*Revista Latinoamericana de Microbiología* **(2000) 42:101-109**
Asociación Latinoamericana de Microbiología

# Variation of Peripheral Blood BoCD2$^+$, BoCD4$^+$, and BoCD8$^+$ Lymphocytes, the BoCD4:BoCD8 index and IgG Antibodies in Bovines Infected and Rechallenged with Isolates of *Anaplasma marginale* of Mexican Origin.

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**ABSTRACT. Peripheral blood (PB) BoCD2$^+$, BoCD4$^+$, and BoCD8$^+$ lymphocyte levels, the BoCD4:BoCD8 index (CDIndex) and levels of IgG antibodies to *Anaplasma marginale* were assessed by flow cytofluorometry and ELISA, respectively, in four bovines during infection and reinfection with mexican *A. marginale* isolates; and in four other animals which served as noninfected controls. During primary infection, the CDIndex and antibody levels increased after the rickettsemia dropped and there was a strong correlation between the CDIndex and the IgG titres. Upon rechallenge, the CD Index and IgG titres did not change significantly with respect to the values observed at the beginning of the study. Additionally, PB lymphocyte levels were estimated in 12 normal intact bovines. The results indicate that BoCD4+ lymphocytes help in the control of infection with mexican *Anaplasma* isolates in the primary infection and that the IgG antibodies play a minor role in protection from infection.**
**Key words: *Anaplasma marginale*, Lymphocyte Levels, Bovines.**

**RESUMEN. : Los niveles de linfocitos BoCD2$^+$, BoCD4$^+$, y BoCD8$^+$ de sangre periférica (SP) el índice BoCD4:BoCD8 (IndiceCD) y los niveles de anticuerpos IgG a *Anaplasma marginale* fueron determinados por citofluorometría de rayo láser y ELISA, respectivamente, en cuatro bovinos durante la infección y reinfección con aislados mexicanos de *A. marginale*; y en otros cuatro animales que sirvieron como testigos no infectados. Durante la infección primaria, el Indice CD y los niveles de anticuerpos se incrementaron después de que la rickettsemia disminuyó y hubo una fuerte correlación entre el IndiceCD y los títulos de IgG. En la reinfección, el IndiceCD y los títulos de IgG no cambiaron significativamente con respecto a los valores observados al inicio del estudio. Adicionalmente, los niveles de linfocitos de SP se determinaron en 12 bovinos normales intactos. Los resultados indican que los linfocitos BoCD4$^+$ ayudan en el control de la infección por aislados mexicanos de *Anaplasma* y en la producción de anticuerpos IgG a *Anaplasma* en la infección primaria y que los anticuerpos juegan un papel menor en la protección contra la infección.**
**Key words: *Anaplasma marginale*, Niveles de Linfocitos, Bovinos.**

## INTRODUCTION

The etiologic agent of bovine anaplasmosis, a disease characterized by severe anemia, abortions and high mortality, is the intraerythrocytic rickettsia *Anaplasma marginale* a parasite that is biologically transmitted by ticks of the Ixodidae family in nature, and mechanically by hematophagous Diptera of Tabanidae family.[23] Although *A. marginale* infection induces a humoral immune response,[33,27] transfer of sera or colostral antibodies to susceptible animals doesn't confer protection.[31,18,43] Animals infected for the first time with virulent strains of *A. marginale* develop a strong cellular immune response that corresponds to the development of clinical signs; once the animal recovers from the acute phase of the infection it maintains a vigorous cellular immunity and remains clinically protected and immune to reinfection, although, more often than not, the animal becomes a sub-clinically infected carrier.[34] Crude *A. marginale* antigens have been used to evaluate the cellular immune responses.[7,8,10]

The protective role of cellular immunity in other intracellular infections such as those induced by *Cowdria rumi-*

*nantium*,[13,38,28] *Rickettsia tsutsugamushi*,[29,30] *Ehrlichia risticii*,[39,40] *Theileria parva*,[2] *Plasmodium* spp.[36] and other parasitic protozoans[37] has been properly stressed. It is not only the macrophages which[20] play an important role in the protection against intracellular pathogens[35,17] but also NK cells and Th lymphocytes. So important are the latter that Th1 clones have been proposed as probes to identify and characterize protective antigens and immune responses.[3,4]

Since data is lacking which would permit the correlation of the changes in the ratios of the bovine's peripheral blood T cell subpopulations with the concentration of anti-*Anaplasma* IgG antibodies in cattle with anaplasmosis we sampled bovines experimentally inoculated and reinoculated with viable Mexican isolates of *A. marginale* with the aim of determining if a relationship exists.

