Revista Científica, FCV-LUZ / Vol. XVII, Nº 1, 7 - 14, 2007

ANALYSIS OF THE IMMUNE RESPONSE OF INFECTED BOVINES WITH *Anaplasma marginale.* IDENTIFICATION OF INFECTION STAGE MARKER ANTIGENS

Análisis de la Respuesta Inmune de Bovinos Infectados con *Anaplasma marginale.* Identificación de Antigenos Marcadores del Estado de la Infección

Salvatore Giardina, Yamilet Hartt and Alena Pance

Lab. de Bioquímica e Inmunología de Hemoparásitos, Dpto. Biología Celular, Universidad Simón Bolívar. Apdo. 89000, Caracas (1081-A), Venezuela. E-mail: sgiard@usb.ve

ABSTRACT

It is hypothesized the existence of marker antigens capable to define early (acute phase) or late (convalescent phase) infection course with *Anaplasma marginale*. Five parasitologically free hybrid calves were splenectomized and experimentally infected with a Zulia isolate of *Anaplasma marginale*. Sera samples were collected before inoculation and during the overall period of the infection. The course of infection was followed through Giemsa stained blood smears and by packed cell volume (PCV). The period of infection was divided into the prepatent phase in which no apparent infection was evident, an acute phase in which a rapid increase of the parasitemia and the hematocrit reduction were conspicuous and a convalescent phase subjectively considered as a marked decrease in parasitemia, after treatment of the infected animals with Oxytetracycline. Western blot assays and immunoplot analyses were performed. The frequency analysis of the detected antigens showed that some of them reacted with normal or prepatent sera, being non-specific cross reaction. Band 63 (p65 kDa) was specific in the acute phase. Bands 188; 68 (p70 kDa) and 58 (p60 kDa) were recognized mainly in the late or convalescent phases. These results also showed that band 18 (p19 or MSP5) was a marker for specific infection with Anaplasma.

Key words: *Anaplasma marginale*, antigens markers, infection course.

RESUMEN

Se hipotetiza que existen antígenos marcadores capaces de definir el curso de la infección con *Anaplasma marginale*, tanto durante la fase temprana (aguda) como la tardía de recuperación (convalecencia) de la infección. Cinco becerros, mestizos, parasitológicamente negativos a *A. marginale*, fueron esplenectomizados y experimentalmente infectados con un aislado Zulia de *A. marginale*. Las muestras de suero fueron colectadas antes de la inoculación y durante todo el periodo de la infección. El curso de la infección fue monitoreado a través de la determinación del hematocrito y de la parasitemia usando frotis sanguíneos teñidos con colorante de Giemsa. El periodo de la infección fue dividido en: fase prepatente, en la que la infección no era evidente, fase aguda en la que un rápido incremento de la parasitemia y reducción del hematocrito son conspicuas y la fase convaleciente, después del tratamiento del animal con oxitetraciclina y en la que se evidencia un claro descenso de la parasitemia. Los sueros colectados fueron usados para los ensayos de Inmunotransferencia y el análisis de las frecuencias de reconocimiento por inmunoplot. El reconocimiento de cada molécula mostró que hay antigenos que reaccionaron con sueros normales o prepatente, es decir, los antigenos de reacción cruzada no específica. Los antigenos marcadores específicos son la banda 63 (proteína p65 kDa) para la fase aguda y las bandas 188; 68 (p70 kDa) y 58 (p60 kDa) para la fase convaleciente. Los resultados obtenidos demuestran también que la banda 18 (p19 kDa o MSP5) es un marcador especifico de infección con *Anaplasma*.

Palabras clave: *Anaplasma marginale*, antígenos marcadores, curso de la infección.

Recibido: 11 / 07 / 2005. Aceptado: 16 / 09 / 2006.

