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Original investigations

Cytological and immunological responses to *Babesia divergens* in different hosts: ox, gerbil, man

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Abstract. A continuous in vitro culture system for *Babesia divergens* was initiated from a human isolate. It was maintained through 305 subcultures for 3 years using a low concentration of serum and a low haematocrit, with no decrease in the initial virulence. This in vitro system enabled the routine culture of all human and bovine *B. divergens* isolates thus far tested, with a mean parasitaemia level of 30%–40%. Different cytological aspects observed in the same isolate by optical and electron microscopy were described in parasitized ox, gerbil and human erythrocytes. The sequence of *B. divergens* antibody responses was determined in man and ox, enabling the precise identification of major *B. divergens* antigens as candidates for vaccines.

Babesiosis is a haemoprotozoan disease transmitted by tick bite to a wide variety of wild and domestic animals, including cattle, horses, dogs and rodents. Human babesiosis is rare but is being increasingly recognized in the United States and in Europe (for review see Healy and Ristic 1988).

Babesia divergens, a bovine parasite, is responsible for most cases of human babesiosis in Europe (Gorenflot 1988; Gorenflot et al. 1990). The Mongolian gerbil *Meriones unguiculatus* has proved to be highly susceptible to *B. divergens*: the parasite could be maintained by serial syringe passages (Lewis and Williams 1979). Several assays to maintain *B. divergens* in in vitro culture have been performed using the microaerophilous stationary-phase system (MASP) according to Levy and Ristic (1980); low parasitaemia levels (5%–10%) were obtained with bovine erythrocytes (Väyrynen and Tuomi 1982). Slightly higher parasitaemia levels were achieved by replacing culture medium every 8 or 12 h and, to reduce labour associated with maintenance, it was suggested that the culture be temporarily stored at 4° C (Konrad et al. 1985).

Morphological differences have been reported in *B. divergens*-infected erythrocytes from normal (ox), and unusual hosts such as hamsters and rats (Canning et al. 1976), gerbils (Entrican et al. 1979; Lewis et al. 1980; Liddell et al. 1980; Gray et al. 1985), chimpanzees (Garnham and Bray 1959) and humans (Gorenflot and Piette 1976).

Two additional cases of human babesiosis observed in France in 1987 and 1988 gave us the opportunity (a) to develop a method of long-term in vitro culture of *B. divergens* in human erythrocytes that resulted in high levels of parasitaemia that routinely reached 30%–40%, (b) to perform a comparative cytological study of *B. divergens* from the same isolate in man, gerbil and ox and (c) to determine for the first time the specificity and the chronology of the response of human-specific and bovine-specific antibodies induced by the same isolate of *B. divergens*.

Materials and methods

In vitro culture of human and bovine Babesia divergens *isolates*

Two splenectomized patients who developed severe babesiosis in France were successfully treated by blood exchange and subsequent chemotherapy (Gorenflot et al. 1990). At the beginning of the blood exchange, parasitized human erythrocytes were collected and injected into a splenectomized and immunosuppressed Friesan cow. Another aliquot of parasitized human red blood cells was mixed

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Abbreviations: EDTA, ethylenediaminetetraacetate; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

with healthy human erythrocytes (blood group 0) for an in vitro culture according to the method of Trager and Jensen (1976), with some modifications, and/or using a semi-defined medium without serum (French patent CNRS/ANVAR 89-16890). For subcultures, parasitized human erythrocytes were diluted with human red blood cells to obtain parasitaemia levels ranging from 0.1% to 1%. Aliquots were dispatched either in culture flasks or in 96- or 24-well plates. The flasks were flushed with a gas mixture of 6% O_2, 3% CO_2 and 91% N_2 or were maintained in an atmosphere containing 5% CO_2 at 37° C. The two isolates were designated as *B. divergens* Rouen 1987 and *B. divergens* Le Mans 1988.

Bovine *B. divergens* isolates from different geographic areas in France [7107B (Bourgogne) and 3503A (Bretagne) isolates, Ecole Nationale Vétérinaire de Nantes, France] and Europe (Weybridge, Y5 and Munich isolates; kindly donated by Rhône-Mérieux S.A., Lyon, France) were also used. The parasitized bovine or gerbil erythrocytes were used to initiate in vitro culture in human red blood cells.

Electron microscopy

Erythrocytes from man, ox and gerbil infected with the same *B. divergens* isolate were fixed in 1% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.3) for 3 h, at 4° C. After centrifugation (1,200 *g* for 10 min at 4° C), the pellets were washed twice in the same phosphate buffer, post-fixed in 1% osmium tetroxide for 1 h at 4° C and dehydrated in ethanol. For transmission electron microscopy (TEM), erythrocytes were embedded in Epon 812. Ultrathin sections stained with uranyl acetate and lead citrate were examined in a JEOL 100S microscope (JEOL Ltd., Japan) at 80 kV. For scanning electron microscopy (SEM), fixed and dehydrated erythrocytes were resuspended in graded amylacetate, critical-point-dried (from CO_2), gold-coated in a JEOL JFC 1100 sputter coater and observed in a JEOL JSM 35 CF microscope at 18 kV.

