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"CARA INCHADA" AND CELLULAR IMMUNITY IN CATTLE[1]

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Foram realizados estudos para verificar os possíveis efeitos da periodontite dos bezerros ("cara inchada") sobre a imunidade celular, utilizando-se a determinação da aderência, fagocitose e quimiotaxia de *Bacteroides melaninogenicus* frente a granulócitos de animais com e sem lesões da enfermidade. A aderência de *B. melaninoganicus* foi significativamente inferior quando testada frente a granulócitos isolados de bezerros afetados pela "cara inchada" (CI). Da mesma forma, a fagocitose de *B. melaninogenicus* por polimorfonucleares (PMN) apresentou-se diminuida em animais com CI. Por outro lado, frente a *B. melaninogenicus*, o índice leucotático de PMN de bezerros com CI e de animais sadios do mesmo rebanho, pareceu levemente superior quando comparado com o de animais de área indene.

TERMOS DE INDEXAÇÃO: "Cara inchada" dos bovinos, doença peridentária, periodontite, imunidade celular, Brasil.

ABSTRACT.- Attempts were made to study possible effects of the periodontal disease "cara inchada" (CI) on the cellular immunity of cattle, using adherence-, chemotaxis- and phagocytosis-determinations. Adherence of *Bacteroides melaninogenicus* to bovine granulocytes was significantly decreased in animals with CI. Phagacytosis of *B. melaninogenicus* by polymorphonuclear granulocytes (PMN) was also decreased in CI-diseased animals. Chemotaxis of the granulocytes appeared to be slightly increased in animals with CI and animals without CI coming from CI-affected herds.

INDEX TERMS: "Cara inchada" of cattle, periodontal disease, periodontitis, cellular immunity, Brazil.

INTRODUCTION

"Cara inchada" of cattle (CI) is a periodontal disease occurring in Zebu breeds in West Central Brazil. The initial CI lesions develop during eruption of praemolar teeth. The disease could lead to loss of teeth, consequently to malnutrition and sometimes to death. As to the etiology of CI a possible nutritional deficiency could not be established (Döbereiner et al. 1990, Moraes et al. 1994, Rosa & Döbereiner 1994). The findings of Blobel et al. (1984) proved the presence of black pigmented *Bacteroides melaninogenicus* and *Actinomyces pyogenes.* Bacteroides produced collagenase, sulfatase, hyaluronidase and other "spreading" enzymes (Dutra et al. 1986) of pathogenic significance. This led to further studies on adherence, phagocytosis and chemotaxis of *B. melaninogenicus* to granulocytes of CI-affected and non-affected cattle.

MATERIALS AND METHODS

Selection of animals. Calves from a farm with CI in Mato Grosso do Sul, Brazil, were taken for the studies. Of these 14 were affected with CI (I) and 14 did not show periodontal lesions (II). Furthermore, 24 healthy animals from a farm with no history of CI in São Paulo served as controls (III). All calves were in the age range between 4 and 8 months.

Preparation of granulocytes. Blood was taken by puncture of the *V. jugularis* from the above-mentioned calves. Titriplex III, (EDTA 25 mM, Merck Darmstadt, FRG) served as anticoagulant.

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The polymorphonuclear granulocytes (PMN) were isolated by Histopaque® (Sigma Deisenhofen, FRG) separation (Steigbigel et al. 1974). The remaining red blood cells were lysed by resuspending the cell pellet in 0.87% NH_4Cl, pH 9.4, followed by 1 washing in Hank's balanced salt solution (HBSS, 10 min at 1000xg). Then the suspensions of the PMN were adjusted to a final concentration of $5x10^6$ cells/ml. Their viability was checked by trypan blue exclusion (0.01% final concentration).

Adherence assays. These were conducted and evaluated by fluorescence microscopy according to the methods of Valentin-Weigand et al. (1987). Fluorescein isothiocyanate (FITC)-stained *B. melaninogenicus* (10^8 bacteria/ml) and PMN (10^6/ml) were incubated for 60 min at 37°C in a shaking water bath at 60 rpm. After washing the PMN (10 min at 1000xg) droplets of the PMN were placed on microscopic glass slides and studied under oil immersion of a Zeiss ultraviolet microscope (1000x magnification). The results were expressed as means of adherent *B. melaninogenicus*/PMN.

Chemotaxis assays. Chemotaxins were produced by incubating serum with yeast zymosan (5mg/ml, 30 min at 37°C) which cleaves the 3rd and 5th component of complement (Ward & Maderazo 1980). Chemotaxis was carried out in modified Boyden acrylic chambers, using Sartorius cellulose nitrate filters with an average pore size of 5 microns (Boyden 1962). The upper compartment of the chamber received 0.5 ml of the PMN suspension ($2.5x10^5$ PMN/ml), the lower compartment 0.5 ml of the chemotaxins (20 µl serum in 480 µl HBSS). The chambers were incubated for 2 h at 37°C in a humidified incubator. Then the filters were removed, fixed in 98% ethanol (10 s), stained with haematoxylin (3 min) and dehydrated in absolute ethanol (10 min). Subsequently, the filters were cleared in xylene (10 min), mounted on microscopic glass slides with Caedax® (Merck) and covered with a thin cover slip. The chemotactic migration was quantitated by adding the number of PMN in focus at intervals of 10 microns from the top of the filter to the distal surface. The counts were started at 20 µm to minimize errors resulting from a possible spontaneous migration. The number of cells counted at each level was multiplied by the distance of that level from the proximal surface. The products obtained were added and the sum, divided by total number of the cells, yielded the "leucotactic index"[LI] (Ward & Maderazo 1980). Suspensions of PMN without chemotactic factors served as controls.