INTRODUCTION

Anaplasmosis is a disease of cattle and other ungulates caused by *Anaplasma marginale*, an ehrlichial member of the Order Rickettsiales [5]. The infection may course in a chronic way, in which animals are asymptomatic or in an acute onset

which results in high fever, severe anemia, weakness and anorexia that can rapidly progress to death. *A. marginale* is an obliged intraerythrocytic parasite, whose cycle in the vertebrate has already been described [8]. Diagnosis of the causative organism during acute phase is easily performed by direct examination of Giemsa-stained blood smears under light microscope. However, the sensitivity of the direct diagnostic methods has not a good level of confidence for the detection of the carrier or convalescent phase of the *Anaplasma* infection. So, serologic methods are essential in diagnosis and eradication programs of the carrier stage. Several different serological techniques have been proposed: complement-fixation test [4], fluorescent antibody [18,26], capillary tube agglutination test [28], rapid card agglutination test [2], indirect fluorescent antibody test [10,17] and enzyme linked immunosorbent assay (ELISA) [11,25,30,32,33]. All these methods are highly sensitive and specific for detecting carrier stage infections. A number of studies using Western blot analysis have been conducted, allowing the identification of antigens from different North American, Japanese, Venezuelan and Brazilian isolates of *A. marginale* [1,13,14,21,22,24] using sera withdrawn at the post-peak period. Although a huge amount of different diagnosis systems has been developed during the past years, accurate differentiation between acute or convalescent infection is still complicate. In this paper it is analyzed the humoral immune response to antigens, of a Venezuelan isolate of *Anaplasma*, during the acute and convalescent periods of the infection. The identified antigens may be considered valuable markers to predicting the stage of the infection.

MATERIALS AND METHODS

Experimental animals

Five male, splenectomized, hybrid calves (Holstein - Zebu and Brown Swiss - Zebu), 6 months up to one year old, *Anaplasma marginale*-free were used. The animals were kept under animal care facilities in vector-controlled conditions.

Experimental infections

Two ml (1x10^9 parasites) of the infected blood was intravenously and intramuscularly inoculated to each calves. Heparinized blood was withdrawn from an *Anaplasma*-infected cow at the peak of parasitemia and cryopreserved under liquid nitrogen. The course of the infection was followed by determining the percentage of parasitized erythrocytes every other day by Giemsa-stained and by packed cell volume (PCV). The animals were treated with Oxytetracycline LA (200 mg/ml, according with the directions of the producer, Pfizer Corp, USA) at the peak of parasitemia

Antigen Preparation

Purified *Anaplasma* bodies were used as antigen. *Anaplasma* bodies were purified as a modification of the method described by Wunderlich et al, [34]. Briefly, erythrocytes were washed once with TS buffer [10 mM Tris-HCl, pH 7.4, 150 mM NaCl]. The buffy coat and the upper third part of the packed red blood cells [RBC] were discarded. To eliminate the contaminating bovine immunoglobulin opsonizing RBC, they were washed once with GS buffer [50 mM Glycine pH 3.5, 150 mM NaCl] and again with TS buffer. Packed RBC were resuspended in an equal volume of 10% (w/v) glycerol in TS buffer. The suspension was mixed and incubated for 2 minutes at room temperature. Then three volumes of hypotonic buffer containing protease inhibitors [TSH-PI Buffer: 10 mM Tris-HCl, pH 7.4, 20 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM α-amino-caproic acid and 1 mM Benzamidine] were added, mixed and centrifuged at 1650 x g at 4°C [Sorvall, RC-5B, GSA rotor]. The supernatant was discarded and the pellet was washed with the same buffer until the hemoglobin was eliminated. Pellet containing purified *Anaplasma* bodies was resuspended in TS-PI Buffer, aliquoted and preserved under liquid nitrogen.

Sera

Normal sera were collected from the animals before they were experimentally infected. Immune sera were collected from infected animals throughout the period of infection, from the day 2 or 3 post inoculum, in which parasitemia was higher than 2%, until the animals recovered from the parasitemia (range: 19 to 41 days). All the sera were aliquoted and stored at -20°C.

SDS-Polyacrylamide Gel Electrophoresis

Preparative SDS-Page was performed following Fairbanks et al. technique [6]. Samples were suspended in electrophoresis sample buffer (50 mM Tris-HCl, pH 7.5, 2.0% SDS, 2.0% β-mercaethanol, 15% Glycerol, 0.01% Bromophenol Blue), boiled for 5 min, and cooled on ice. The antigenic preparations were then loaded on a 10% gel with a 5% polyacrylamide stacking gel [miniprotean II, Bio Rad], electrophoresed at 50 mA, until the tracking dye reached de positive pole. The gel was stained with Coomassie Brilliant Blue and compared with molecular weight standards (Bio-Rad, broad range) or transferred to nitrocellulose filter for Western blot analysis.