[^{35}S]-methionine radiolabelling of Babesia divergens

Metabolic radiolabelling of the *B. divergens* Rouen 1987 isolate was performed with [^{35}S]-methionine (>1,000 Ci mmol^{-1}; Amersham International plc, Amersham, UK) at 15 µCi ml^{-1} in a methionine-free medium (MEM; Gibco Ltd., Scotland) adapted to *B. divergens* growth: 2 m*M* glutamine and 25 m*M* HEPES (pH 7.35) supplemented with 10% human serum. In order to cover a complete growing cycle, the cells were incubated for 10–12 h at 37° C. After incubation, cells were collected by centrifugation at 1,000 *g* for 5 min at 4° C, extensively washed in serum-free culture medium and solubilized in a Triton lysis buffer comprising 2% Triton X-100, 0.6 *M* KCl, 5 m*M* ethylenediaminetetraacetate (EDTA), 3 m*M* phenylmethylsulfonyl fluoride (PMSF), 1% aprotinin and 2.5% iodoacetamide in TRIS-bufferd saline [TBS: 0.14 *M* NaCl, 10 m*M* TRIS-HCl (pH 7.8)]. The extract was maintained on ice for 1 h and then centrifuged at 50,000 *g* for 20 min at 4° C. The Triton X-100 insoluble pellet was discarded and the supernatant containing the radiolabelled parasite extract was used immediately or kept at −80° C.

Sera

Human sera were collected from the two French patients during both the acute phase of babesiosis and subsequent months: 11 months for the first patient (*B. divergens* Rouen 1987) and 1 month for the second (*B. divergens* Le Mans 1988). Control sera were obtained from healthy donors with a negative serology to *B. divergens*. Bovine antisera were obtained from a 2-year-old ox that had been intravenously infected with 10^{11} human erythrocytes parasitized with *B. divergens* Le Mans 1988. The preimmune serum of the ox was used as a control.

Immunoprecipitation assays

Aliquots of radiolabelled antigens (10^6 cpm) were mixed with 7 µl of each serum and then incubated overnight at 4° C with constant stirring. The antigen-antibody complexes were precipitated by the addition of 70 µl protein A-Sepharose CL4B beads (Pharmacia; Uppsala, Sweden) diluted 1:1 (v/v) in TBS (pH 7.4). The complexes were washed four times with the washing buffer [2% Triton X-100, 0.6 *M* KCl, 5 m*M* EDTA in TBS (pH 7.8)] and then twice with TBS by centrifugation at 3,000 *g* for 5 min. After the removal of excess buffer, the immunoprecipitates were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) and were then run on 12.5% acrylamide gels, which were autoradiographed using standard procedures. The molecular-weight standards (14–200 kDa) were supplied by Amersham and the Kodak X-OMAT XAR-5 film was obtained from Eastman Kodak Company (Rochester, USA).

Results

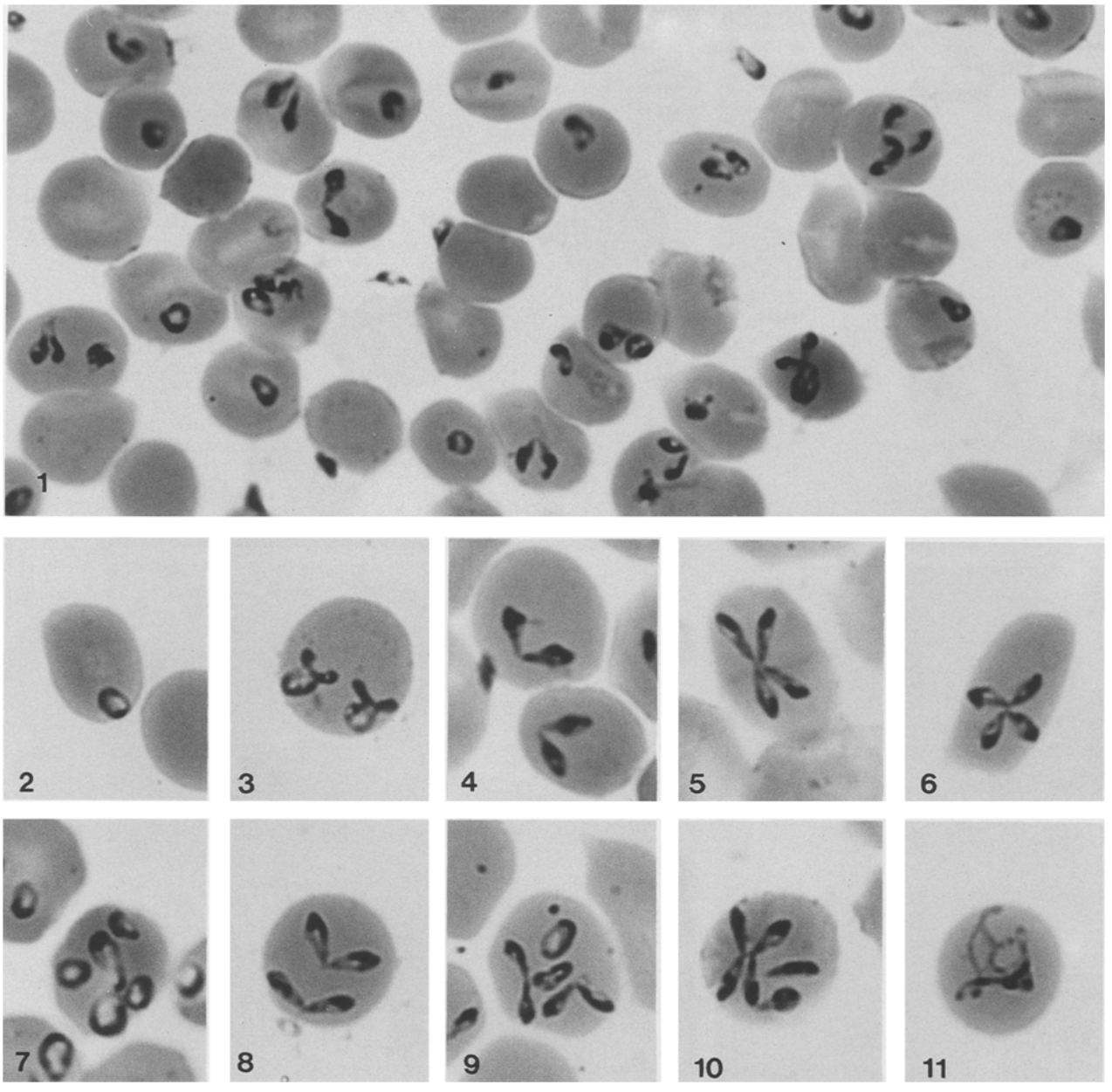
Long-term in vitro culture of Babesia divergens *in human erythrocytes*

