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Studies on the role of complement in the *in vitro* invasion of bovine erythrocytes by *Babesia bovis*

Etude sur le rôle du complément sérique dans l'infection *in vitro* des érythrocytes par *Babesia bovis* – L'inactivation par la chaleur de sérum bovin utilisé dans les cultures en phase stationnaire micro-aérophiles (PSMA) n'influence pas de manière significative la multiplication de *B. bovis*. Le traitement du sérum par le zymosan n'altère ni le degré ni la cinétique de croissance de *B. bovis*. Par contre, les agents chélateurs, qui déprime les ions Ca^{++} et Mg^{++} de façon significative, inhibent la multiplication du parasite dans le système PSMA. L'élution des produits de conversion du C3 à partir d'érythrocytes sensibles n'affecte pas le degré de la parasitémie. On a également montré que les exo-antigènes de *B. bovis* étaient hautement anti-complémentaires, et que cette activité était liée à la dose utilisée. En conclusion, la dégradation du complément par *B. bovis* au cours de sa croissance en culture ne favorise pas l'invasion par le parasite. Celui-ci requiert des ions Ca^{++} et Mg^{++} pour se trouver dans les conditions optimales d'une invasion complément-indépendante des érythrocytes sensibles par les mérozoites. Ces ions peuvent jouer un rôle essentiel dans l'intégrité de la membrane du mérozoite et/ou de l'érythrocyte hôte. La relation générale entre le complément sérique et l'aptitude au développement de l'infection *in vivo* ou *in vitro* des différents protozoaires fait l'objet d'une brève discussion. *Mots clés : Babesia bovis* – Infection expérimentale – Erythrocytes – Complément sérique.

INTRODUCTION

Infections with a number of intraerythrocytic parasites, including *Babesia* species, are often associated with a variety of immunopathological manifestations including consumption of complement (1, 3, 6, 8, 9, 12). In addition to the direct interaction between parasites and/or their products with complement in the induction of lesions, *e.g.*, glomerulo-nephritis associated with *B. rodhaini* infection in the rat, drastic reduction in serum complement has been observed (3), and may contribute to increased susceptibility to other disease agents. Investigation of erythrocyte invasion of *B. rodhaini* has resulted in the putative conclusion that C3 and C3b are required in the invasion process (3). Independent observations on *B. rodhaini*, however, have recently concluded that complement does not play a role in infection of BALB/c mice by this parasite (10).

Although infection of cattle with *B. bovis* induces hypocomplementemia (7), metabolic, physiologic and immunologic events confound the *in vivo* studies. Utilizing the microaerophilous stationary phase (MASP) culture system for the continuous cultivation of *B. bovis* (5), we sought to determine whether complement was involved in erythrocyte invasion, and wether parasite products released in culture would directly or indirectly interact with complement *in vitro*, via the classical or alternate pathway.

MATERIALS AND METHODS

Parasite and *in vitro* culture

A Mexican isolate of *B. bovis* was inoculated into a splenectomized calf and the parasites introduced into the MASP culture system of LEVY and RISTIC (5). Briefly, the complete media (BTCM4) consisted of 40 p.100 adult bovine serum and 60 P. 100 M199 supplemented with Hepes and antibiotics. Bovine erythrocytes from defibrinated blood were added to a final concentration of 8-10 p. 100 and the pH adjusted to 7.0. Media was replaced daily and subcultures were effected by diluting infected cultures with normal culture containing only uninfected erythrocytes every 2 to 4 days. At the time of subculture, the percent parasitized erythrocytes (PPE) was reduced 10 to 20-fold. Serum was obtained by rapid defibrination of bovine blood by shaking with glass beads followed by centrifugation at 1 500 g for 15 min at 4 °C. The collected serum was used immediately or quickly frozen at

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−70 °C in order to preserve labile elements. Media was prepared daily immediatly prior to use, and erythrocytes were collected on the day used. Erythrocyte-free merozoites were prepared from CO_2 deprived MASP cultures as previously described (5).