Western blot

Western blot analysis of separated proteins was done following Towbin et al. method [31]. After separation, proteins were transferred to 0.45 µm pore size nitrocellulose filter (100 V, for 45 min) in transblot buffer (25 mM Tris, 190mM glycine and 20% methanol). The membranes were blocked for 2 hours with buffer A (100 mM PBS, pH 7.4, 0.05% Tween-20) with 1% gelatin, washed three times with buffer A and incubated with anti-anaplasma bovine serum diluted 1:100 in buffer A. Bound antibodies were detected with horseradish peroxidase-conjugated rabbit anti bovine IgG (Sigma Chem Co.), followed by incubation in a substrate solution containing 1.5 ml of 3

mg/ml 4-Cl-α-naphtol in methanol, 7.5 ml of PBS and 5 µl of 30% H_2O_2, until the antigenic bands became visible.

Immunoplots

Computer analyses and graphic procedures of Western blots were done according the method developed by Larralde *et al.* [15]. Bands were numbered as the midpoint of 5 kDa range.

RESULTS AND DISCUSSION

A positive serodiagnosis indicates that the infected animal has responded immunologically to the parasite. Finding markers that define the state of the infectious process allows controlling the application of efficient prophylactic systems, diminishing treatment costs and the problems associated to the generation of antibiotic resistance. Animals in acute phase would require a suitable monitoring and treatment with specific drugs, whereas an animal group in single convalescent stage requires only monitoring, since its immune system has controlled the infection and have surpassed the critical phase of the disease.

The course of the experimental infections was different in all the five animals in this study. FIG. 1 shows that there was a variable prepatent period ranging from 12 to 24 days and after this stage there was a rapid increase of parasitemia. The maximum parasitemia reached in each animal was 11% (animal Nº 5044), 15% (7099), 49% (5042) and 51%, (6147 and 7101). A carrier stage after oxytetracycline treatment was established.

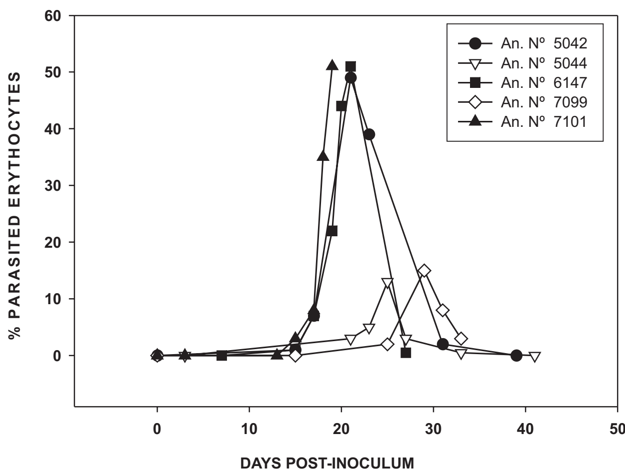

FIGURE 1. COURSE OF THE *ANAPLASMA MARGINALE*-PARASITEMIA (VENEZUELAN ISOLATE) IN FIVE BOVINES EXPERIMENTALLY INFECTED. ANIMALS WERE TREATED WITH OXYTETRACYCLINE AT THE PEAK OF PARASITEMIA / CURSO DE LA PARASITEMIA DE CINCO BOVINOS INFECTADOS CON *ANAPLASMA MARGINALE* (AISLADO VENEZOLANO). LOS ANIMALES FUERON TRATADOS CON OXITETRACICLINA EN EL PICO DE LA PARASITEMIA.

However, following the initial parasitemia, relapses appeared with successively descending parasitemia but with a similar effect on the PCV reduction.

The control of any infectious disease depends on the discrimination between infected and non-infected individuals. Direct methods are easy to perform and useful when many of the clinical manifestations are evident and pathophysiological effects are established, but often fails to identify affected individuals at the early stage of infection or at the carrier stage when the symptoms are not conspicuous. The requirement of proficient methods to detect early infections is common to many infectious diseases, in order to prevent pathophysiological damages.