The *B. divergens* Rouen 1987 isolate has been maintained in long-term in vitro culture since 1987; by December 1989, 305 subcultures had been completed. Pooled human sera were decomplemented for 30 min at 56° C. As serum concentrations of between 5% and 20% (v/v) enabled parasite growth, a concentration of 10% (v/v) was chosen. The influence of haematocrit was also tested by using 25-cm^2 culture flasks containing 5 ml culture medium; 2.5%–7.5% (v/v) haematocrits were usable, and a 5% haematocrit was selected for usual cultures. The increase in parasitaemia level was correlated with a progressive darkening of the erythrocytes in the culture flask. Few differences were observed between growth rates of the two human isolates; a minimal growth rate (× 3 per 24 h) was always observed at parasitaemia levels of <30%. When parasitaemia reached 25%–40%, a subculture was performed by dilution to 0.1%–1% for maintenance. The overlying medium was removed daily as soon as parasitaemia had reached 5%; by this method a parasitaemia level of ≥60% could be obtained (Fig. 1). Under these conditions, free extracellular merozoites were rarely observed during the exponential phase of growth.

In vitro subcultures of the *B. divergens* Le Mans 1988 isolate showed similar values. In vitro cultures of two *B. divergens* French bovine isolates (7107B and 3503A) and of three isolates (Weybridge, Y5 and Munich) from different European geographic areas were successfully initiated by mixing normal human red blood cells with bovine or gerbil erythrocytes. After a few subpassages, the animal erythrocytes disappeared and the isolate was stabilized.

Cytology of the Babesia divergens *Rouen 1987 isolate in its different host erythrocytes*

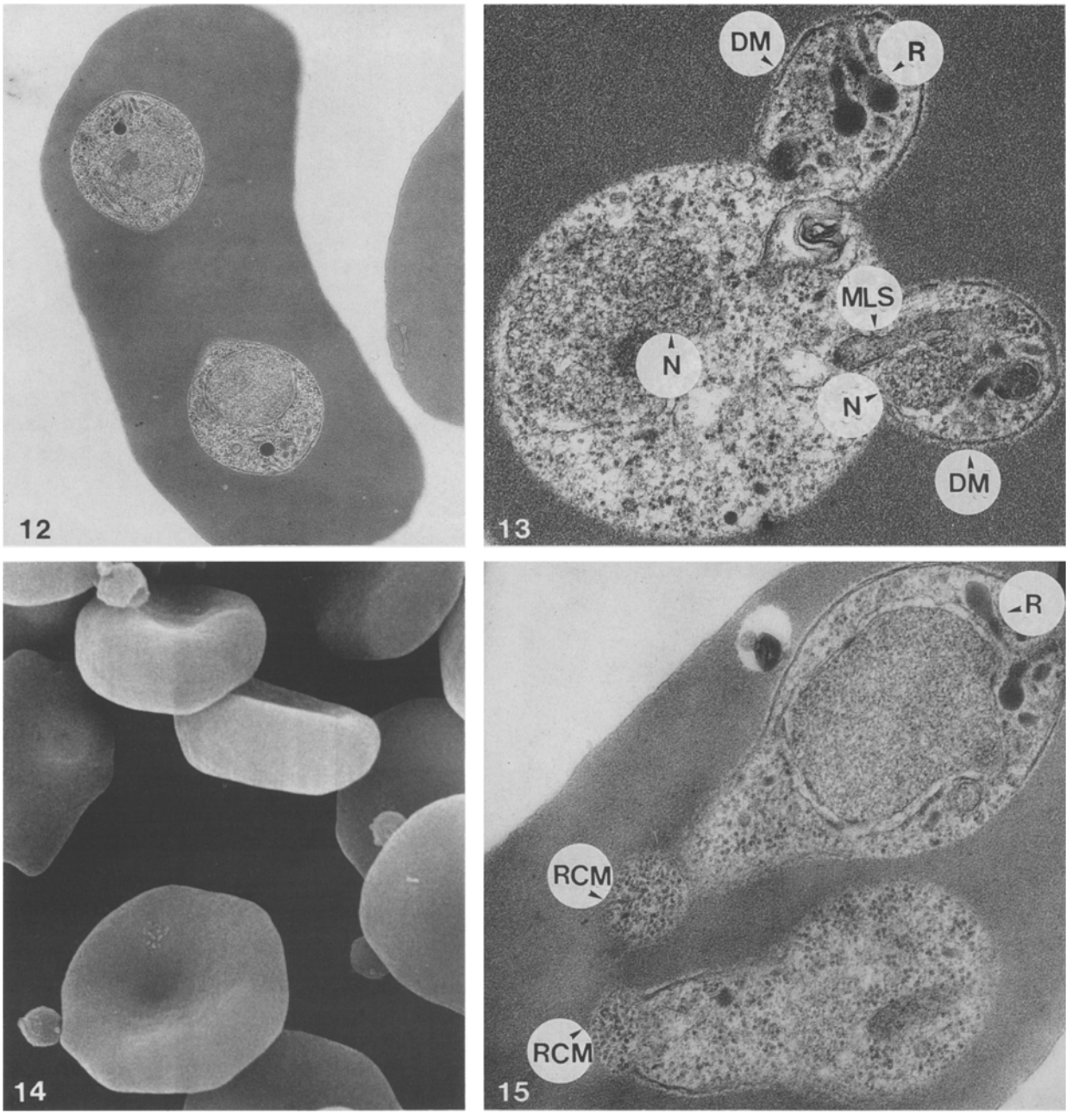
Human erythrocytes. In human red blood cells, *B. divergens* was usually located in a (sub)central position. Using light microscopy, different stages were observed, includ-



