesia divergens* Rouen 1987, adapted to RPMI 1640 (25 mM HEPES) without serum. Parasites were grown in medium alone (◇), medium plus 3 × vitamins (RPMI–Vit; 3 × ; △), medium plus 1 g/l glutathione (RPMI–Glut; 1 g/l; □), or medium with both vitamins and glutathione (RPMI–Vit–Glut; ▲). Each bar represents the mean ± S.D.

concentration, a PPE of 37% could be obtained (Fig. 3). A routine PPE of 39% was obtained at an Albumax I concentration of 10 g/l (data not shown). Since average PPE differences between supplementation with 5 or 10 g/l of Albumax I were very small (data not shown), we chose to supplement RPMI with 5 g/l Albumax I. Thus, the RPMI–Albumax medium allowed continuous growth of *B. divergens* with PPE and multiplication index comparable to those obtained with RPMI–10% HS.

The RPMI was also supplemented with BSA–Cohn's Fraction V (RPMI–BSA–FV). From the lowest concentration to the 10 g/l concentration, the average PPE gradually increased while above 10 g/l parasitaemia did not increase further (data not shown). Therefore, the optimal concentration of this supplementation was 10 g/l. The *B. divergens in vitro* culture was continuous with RPMI–BSA–FV with a 33% routine PPE (Fig. 3).

Supplementation of RPMI with the protein moiety of Albumax and BSA–FV

RPMI was supplemented with commercial fatty acid-free BSA and with albumin extracted from Albumax I, at a protein concentration which previously proved to be optimal for parasite growth, 10 and 5 g/l respectively. To allow a better comparison, we used the same protocol as for commercial free-fatty acid-BSA to delipidate protein from Albumax (Chen, 1967).

When the culture medium consisted only of RPMI plus 10 g/l fatty acid-free albumin (RPMI–BSA), the PPE did not exceed 1%, the culture was not continuous and the erythrocytes were lysed. Similarly, the supplementation of the culture medium with proteins extracted from Albumax I (RPMI–Alb), at a 5 g/l concentration had a haemolytic effect on the erythrocyte leading to a complete disappearance of the parasites.

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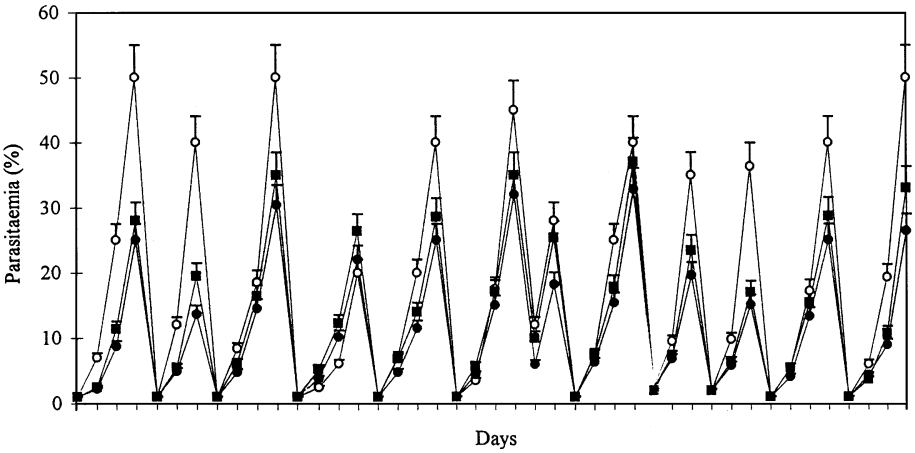

Fig. 3. *In vitro* growth of *Babesia divergens* Rouen 1987 in RPMI–10 % HS (○), RPMI supplemented with 10 g/l BSA–Cohn's Fraction V (RPMI–BSA–FV; ●), or with 5 g/l Albumax I (RPMI–Alb; ■). Each bar represents the mean ± S.D.