Immune response

Detailed Western blot patterns of the *A. marginale* antigens recognized by sera from experimentally infected calves, beginning on the day of inoculation, are shown in FIG. 2. The polypeptide bands recognized by the various sera included antigens between 300 and 15 kDa.

The humoral immune response measured as the number of antigenic bands recognized in Western blot as a function of time post-infection shows a sigmoid behavior. There appears to be a variable lag period, in which few bands were recognized while the infection was prepatent. This period is followed by a fast increase in the number and intensity of the bands, which go along with the rise of parasitemia. Surface molecules, related with the process of recognition and invasion of the parasite to the target cell, are among those initially recognized. During the intraerythrocytic cycle of the parasite [8], the bovine immune system is challenged with an increasing number of antigens related to metabolic, proliferative and parasite destruction processes. Therefore, the number of new antigens would increase to a maximum of approximately 25. Finally a plateau is reached for all bovines, in which the antigenic recognition remained stable, except for 7101, which showed a poor immune response and died at peak of parasitemia.

The humoral immune response of the bovine to Anaplasma antigens correspondingly follows the course of the parasitemia. However, animals that are poor responders would not survive to the increase of parasitemia due to the improper control of the parasite multiplication and its pathophysiological and autoimmune consequences [9].

Immunoplots

Using Western blot we identified twenty five antigens. Fifteen of them (18, 33, 38, 43, 58, 63, 68, 73, 78, 83, 88, 103, 188, 198 and 203 kDa) were recognized by 20% of the sera from experimental infected animals. This result is compatible with those reported by others [1, 13, 14, 16, 21-23].

In order to distinguish which bands were recognized by control, acute and convalescent sera, frequency plots were

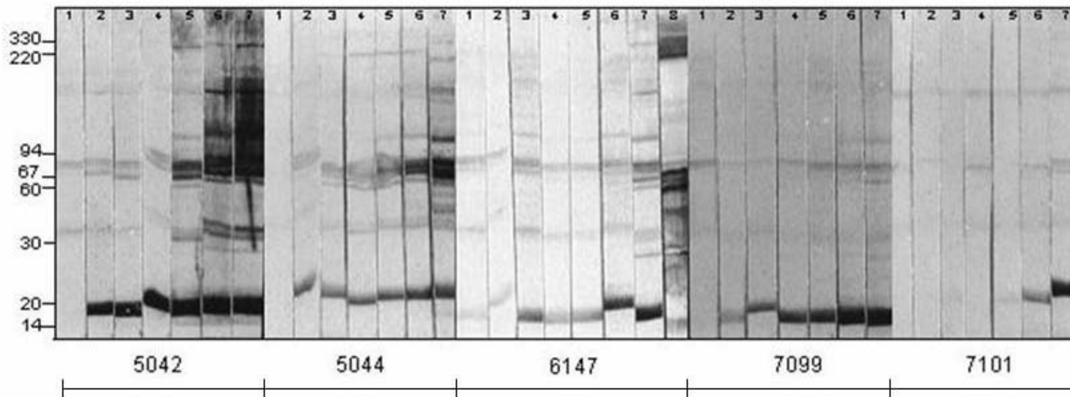

FIGURE 2. WESTERN BLOTS PATTERN OF INDIVIDUAL SERUM (1:100 DILUTION) WITHDRAWN AT DIFFERENT TIME FROM EXPERIMENTAL INFECTED BOVINES. NORMAL SERA CORRESPOND TO NO. 1, FOR EACH ANIMAL. IMMUNE SERA CORRESPOND TO STRIP Nº 2 TO 7 OR 8 FOR EACH ANIMAL. SERA WERE WITHDRAWN AT THE SAME DAYS POST-INOCULUM TO ASSAY FOR PARASITEMIA (SEE FIG. 1 FOR DETAILS). X AXE: ANIMAL NUMBER; Y AXE: RELATIVE MOLECULAR WEIGHT / **PATRONES DE SUEROS INDIVIDUALES EN ENSAYOS DE WESTERN BLOTS. LOS SUEROS (DILUCIÓN 1:100) FUERON TOMADOS A TIEMPOS DIFERENTES EN CADA ANIMAL INFECTADO EXPERIMENTALMENTE. LOS SUEROS NORMALES CORRESPONDEN A LOS Nº 1 DE CADA ANIMAL. LOS SUEROS INMUNES CORRESPONDEN A LOS Nº 2 A 7 U 8 PARA CADA ANIMAL. LOS SUEROS FUERON TOMADOS AL MISMO DÍA POST-INÓCULO PARA EL ENSAYO DE PARASITEMIA (VER FIG. 1 PARA DETALLES). EJE X: NUMERO DE ANIMAL. EJE Y: PESO MOLECULAR RELATIVO.**