Figs. 1–11. May Grünwald-Giemsa-stained smears of the *B. divergens* culture in human erythrocytes (Fig. 1, ×2,050; Figs. 2–11, ×2,500): **Fig. 1** high parasitaemia after 3 days of in vitro culture; **Fig. 2** ring; **Fig. 3** budding stages; **Fig. 4** paired uniform parasites; **Figs. 5, 6** tetrad forms; **Figs. 7–10** different aspects of polyparasitism; **Fig. 11** filamentous parasites (crisis forms)

ing rings (Fig. 2); dividing parasites with two budding merozoites (Fig. 3); piriform, divergently paired parasites assembled by their fine extremity (Figs. 4, 8); tetrad forms, with four piriform *Babesia* assembled by their fine extremity (Figs. 5, 6), representing $< 5\%$ of the parasitic forms; and filamentous forms occurring at very high levels of parasitaemia (Fig. 11) and probably corresponding to crisis forms, since their number increased with exhaustion of the culture media. Most of the parasitized erythrocytes contained up to five parasites (ring and/or piriform) and a few contained more (Figs. 7, 9, 10). Polyparasitism could occur more frequently especially at the beginning of mixed cultures (gerbil/man or ox/man).

As revealed by TEM, *B. divergens* did not protrude at the cell surface of human erythrocytes (Fig. 12). After invasion, merozoites were transiently enclosed in a parasitophorous vacuole, but trophozoites and later stages were limited by one single plasma membrane that was directly in contact with the erythrocyte cytoplasm (Figs. 13 and 15). In dividing trophozoites, the two cytoplasmic buds were limited by a plasma membrane that was underlaid by two cytomembranes, which showed vesicular organization. These buds corresponded to future merozoites. In their apex, rhoptries, microsomes and part of a mitochondria-like structure could be observed (Fig. 13). As the differentiating merozoites enlarged, the trophozoite decreased in size until only a

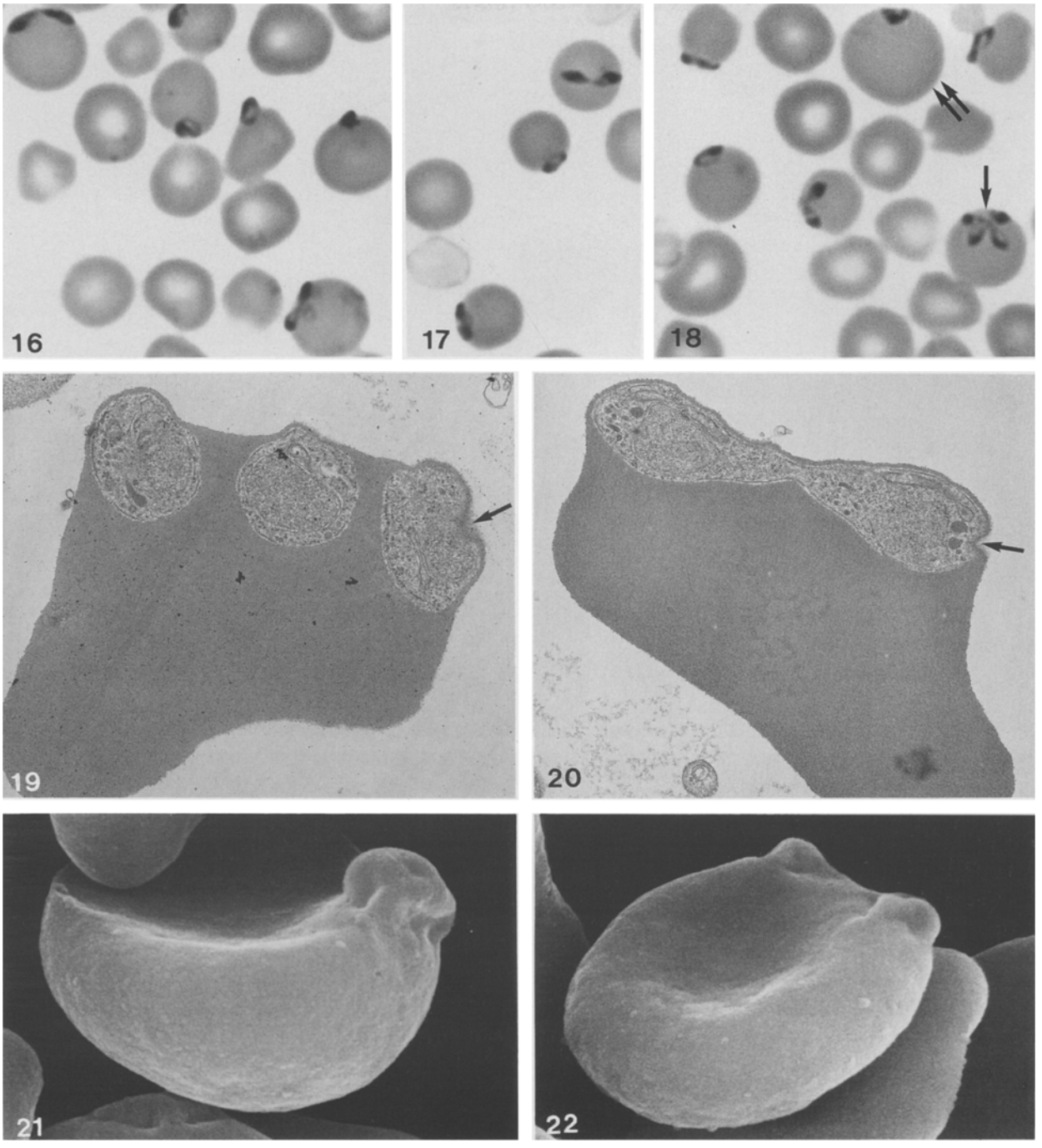


Figs 12–15. Electron microscopy of *B. divergens* Rouen 1987 from in vitro culture. TEM: **Fig. 12** two intraerythrocytic merozoites in a (sub)central position (×16,000); **Fig. 13** trophozoite with two budding merozoites (×36,200); **Fig. 15** piriform parasites – note the residual cytoplasmic mass at the posterior end of the merozoites (×37,700). SEM: **Fig. 14** merozoites attached to human erythrocytes (×6,000). *DM*, double membrane; *MLS*, mitochondria-like structure; *N*, nucleus; *R*, rhoptries; *RCM*, residual cytoplasmic mass