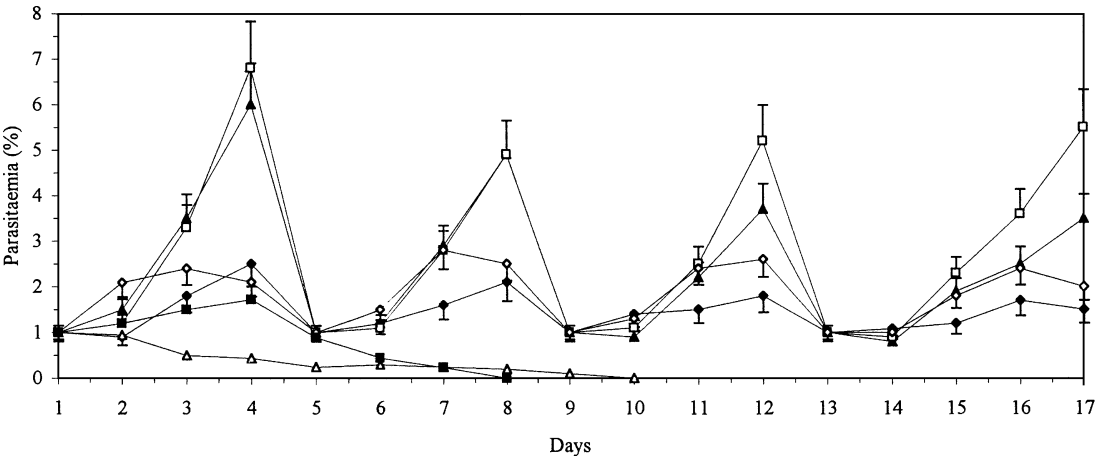

Fig. 4. Effect of free lipids or lipids loaded on albumin on the *in vitro* growth of *Babesia divergens* Rouen 1987. Parasites were grown in medium alone (RPMI; ◇), medium supplemented with 10 mg/l serum lipids (RPMI–Lip; ▲), medium supplemented with 10 mg/l lipids extracted from Albumax I (RPMI–LipAlb; ■), medium supplemented with 4 g/l BSA loaded with linoleic acid (RPMI–BSA–Lin; ◆), medium supplemented with BSA loaded with 4 fatty acids (RPMI–BSA–4FA; △), or medium supplemented with BSA loaded with 100 mg/l lipids extracted from serum (RPMI–BSA–Lip; □). Each bar represents the mean ± S.D.

Supplementation of RPMI with different lipid fractions

The *B. divergens* culture was supplemented with lipids extracted from Albumax I (RPMI–LipAlb). The optimal concentration was found to be 10 mg/l. In this case, the maximum PPE obtained was 1·5 % and the culture was not continuous (Fig. 4). Higher concentrations did not give better results and induced haemolysis of the erythrocytes from a concentration of 100 mg/ml.

Serum lipids extracted from human serum were added without any carrier in RPMI (RPMI–Lip). The culture was continuous and a maximum PPE of 6 % was reached at an optimal concentration of 10 mg/l. However, after several days, the routine PPE decreased to 3·5 % (Fig. 4). Lower concentrations prevented a continuous *in vitro* culture of *B. divergens* and the parasite died. In the range 10–100 mg/l there was no significant difference in average PPE. High concentrations of lipids (500 mg/l) had a strong deleterious effect by haemolysing the erythrocytes (data not shown).

Even if some serum lipidic fractions added to the basal medium without any carrier (RPMI–Lip) improved the *in vitro* culture of *B. divergens*, the routine PPE (3·5 %) was low in comparison with the PPE obtained in RPMI–10 % HS.

Supplementation of RPMI with fatty acid-free albumin loaded with different lipid fractions

BSA loaded with linoleic acid was added to the basic medium. The culture of *B. divergens* was maintained for several weeks but this supplementation did not improve the growth of the parasite, in comparison with the parasite culture in the basal medium. A maximum PPE of 2·5 % was obtained with a linoleic acid-loaded albumin at the optimum concentrations of 4 g/l BSA and 40 mg/l linoleic acid (Fig. 4).