performed. FIG. 3 shows the plot of the recognition frequency of bands by acute sera ($F_{(Bi)}$acute) versus recognition frequency of bands by control sera ($F_{(Bi)}$control). Bands that reacted with control sera plotted above the midline, while those bands that reacted with acute sera plotted below the midline. On the other hand, there are bands (163*, 161 and 223) which reacted only with control sera and plotted on Y-axis, although others (23*, 28* and 63*) reacted only with acute sera but not with normal sera plotted on the X-axis. Bands 38 and 83 has high recognition frequency by both control and acute sera; bands 73* and 78 have high recognition frequency by acute sera but also they are recognized by more than 50% of the control sera. The low molecular weight band 18 has a high frequency of recognition by sera from acute animal but low frequency by normal sera.

Likewise FIG. 4 shows the frequency of band recognition by convalescent sera $F_{(Bi)}$ convalescent) versus control sera ($F_{(Bi)}$control). Again, the most relevant antigens to the condition of convalescent or carrier stage of infection plotted on the X-axis, were 28*, 113, 123 and 178, 48*, 63, 103 and 278, 23* and 165 and 98*, 143, 148, 153 and 263. Those which reacted with control sera plotted above the X-axis, including the 18 polypeptide which reacted with all convalescent sera and with less than 30% of the control sera, whereas the 38 and 83 polypeptides showed the highest frequency of reaction with both the convalescent and the control sera. Other bands, such as the 73 and 188, have been recognized with high frequency by convalescent sera and with medium frequency by normal sera. The 58 and 33 bands have high frequency of recognition by convalescent sera and low with normal sera.

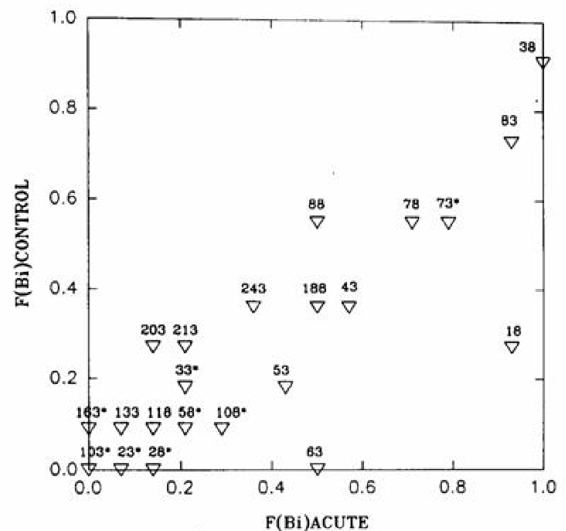

FIGURE 3. FREQUENCY OF INDIVIDUAL BAND RECOGNIZED BY CONTROL SERA AGAINST FREQUENCY OF SAME BAND RECOGNIZED BY IMMUNE SERA AT THE ACUTE PHASE. ANTIGENIC BANDS LABELED WITH * SHARE FREQUENCY WITH THE FOLLOWING BANDS: 163*: 163 AND 223; 108*: 108, 173 AND 228; 103*: 103,143 AND 278; 73*: 73 AND 198; 33*: 33 AND 68; 28*: 28, 113, 123 AND 178; 23*: 23, 48, 93, 148, 248 AND 263 / **FRECUENCIA DE BANDA INDIVIDUAL RECONOCIDA POR LOS SUEROS CONTROL CONTRA LA FRECUENCIA DE LA MISMA BANDA RECONOCIDA POR LOS SUEROS INMUNE DURANTE LA FASE AGUDA. LAS BANDAS ANTIGÉNICAS MARCADAS CON * COMPARTEN FRECUENCIAS CON LAS SIGUIENTES BANDAS: 163*: 163 Y 223; 108*: 108, 173,Y 228; 103*: 103, 143 Y 278: 73*: 73 Y 198; 33*: 33 Y 68; 28*: 28, 113, 123 Y 178; 23*: 23, 48, 93, 148, 248 Y 263.**

