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NUCLEIC ACID LEVELS IN *Anaplasma marginale* DURING EXPERIMENTALLY INDUCED BOVINE ANAPLASMOSIS

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The DNA and RNA concentrations in uninfected and *Anaplasma marginale*-infected bovine erythrocytes were determined. Bovine anaplasmosis was experimentally induced and the nucleic acid levels were followed during the development of the *A.marginale* infection. Two distinct growth stages were found, the "multiplication" stage and the "transfer" stage. Based on this hypothetycal developmental cycle, the amount of DNA and RNA per *Anaplasma* initial body was estimated as 73.1 x 10^{-3} pg and 45.7 x 10^{-3} pg repectively.

The rickettsia *Anaplasma marginale* is the causative agent of bovine anaplasmosis, an infectious and transmissible disease characterized by intraerythrocytic parasitism and progressive hemolytic anemia. After the biological or mechanical transmission through the arthropod vectors, several disease periods are associated with the pathogenesis of anaplasmosis: the incubation, with a very low number of parasites in the circulating blood; the acute phase, during which the parasitemia increases rapidly and a severe anemia develops; and finally, the convalescence period in those animals that survive the infection. The last stage is followed by the establishment of a chronic phase during which low parasitemia may persist indefinitely (1). The intraerythrocytic

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cycle of this parasite has been studied by electron microscopy (2,3) and it is well established that it replicates by binary fission (1,4). A hypothetical developmental cycle was formulated by Ristic (1) based on the frequency of occurrence of Anaplasma and its location in the infected erythrocytes. Four developmental stages of the organism were postulated: the early stage, with single initial bodies in close proximity of the erythrocyte membrane; the mixed population stage, with some of the initial bodies appearing to be free in the cytoplasm; the vigorous growth and transfer stage, where multiple infections of individual erythrocytes were commonly observed; and, the massive multiplication stage, with a predominancy of marginal Anaplasma bodies in the cell population. The purpose of the present work is to further study the life cycle of this microorganism in the mature erythrocytes by measuring the nucleic acid levels in the various growth stages during the development of the A.marginale infection.

Anaplasma DNA and RNA have been demonstrated by histochemical staining methods (5,6) as well as the ability of these microorganisms to synthesize DNA and proteins during short-term culture conditions (7,8). The Anaplasma DNA isolated from marginal bodies was found to be double stranded and to contain 51 moles % of guanine plus cytosine (9). In this study we isolated the red blood cells from non-infected and from Anaplasma-infected bovines and then, quantified the extracted DNA and RNA. The nucleic acid levels were followed during the course of the infection and the correlation among these values and the circulating parasitemia in the bovine erythrocytes allowed us to study the intraeythrocytic multiplication cycle of the microorganism and to have an estimate of the concentrations of DNA and RNA present in the Anaplasma marginal bodies.

MATERIALS AND METHODS

Control and experimental calves were maintained under tick-free conditions (Experimental Station, Fac. Cienc. Vet., Univ. Central de Venezuela). Two splenectomized calves -Holstein, male, ten and twelve month old- were infected with A.marginale as previously described (10). Blood was collected at various times during the course of the infection. The red blood cells were lysed with saponin (11) and centrifuged at 40,000 xg for 10 minutes. The pellet was washed repeatedly by resuspension and centrifugation with hypotonic buffer (125 mM NaCl, 10 mM TRIS-HCl pH 7.4) until free of hemoglobin. Nucleic acids were extracted essentially as described by Hanson and Phillips (12). The pellets were resuspended in hypotonic buffer and perchloric acid was added to a final concentration of 0,25N. After homogenation and a 30 minutes incubation period on ice, the suspension was centrifuged at 10,000xg for 10 minutes. The pellet was extracted with 0,5N perchloric acid at 70°C for 15 minutes and centrifuged at 5,000xg for 10 minutes at 20°C. The acid treatment was repeated twice and the supernatants were pooled. Total nucleic acids in the samples were estimated at 260 and 280 nm (13). DNA from Kluyveromyces fragilis was used as standard. DNA was determined with the diphenilamine reaction (14) and the orcinol method (14) was used for the estimation of RNA. The effect of saponin in the reactions was checked and proved not to interfere with both methods. To present the results as the concentration nucleic acids per infected cell, the values were expressed per ml of packed red cells (15 min at 1,650xg) corrected for the number of cells in such volume and for the parasitemia (percentage of the red cells infected) of the sample.

RESULTS

Table I shows the average concentrations of total nucleic acids, DNA and RNA found in the control and in the parasitized bovine red cells. The results are expressed both, per ml of packed red blood cells and per red cell. In the infected samples the values were corrected for the parasitemia of the circulating blood. A considerable variation was found between the different samples analyzed as can be observed from the standard deviation of the average values. The average nucleic acid concentrations for the bovine red cells and for the Anaplasma-infected red cells were 0.0132 and 0.174 pg/cell respectively. A good correspondence was found in the values of total nucleic acids when estimated with the UV absorption method or by adition of the values from the individual determinations of DNA and RNA.

Table I. DNA, RNA and total Nucleic Acid concentrations of mature bovine erythrocytes and A.marginale-infected bovine erythrocytes. The results are presented as the means corrected by the standard deviation. Figures in parentheses indicate the number of different samples analyzed.

