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PHAGOCYTOSIS OF *Anaplasma marginale* INFECTED AND UNINFECTED ERYTHROCYTES BY BOVINE PERIPHERAL BLOOD LEUKOCYTES

Fagocitosis de eritrocitos no infectados e infectados con *Anaplasma marginale* por leucocitos bovinos

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ABSTRACT

The process of phagocytosis carried out by calf peripheral blood leukocytes (PBLs) on *Anaplasma marginale* infected or uninfected red blood cells (RBCs) in front of four different bovine sera was evaluated *in vitro*. Eight tests were run in a microplate containing PBLs, normal bovine erythrocytes (NBE) or *A. marginale* infected bovine erythrocytes (IBE), and either normal bovine serum (NBS), normal calf serum (NCS), vaccinated immune serum (VIS) or immune serum (IS). Erythrophagocytosis was detected in all tests that had IBE except in the test with NCS, and it was not detected in tests that had NBE, except in the test with NBS. The addition of IS and IBE to PBLs increased significantly ($P < 0.01$) the percentage of erythrophagocytosis when it was compared with the percentage detected in the tests that used NBS, VIS or IBE respectively. A 4% and 9% phagocytosis was detected in both tests performed with NBS. PBLs phagocytosed almost three times more NBE than IBE. In agreement with previous reports this study shows that IS, probably through specific immunoglobulins, mediates and enhances the process of erythrophagocytosis observed *in vitro*, which *in vivo* leads to severe anaemia; whereas the detection of phagocytosis in both tests with NBS would be explained either by a cross-reaction against *A. marginale* epitopes or by the presence of autoantibodies against cattle RBCs in the NBS or by other mediators like C3b. In conclusion, erythrophagocytosis, appears to be an antibody-dependent mechanism greatly responsible for the etiology of the severe anemia observed during the prepatent and patent periods of cattle anaplasmosis.

Key words: *Anaplasma marginale*, erythrophagocytosis, cattle, leukocytes.

RESUMEN

El proceso de fagocitosis efectuado por leucocitos bovino (PBLs) sobre eritrocitos (RBCs) no infectados o infectados con *Anaplasma marginale* y en presencia de cuatro tipos de sueros bovinos fue evaluado *in vitro*. Ocho ensayos se efectuaron en una microplaca que contenía PBLs, eritrocitos bovinos normales (NBE) o eritrocitos infectados con *A. marginale* (IBE), o bien suero normal bovino (NBS), suero normal de becerro (NCS), suero de un bovino vacunado con antígenos de *A. marginale* (VIS) o suero inmune a *A. marginale* (IS). Eritrofagocitosis fue detectada en todos los ensayos que tenían IBE, excepto en el ensayo con NCS, y este proceso no fue detectado en los ensayos que tenían NBE, excepto en el ensayo con NBS. La adición de IS y IBE a los PBLs aumentó significativamente ($P < 0,01$) el porcentaje de eritrofagocitosis cuando este valor fue comparado con el porcentaje detectado en los ensayos que usaron NBS, VIS o IBE respectivamente. Un 4% a 9% de eritrofagocitosis fue detectada en dos ensayos realizados con NBS. Los PBLs fagocitaron casi tres veces más NBE que los IBE. En concordancia con anteriores publicaciones este estudio muestra que el IS, quizás mediante inmunoglobulinas específicas, media e incrementa el proceso de eritrofagocitosis observado *in vitro*, el cual *in vivo* conduce a anemia severa; mientras que la detección de fagocitosis en ambos ensayos con NBS se explicaría por una reacción cruzada con epitopes de *A. marginale* o por presencia de autoanticuerpos en el NBS contra los RBCs del bovino o por otros mediadores como el factor C3b del complemento. En conclusión, la eritrofagocitosis parece ser un mecanismo dependiente de los anticuerpos y sería responsable por la etiología de la severa anemia observada frecuentemente durante las fases prepatente y patente de la anaplasmosis.

Palabras clave: *Anaplasma marginale*, eritrofagocitosis, bovinos, leucocitos.