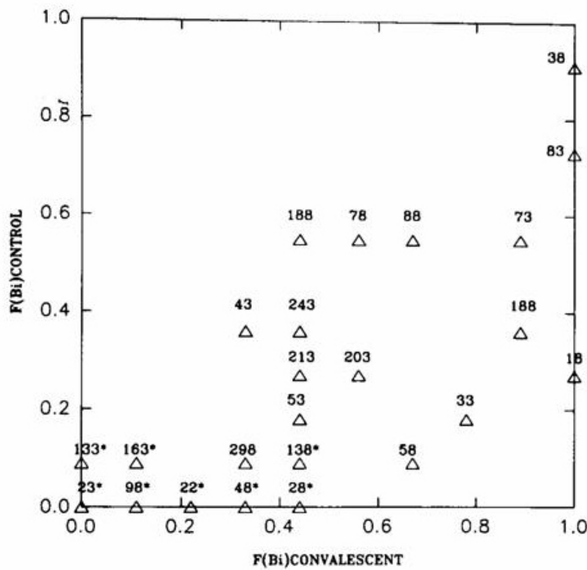

FIGURE 4. FREQUENCY OF INDIVIDUAL BAND RECOGNIZED BY CONTROL SERA AGAINST FREQUENCY OF SAME BAND RECOGNIZED BY IMMUNE SERA AT THE CONVALESCENT PHASE. ANTIGENIC BANDS LABELED WITH * SHARE FREQUENCY WITH THE FOLLOWING BANDS: 163*: 163 AND 223; 108*: 108, 173 AND 228; 103*: 103,143 AND 278; 73*: 73 AND 198; 33*: 33 AND 68; 28*: 28, 113, 123 AND 178; 23*: 23, 48, 93, 148, 248 AND 263 / FRECUENCIA DE BANDA INDIVIDUAL RECONOCIDA POR LOS SUEROS CONTROL CONTRA LA FRECUENCIA DE LA MISMA BANDA RECONOCIDA POR LOS SUEROS INMUNES EN LA FASE CONVALECIENTE. LAS BANDAS ANTIGENICAS MARCADAS CON * COMPARTEN FRECUENCIAS CON LAS SIGUIENTES BANDAS: 163*: 163 Y 223; 108*: 108, 173 Y 228; 103*: 103,143 Y 278; 73*: 73 Y 198; 33*: 33 Y 68; 28*: 28, 113, 123 Y 178; 23*: 23, 48, 93, 148, 248 Y 263.