small, residual cytoplasmic mass remained bound to their posterior extremities (Fig. 15). Sometimes small vesicles were observed in the erythrocyte cytoplasm. Merozoites were seen to invade erythrocytes by internalization of erythrocyte membrane via a moving junction (for review see Igarashi et al. 1988).

As shown by SEM, only a few infected erythrocytes showed an irregular form. Sometimes one or more merozoites were attached to one erythrocyte (Fig. 14). Similar cytological features were found in in vitro cultures of human erythrocytes with *B. divergens* Le Mans 1988 and bovine isolates.

Bovine erythrocytes. When the level of parasitaemia in the ox infected with patient erythrocytes reached about 17%, cytological studies were performed. After its penetration into the bovine erythrocytes, *B. divergens* appeared in a peripheral location, as usually observed in natural bovine infections. Ring or paired piriform parasites formed protrusions at the periphery of the red blood cell (Fig. 16), only occasionally occupying a central position (Fig. 17). Polyparasitism (>2 parasites in 1 erythrocyte) was infrequent, and tetrad forms were extremely rare (<1 in 5,000 parasitized erythrocytes) (Fig. 18).

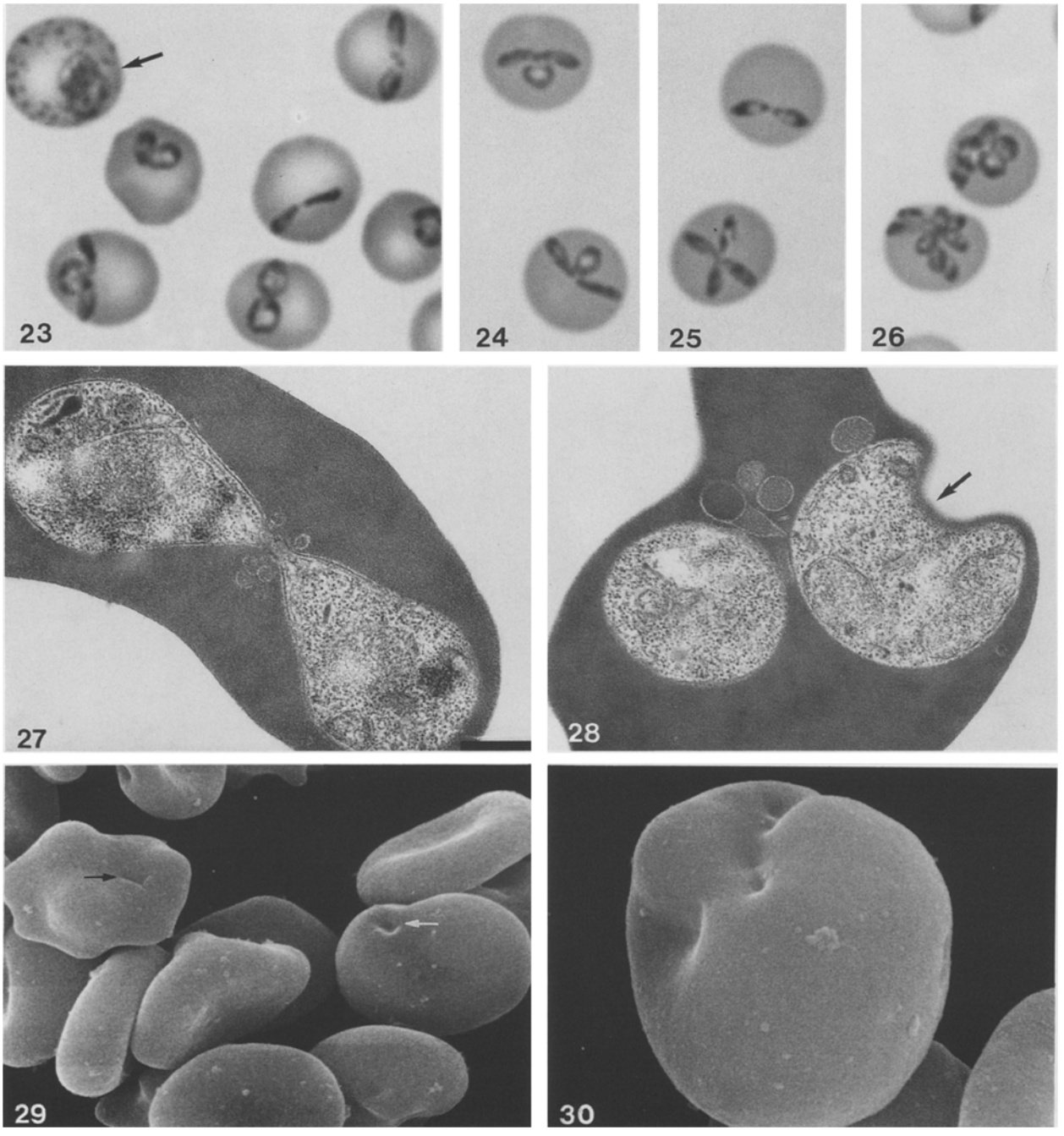


Figs. 16–22. Morphological aspects of *B. divergens* Rouen 1987 in bovine erythrocytes. Light microsopy: **Figs. 16–18.** May Grünwald-Giemsa-stained smears (×2,500). Single or paired parasites are usually in a peripheral position. Note the tetrad form in the periphery of an erythrocyte (*arrow*) and the peripheral position of the parasite even in a large bovine erythrocyte (*double arrow*). TEM: **Fig. 19** parasites in peripheral position (×18,800); **Fig. 20** geminated parasites close to the erythrocyte membrane (×14,500). Note the small invagination of the red blood cell in front of the apex of the merozoite (*arrow*). SEM: **Fig. 21** *B. divergens* protruding at the host-cell surface (×16,000); **Fig. 22** geminated parasites (×14,500)

