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ABNORMAL BOVINE ERYTHROCYTE MEMBRANE PROTEINS AND GLYCOPROTEINS DURING AND AFTER INFECTION WITH *ANAPLASMA MARGINALE*

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The erythrocyte membrane proteins from normal and Anaplasma-infected bovine blood have been compared. Two distinct new polypeptides were present in membranes from acutely infected cells. The glycoprotein pattern was also altered: in addittion to the three main bands observed in normal cells, there were four new bands present which were glycosylated. The normally found membrane glycolypeptide (250000 D) was missing. The role of these protein alterations in relation to the infectious process is discussed.

INTRODUCTION

Bovine Anaplasmosis is a tick-transmited disease of cattle living in exposed areas. Among other hemoparasites, it represents a barrier for the proper development of cattle in tropical and subtropical regions, causing economic loss due to a marked decrease in productivity (1,2). There species of the causative agent, a rickettsia, have been described: *Anaplasma marginale*, highly virulent; A. *centrale*, first described in South Africa and less pathogenic; and *Paranaplasma caudatum*, thought to be a morphologic and antigenic variant of the first (3,4). Several attemps have been made to produce suitable vaccines, with an apparent success when using attenuted A. *marginale* (5,6) as antigen. These studies as well as those of Schroeder *et al.* (7) have pointed out that the efficiency of the vaccine seems to depend, at least partially, on the local *Anaplasma* strains present Therefore, the identification and characterization of the involved antigens appears to be of interest not only in the design of vaccines but for the better understanding of the disease. Moreover, structural modifications of antigenic relevant erythrocyte membrane proteins may result as a consecuence of the host-parasite relationship. It has been described that other hemoparasites such as *Plasmodium*

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and *Babesia* produce parasite-induced modifications on the surface proteins and glycoproteins of infected erythrocytes. The biochemical knowledge of these alterations is particulary important in Anaplasmosis since a correlation between cell-mediated immunity and protection has been shown (5). As *Anaplasma* is an intraerythrocytic living organims, the host cell membrane may play a crucial role in the development of the immune response to infection. In this study we have tried to detect such membrane protein alterations by studying the electrophoretic behavior of isolated bovine erythrocyte membranes from infected and uninfected animals.

MATERIALS AND METHODS

All the chemicals used throughout the experiments were from the highest quality availabe: Sigma, BDH, Merck, Hopkins and Williams, Bio Rad, Calbiochem and Fisher Co. The electrophoretic molecular weight standars used were from Pharmacia Fine Chemicals. *Experimental Animals.* Eleven animals (male and famale), *Bos taurus* of mixed breeds, three months to two years old were maintained under tick-free conditions. (Enormous Animals Hospital*, Veterinary Faculty,Maracay). These animals were divided into two groups. Three of them were splenectomyzed, and Anaplasmosis was induced by subcutaneous injection of 10ml of blood from an acutely infected calf. The rest of them were kept under the same conditions and used as control animals. Blood was collected intravenously into an anticoagulant solution (0.3% Citric Acid, 2.6% Sodium Citrate, 0.2% Sodium Fosfate and 2.5% Dextrose) and parasitemia was determined by examination of blood smears stained with Giemsa stain. Similarly, samples of blood were also collected from free-living animals in endemic areas (six) as well as from 'recovered' animals (five). Both groups of animals have parasitemia levels of 1 to 2%. *Isolation of Membranes.* Red cells were concentrated by centrifugation, plasma and "buffy coat" removed and then washed three times by repeated resuspension and centrifugation in bovine isotonic solution (0.125 M-Sodium Chloride, 20 mM-Sodium Fosfate, pH 7.3 at 37°C). Cells were lysed in 30 volumes of cold distilled water, membranes isolated according to Dodge *et al.* (8) and protein concentration estimated folowing the Lowry

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et al. method (9). *SDS* - Polyacrylamide Gel Electrophoresis.* The method used was esentially that described by Fairbanks *et al.* (10). The gels were stained with Coomassie brilliant Blue for protein detection. Glycoproteins were visualized using the modified periodic-acid Schiff base (PAS) procedure described by Neville *at al.*(11). The gels were finally scanned with a Beckman Model 25 spectrophotometer with an adapted linear recorder.