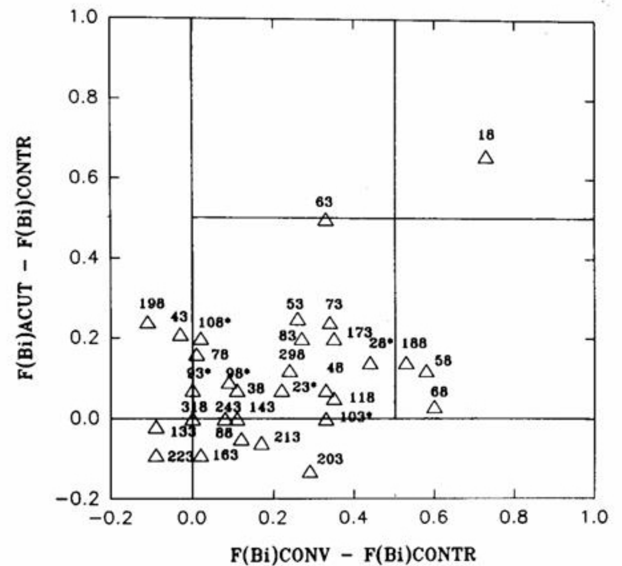

FIGURE 5. DIFFERENCE OF FREQUENCY OF INDIVIDUAL BAND RECOGNIZED BY ACUTE SERA LESS THE FREQUENCY OF RECOGNIZING OF NORMAL SERA VERSUS FREQUENCY OF THE SAME BAND RECOGNIZED BY IMMUNE SERA AT THE CONVALESCENT PHASE LESS THE FREQUENCY OF RECOGNIZING OF NORMAL SERA. ANTIGENIC BANDS LABELLED WITH * SHARE FREQUENCY WITH THE FOLLOWING BANDS: 163*: 163 AND 223; 108*: 108, 173 AND 228; 103*: 103,143 AND 278; 73*: 73 AND 198; 33*: 33 AND 68; 28*: 28, 113, 123 AND 178; 23*: 23, 48, 93, 148, 248 AND 263 / DIFERENCIA DE FRECUENCIA DE BANDAS INDIVIDUALES RECONOCIDAS POR LOS SUEROS EN ESTADO AGUDO MENOS LA FRECUENCIA DE RECONOCIMIENTO DE LOS SUEROS NORMALES CONTRA LA FRECUENCIA DE LA MISMA BANDA RECONOCIDA POR LOS SUEROS INMUNES EN LA FASE CONVALECIENTE MENOS LA FRECUENCIA DE RECONOCIMIENTO POR LOS SUEROS NORMALES. LA BANDA ANTIGÉNICA MARCADA CON * COMPARTEN FRECUENCIAS CON LAS SIGUIENTES BANDAS: 163*: 163 Y 223; 108*: 108, 173 Y 228; 103*: 103,143 Y 278; 73*: 73 Y 198; 33*: 33 Y 68; 28*: 28, 113, 123 Y 178; 23*: 23, 48, 93, 148, 248 Y 263.

By single frequency analysis (FIG. 6) it was concluded that the most relevant antigens are those of rMW 188, 88 (p86), 83 (p80), 73 (p70) and 38 (p37) kDa. Bands 38 and 83 show the highest frequency of recognition. Furthermore, control sera also recognized these proteins with frequencies higher than 0.9 and 0.7 respectively; the only plausible explanation is that animals were not seronegative, because of transplacentarial transmission of Anaplasma [26, 29]. Sera from these animals contained antibodies that recognize these bands even three days after inoculation, without any evidence of parasitemia.

Furthermore, in order to distinguish which antigenic bands are recognized by both the acute and the convalescent period but not to the normal sera, a plot of frequency differences was built. FIG. 5 shows bands which reacted with both acute and convalescent sera plotted at the upper right quadrant, in fact, there it was founded only the 18 band; likewise those bands which mainly reacted with convalescent sera plotted at the lower right quadrant. In this group it was found 33* and 68, 58 and 188 bands. This analysis allowed to make predictions regarding the possible behavior of different protein bands. The 18 the (p19 kDa), which plots in the upper right quadrant, has been cloned and expressed in the laboratory [25] and is used in diagnosis based on an ELISA assay. Using recombinant p19, Reyna-Bello *et al.* [25], found that calves experimentally infected with *A. marginale* developed serum antibodies starting day 21 after experimental infection with maximum reactivities on day 28. When sera from field animals were used, a 46.7% were positive. This correlates very well with previous results (57.7%) of seroprevalence using indirect immunofluorescence assay [12].

Band 103 (MSP1a/MSP1b complex) reacted weakly with convalescent sera but not with normal sera. The results of this work show (FIG. 6) that it elicit an immune response in less than 40% of the Venezuelan animals in the convalescent period. McGarey and Allred [19], reported that antibodies against 105 (MSP1a), 100 (MSP1b), kDa Anaplasma proteins completely or