TEM confirmed the location of the parasite just beneath the erythrocyte plasma membrane, and no parasitophorous vacuole was identified (Figs. 19, 20). Small vesicles were encountered in the vicinity of some parasites, and small invaginations of the erythrocytic membrane could be observed at the apex of merozoites (Fig. 20).

By SEM, well-limited areas (diameter, 1 μm) with a "brain-like" surface were observed on the erythrocyte membrane; they indicated the position of the underlying parasite (Figs. 21, 22).

Gerbil erythrocytes. In gerbil erythrocytes, rings, paired piriform parasites and tetrad forms of *B. divergens*



Figs. 23–30. Morphological aspects of *B. divergens* Rouen 1987 in gerbil erythrocytes. Light microscopy: **Figs. 23–26.** May Grünwald-Giemsa-stained smears (×2,500). Cytological features are similar to those seen in in vitro culture. Some erythrocytes exhibit polyparasitism higher than that observed in man. Reticulocytes could also be infected by *B. divergens* (*arrow*). TEM: **Figs. 27, 28** (×23,000) (sub)central position of *B. divergens* – note the presence of numerous vesicles in the host-cell cytoplasm and the local invagination of the erythrocyte membrane (*arrow*). SEM: **Figs. 29, 30** *B. divergens*-induced scratches (*arrows*) on gerbil erythrocytes. (Fig. 29, ×6,000; Fig. 30, ×14,500)

Rouen 1987 were (sub)centrally located (Figs. 23–26) and dividing forms were frequently seen (Fig. 24). Parasites displayed no preference for reticulocytes (stippling or polychromatophile erythrocytes) (Fig. 23). Polyparasitism was common and, with red blood cells often containig up to ten parasites and sometimes more (Fig. 26); such multiply infected erythrocytes were spheroid.

By TEM, numerous vesicles were found in the host-cell cytosol close to the link between the paired piriform parasites (Fig. 27). Structures similar to Maurer's clefts of *Plasmodium* could sometimes be identified (Fig. 28). The (sub)central position of the parasites did not preclude contact between the parasite and the red cell membrane with local invagination of the erythrocyte membrane (Fig. 28). As seen by SEM, such invaginations looked like scratches (Figs. 29, 30).