## MATERIALS AND METHODS

***A. marginale* isolates.** The Mexican *A. marginale* isolates used in the study were collected from infected cattle in Mexico's Morelos State (MEX-17-029-01) and in the Tizimin district of the State of Yucatán (MEX-31-096-01) and maintained as stabilates. The infected erythrocytes were suspended 1:1 with 31.2% (w/v) dimethylsulfoxide in phosphate-buffered saline, pH 7.2 and were stored in liquid nitrogen.[24] Before use each isolate was passed serially in two splenectomized immunosupressed bovines.

**Experimental animals.** Eight Holstein breed calves, 12-18 months old were obtained from the State of Chihuahua. The animals were confirmed to be free of tuberculosis by the TB test, brucellosis by serologic tests, babesiosis by the indirect immunofluorescent test and anaplasmosis by ELISA. Giemsa stained blood films were used to confirm the absence of hemoparasites. The animals were kept in isolation in pens with concrete floors. Animals consumed food and water *ad libitum.* Following experimental infection of cattle with *A. marginale* isolates data on parasitemia was collected every other day. Four animals were inoculated with 1 x $10^6$ *A. marginale* infected erythrocytes (MEX-17-029-01 isolate) and four non-infected normal bovines were maintained as control (C). The CD Index, the percentage of parasitized erythrocytes (PPE), the packed cell volume (PCV), and the temperatures (T) were evaluated on the same animals at 0, 12, 25, 34, 41, 90, 93, 103, 111, 118 and 124 days. The four infected bovines were infected again, 90 days after the first infection, with 2.6 X $10^8$ *A. marginale* PE (MEX-31-096-01 isolate).

**Percentage of parasitized erythrocytes (PPE), packed cell volume (PCV) and temperature (T).** Blood samples collected from the animals were used for the preparation of Giemsa-stained blood films to assess PPE by light microscopy[9] and for determination of PCV (recorded as percentage) by standard methods.[1] The measurement of temperature (°C) was carried out using a standard rectal thermometer.

**Peripheral blood T-cells.** The peripheral blood T lymphocytes in the blood samples collected at 0, 12, 25, 34, 41, 90, 93, 103, 111, 118 and 124 days were characterized by flow cytometry (FC), using anti-BoCD2$^+$, anti-BoCD4$^+$ and anti-BoCD8$^+$ mouse monoclonal antibodies (mAb) (Serotec, England). Briefly, to 100 µl of blood collected in tubes containing EDTA, 10 µl (diluted 1:100 in PBS) of anti-BoCD2$^+$, BoCD4$^+$ or BoCD8$^+$ mAb were added and the mixture incubated for 30 min at 4$^o$C in the dark, then 20 µl (diluted 1:100 in PBS) of goat serum anti-mouse immunoglobulines, conjugated to fluorescein isothiocyanate-(Sigma, Missouri, USA) were added and the mixture incubated again for 30 min. at 4$^o$C in the dark. Next two ml of lysis solution (Becton Dickinson, San Jose, CA, USA) were added and then this mixture was incubated for 15 min at room temperature. Subsequently the sample was centrifuged at 380 x *g* for 5 min at 4$^o$C, and the pellet obtained was resuspended with 2 ml of PBS and centrifuged at 380 x *g* for 5 min at 4$^o$C; finally the supernatant was decanted and 0.5 ml of a paraformaldehyde solution at 0. 5% was added and the sample was kept at 4$^o$C in the dark for no more than three days. FC was assessed with a FACScan (Becton Dickinson, San Jose, CA, USA) analyzer. The data was analyzed using the software Lysis II System from Becton-Dickinson (San Jose CA, USA) and recorded as percentages. Using the T-cell values obtained, the BoCD4: BoCD8 index (CDIndex) was calculated (BoCD4 value divided by the BoCD8 value). Both as a source of data and to develop our technique the peripheral blood BoCD2$^+$, BoCD4$^+$ and BoCD8$^+$ values, and the CDIndexes were determined in 12 normal intact bovines aged 12-18 months old before the studies of the effects of infection were undertaken.

**T-cells/ml of peripheral blood.** The numbers of BoCD2$^+$, BoCD4$^+$ and BoCD8$^+$ lymphocytes per ml of peripheral blood in the infected animals were calculated using 100 µl samples of peripheral blood/animal. The RBC's were lysed and then the total number of leukocytes counted. The proportion of BoCD2$^+$, BoCD4$^+$ or BoCD8$^+$ cells were determined as described above following fluorescent staining of the leukocyte population, and then by multiplying the % of BoCD2$^+$, BoCD4$^+$ or BoCD8$^+$ in the sample times the total number of leukocytes recovered per ml of blood the numbers of leukocytes of each type per ml of blood were estimated.