Treatment of non-infected erythrocytes

Freshly collected bovine erythrocytes recovered from defibrinated blood were washed 3 x in 10-20 volumes of M199 pH 7.0 to reduce contamination by buffy-coat cells and serum. These washed cells were positive for bound complement as determined by a direct fluorescent antibody test utilizing goat anti-bovine C3 (MILES, ELKHART, Indiana). Substitution of M199 pH 6.0 for the second wash resulted in elution of bound complement from erythrocyte surface (low pH wash). This was confirmed by immunofluorescent tests using monospecific conjugated FITC-anti-bovine C3 (MILES, Indiana).

Serum treatments

Adult bovine serum (ABS) was decomplemented by heat inactivation (56 °C/30 min) or incubation at 37 °C with 0.5 p. 100 zymosan (Sigma) followed by centrifugation and filtration (0.45 μ). The early complement components were stabilized under predetermined conditions using a solution of 8.3 g potassium iodide and 0.25 g iodine in 10 ml of 0.1 MPBS pH 6. Ten microliters of the iodine solution was added to an equal volume of serum, and the mixture incubated for 5 min at 4 °C followed by addition of 1 mg glucose/ml of serum to effect neutralization of the iodine. The final pH of these and all cultures was adjusted to 7.0.

Titration of residual total hemolytic complement

A modification of KABAT and MEYER 's procedure was used as previously described (4) using sensitized rabbit erythrocytes (EA_{rab}). Values were expressed as 50 p. 100 hemolytic units (CH_{50}).

Assay of C3 conversion products

The method of GRABAR and WILLIAMS (2) immunoelectrophoresis was used. Fresh bovine serum and zymosan-treated bovine serum were used as reference standards.

Assay for anti-complementary activity (AC)

A zymosan-treated duplicate was used in the assay. Anti-complementary activity in aliquots treated with MASP supernatants were expressed relative to zymosan-treated samples. The AC was thus recorded as a zymosan equivalent unit (*i.e.,* relative to the degree of conversion observed when a duplicate serum sample was treated with zymosan at a final concentration of 0.5 p. 100).

EXPERIMENTS

Eight wells of a 96 well flat-bottomed tissue culture tray (Bellco) were used for each treatment with a culture volume of 0.2 ml. Three of the cultures were randomly sampled at each sampling time. The percent parasitized erythrocytes (PPE) was determined by examining 500-1 000 erythrocytes on methanol fixed Giemsa-stained blood films at 1 000 ×. Supernatants used in the detection of residual complement were removed from several wells, pooled, centrifuged at 200 g for 10 min at 4 °C and quickly frozen at −70° C until assayed within one week of collection. Supernatants examined for anticomplementary activity were similarly treated, but were heat-inactivated prior to storage. Prior to inoculation into test cultures infected cells were washed to remove any residual serum components. Initially, erythrocytes from such cultures were resuspended in 10 to 20 volumes M199 pH 7.0 and sedimented at 400 g for 10 min at 4 °C. Following 2 such washes the erythrocytes were resuspended in the homologous test media and inoculated into uninfected cultures.

RESULTS

Cold centrifugation resulted in altered parasite-growth kinetics. Additionally damaged organisms exhibited altered Giemsa staining characteristics. Therefore, washing of infected erythrocytes prior to subculture was accomplished simply by removing the supernatant from active cultures and replacing it with fresh complete medium. The cells were allowed to settle while in the incubator and the process was repeated. The 3 changes

consisted of homologous test medium. Uninfected bovine erythrocytes were undamaged following extensive cold centrifugation and supported growth as well as unwashed erythrocytes.

Growth rates of B. bovis in vitro were similar when either fresh adult bovine serum (ABS), or heat inactivated ABS was incorporated into the complete medium (Table I). Parasitized erythrocytes subjected to centrifugation prior to introduction into test cultures showed an initial growth lag when either heat-treated or fresh sera was used and better growth between 2 and 4 days was observed in cultures containing fresh ABS (Table II). Stabilization of early complement components did not enhance the rate of infectivity by B. bovis (Table III). When cultures were prepared with erythrocytes devoid of membrane-bound C3, fresh ABS, or serum preincubated with zymosan (0.5 p. 100) to remove complement, no differences in growth rates were observed over a period of 48 h by which time the PPE increased an average of 48-fold in all culture treatments (Table IV). Additional adsorption of zymosan-treated serum with complement-eluted erythrocytes prior to their incorporation into complete media did not affect these results.