INTRODUCTION

Bovine anaplasmosis is an arthropod-borne hemoparasitic disease caused by the intraerythrocytic rickettsia *Anaplasma marginale*. Fever, severe anemia, icterus, high levels of rickettsemia, reduction of milk production, and even death, are the main symptoms of this disease in cattle [17]. The only known cells used for development of *A. marginale* are bovine erythrocytes. After initial bodies of *A. marginale* invade red blood cells (RBCs) the number of infected cells increases geometrically during the incubation period (prepatent period) of anaplasmosis, simultaneously the reticuloendothelial system removes *A. marginale* infected and uninfected RBCs at 8 to 10 times the normal rate, producing a frequently fatal anemia [4, 9]. Destruction of *A. marginale* infected or uninfected RBCs is caused by phagocytosis resulting in development of icterus without hemoglobinemia and hemoglobinuria [9]. The mean incubation period for bovine anaplasmosis is about 28 days when *A. marginale* parasitemia is initially detected in thin blood films. During the patent period parasitemia increases geometrically and it is common to see animals with 15% to 75% parasitemia [18]. Some studies carried out four decades ago on the process of erythrophagocytosis, as the main cause of acute anemia observed in bovine anaplasmosis, concluded that immunoglobulins elicited against *A. marginale* antigens acted as opsonizing antibodies, which both attracted phagocytic cells and enhanced the process of erythrophagocytosis [10-12], and second, that biochemical and biophysical alterations of erythrocyte membranes would elicit autoantibodies against *A. marginale* infected or uninfected erythrocytes, which act as mediators of erythrophagocytosis [3, 11, 20]. The opsonins, that mediate and enhance the process of erythrophagocytosis during bovine anaplasmosis were characterized as IgM and IgG molecules whose maximum serum titres coincided with the highest crisis of anemia [14]. In general, it can be stated that in bovine anaplasmosis the host immune system plays a primary role in the pathogenesis of acute anemia.

The antibody kinetic during anaplasmosis was previously studied [8] and it was observed a macroglobulinemia peak characterized by two thirds antibodies of the IgM class and one third of the IgG class. It is well known that these immunoglobulins and complement fractions, like C3b, play a synergistic role during macrophage phagocytosis, and it was recently proposed that even though antibody alone is not sufficient for protection against *A. marginale* infections, immunoglobulins are required to provide specificity for macrophage phagocytosis, as shown by *in vitro* opsonization assays with antibody of cattle immunized with MSP1 [1, 15]. Due to the following facts: 1) that bovine serum levels of IgM increase one week before the highest peak of *A. marginale* parasitemia [8], 2) sudden peaks of IgM were recently reported just before the peak of parasitemia in *A. marginale* experimentally infected calves [16], and 3) that isotypes of serum IgM participate more efficiently

than IgG in functions like opsonization, complement activation, and agglutination of foreign microorganisms [21], it was evaluated *in vitro* the process of erythrophagocytosis in front of four different bovine sera and in eight different systems.

MATERIALS AND METHODS

Animals

Two Holstein calves, 4 to 5 months old, were obtained from a dairy farm located at an arid zone where cattle hemoparasitic diseases have rarely been diagnosed. Previously, serum from both calves had been analyzed for *Anaplasma* antibodies using a modified indirect immunofluorescence assay (IFA) [13], with negative results. Both animals were kept in a fly-proof isolated box. One calf was kept as a donor of normal bovine erythrocytes (NBE), peripheral blood leukocytes (PBLs) and serum without *Anaplasma* antibodies, named normal calf serum (NCS), whereas the second calf was splenectomized, three months later infected with an isolate of *A. marginale*, and it was the source of *A. marginale* infected bovine erythrocytes (IBE) when it had a parasitemia over 30%.

Bovine sera

Sera with antibodies against *Anaplasma* were collected from two sources: a) immune serum (IS) was separated from blood of a 2-year-old steer which had suffered acute anaplasmosis. This IS was tested by IFA and it had an anti-*Anaplasma* titre of 1:1280 and b) serum was separated from blood from a heifer which had been vaccinated with *A. marginale* soluble antigens (collected from the supernatant of *A. marginale* short term cultures [7]) and had by IFA an anti-*Anaplasma* titre of 1:640 (VIS). Normal bovine serum (NBS) or control serum was obtained from a Holstein heifer negative to *A. marginale* antibodies by IFA. All sera were kept frozen at -30°C before using.

Separation of PBLs

Peripheral blood was collected in two 20 mL syringes containing EDTA. Blood was centrifuged at $3,000 \times g$ for 10' at 4°C . Buffy coats were layered onto 20 mL of Ficoll and centrifuged at $1,500 \times g$ for 30' at 4°C . The layers with the concentrated PBLs were collected, pooled and washed in phosphate buffer saline (PBS), pH 7.2 at $1,500 \times g$ for 10'. The pelleted cells were resuspended in 2 mL of new PBS, counted in a hemocytometer chamber, and adjusted to a concentration of approximately 100,000 PBLs per 100 μL of PBS.