**Purification of *A. marginale* organisms.** Free *A. marginale* were obtained from bovine erythrocytes infected with the MEX-31-096-01 isolate of *A. marginale* according to the method described by Palmer and McGuire.[31] Briefly, 4 x $10^9$ parasitized eryhtrocytes were washed three times by centrifugation at 27,000 x *g*. Before each washing the pellet from the previous centrifugation was suspended in 40 ml of RPMI 1640 media containing 2 mM L-glutamine and 25 mM HEPES. The sediment from the final centrifugation was resuspended in 35 ml of media, disrupted by 2 min of sonication at 50 W, and washed by sus-

pension and centrifugation two times at 1650 x *g* for 15 min. The amount of protein in the preparations was determined according to Lowry et al.[25]

**ELISA**. Serum samples were obtained before infection with *A. marginale*, and at 12, 25, 34, 41, 90, 93, 103, 111, 118 and 124 days after infection. IgG anti-*A. marginale* antibody levels were measured by ELISA using *A. marginale* organisms as antigen, the organisms were washed with sodium dodecyl sulfate (SDS) then exposed to the bovine serum according to Winkler et al,[41] then exposed to rabbit serum anti-bovine IgG alkaline phosphatase-conjugate (Sigma, Missouri, USA) in p-Nitrophenyl Phosphate, Disodium (pNPP, Sigma, Missouri, USA) as substrate. After additional washing readings were carried out in an ELISA reader (Multiskan Plus, Labsystems, Finland) at $OD_{405}$. Those sera, diluted 1:100, that gave OD lectures higher than 0.200 (mean + two standard deviations of the results obtained with sera from *A. marginale* free cattle) were considered as positive to anti-*A. marginale* antibodies. This ELISA test detects antibodies to cross-reactive antigens as well as unique antigens to both of the *A. marginale* strains employed (García-Ortíz M. A. personal communication).

**Statistical analysis.** Data were analyzed with Student's *t*-test and a linear regression analysis.

## RESULTS

The mean values ± SE of peripheral blood $BoCD2^+$, $BoCD4^+$, $BoCD8^+$ cells, and the BoCD4:BoCD8 Indexes calculated from studies of the blood of the 12 normal bovines are shown in Table 1. They were respectively of 45.9 ± 3.2, 30.2 ± 1.5, 15.9 ± 1.0 and 2.0 ± 0.1.

In the infected bovines, the $BoCD2^+$, $BoCD4^+$ and $BoCD8^+$ values ranged from 40.4-49.8%, 24.4-33.6%, and 10.7-15.2%, respectively, during primary infection; and between 43.5-48.5%, 28.4-31.9% and 13.4-16.2% during reinfection (Fig. 1). In the control, uninfected animals the $BoCD2^+$, $BoCD4^+$ and $BoCD8^+$ ranged between 39.8-