TABLE I Kinetics of growth of* B. bovis *in fresh and decomplemented serum (56 °C for 30').

	PPE (± SD)	
	Fresh	D°
To	0.4 p. 100	0.4 p. 100
Day 1	1.2 ± 0,2	1.2 ± 0.3
Day 2	7.3 ± 0,7	6.8 ± 1.4
Day 3	13.3 ± 1,9	12.8 ± 1.7

No significant differences between treated control cultures.

TABLE II Growth of* B. bovis *with fresh and heat-inactivated serum (+ centrifugation).

Day	Fresh serum (PPE) + SD	Inactivated serum (PPE) + and cold centrifugation
0	0.4 ± 0,1	0.4 ± 0.1
1	0.9 ± 0,4	0.9 ± 0.7
2	6.1 ± 1,2	1.9 ± 0.7
3	7.6 ± 1,1	2.0 ± 0.1
4	18.3 ± 5,6	2.5 ± 1.2

TABLE III The role of early complement components (1, 4, 2) on growth of* B. bovis *in vitro : relative percent parasitized erythrocytes (PPE)*.

Time	Control culture	Early complement stabilized
0 h	3.4	3.4
24 h	12.6 ± 2,3	9.1 ± 0.4

** Mean of triplicate cultures ± S.D.*
No significant difference was observed.

TABLE IV Complement requirement for* B. bovis *in vitro.

Treatment	C3 Conversion products	IP	FP (48 h) Percent
1) Fresh serum - 4 °C	+	0.4	16.0
2) Fresh serum - 37 °C	+ +	0.4	18.8
3) Serum + 0.5 p. 100 zimosan	+ + +	0.4	22.0
4) Serum + 0.5 p. 100 zimosan + absorption with washed erythrocytes	±	0.4	20.5

± = trace ; + = weak ; + + = intermediate ; + + + = high concentration.
NOTE : Erythrocytes were washed 3 times in M199 pH 6.0 to elute bound C3 complement conversion products as determined by IFA. In treatment n° 3, serum was depleted of C3 and C3 conversion products by treatment with zymosan and further absorption with normal bovine rbc followed by centrifugation, before the C3-depleted serum was used in culture. There was no significant difference between PPE in different treatment groups.

By initiating cultures with isolated erythrocyte-free merozoites, we examined the penetration efficiency of erythrocytes and subsequent short-term development of the parasites. Serum decomplementation by heat inactivation, chelation, or exposure to soluble parasite products did not affect erythrocyte penetration by merozoites or the subsequent intraerythrocyte growth.

Filtered supernatants from infected cultures were highly anticomplementary whereas only low baseline anticomplementary activity was detected in control supernatants. M199 and complete media did not show any AC activity, but low AC activity was also associated with lysate, stroma, and control supernatant (Fig. 1) ; whereas, supernatants from infected cultures were highly anticomplementary. The anticomplementary activity of the culture supernatant was dose dependent (Fig. 2) and progressively depleted complement from culture media (Fig. 3).

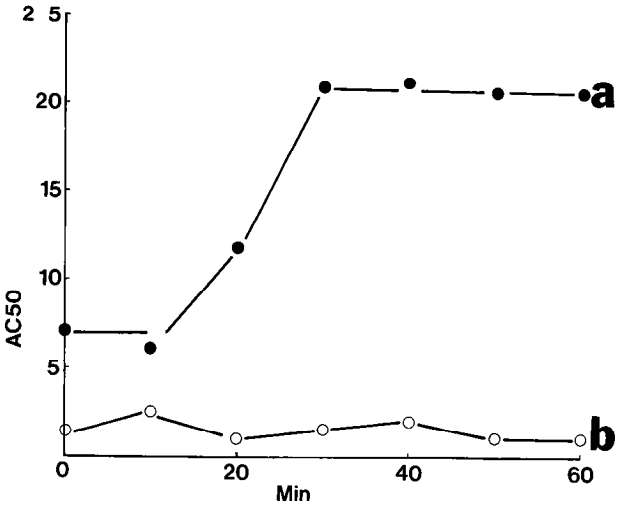

Fig. 1 : Kinetics of AC activity in B. bovis *infected (a) and non-infected cultures (b).*