Bovine erythrocytes

A. marginale infected and uninfected RBCs were drawn from the jugular vein of experimental calves respectively. RBCs were washed three times by centrifugation in PBS, pH 7.2 at $3,000 \times g$ for 15' before being used.

Erythrophagocytosis in vitro tests

These were carried out in 96-wells polystyrene microplates (Becton Dickinson Co., USA) where eight tests were performed. The amounts of bovine sera or cells put in each well were as follows: a) NBS, NCS, VIS or IS: 20 µL, b) PBLs: 50µL with 50,000 leukocytes, c) NBE or IBE: 50 µL with 1×10^6 RBCs. All the components of each test were gently mixed and the microplate was incubated for 1 hour at 37°C in a 5% CO₂ and 95% air atmosphere. After incubation thin blood films were prepared by duplicate from each well, stained with Giemsa, and microscopically studied at 100X. The percentage of erythrophagocytosis (%E) was calculated as follows: %E = number of PBLs containing phagocytosed RBCs/100 PBLs counted in a blood film. Statistical analysis was done using the chi-square test in the EpiINFO 2000 program, version 1.0 and differences in all tests were considered significant when $P < 0.01$.

RESULTS

The results of the erythrophagocytosis tests are presented in TABLE I. Bovine PBLs showed phagocytic activity in all tests that had *A. marginale* infected erythrocytes (IBE) except in the test with NCS. On the contrary, erythrophagocytosis was not observed in tests which had normal bovine erythrocytes (NBE) except in the test with NBS. The %E by PBLs was significantly increased, 42.0% ($P < 0.01$), when bovine immune serum (IS) and IBE were added to the test (TABLE I) compared with 9% and 5% phagocytosis in the tests that used NBS, VIS, and IBE respectively. Moreover, a 4.0% and 9.0% erythrophagocytosis was detected in the tests performed with NBS (control serum) either in the presence of NBE or IBE, and these results were unexpected since as it was previously stated that appropriate immunoglobulins are required to pro-

vide specificity for macrophage phagocytosis [1, 15]. Besides, it is shown in Table I that PBLs phagocytosed not only *A. marginale* infected RBCs but also uninfected bovine RBCs (FIG. 1), e.g. in the test with IS and IBE from a total of 93 phagocytosed RBCs 24 were *A. marginale* positive or infected, whereas 69 were negative. In general, PBLs phagocytosed a greater amount of uninfected RBCs than *A. marginale* positive or infected RBCs.

DISCUSSION

This study shows that the addition in vitro of IS and IBE to PBLs collected from an *A. marginale*-free calf enhanced significantly ($P < 0.01$) the phagocytosis of *A. marginale* infected and uninfected RBCs, which is in agreement with previous works on the pathogenesis of anemia in cattle anaplasmosis [10, 11, 12]. Similar observations on the role of immunoglobulins on opsonization, macrophage attraction and phagocytosis have been demonstrated in other hemoparasites [2, 6, 23]. Erythrophagocytosis was not detected in the test NCS/IBE/PBLs which probably means that serum from the donor calf (NCS) lacked anti-*Anaplasma* antibodies either elicited by active infection or by passive transfer from the dam. Conversely, the detection of erythrophagocytosis in both tests using normal bovine serum (NBS) would be explained by any of these possibilities: a) the NBS had immunoglobulins that cross-reacted against some *A. marginale* epitopes which triggered the phagocytic activity, b) The heifer donor of NBS had developed an autoantibody against cattle RBCs, and c) It is suggested that erythrophagocytosis in the presence of NBS may be mediated by C3b or perhaps by the cell surface receptor CD36 in a similar way as it was recently proved in *Plasmodium*-infected RBCs not opsonized by either antibodies or complement, which were recognized and phagocytosed

TABLE I
PERCENTAGE OF ERYTHROPHAGOCYTOSIS DETECTED IN CALF PBLs ASSESSED IN EIGHT *in vitro* TESTS
FROM A 2-YEAR-OLD STEER WHICH HAD SUFFERED ACUTE BOVINE ANAPLASMOSIS