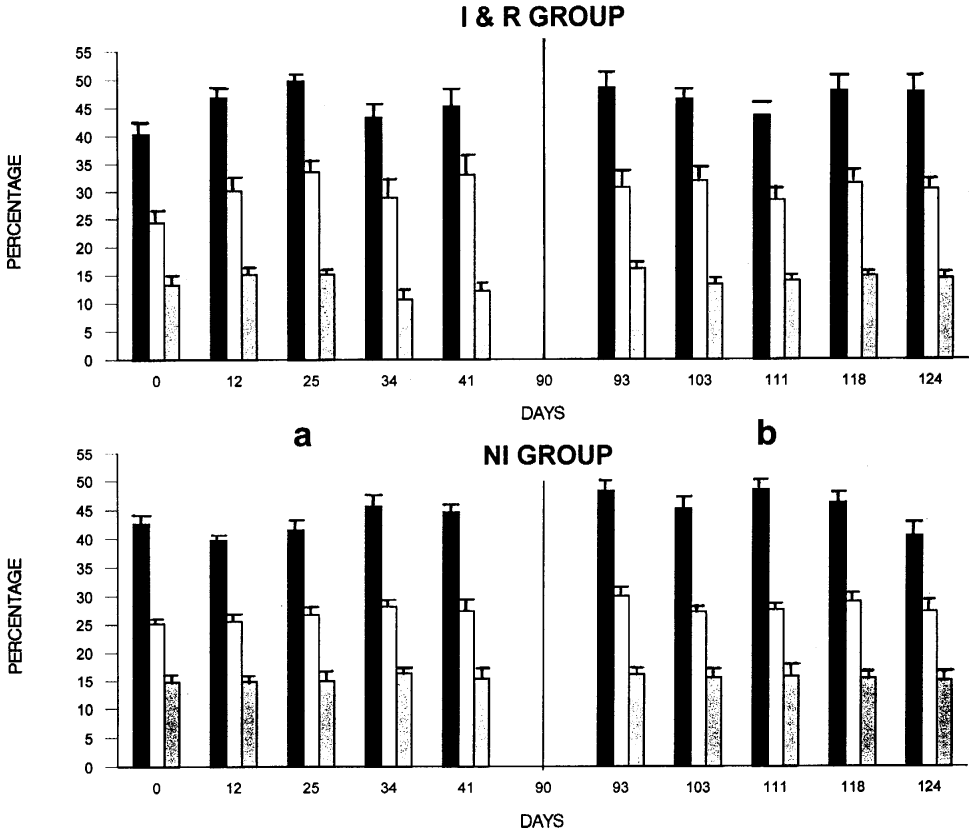


Fig. 1. Fluctuation of peripheral blood $BoCD2^+$, $BoCD4^+$ and $BoCD8^+$ T cells in two groups of bovines: Infected and Re-challenged (I&R), and Noninfected (NI). (a) Infection at day 0 with $10^6$ *A. marginale* (isolate MEX-17-029-01) parasitized erythrocytes/animal; (b) rechallenge at day 90 with 2.6 $x10^8$ *A. marginale* (isolate MEX-31-096-01)) parasitized erythrocytes/animal. Black bars: $BoCD2^+$, white bars: $BoCD4^+$, gray bars: $BoCD8^+$. Each bar represents the mean ± SEM of four bovines. Days 93, 103, 111, 118, and 124 are days 3, 10, 18, 25, and 31 after rechallenge, respectively.

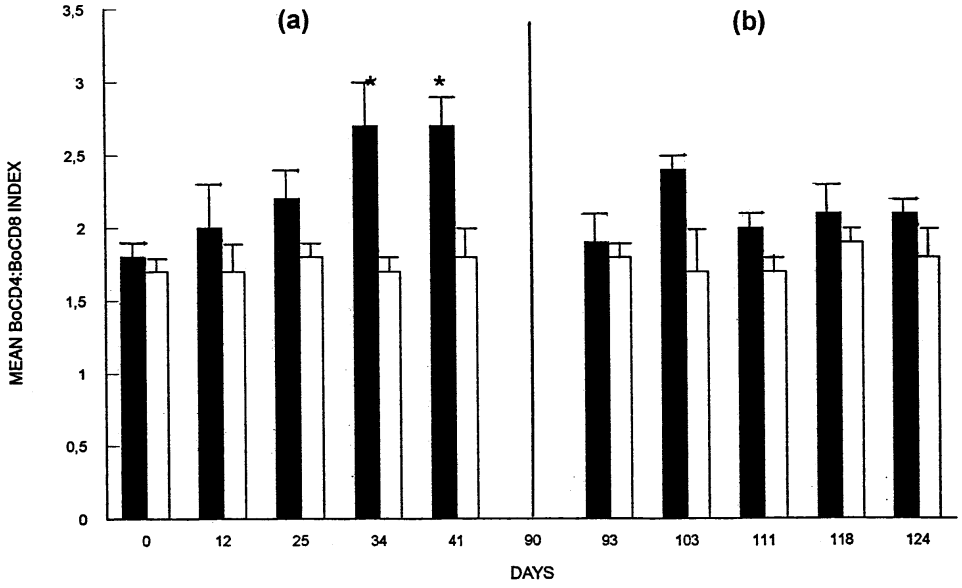


Fig 2. Fluctuation of the BoCD4:BoCD8 Index (% peripheral blood BoCD4$^+$/% peripheral blood BoCD8$^+$ T cells) in bovines infected and rechallenged with *Anaplasma marginale.* (a) Infection at day 0 with $10^6$ *A. marginale* (isolate MEX-17-029-01) parasitized erythrocytes/animal; (b) rechallenge at day 90 with 2.6 x$10^8$ *A. marginale* (isolate MEX-31-096-01) parasitized erythrocytes/animal. Black bars: infected animals, white bars: noninfected animals. Each bar represents the mean $\pm$ SEM of four bovines. Days 93, 103, 111, 118, and 124 are days 3, 10, 18, 25, and 31 after rechallenge, respectively. * $p < 0.05$ with respect to initial values, including those of Table 1, and to days 3, 10, 18, 25 and 31 after rechallenge.