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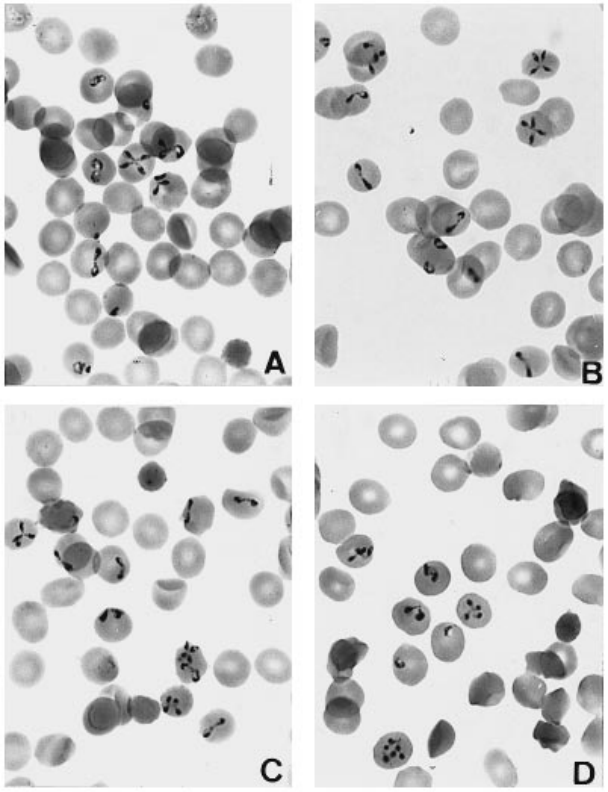

Fig. 5. Light microscopy studies showing the cytological characteristics of *Babesia divergens* in different culture media. (A) RPMI–10HS; (B) RPMI–BSA–FV; (C) RPMI–Albumax; (D) RPMI. Erythrocyte smears were stained by Diff-Quick.

The BSA was also loaded with a mixture of the major plasma fatty acids (palmitic, stearic, oleic and linoleic acid). Whatever the tested concentration, the PPE fell rapidly, never exceeding 1 % and the *in vitro* culture was not continuous (Fig. 4).

In a last set of experiments, albumin was loaded with the total lipid fraction of human serum (RPMI–BSA–Lip) or the lipid fraction of Albumax (RPMI–BSA–LipAlb). In these conditions, routine parasitaemia increased significantly when compared with culture in the presence of RPMI. An optimal concentration of 100 mg/l of lipids allowed the culture to reach a routine PPE of 7 % and the *in vitro* culture was continuous (Fig. 4). The growth of *B. divergens* was similar when we loaded lipids extracted from Albumax I (100 mg/l) on fat-free bovine serum albumin (Fig. 4).

Cytological characteristics of the parasitic forms

All the parasitic forms of the intra-erythrocytic cycle were observed in RPMI–BSA–FV and RPMI–Albumax: namely rings, geminated forms and tetrad forms; no significant differences could be observed in the cytological characteristics of the parasites compared to that in RPMI–10 % HS (Fig. 5). Moreover, no haemolysis or erythrocytic alterations were observed when using RPMI, RPMI–BSA–FV or RPMI–Albumax for the *B. divergens in vitro* culture.

The same observations have been made regarding RPMI 1640 supplemented with vitamins (RPMI–Vit), with serum lipids (RPMI–Lip), with linoleic acid–albumin (RPMI–BSA–Lin) or with lipids from serum or Albumax loaded on delipidated BSA (respectively RPMI–BSA–Lip and RPMI–BSA–LipAlb).

DISCUSSION

The development of serum-free chemically defined culture medium for *Babesia* sp. is fundamental for the study of cell biology, physiology and also for some specific studies such as the identification of exoantigens released into the culture medium. Soon after the development of an efficient *in vitro* culture of *B. divergens* (Gorenflot *et al.* 1991), studies were made on the elaboration of a semi-defined medium based on supplementation with HDL (Valentin *et al.* 1991). Recently, Zweygarth *et al.* (1995*b*) have described the *in vitro* cultivation of bovine *Babesia* species in a serum-free medium where serum was replaced by bovine lipoproteins and 2 % Nutridoma–SR. Similarly, for the culture of *Babesia equi* (now considered as a *Theileria* species) Holman *et al.* (1994) have diminished the percentage of serum by using a medium containing 20 % foetal bovine serum and 1 g Albumax I/l and Zweygarth *et al.* (1996) have replaced horse serum by bovine lipoproteins and bovine serum albumin. Except for these trials, all *Babesia* species so far tested were cultivated in media supplemented with 10–40 % canine, bovine or equine serum (Moreau & Laurent, 1984; Vega *et al.* 1985; Holman *et al.* 1993, 1994; Zweygarth, Just & De Waal, 1995*a*). Thus, the present study was aimed at developing a serum-free medium for the continuous culture of *B. divergens*.