	Bovine Erythrocytes	
Nucleic Acids	Uninfected[a]	Infected[b]
TOTAL	13.2 ± 2.9 (4)	173.9 ± 106.5 (4)
DNA	5.9 ± 1.3 (4)	107.9 ± 66.2 (6)
RNA	9.4 ± 2.1 (4)	66.3 ± 26.9 (6)

(a) (pg/cell) x 10^{-3}

(b) (pg/infected cell) x 10^{-3}

	Bovine Erythrocytes	
Nucleic Acids	Uninfected[a]	Infected[a]
TOTAL	46.5 ± 10.2 (4)	191.1 ± 51.6 (3)
DNA	20.8 ± 4.8 (4)	118.1 ± 72.3 (6)
RNA	32.8 ± 7.2 (4)	90.3 ± 27.2 (4)

(a) (µg/ml of RBC)

It is to be expected that the concentration of nucleic acids per infected erythrocyte varies during the course of the Anaplasma infection. This could be responsible, at least partially, for the great variability in the data presented in Table I, which corresponds to the average value of all the infected samples studied. Figure 1 presents the results of one of the two experiments in which two suceptible bovines were experimentally infected with A.marginale. The DNA and RNA concentrations were estimated in blood samples taken before the inoculation and at different periods during the course of the disease. This animal died 26 days after the inoculation of the microorganism. At that moment, the circulating parasitemia was about 64%, whereas the other experimental animal survived, and the maximal parasitemia

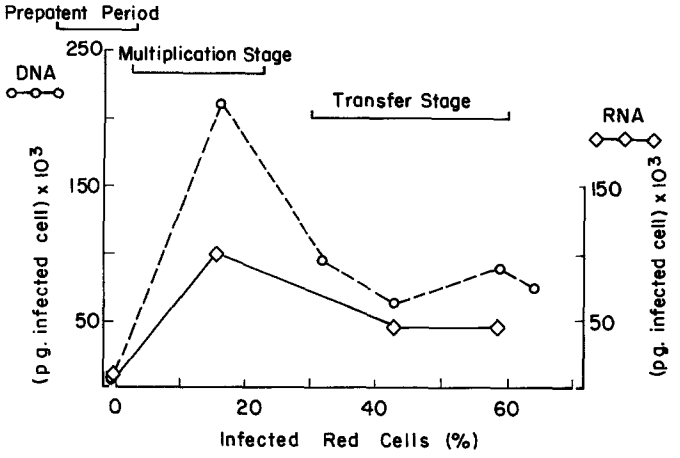

Figure 1. Nucleic acid concentrations during the course of bovine anaplasmosis. Anaplasmosis was experimentally induced in a suceptible bovine and the concentrations of DNA and RNA were estimated by duplicate in the red cell extracts. The results were corrected for the nucleic acids present in the control red cells (uninfected) and for the percentage of infected cells, in order to express the results as pg per infected red cell.

developed was 21%. Both animals showed a similar general pattern. The content of DNA and RNA in the infected erythrocytes was corrected for the basal concentrations of the nucleic acids in the control samples.

DISCUSSION

No data were found in the literature for nucleic acid concentrations of bovine erythrocytes. The presence of reticulocites under our experimental conditions was minimal as checked in blood smears. Therefore the nucleic acids in the control cells may represent residual DNA and RNA from the maturation process of the erythrocytes and/or be a reflection of an heterogeneous population of mature erythrocytes. Nucleated red cells such as chicken erythrocytes have 2.49 pg of DNA per cell

(15) which represents about four hundred times more than the DNA concentration in the bovine red cells. Human leucocytes have a DNA concentration about six hundred to one thousand higher (16) than the concentration found in the bovine erythrocytes (Table I).

Our biochemical evidence seems to support the hypothetical developmental cycle proposed by Ristic (1). The results suggest that the synthesis of nucleic acids is maximal at the early stages of the Anaplasma infection (Fig. 1). This accelerated division of the initial infective units presumably involves the presence of a low number of parasitized erythrocytes with marginal bodies containing several internal subunits. The liberation of these subunits will lead to the infection of other red cells with the concomitant increase in the parasitemia of the circulating blood. If so, this increase in parasitemia not necessarily will be accompanied by an increase in the amount of nucleic acids per infected red cell, which is what Figure 1 seems to suggest. At least two distinct stages of the microorganism seem to be involved during the course of the infection in the bovine. The first involves a period of massive multiplication, and the second, the "transfer" stage, refers to a stage in which the reinfection of other erythrocytes seems to be the predominant event.

The described multiplication cycle supports observations by other authors (4,17) in the sense that marginal bodies with only one subunit are expected to be more abundant during the acute phase of the disease and during the convalescense period. Based on the previous assumption, we were able to estimate the amount of DNA and RNA per Anaplasma subunit as 73.1×10^{-3} pg and 45.7×10^{-3} pg, respectively. The figures could be an overestimation and should be taken only as a first approximation. Anaplasma infections are ussually followed by a recovery period in which, according to Ristic (1) the marginal Anaplasma bodies predominate.

The initial *Anaplasma* body represents the *modus operandi* for initiation and perpetuation of the infection (1). Our study did not include these two stages, the prepatent and the chronic periods of the disease, therefore it is not possible to discard completely the possibility of marginal bodies with more than one internal subunit in our model system.

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