48.4%, 25.2-30%, and 14.8-16.4%, respectively, during the 124 days of the study (Fig. 1). At day 0 the CDIndex was 1.8 for the infected bovines and 1.7 for the controls (Fig. 2). Later, during the multiplication phase of the rickettsia (PPE) in the infected animals (mean of 23.25% infected erythrocytes at 25 days after infection, Fig. 3), the PCV began to diminish (mean 34%, initial mean value 41.8%) and the temperature (mean 40.7 $^o$C) and the CDIndex to increase (2.2) above the initial levels (1.8). At day 34 after infection, when the PPE was descending, the PCV was recovering (mean 16%) and the CDIndex showed a significant increase (2.7, $P < 0.05$) with respect to that of the controls (Fig. 2). At 41 days after infection, when the mean PPE was of 1.2 (Fig. 3) and with a recovering PCV (mean 21%), the CDIndex was still significantly (2.7) above initial values. The number of BoCD4$^+$ lymphocytes per ml of blood, showed increases (not significant) with respect to initial values, at days 12 and 34 after primary infection and at days three and 25 after secondary infection (Table 2).

Significant decreases ($P < 0.05$) in the number of BoCD8$^+$ lymphocytes, with respect to initial values, were observed at days 25, 34 and 41 after primary infection (Table 2); there was also an increase, although not significant, in the number of these lymphocytes at day three after rechallenge. During the period of primary infection the non-infected bovines did not show changes in PCV (data not shown) nor did *A. marginale* appear in their peripheral blood, their T cell population and CDIndexes did not show significant changes during the period (Fig. 2). In the infected animals the IgG anti-*Anaplasma* antibodies in-

Table 1. Mean percentages ($\pm$SEM) of peripheral blood BoCD2$^+$, BoCD4$^+$, and BoCD8$^+$ T cells, and the BoCD4:BoCD8 Index of 12 normal intact bovines, as determined by monoclonal antibodies and laser ray cytofluorometry

| Peripheral blood T-cell | Mean | $\pm$ SEM |
|---|---|---|
| BoCD2$^+$ | 45.9<br>30.2<br>15.9 | $\pm$ 3.2<br>$\pm$ 1.5<br>$\pm$ 1.0 |
| CDIndex | 2.0 | $\pm$ 0.1 |

CDIndex: BoCD4:BoCD8 Index = Percentage of peripheral blood BoCD4$^+$/percentage of peripheral blood BoCD8$^+$ T cells

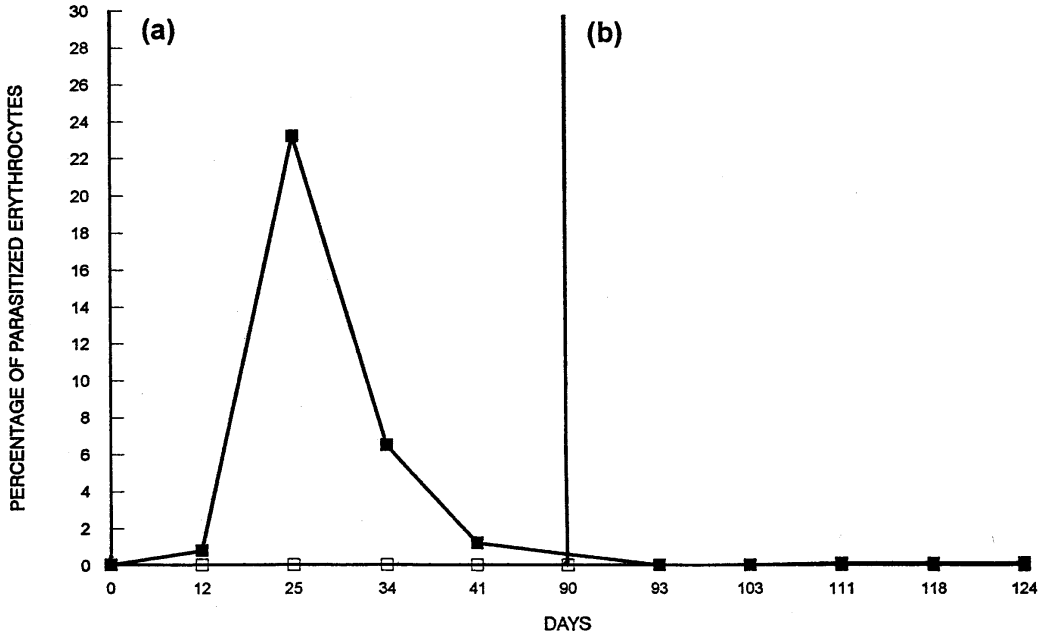


Fig 3. Kinetics of parasitized erythrocytes in bovines infected and rechallenged with *Anaplasma marginale*. (a) Infection at day 0 with $10^6$ *A. marginale* (isolate MEX-17-029-01) parasitized erythrocytes/animal; (b) rechallenge at day 90 with 2.6 x$10^8$ *A. marginale* (isolate MEX-31-096-01) parasitized erythrocytes/animal. Black squares: infected animals, white squares: noninfected animals. Each point represents the mean of four bovines. Days 93, 103, 111, 118, and 124 are days 3, 10, 18, 25, and 31 after rechallenge, respectively.

creased soon after parasitemia began to increase and then dropped after parasites disappeared or were at very low concentrations in the peripheral blood (Fig. 3 and 4). Between days three and 31 after secondary infection (days 93 and 124, respectively), there were no significant changes in T cell population (data not shown), nor in the CDIndexes (Fig. 2) nor in anti-*Anaplasma* IgG antibodies (OD, Fig. 3).