First, we progressively reduced the concentration of human serum from 10 % to 0 % in order to adapt *B. divergens* parasites to growth in the fully synthetic medium RPMI 1640 that only contains amino acids, sugar, some vitamins, but that does not contain any protein, growth factors or lipids. Interestingly, the parasite viability was maintained with a PPE of 3 %. Nevertheless, parasitaemia in the absence of serum, although continuous, remained low as if essential nutrients could be found by the intracellular *B. divergens* parasite, but in a quite limited amount. Further experiments aimed to restore the multiplication index and routine parasitaemias equivalent to those obtained with RPMI–10 % HS by supplementation of RPMI with various nutrients present in the serum.

We noted that vitamins and an antioxidant component (reduced glutathione) added to the

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RPMI medium, alone or in combination, did not improve the parasitic growth. To the contrary, reduced glutathione had a deleterious effect and, it is interesting to note that reduced glutathione, which protects against oxidative stress, has a positive effect on the growth of *Babesia canis* (unpublished results) and *P. falciparum* (Zolg *et al*. 1982). Therefore, the redox state of the *B. divergens*-infected erythrocyte as well as the metabolic pathways involved in its defence against oxidative stress are not exactly similar to those involved in *B. canis* or in *P. falciparum*.

To explain the fact that *B. divergens* can be cultivated in RPMI without any supplementation, we have presumed that some essential nutrients could be adsorbed on the normal erythrocytes providing specific supplements to the parasite. This hypothesis must be discarded because extensive washings did not alter the parasitic development. The parasite probably uses some nutrients directly obtained from the host erythrocyte (cytoplasm or membranes) and not from the plasma molecules bound to the erythrocytic membrane. Nevertheless, the low PPE suggests that the parasite needs additional serum factors for optimal growth. Growth of the parasite within the host erythrocyte and the subsequent formation of 2–4 merozoites requires a large increase of total membranes (mitochondria, rhoptries, micronemes, nuclear envelope…). Thus, the dramatic increase of membranes is associated with a considerable requirement of new phospholipid molecules, either as exogenous pre-formed molecules or *de novo* biosynthesized molecules from endogenous precursors. *B. divergens* is unable to synthesize *de novo* fatty acids and cholesterol but was shown to biosynthesize new phospholipid molecules from exogenous polar heads and fatty acids (Valentin *et al*. 1991). Thus, *B. divergens* parasites probably need to incorporate phospholipids or phospholipid precursors such as polar heads or fatty acids in order to enable optimum growth. Considering these tremendous requirements of new phospholipid molecules, it is unlikely that host erythrocyte lipids alone would be sufficient to allow a comparable growth to that obtained in RPMI–10 % HS. Choline, serine and inositol are abundant polar heads in the RPMI 1640 basal medium, but RPMI 1640 does not contain any pre-formed phospholipids or the fatty acid part (Moore, Germar & Franklin, 1967). Molecules such as human lipoproteins can be involved in the transport of pre-formed phospholipids or neutral lipids to the intracellular parasites; nevertheless only HDL are able to promote the growth of *B. divergens* (near 30 %), but only for a short time, while LDL (low density lipoprotein) and VLDL (very low density lipoprotein) appear to be lethal for *B. divergens* (Valentin *et al*. 1991). This led us to investigate the role of lipids, whether bound to albumin or not, on the continuous growth of *B. divergens*. Albumin has been utilized as a lipid carrier in many serum-free formulations. As the methods of purification and isolation of biologically active components may contribute to their biological function, we tested BSA–Cohn's fraction V (BSA–FV: purified by ammonium sulfate precipitation) and Albumax I (purified by liquid chromatography). Complete lipid-rich albumin fraction such as BSA–FV or Albumax I greatly improved the *in vitro* parasitic growth and the PPE obtained were close to those obtained in RPMI–10 % HS. Moreover, we have been able to adapt *B. divergens* isolates from different geographic areas in RPMI supplemented with Albumax I and finally, for our reference isolate (*B. divergens* Rouen 1987) and other isolates, no differences were observed between RPMI–Alb and RPMI–10 % HS (data not shown). By contrast, the results obtained with Albumax I could not be completely extrapolated to BSA–FV; even after more than 50 subcultures, the PPE remained significantly lower when we added BSA–FV in basal medium instead of 10 % human serum or Albumax I. The better efficiency of Albumax I on parasitic growth might be due to the use of chromatography purification which is softer than the precipitation method.