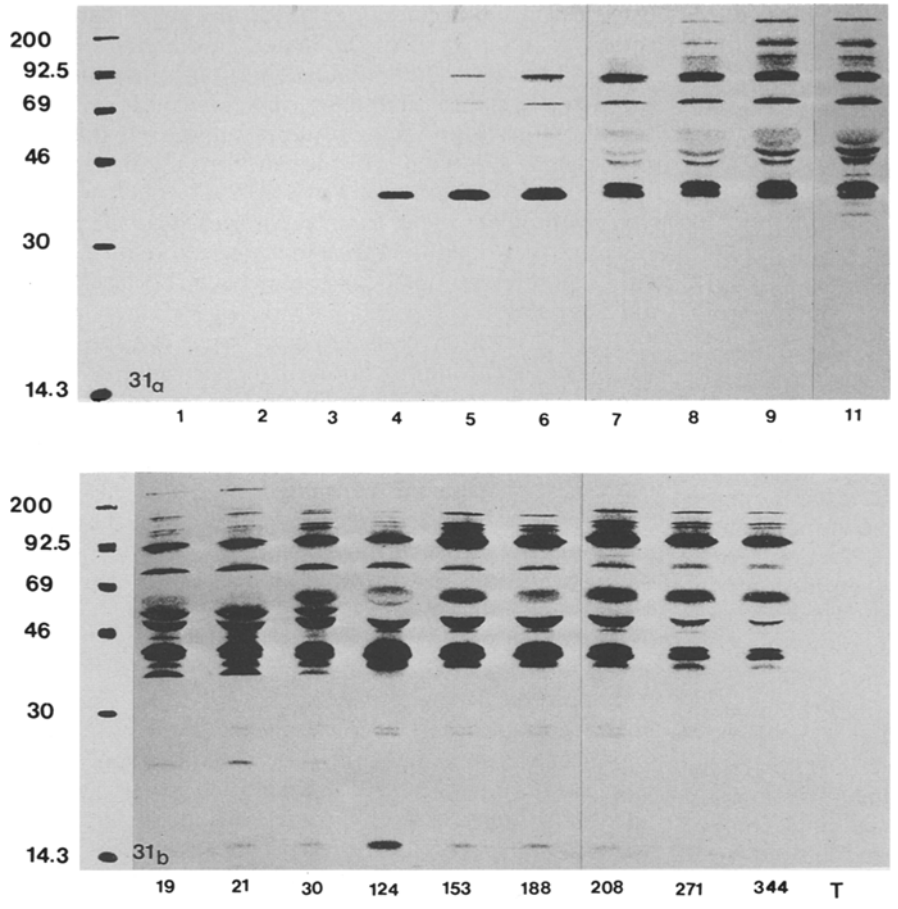
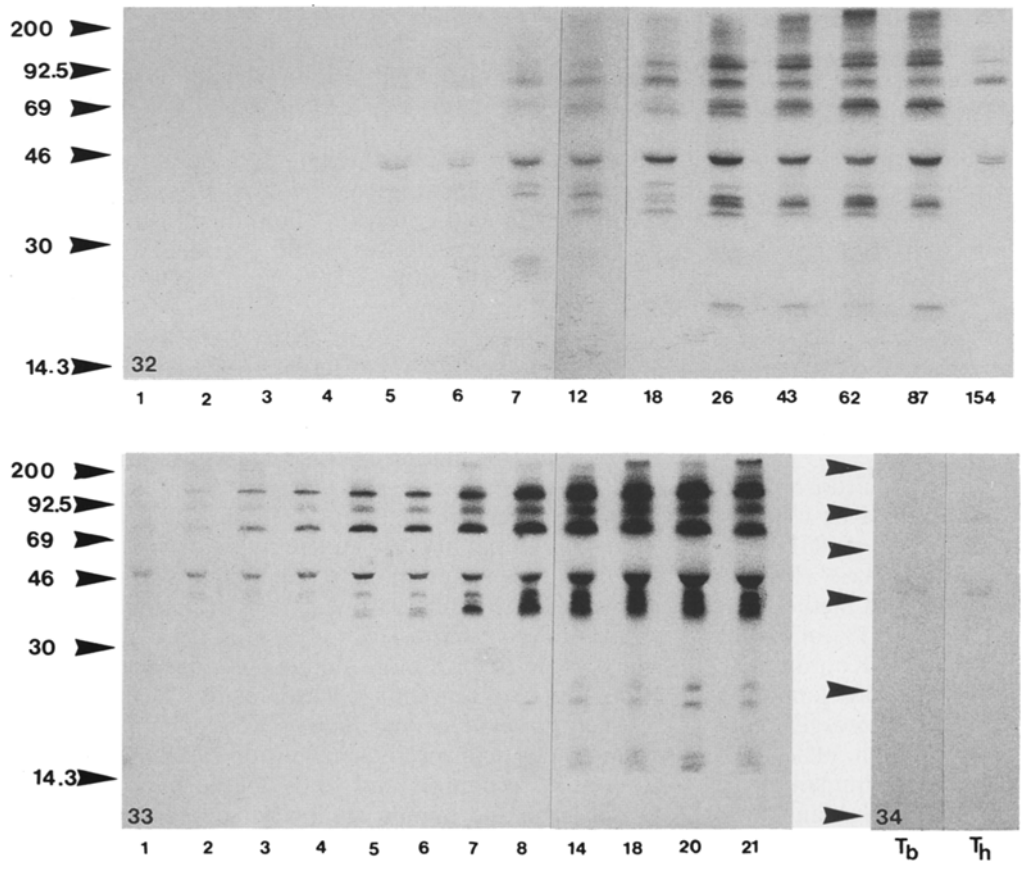


Fig. 31 a, b. Human chronological antibody response against *B. divergens* Rouen 1987: **a** antibody response during the 11 days following blood exchange; **b** antibody response during the subsequent 11 months (days 19–344). *T*, control immunoprecipitation



Figs. 32–34. Comparative immunoprecipitation with sera from an ox and a man infected with *B. divergens* Le Mans 1988. **Fig. 32.** Bovine chronological humoral response (days 1–154 post-infection). **Fig. 33.** Human chronological humoral response (days 1–21 post-hospitalisation). **Fig. 34.** Control immunoprecipitations (T_b, bovine control; T_h, human control)

Chronological antibody responses against Babesia divergens *in man and cow*

The chronology of antibody-mediated immune responses to *B. divergens* in the two French patients (Rouen 1987 and Le Mans 1988) and in the ox (Le Mans 1988) was established in immunoprecipitation experiments using [^{35}S]-methionine-labelled *B. divergens* Rouen 1987. In the man infected with *B. divergens* Rouen 1987, the chronological post-infection humoral response (Fig. 31) displayed aspects: (1) a constant response against a 36-kDa polypeptide (from day 4 post-hospitalization onwards), 74- and 89-kDa polypeptides (from day 5 onwards), a 46-kDa polypeptide (day 7 and thereafter), a group of high-molecular-weight polypeptides (153–186 kDa, from day 8 onwards) and a 37-kDa polypeptide (day 11 and thereafter) and (2) a temporary response against a 16-kDa polypeptide (maximum, from day 21 to 8 months post-infection), a 20-kDa polypeptide (maximum, from day 19 to 1 month post-infection), a 26- to 27-kDa doublet (maximum, from 1 month to 7 months post-infection) and a 235-kDa polypeptide (maximum, from day 8 to 1 month post-infection).

The antibody kinetics was quite similar in the patient and ox infected with the *B. divergens* Le Mans 1988 isolate (Figs. 32, 33); no response was seen in the controls (Fig. 34). In the bovine and human response against *B. divergens* Le Mans 1988, the antibodies against the 46-kDa protein appeared first, followed by the simultaneous emergence of antibodies against both the 72- and 90-kDa polypeptides and a group of proteins with molecular weights of 112–120 kDa. Finally, antibodies against the group of 35- to 38-kDa polypeptides appeared. In both the patient and the ox, it is noteworthy that antibodies against low-molecular-weight proteins appeared later and only transiently (from 1 month to 5 months post-infection).

Discussion

The present work demonstrated that human red blood cells infected with *Babesia divergens* Rouen 1987 and Le Mans 1988 isolates could initiate an efficient, continuous in vitro culture in human erythrocytes; it was also possible to generalize this in vitro culture system to any bovine isolate.