During this period in the control animals T cell population did not change significantly (data not shown), the CDIndexes and the antibody levels did not show any significant changes. These values were also similar at the beginning and at the end of the primary and secondary infections.

There was a strong positive correlation ($r$ = 0.9606) between the BoCD4:BoCD8 Index (CDIndex) and the titers of the anti-*Anaplasma* IgG antibodies (OD) in the primary infection; while there was no correlation ($r$ = -0.0543) between the same parameters in the secondary infection (Table 3).

## DISCUSSION

In the group of 12 normal animals, the mean percentage of peripheral blood BoCD2$^+$ cells (45.9%) was lower than the values published by others, while those of BoCD4$^+$ (30.2%) and BoCD8$^+$ cells (15.9%) were within the range of published values. These published values are 50-70%, 25-35% and 15-25% for BoCD2$^+$, BoCD4$^+$ and BoCD8$^+$, respectively.[26,21] The difference between the values reported by others for BoCD2$^+$ and those of the animals used in this study may be related to genetic differences in the population studied or the particular conditions in which the different animals studied were raised or even due to differences in the monoclonal antibodies used in the different studies. In the infected animals BoCD2$^+$ values ranged between 40.4 and 49.8%, while in the controls they ranged from 39.8 to 48.4% (Fig 1). These values are also lower than those reported by others.

During the primary infection, anti-*Anaplasma* antibodies increased as the infection progressed. Then, when the infection was controlled and the load of antigen decreased the antibody titers dropped. Animals were reinoculated 90 days after primary infection. The reinfection was with a higher dose of *A. marginale* parasitized erythrocytes than was the primary infection (first infection 1 x $10^6$ PE; rechallenge 2.6 x $10^8$ PE). Following this inoculation, there was no significant increase in anti-*Anaplasma* antibodies in the challenged animals (Fig 4). Similar responses have been observed in animals reinfected with bovine babesiosis.[16] In this respect, it is known that the bovine immune response against *A. marginale* effectively controls a chal-

Table 2. Mean (±SEM) values BoCD2$^+$, BoCD4$^+$, BoCD8$^+$ lymphocytes per milliliter of peripheral blood in four bovines infected and rechallenged with *Anaplasma marginale*.

| Days after infection[a] | BoCD2+ x $10^3$ per ml | BoCD4+ x $10^3$ per ml | BoCD8+ x $10^3$ per ml |
|---|---|---|---|
| 0 | 2379 ± 73 | 1519 ± 104 | 915 ± 106 |
| 12 | 2533 ± 473 | 1873 ± 192 | 912 ± 127 |
| 25 | 2151 ± 276 | 1429 ± 184 | 635 ± 76* |
| 34 | 2491 ± 189 | 1650 ± 169 | 676 ± 59* |
| 41 | 2182 ± 136 | 1505 ± 64 | 649 ± 59* |
| 93 (3 a.r.[b]) | 2772 ± 131 | 1861 ± 146 | 985 ± 32 |
| 103 (10 a.r.) | 1714 ± 305 | 1485 ± 33 | 700 ± 79 |
| 111 (18 a.r.) | 2166 ± 215 | 1423 ± 98 | 765 ± 53 |
| 118 (25 a.r.) | 2649 ± 190 | 1666 ± 93 | 941 ± 110 |
| 124 (31 a.r.) | 2359 ± 124 | 1445 ± 50 | 715 ± 33 |

[a]Each bovine was infected with 1 x $10^6$ *A. marginale* (isolate MEX-17-020-01) parasitized erythrocytes. [b]a.r.: after rechallenge with 2.6 x $10^8$ *A. marginale* (isolate MEX-31-096-01) parasitized erythrocytes/animal. * $p < 0.05$ with respect to initial values.