Albumax I and BSA–FV are principally made up of serum albumin, one of the major roles of which is the transport of some lipids. In order to determine the respective role of each of these components (proteins and lipids), we separately tested delipidated albumin and different lipid fractions. Considering that fatty acids constitute one of the building blocks, precursors for phospholipids and neutral lipids, we used albumin loaded with various lipid fractions such as linoleic acid or a mixture of the 4 essential plasma fatty acids. Supplementation of the RPMI medium with BSA–linoleic acid (RPMI–BSA–Lin) or with a mixture of 4 essential fatty acids (RPMI–BSA–4FA) under physiological conditions did not allow an improvement of the parasite growth. In the absence of albumin, the supplementation of the RPMI culture medium with lipids extracted from human serum (RPMI–Lip) or from Albumax I (RPMI–LipAlb) resulted in a PPE similar to those observed in RPMI, demonstrating that the supplementation of basal medium with free lipids is not sufficient for optimal growth. Moreover, when the RPMI medium was only supplemented with the protein fraction extracted from Cohn's fraction V (RPMI–BSA) or albumin extracted from Albumax I (RPMI–Alb), the PPE fell and never exceeded 1 %. These results indicate that proteins alone or the lipids alone, added to the culture medium, are not able to restore the full growth of *B. divergens* or were inhibitory although their concentrations were optimal.

In the final series of experiments, the whole serum lipidic fraction containing unesterified fatty acids,

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phospholipids, triacylglycerol and cholesterol or the Albumax lipidic fraction was loaded on albumin (RPMI–BSA–Lip or RPMI–BSA–LipAlb) and added to RPMI 1640. This supplementation increased the routine PPE to 7%. Thus, whereas proteins or lipids added separately are not capable of improving parasite growth, their simultaneous addition significantly increases the PPE. Thus, albumin acts as lipid carrier allowing an efficient presentation of lipids to the parasite. Moreover, considering that Albumax does not contain any preformed phospholipids (as in HDL), and that the erythrocyte lacks any machinery to biosynthesize phospholipids *de novo*, we can deduce, in agreement with results obtained by Valentin *et al*. (1991), that *B. divergens* has the capacity to biosynthesize phospholipids from pre-built blocks that are polar heads and plasma fatty acids. However, the addition of fatty acid-loaded BSA was not able to restore the PPE and only albumin loaded with lipids extracted from serum and Albumax I was able to increase PPE significantly. These results indicate that the balance of the added fatty acids was not in agreement with the *B. divergens* requirement or that more specific lipids were necessary.

In this paper, we showed that Albumax I and BSA–FV can replace serum and that a combination of lipids and albumin is necessary for the growth of *B. divergens*. However, since full growth was not restored, other key components are certainly also involved in the optimal growth of *B. divergens*. Nevertheless, as 10% human serum could be completely replaced by Albumax I, such components could be carried by albumin and the differences observed between Albumax I and BSA–FV proved that the methods of isolation of biologically active components are critical to their biological function in cell culture systems. The replacement of serum by Albumax I offers many advantages, for example (i) no drug, eventually contained in serum used for conventional culture, can influence the development of the parasite, (ii) the prevention of any risk of infection with viruses for those handling the cultures, (iii) a standardization of *in vitro* chemosensibility tests among the different laboratories and (iv) an easier purification and identification of the parasite exoantigens.

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