Phagocytosis assays. Suspensions of acridinorange-labelled *B. melaninogenicus* were prepared according to the method of Verhoef et al. (1977). To 225 µl of each suspension (10^9 cells/ml), 25 µl serum were added. The suspensions were incubated for 30 min at 37°C in a shaking water bath. Then 100µl of the opsonized bacteria were added to 100µl of the PMN suspension in Eppendorf tubes. The capped tubes were rotated for 2 h at 37°C in a shaking water bath. Phagocytosis was stopped by flooding the suspension with 200 µl of a acridinorange solution (20 µl/ml) and 800 µl ice-cold HBSS. Immediately after washing the PMN (twice in ice-cold HBSS at 1000xg) the pellets were resuspended in 50 µl HBSS. Drops of the resuspended cells were studied on microscopic glass slides under oil immersion in a Zeiss ultraviolet microscope (1000x magnification). The results were expressed as means of intracellularly killed (red) and live (green) bacteria after counting respectively 50 PMN.

RESULTS

Adherence. PMN from calves with CI from an affected herd (I) yielded the lowest adherence values, followed by those calves with no peridontal lesions, but of the same herd (II). In contrast, CI-negative animals from a CI-free herd (III) yielded the highest rates of bound *B. melaninogenicus*/PMN (Table 1).

Table 1. Rates of adhered *B. melaninogenicus*/PMN

I	II	III
n=14	n=14	n=24
0.54	1.2	1.5

Chemotaxis. All PMN responded to the chemotactic gradients. PMN migration through the filter was 70 µm, spontaneous migration only 10 µm. PMN of the CI-positive calves (I) revealed an average LI of 30.1 and those from animals without peridontal lesions but from the same herd yielded an LI of 29.1 (II). PMN of the healthy control group (III) had an LI of 24.8. Thus, chemotaxis of PMN from CI-positive animals appeared to be slightly increased in comparison to that of PMN from healthy calves (Table 2).

Table 2. Leucotactic index (LI) rates[a]

I	II	III
n=14	n=14	n=24
30.1	29.1	24.8

[a]Chemotaxis: LI 20 (average migration of PMN in the filter).

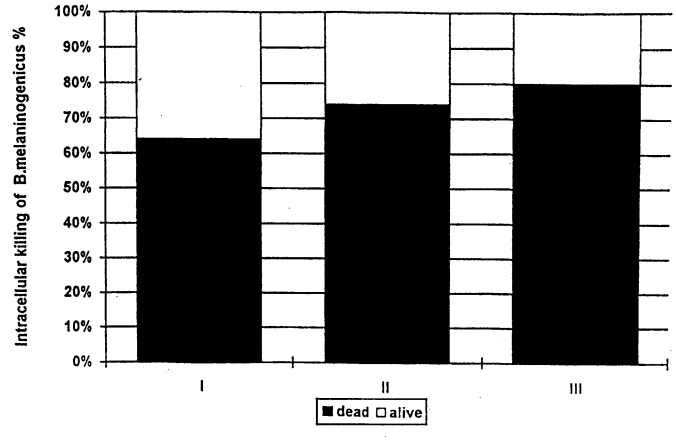

Fig. 1. Intracellular killing of *B. melaninogenicus* by PMN of CI positive-animals (I), animals from the same herd with no signs of the disease (II) and animals of the CI-negative control group (III).

Phagocytosis. The PMN from calves with CI revealed the lowest intracellular killing rates followed by those from CI-free animals of the same herd. The PMN from the CI-negative control group yielded the highest intracellular rates. (Fig. 1)

DISCUSSION

The etiology and the pathogenic mechanisms of CI are still not completely known. Phagocytic cells such as granulocytes play an essential role in host defense against bacterial

pathogens. Their decreased functions in either adherence, chemotaxis or phagocytosis could be associated with predispositions for bacterial infections. The deficiencies might also constitute a predisposition for CI, the Chediak syndrome (Tempel et al. 1972), the localized juvenile peridontitis (Van Dyke et al. 1985), or the bovine adhesion deficiency "BLAD" (Takahashi et al.1987). It was demonstrated that CI-affected animals revealed the lowest adherence of *B. melaninogenicus* to PMN. The phagocytic and bactericidal functions of PMN from these animals were found to be less effective than those of the healthy control group. Thus, it may be concluded that the deficiencies of the granulocytes in adherence and phagocytosis possibly constitute predisposing factors in the pathogenesis of CI.

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