Table 3. Linear regression analysis (coefficient of correlation: *r*) between PPE - CDIndex, PPE - O.D., and CDIndex - O.D. in bovines (n=4) infected and rechallenged with *Anaplasma marginale*[a].

| Type of Infection | PPE-CDIndex | PPE-OD | CDIndex-OD |
|---|---|---|---|
| Primary[b] | 0.4173 | 0.1817 | 0.9606 |
| Secondary[c] | -0.2439 | -0.1299 | -0.0543 |

PPE: Percentage of parasitized erythrocytes; CDIndex: BoCD4:BoCD8 Index = Percentage of peripheral blood BoCD4/ percentage of peripheral blood BoCD8; O.D.: optical density at 405 nm. [a]Based on data from fig. 1 and 3. [b]1 X $10^6$ *Anaplasma marginale* (isolate MEX-17-029-01) parasitized erythrocytes (PE) per animal at day 0. [c] 2 X $10^8$ *A. marginale* (isolate MEX-31-096-01) PE per animal at day 90.

lenge dose of > $10^8$ $ID_{100}$ but cannot clear a low level, microscopically undetectable (< $10^7$ parasitized erythrocytes per ml), persistent infection.[32,23] It should also be pointed out the strong correlation ($r$ = 0.9696) obtained between the BoCD4:BoCD8 Index and the anti-*Anaplasma* antibody titer (expressed as OD) in the first infection and the lack of correlation ($r$ = -0.0543) in the secondary infection.

This observation indicates that BoCD4$^+$ cells were cooperating in the production of anti-*A. marginale* antibodies in the primary infection.

Animals were protected against reinfection with a heterologous *A. marginale* isolate (Tizimin isolate) in spite of the fact that they had low levels of anti-*A. marginale* antibodies (Fig. 4). It is worth to mention that, in other study, this same isolate was inoculated in four naive animals which developed anaplasmosis three weeks later (García-Ortíz M. A., data not published). However, we do not know if after rechallenge even the low titers did not provide enough antibodies to inhibit a buildup of parasites in the blood by binding to the challenge dose of parasites and clearing it so efficiently that they did not induce an anamnestic response strong enough to cause an increase in antibody titer. In a previous study, IgG anti-*A. marginale* antibody titers did not significantly increase during a 33 day observation period after homologous or heterologous *A. marginale* reinfection. These observations as those of Gale et al[18] in which they demonstrated the inability of *A. marginale* immune cattle sera to confer protection in passive-transfer experiments all suggest that a cellular immune response is largely responsible for the protection observed.

Protective immunity in other rickettsial infections depends on activated macrophages phagocytizing and killing the microorganisms.[29,30] Phagocytosis is certainly an important mechanism in immunity against *A. marginale*. The

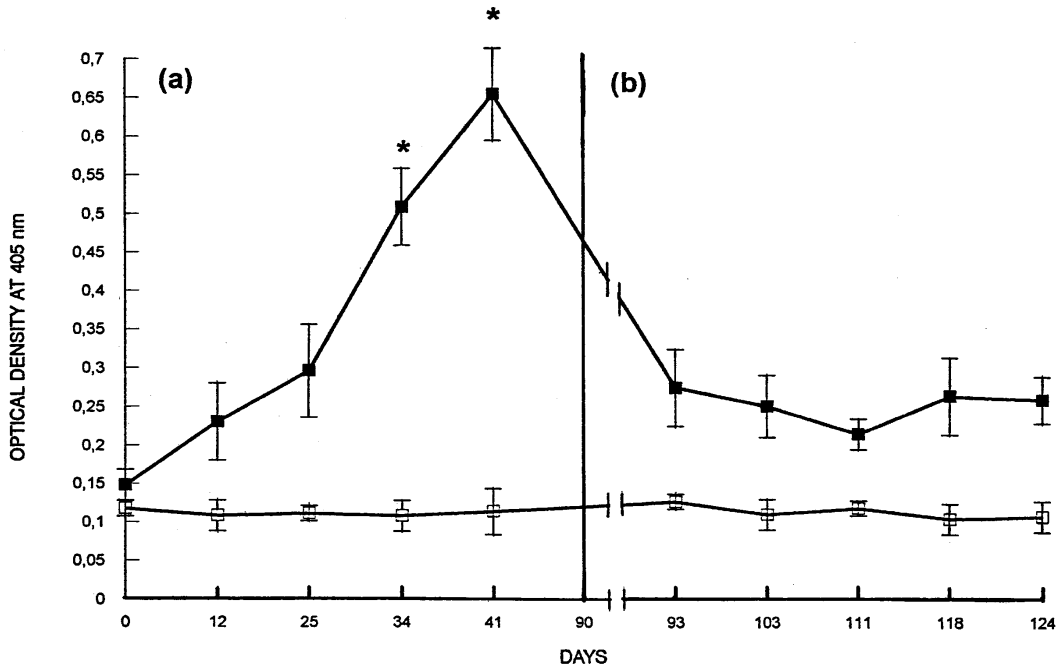


Fig. 4. Kinetics of IgG anti-*A. marginale* antibodies in bovines infected and rechallenged with *Anaplasma marginale* as determined by ELISA. (a) Infection at day 0 with $10^6$ *A. marginale* (isolate MEX-17-029-01) parasitized erythrocytes/ animal, (b) reinfection at day 90 with 2.6 x$10^8$ *A. marginale* (isolate MEX-31-096-01) parasitized erythrocytes/animal. Black squares: infected animals, white squares: noninfected animals. Each point represents the mean ± SEM of four bovines. Days 93, 103, 111, 118, and 124 are days 3, 10, 18, 25, and 31 after rechallenge, respectively. * $p < 0.01$ with respect to initial values and to days 3, 10, 18, 25, and 31 after rechallenge.