RESULTS

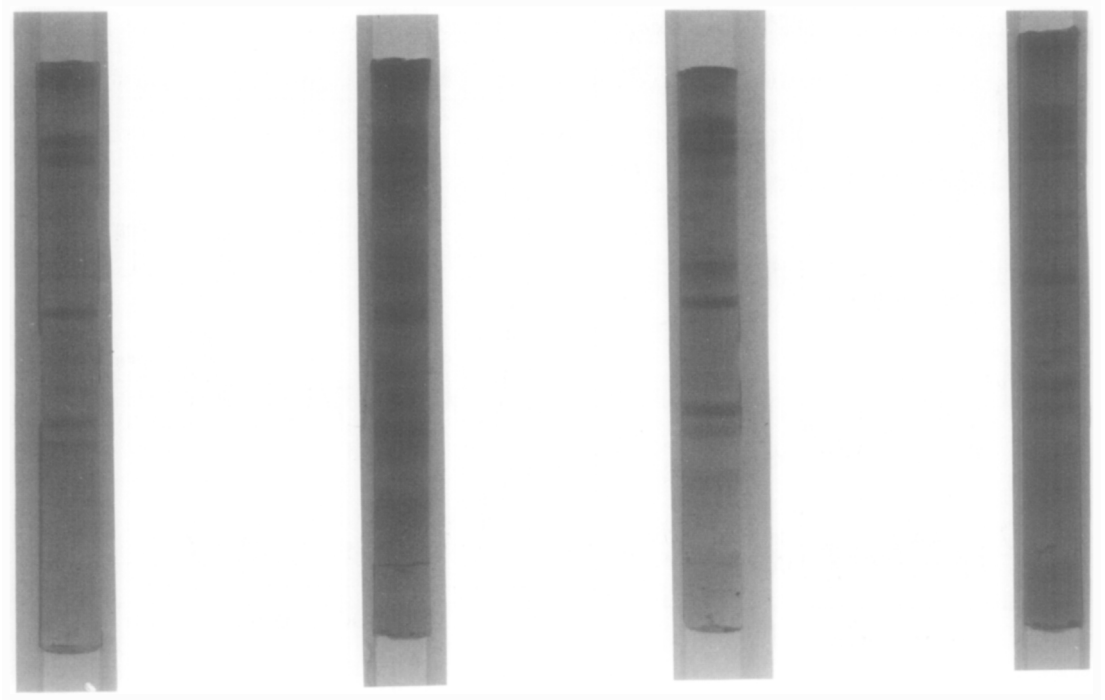
Erythocyte Membrane Proteins. For the study of the major membrane proteins present in experimental and control erytrocytes, samples of blood were simultaneously processed after 30 to 35 days of the initial *Anaplasma* inoculation. This stage corresponded to the acute phase of the disease. Under our experimental conditions the parasitemia levels obtained were between 10 and 20%.

The separation pattern obtained after SDS-polyacrilamide gel electrophoresis is shown in Figs 1A and B. Control bovine erythrocytes (Fig.1A) exhibited sixteen Coomassie Blue-stained polypeptides, with molecular weights ranging from 290000 to 21000 Dalton. The results obtained are in agreement with those described by Emerson and Kornfeld (12). We are able to resolve two low molecular weight extra bands located at the Hemoglobin region since we used hemoglobin-free ghosts erythrocytes in the present study. Electrophoresis carried out with membranes containing significant amounts of the pigment produced the expected results.

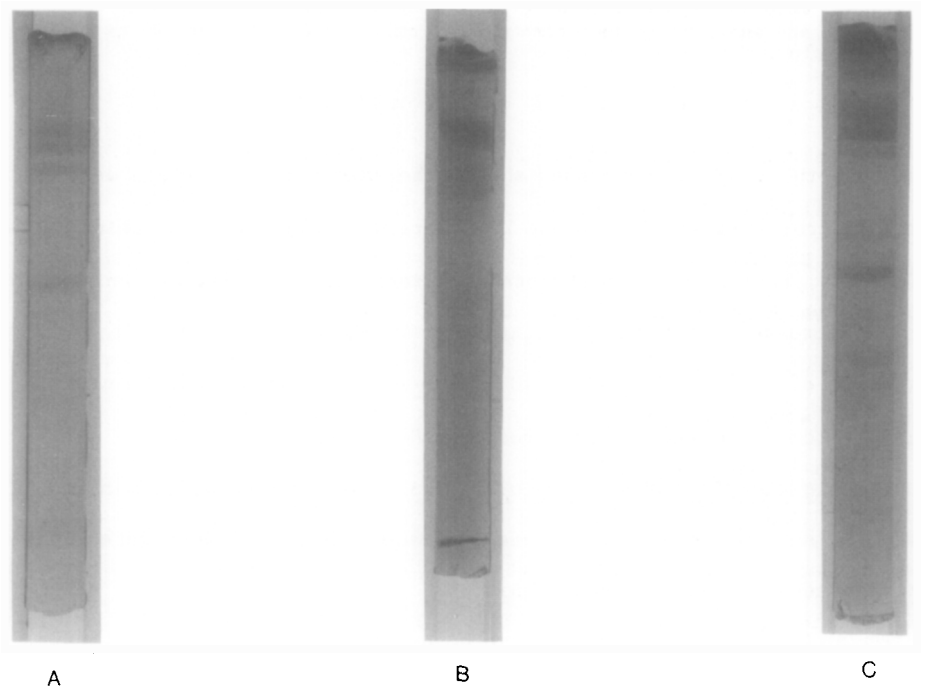
Differences were observed when infected and control cells were compared (Fig.1A and B). Two distinct new polypeptide bands were obtained with the infected cells. These polypeptides had an apparent molecular weight of 155000 and 140000 Dalton respectively (bands shown with arrows in Fig.1B). In addition, some minor alterations were repeatedly observed; bands 4 and 10 in the infected cells had a lower relative intensities (Fig.1B) than the control cells.