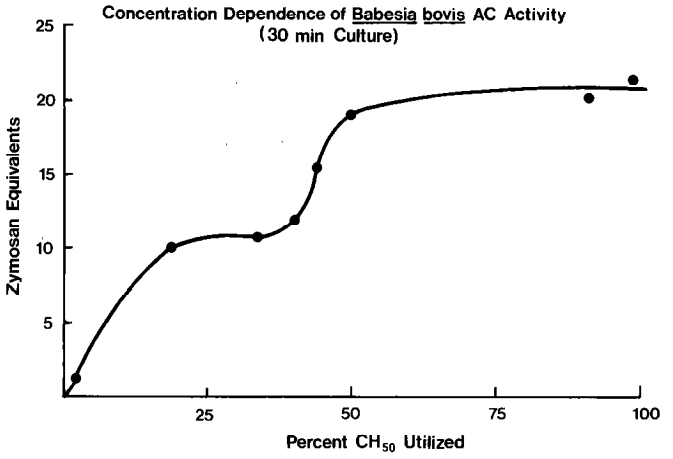

Fig. 2 : Dose-dependence of B. bovis *AC utilized in relation to* CH_{50} *in MASP cultures.*

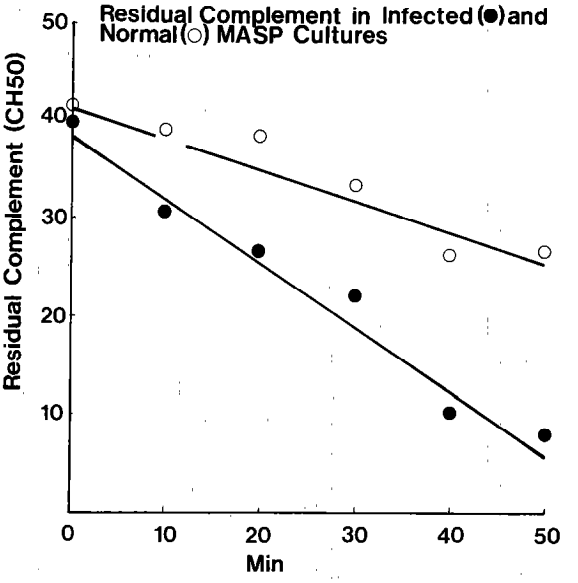

Fig. 3 : Residual hemolytic complement activity in MASP cultures.

DISCUSSION

The critical requirement for complement in the invasion of erythrocytes has been systematically studied in *B. bovis* MASP cultures. Our observations demonstrate that the classical and alternate pathways are activated by soluble babesia products *in vitro*, but that this phenomenon is neither a prerequisite, nor does it potentiate the invasiveness of the organism for susceptible target cells. Nevertheless, the transitory influence of chelating agents such as EGTA and EDTA which block classical and alternate pathway, superficially suggests that complement is essential for invasion of the erythrocyte (KAKOMA and LEVY, unpublished data). If that were so heat-inactivation and zymosan treatment, which directly affect complement would have significant influence on the infectivity of this organism. There is evidence to show that Ca^{++} supplemented, decomplemented serum is capable of supporting normal growth of *B. bovis* in MASP cultures. Our *in vitro* observations are consistent with a recent report (10) that complement does not play any significant role in promoting *B. rodhaini* infection contrary to previous reports (3). Our observations during separation of bovine serum on glass beads showed that the procedure leads to substantial binding of C3 conversion products onto homologous erythrocytes in an antibody independent fashion. We utilized this model to investigate whether erythrocytes carrying C3 conversion products were more susceptible to *in vitro* infection. There was no difference between cultures with C3-free erythrocytes and those maintained in zymosan activated serum demonstrably containing erythrocyte-bound and excess C3 conversion products in media.