efficiency of phagocytic function is adversely affected both by splenectomy[22] and by the artificial suppression of lymphocyte functions.[12] This indicate a role for cellular immunity in its classical form in the control of anaplasmosis. An explanation to our results could be that the cellular immune response acted efficiently, possibly in the spleen, impeding the multiplication of the microorganisms after secondary infection and thus preventing significant antigenic stimulus to the humoral system. It is likely that splenic macrophages were activated by cytokines released from *A. marginale*-sensitized BoCD4$^+$ cells (probably Th1), and these in turn became armed with anti-*A. marginale* antibodies and killed and phagocytized the rickettsia as it has been proposed.[5,6,32] It has also been suggested that an antibody-independent mechanism of rickettsemia control might operate to end the acute phase of anaplasmosis.[42]

The increase over initial values of the BoCD4:BoCD8 index observed in the blood of bovines during primary infection, may indicate an increased activity of BoCD4$^+$ T-cells (note changes in the index on days 12 and 34 in Table 2). These changes suggest an important role for these lymphocytes in the immunologic control of *A. marginale* infection. It has been proposed in a similar way that the major cellular immune responses of cattle against *Cowdria ruminantium* infection are mediated in part by BoCD4$^+$ T cells.[28] In the present study, it was also noticed that there was a significant decrease with respect to initial values in the number of BoCD8$^+$ T cells, at days 25, 34 and 41 after primary infection (Table 2). This decrease may have contributed to the significant increase in the BoCD4:BoCD8 index observed on days 34 and 41 (Fig. 2). An increase in the BoCD4:BoCD8 index has been shown to occur in cattle infected with *Trypanosoma congolense* during the first parasitemia peak also.[14] We could ask if during bovine primary *A. marginale* infection BoCD8$^+$ T cells migrated from the peripheral blood to the spleen to be sensitized there and to act against the microbe? We do not at present have an answer but at the moment, we do know that in other studies it has been demonstrated that clearance of spotted fever group rickettsiae from endothelial cells requires immune CD8$^+$ T lymphocytes, which might exert an antirickettsial effect by releasing cytokines such as IFN-γ and/or acting as cytotoxic T lymphocytes.[15] Our results indicate that both of the Mexican *A. marginale* isolates used in the study are antigenically related. As however bovines immunized with a commercial inactivated vaccine against *A. marginale* (PlazVax$^R$, containing *A. marginale*, of Mississippi origin) were not protected when challenged either experimentally[11] or naturally (Figueroa J. V. personal communication) with Mexican isolates of *A. marginale* there may be considerable variation among *Anaplasma* worldwide. It is possible that the vaccine did

not have the same antigens as the Mexican isolates or of course it simply may not be an effective vaccine. An effective method for evaluating vaccines apart from the classic challenge system would be valuable. As it has been shown that Th1 lymphocytes play a major role in protection against intracellular pathogenic microorganisms, recently reviewed.[15,31] Also, it has been proposed that those antigens from *A. marginale* that induce Th1 responses (BoCD4$^+$cells) they have a potential role as protective immunogens against *A. marginale* infection.[19]

The results of this study provide additional data on bovine T lymphocyte subsets and also indicate that: 1) They could possibly be used as probes to evaluate vaccine candidates, 2) BoCD4$^+$ lymphocytes help to control *A. marginale* infection with isolates from mexican origin and help to produce IgG antibodies against the rickettsia, and 3) that antibodies play a minor role in protection against infection with mexican isolates of *A. marginale*.

In general, the findings support the notion that, in bovine anaplasmosis, the major portion of protection is conferred by cellular immune responses. However further work is required to define the mechanisms which carry out this protection.

## AKNOWLEDGEMENTS

This study was supported by grant K009-B9710 from CONACyT. Authors express their gratitude to Dr. Julius P. Kreier (Emeritus professor, retired, The Ohio State University, Columbus , OH) and to Dr. Cynthia L. Baldwin (University of Massachusetts at Amherst, Amherst, MA) for critical comments.

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