With the results obtained with experimentally-induced infected animals, it was decided to examine the red cell membranes of animals living in areas known to be endemic for Anaplasmosis (free-living animals) and those from animals that have survived the *Anaplasma* infection under our experimental conditions ("recovered"

* SDS: Sodium dodecyl sulphate



PHOTOGRAPHS OF COOMASSIE - BLUE - STAINED GELS



PHOTOGRAPH OF PAS - stained gels

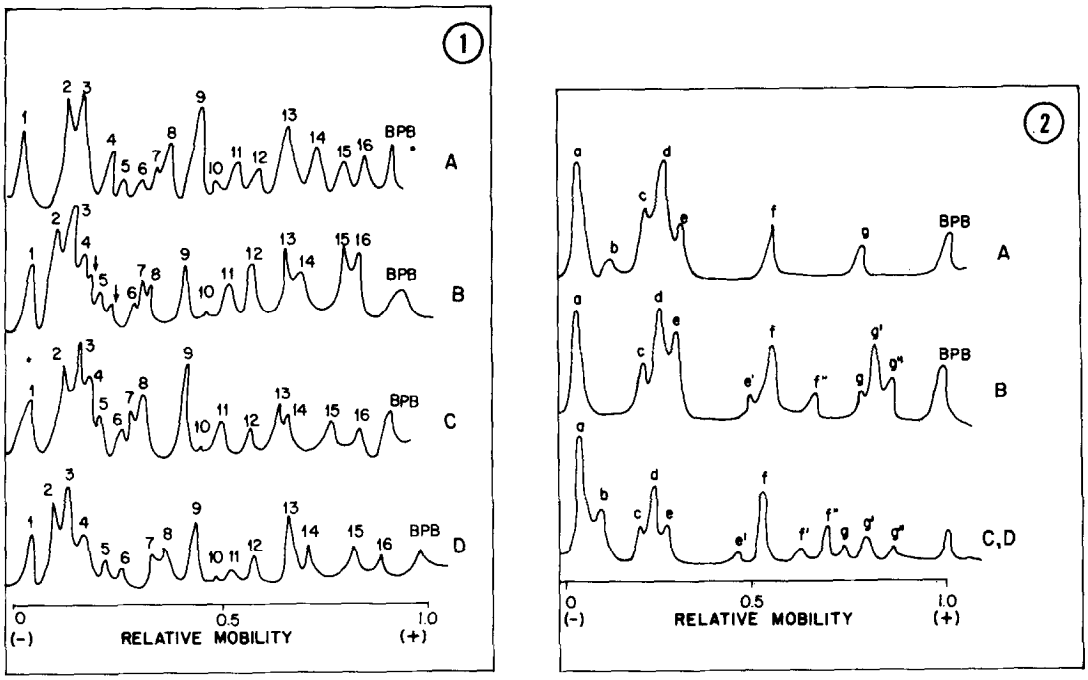


Fig. 1 Dodecyl sulphate - polyacrylamide gel patterns of bovine erythrocyte membrane proteins (200 µg) prepared from (A) Control animals (B) Experimentally infected animals (high parasitemia stage) (C) 'Free-living' animals, and (D) 'Recovered' animals; stained with Coomassie Blue and analyzed by densitometry (552 nm).