Thus *B. bovis* is a potent C activator as judged by the dose-dependent AC activity, but the need for Mg^{++} and Ca^{++} may relate to maintaining the integrity

of the membrane of the merozoite and/or the susceptible erythrocytes. This observation does not contradict the observed hypocomplementaemia in *B. bovis* infected cattle (7) since the latter is a secondary rather than a primary manifestation following the establishment of high parasitemia. However, the AC activity appears to be a result of invasion activity rather than a primary potentiating event in infectivity of susceptible erythrocytes as reported for *B. rodhaini* (11). Nevertheless, the possibility that requirements for complement vary according to the particular *Babesia* species, cannot be precluded at this stage.

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LEVY (M. G.), KAKOMA (I.), CLABAUGH (G.), RISTIC (M.).
Studies on the role of complement in the *in vitro* invasion of bovine erythrocytes by *Babesia bovis*. *Rev. Elev. Méd. vét. Pays trop.*, 1986, **39** (3-4) : 317-322.
The growth rate of *Babesia bovis* is not significantly influenced by heat-inactivation of bovine serum used in microaerophilous stationary phase (MASP) cultures. Zymosan treatment of the serum did not alter the degree and kinetics of growth of *B. bovis*, but chelating agents which deplete Ca^{++} and Mg^{++} significantly inhibited the growth of the organism in the MASP system. Elution of C3 conversion products from susceptible erythrocytes did not affect the degree of parasitization. It was further demonstrated that exoantigens of *B. bovis* were highly anti-complementary (AC) and that the AC activity was dosedependent. It is concluded that the complement consumption by the growing *B. bovis* in culture does not facilitate the invasiveness of the parasite and that the organism requires Ca^{++} and Mg^{++} ions for optimal conditions for a complementindependent merozoite invasion of susceptible erythrocytes. These ions may be crucial in the membrane integrity of the merozoite and/or the host erythrocyte. The overall relevance of serum complement in facilitating the *in vitro* or *in vivo* infectivity of various protozoa is briefly discussed. *Key words : Babesia bovis* – *In vitro* invasion – Erythrocytes – Serum complement.

LEVY (M. G.), KAKOMA (I.), CLABAUGH (G.), RISTIC (M.). Estudio sobre el papel del complemento serico en la infestación *in vitro* de los eritrocitos por *Babesia bovis*. *Rev. Elev. Méd. vét. Pays trop.* 1986, **39** (3-4) : 317-322.
La inactivación por el calor de suero bovino utilizado en los cultivos micro-aerofilos en fase estacionaria (CMAFE) no influencia de modo significativo la multiplicación de *B. bovis*. El tratamiento por el zymosan no modifica el grado o la cinética de crecimiento de *B. bovis*. En cambio, los agentes quelatores que deprimen los iones Ca^{++} y Mg^{++} de modo significativo inhiben la multiplicación del parásito en el sistema CMAFE. La elución de los productos de conversión del C3 a partir de eritrocitos sensibles no cambia el grado de la parasitemia. Se mostró tambien que los exo-antigenos de *B. bovis* eran muy anti-complementarios y que esta actividad dependía de la dosis utilizada. En conclusión, la degradación del complemento por *B. bovis* durante su crecimiento en cultivo no favorece la invasión por el parásito ; el cual necesita iones Ca^{++} y Mg^{++} para estar en las condiciones óptimas de una invasión complemento-independiente de los eritrocitos sensibles por los merozoitos. Dichos iones pueden desempeñar un papel esencial en la integridad de la membrana del merozoito y/o del eritrocito huesped. Se discute de la relación general entre el complemento serico y la aptitud para el desarrollo de la infección *in vivo* o *in vitro* de los diferentes protozoarios. *Palabras claves : Babesia bovis* – Infestación experimental – Eritrocitos – Complemento serico.

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