Tests <i>in vitro</i>	Erythrophagocytosis ^a (%E)	Nº of phagocytized RBCs (<i>A. marginale</i> + / <i>A. marginale</i> -)
NBS/NBE/PBLs	4.0 (4/100)	9 (0/9)
NBS/IBE/PBLs	9.0 (9/100)	11 (4/7)
NCS/NBE/PBLs	0 (0/100)	0
NCS/IBE/PBLs	0 (0/100)	0
VIS/NBE/PBLs	0 (0/100)	0
VIS/IBE/PBLs	5.0 (5/100)	14 (8/6)
IS/NBE/PBLs	0 (0/100)	0
IS/IBE/PBLs	42.0 ^b (42/100)	93 (24/69)

^a %E = Nº of PBLs containing phagocytosed RBCs/100 PBLs counted in a blood film. ^b $P < 0.01$. %E: percentage of erythrophagocytosis. NBE: normal bovine erythrocytes (control). IBE: infected bovine erythrocytes from a steer with 35% *A. marginale* parasitemia. PBLs: peripheral blood leukocytes. NBS: normal bovine serum. NCS: normal calf serum. VIS: immune serum from a heifer vaccinated with *A. marginale* soluble antigens. IS: immune serum.

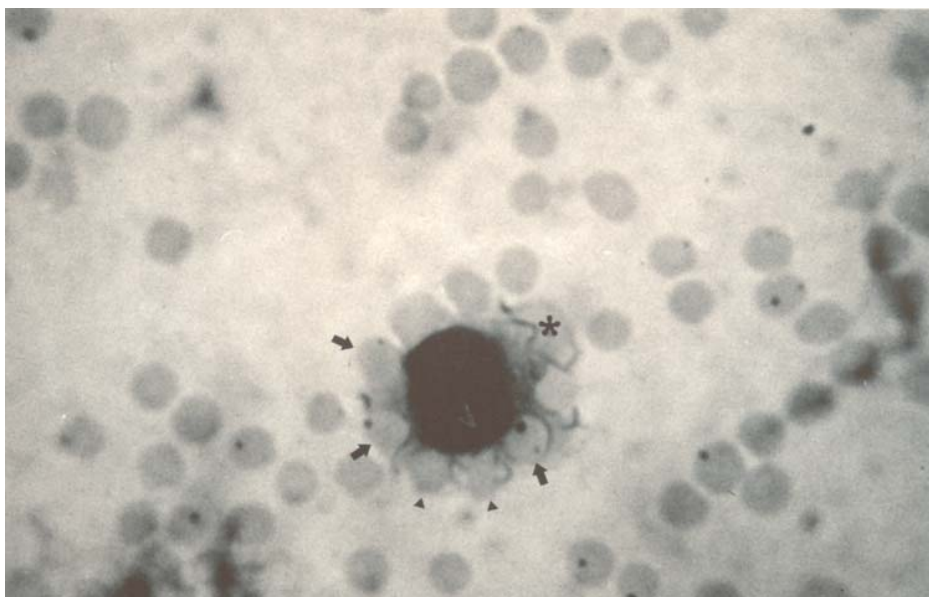


FIGURE 1. PHAGOCYTOSIS OF *Anaplasma marginale* INFECTED (ARROWS, ↑) AND NONINFECTED (ARROW HEADS, ▲) ERYTHROCYTES BY A PBL (*), PROBABLY A MONOCYTE. THIS THIN BLOOD FILM WAS PREPARED FROM TEST IS/IBE/PBLs. 100X.

by macrophages and monocytes via the cell surface receptor CD36 [19]. PBLs phagocytosed *in vitro* almost three times more *A. marginale* uninfected RBCs than infected ones (TABLE I), which is in agreement with the process that occurs *in vivo* during the prepatent period of bovine anaplasmosis leading to severe anaemia [9, 18]. Other authors have demonstrated that erythrophagocytosis of *A. marginale* infected or uninfected RBCs is also mediated by autoantibodies against those RBCs, which triggers the process of phagocytosis and it causes severe anaemia [3, 5, 20]. Two unanswered questions are raised by this process: 1) What is the fate of phagocytosed initial bodies of *A. marginale*?, and 2) Do initial bodies escape from phagolysosome vacuoles and invade other RBCs? These questions should motivate further research on this field.

CONCLUSIONS

Erythrophagocytosis in cattle with anaplasmosis appears to be an antibody-dependent mechanism which seems to participate in the etiology of the severe and even fatal anemia observed during the prepatent and patent periods of this disease; nonetheless, erythrophagocytosis in an *A. marginale* chronic "carrier" cattle would work as one of the protective mechanisms with the aim of keeping under control its parasitemia during phases of rickettsemia.

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