Fig. 2. Dodecyl sulphate - polyacrylamide gel patterns of bovine erythrocyte membrane proteins (400 µg) stained with Periodic Acid-Schiff Reagent and analyzed by densitometry (547 nm). (A) (B) (C) and (D) as Fig.1.

animals). Both groups of membranes showed the same general pattern described before for the control animals (Fig.1C and D). There was only one observatin that was consistently present in all membranes analyzed: the "free-living" animals showed a marked increase in the relative intensity of Band 8 (Fig.1D). The previous observation of a decrease in Band 10 in experimental animals is maintained in membranes from "free-living" and "recovered" animals.

Erythrocyte Membrane Glycoproteins. The involvement of membrane glycoproteins in such modified erythrocyte membranes was also investigated because of their relevance in raising inmunogenicity.

Gels were run under the same conditions described before except for the amount of membrane protein analyzed (400 µg). The results are shown in Fig.2. Control samples (Fig.2A) showed seven PAS stained polypeptide chains with estimated

Table I. Molecular weights of the erythrocyte membrane glycoproteins found in the three experimental animal groups. The molecular weight was estimated after SDS-polyacrylamide gel electrophoresis by comparison of the relative mobilities with those of known molecular weight proteins (Pharmacia Fine Chem.). The relationship between the Coomassie Blue and the PAS-stained polypeptides according to their calculated molecular weight is presented.

POLYPEPTIDES	GLYCOPROTEINS	CALCULATED MOLECULAR WEIGHT		
		CONTROL ANIMALS	INFECTED ANIMALS	
			'Recovered'	'Acute Phase'
1	a	300.000	300.000	300.000
2	b	260.000	240.000	—
3	c	190.000	190.000	190.000
4	d	170.000	170.000	160.000
5	e	145.000	150.000	145.000
9	e'	—	86.000	90.000
10	f	70.000	70.000	60.000
12	f'	—	56.000	—
13	f"	—	40.000	40.000
14	g	37.000	34.000	35.000
15	g'	—	27.000	28.000
16	g"	—	23.000	21.000

molecular weights from 300000 to 32000 Dalton. The three major components (a,d, and f in Fig.2A) had an apparent molecular weight of 300000, 120000 and 76000 D.

Infected cells (Fig.2B), in addition to the three main bands, showed the presence of four new bands (bands e', f", g' and g"). The glycopolypeptide g' (28000 D) seems to be present at a relative high concentation as observed from the spectrophometric scann. Band b was missing in these cells.

Erythrocyte membranes from "free-living" and "recovered" animals also showed alterations in their sugar components (Fig.2C): A total of 12 bands were detected; one of them, band f', was not present as glycoprotein in either control or experimental infected red cells.

Table I shows the relationship between the Coomassie Blue and the PAS-stained polypeptides according to their apparent molecular weights. It can be seen, that at the final stage of the infection process ("recovered" animals) the polypeptide bands 9, 12, 13, 15 and 16 were glycosylated.

DISCUSSION

The survival of parasitic rickettsias in the interior of red cells implies a series of adaptations as a result of the rickettsia-erythrocyte interaction

The adaptations may involve permanent and/or progressive alternating changes in the structure and physiology of both, the host cell and the invader. Such changes are related, in some way, to the plasma membrane or cell walls of interacting structures (13).

The alterations in erythrocyte membrane proteins in *Anaplasma* infected bovines described here seem to be related to the active presence of the rickettsia during the high parasitemia stage or to be a consecuence of the infection process itself as in the case of "free-living" and "recovered" animals. The origin of the two new high molecular weight polypeptides in acutely infected red cells is not fully understood. We suggest that they may represent the parasite itself or a temporal change, presumably of antigenic importance during the course of the infectious process. The first possiblity implies a close relationship between *Anaplasma* and the erythrocyte membrane. There are some reports suggesting that the *Anaplasma* "limiting membrane" is erythrocyte-derived (14-15) but this does not involve necessarily a permanent association. In our laboratory, electron microscopy studies of such infected cells seem to exclude a permanent interaction, at least in *Anaplasma* mature stages (S.Giardina, manuscript in preparation). In other hemoparasite-caused diseases, such as malaria and babesiosis, similar results have been described. Changes in the relative intensity, phosphorylation pattern and insertion of new polypeptides are postulated to be caused by different *Plasmodium* species (16,17). Surface proteins and glycoproteins are altered during infection with *Babesia bovis* (18). Nevertheless, the possibility of parasite contamination in our erythrocyte membranes should not be completely excluded since, at least in *Plasmodium*, another intraerythrocytic parasite, some difficulties in isolating the parasite free from contaminating erythrocyte proteins have been reported (19).