Since *Plasmodium falciparum* in vitro culture according to Trager and Jensen (1976) could not be used for *B. bovis* (Erp et al. 1978), Levy and Ristic (1980) developed the microaerophilous stationary-phase (MASP) method. When applied to *B. divergens*, this method was reported to give parasitaemia levels of $<15\%$ in bovine erythrocytes (Väyrynen and Tuomi 1982; Konrad et al. 1985). In contrast with previous results, our long-term in vitro culture (>305 subpassages) of *B. divergens* routinely exhibited parasitaemia levels of about 30%–40%. This high parasitaemia was not due to the human origin of the *B. divergens* Rouen 1987 and Le Mans 1988 isolates, since every European bovine isolate thus far tested could infect human erythrocytes, with high parasitaemia and only slight differences in the growth rates (data not shown). The optimal conditions of our culture included a haematocrit and a serum concentration lower than those used in previous studies; moreover, the high parasitaemia observed with our in vitro culture system transgressed the host specificity of *B. divergens* for bovine erythrocytes. This transgression of host specificity was previously identified with in vitro culture of *B. bovis* with sheep, goat, horse or rabbit erythrocytes (Ristic and Levy 1981).

After 2 years of continuous in vitro culture, there was no apparent modification of the virulence of *B. divergens* Rouen 1987 as demonstrated by the injection of 10^4 parasitized erythrocytes in gerbils (data not shown). The persistence of virulence in this isolate might have resulted from the homologous erythrocyte-serum conditions used in our system, in contrast with the attenuation of virulence in *B. bovis* obtained under the heterologous conditions previously described by Yunker et al. (1987).

The cytological characteristics of the human or bovine *B. divergens* isolates maintained in in vitro culture in human erythrocytes were similar to those observed during the acute phase of *B. divergens* babesiosis in man: a (sub)central position of the different stages, polyparasitism and the occurrence of tetrads. When parasitized human erythrocytes were injected into an ox, *B. divergens* recovered its typical peripheral location and, in contrast, the five bovine isolates passaged in culture became cytologically undistinguishable from the human isolates. This clearly demonstrated the influence of the host erythrocyte on the cytological aspects of enclosed parasites. Parasite-host-cell relationships appeared to vary between hosts. However, despite the high polymorphism of *B. divergens* in different hosts (man, ox, gerbil), no major ultrastructural changes in the parasites were observed. In contrast, erythrocyte membrane alterations were clearly observed, especially in *B. divergens*-infected bovine erythrocytes, whose "brain-like" surfaces appeared to be quite similar to the "metabolic window" of *P. berghei* (Bodammer and Bahr 1973; Gorenflot et al. 1981).

The process of penetration of *Babesia* merozoites into red blood cells from different hosts involved a moving junction similar to that previously described in *P. knowlesi* (Aikawa et al. 1978). However, in contrast to *Plasmodium*, the lifetime of the parasitophorous vacuole of *B. divergens* is short and the parasite rapidly reaches the erythrocyte cytosol, as has been shown for *B. microti* (Rudzinska et al. 1976; Rudzinska and Trager 1977). The resulting situation in *B. divergens* is the absence of the compartments with different pH previously observed in *P. falciparum* (for review see Cabantchik 1989), which could involve more simple exchange mechanisms in the supply of nutrients to the parasite. The differences between *Plasmodium* and *Babesia* were enhanced by the evidence of a less specific recognition mechanism in the latter that recognizes and leads to the internalization of ox, gerbil and human erythrocytes, whereas *P. falciparum* exhibits a very marked host-cell specificity, as

recently described (Mitchell et al. 1986; Perkins and Holtz 1988). The present, efficient in vitro culture could open new possibilities for research in *B. divergens* on the physiological and biochemical levels, i.e. interactions between piroplasms and the erythrocyte cytoskeleton could be investigated to obtain a better understanding of the varying locations of this parasite in red blood cells.

Clinical and antibody responses to *B. divergens* in vaccinated calves or in acute bovine babesiosis are well documented. (Christensson 1987; Christensson and Moren 1987). In contrast, little is known about the babesial antigens involved in the protective immune response. The development of this efficient continuous-culture system and of the use serial sera from patients and oxen enabled us to characterize for the first time the major antigens of the *B. divergens* antibody-mediated immune response. Several proteins seemed to be involved in the humoral response to this parasite. The major proteins that induced antibodies in the three host sera tested included a group of polypeptides (35–38 kDa), a 46-kDa protein, a polypeptide with a molecular weight of around 72 kDa and higher-molecular-weight proteins.

Using an indirect immunofluorescence assay (IFA), kinetic studies of the humoral response to *B. divergens* (unpublished results) showed that in the cow infected with *B. divergens* Le Mans 1988, antibodies appeared on day 6 following infection, reached and persisted at the maximal level from day 15 to day 21 and decreased thereafter. Immunoprecipitation studies revealed that these antibodies were more easily detected when the IFA titers had declined to low levels. Similar antibody responses were observed with serum from the patient with *B. divergens* Rouen 1987 babesiosis. Titers peaked by day 10 and reached a plateau, where they persisted for 8 weeks. As in the bovine host, no correlation was seen between immunoprecipitated parasite antigens and the IFA titers. At 1 year post-infection, the IFA titers had returned to near baseline, whereas the immunoprecipitated *B. divergens* antigens had remained at a high level. These results suggest that the high degree of immunity to *B. divergens* following an acute infection probably depend more on the high specificity of only some *Babesia* antibodies than on a high total level of anti-*B. divergens* immunoglobulins.

The present in vitro culture system provides a better tool for cytological, biochemical and immunological investigations of *B. divergens* and can facilitate research on the chemotherapeutic efficacy of drugs. The most practical application of this culture system will involve the production of immunogens, and it is reasonable to anticipate that the high parasitaemia levels obtained in in vitro culture will provide large amounts of parasitic exoantigen candidates for vaccines.

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