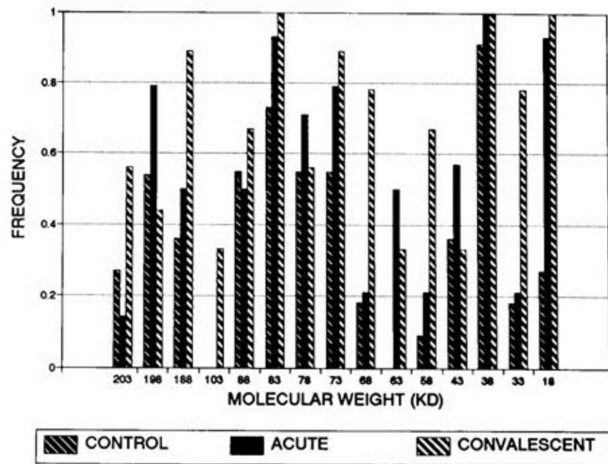

FIGURE 6. SINGLE FREQUENCY OF IMMUNE RECOGNITION OF RELEVANT BANDS BY BOVINE SERA FROM NORMAL, ACUTE AND CONVALESCENT STAGES OF ANAPLASMA INFECTION / FRECUENCIA SIMPLE DE RECONOCIMIENTO INMUNE DE BANDAS RELEVANTES POR SUERO BOVINO DE LOS ESTADIOS NORMAL, AGUDO Y CONVALECIENTE.

partially inhibited hemagglutination of bovine erythrocytes. They proposed MSP1 proteins as the adhesins. Likewise, McGarey *et al*. [20], showed that recombinant *E. coli* expressing MSP1a and MPS1b on its surface adhere specifically to bovine erythrocytes, and concluded that those polypeptides have functions as adhesins on *A. marginale* initial bodies. Recently, MSP1a and MSP1b have been reported as gut tick cell adhesins [3, 7]. Other bands 33* (p31), 68 (p70) and 58 (p60) plots in the lower right quadrant. In other studies, these polypeptides (p70, p60 and p31) bound with high affinity to the surface proteins of normal bovine, sheep, goat and deer red blood cells, but not with species refractory to Anaplasma infection (human, horse, rabbit, rat and mouse red blood cells) (Ñañez and Giardina, personal communication). This suggests that p70, p60, and p31 may have adhesin activity. These polypeptides show very low antigenic activity with normal and acute sera. They are only recognized by sera withdrawn during the convalescence period, initiated after parasitemia peak and antibiotic treatment in our experimental animals. The adhesin activity of these proteins involved in the adhesion-invasion process may explain the low inmunogenicity observed in the obtained results.

Antigens that were recognized by acute sera plotted at the upper left quadrant, there it was found only the 63 band. This band is a marker antigen for acute anaplasmosis. In the lower left quadrant it was found many bands with a common feature, which react very frequently with control sera, including the 38 polypeptide.

From the point of view of diagnosis, the ideal system should be able to differentiate an acute ascending parasitemia from a controlled convalescent stage of infection of an animal. The frequency analysis may be used to design a diagnostic system with three purified or recombinant bands, in which a positive reaction with band 18 (p19) would demonstrate specific *Anaplasma*-infection, a positive immune reaction with band 63 (p65) would indicate that the animal is in an acute stage and immune recognition of band 33 (p33), but not to band 63 (p65), would be indicative that the animal is in the convalescent period. The use of a combination of recombinant antigens may improve serodiagnosis and eventually discriminate between acute and chronic infection. Serodiagnosis is easy to perform, sensitive, specific and of low cost, comparing with direct detection of the parasite by molecular techniques. However it is necessary to test a higher number of animals and to perform infection with other isolates to confirm these results.

CONCLUSIONS

The course of the humoral immune response of several *A. marginale*-infected calves were studied using Westernblot and Immunoplot analyses.

Antigens markers for the conditions of infected cows, acute and convalescent stages were founded.

The p19 kDa (band 18) is the antigen that define the condition of *A. marginale*-infected cow.

The p65 kDa (band 63) is the antigen marker for acute stage of anaplasma infection.

The p31, p60 and p70 (bands 33, 58 and 68) are the antigens markers for the convalescent stage. The protein p60 is the heat shock protein 60 (HSP-60) and p70 is the HSP-70 of *A. marginale*.

ACKNOWLEDGEMENT

This work was partially supported by grants G-97000367 and G-98003462 from FONACIT and GID-21 from Research and Development Dean of the Simón Bolívar University.

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