Changes in relative concentration of membrane proteins may be interpreted as result of an specific host-parasite interaction. Thus, it is possible to speculate that *Anaplasma* may have a specific protein interaction site at the red cell membranes. Penetration could be facilitated through the active attack of membranes by proteolytic enzymes. Such proteolytic enzyme have been demonstrated to act during the cell penetration in other parasitic rickettsia, *Chlamydia psittaci* (20).Although

with the present knowledge the latter hypothesis is quite speculative, we do think that our experimental results are compatible with it. The "attachment site" could involve bands 2,4 and 10, or it is possible that these bands are located on its vicinity. All these bands are glycosylated (some of them could be subunits of the same protein) and apparently represent structural modifications that remain after the acute phase of the disease.

At the present, we are conducting experiments to prove if they are surface-exposed proteins. Thus, these structural modifications could be a result of the invasion process. The other changes involved, glycosylation of Bands 9, 13, 15 and 16 could be related or associated to the coinfectious immunity described in Anaplasmosis (21,22). As it is known, in this and in other parasite related diseases, a "tolerant symbiosis" is established which allows the parasite to persist in an immonollogical hostile host (22). The complete acceptance of this hypothesis requires more experimentation.

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REFERENCES

1. Bram,R.A. (1975) Rev. Mund. Zoot. 16 : 1-5.
2. Uilemberg,G (1976) Rev. Mund. Zoot. 17 : 8-15.
3. Ristic, M. (1968) Anaplasmosis in Infectious Blood Disease of Man and Animals. S. Weinman and M. Ristic, Eds. pp: 473-542, Academic Press, N.Y.
4. Carson,C.A., Weisiger, R.M., Ristic,M., Miurnur, J.C.and Nelson,D.R. (1974) Am. J. Vet. Res. 35 : 1529-1531.
5. Ristic, M. and Nyimdo, M. (1973) Proc. 6th Natl. Anaplasmosis Conf., Las Vegas pp : 60.
6. Osorno, B.M., Solana,P.M., Pérez,J.M. and Trujillo, R.L. (1975) Am. J. Vet. Res. 36 : 631-634.
7. Schroeder, W., Rivas,C., Benítez,M. and López, B. (1971) Anaplasmosis, Prevención y Control. Ministerio de Agricultura y Cría. Centro de Investigaciones Veterinarias, Venezuela.
8. Dodge,J.T., Mitchell, C. and Hanchan, D.J. (1963) Arch. Biochem. Biophys. 100 : 119-130.

9. Lowry,O., Rosebrough, N., Farr, L. and Randall, R. (1975) J. Biol. Chem. 193 : 265-275.
10. Fairbanks, G., Steck,T.L. and Wallach, D. (1971) Biochem. 10 : 2606-2617.
11. Neville, D. and Glossman, H. (1974) Methods in Enzymology Vol XXXII pp: 92-102. Academic Press, N.Y.
12. Emerson, W. and Kornfeld, S. (1976) Biochem 15 : 1697-1703.
13. Wallach, D.F. and Schmidt-Ullrich, R. (1980) The Host-Invader Interplay, pp : 3-14.
14. Simpson, C.F., Killing, J.M. and Love, J.N. (1967) Am. J. Vet. Res. 28 : 1055-1065.
15. Francis, D.H., Kinden, A. and Buening, G.M. (1979) Am. J. Vet. Res. 40 : 777-782.
16. Yuthavong, Y., Wilairat, P., Panijan, B., Potiwan, C. and Beale, G.H. (1979) Comp. Biochem. Physiol. 63B : 83-85.
17. Chaimaneé, P. and Yuthavong, Y. (1979) Biochem. Biophys. Res. Commun. 87 : 953-959.
18. Howard, R.J., Rodwell, B.J., Callow, L.L. and Mitchell, G. (1980) J. Protozool. 27 : 241-247.
19. Sherman, I.R. and Jones, L.A. (1979) J. Protozool. 26 : 489-501.
20. Stokes, G.V. (1974) J. Bacteriol. 118 : 616-620.
21. Jones, T.C. (1974) J. Reticuloendothelial Soc. 15 : 439-441.
22. Ristic, M. (1976) Vet. Parasitol. 2 : 31-47.