IMMUNOLOGICAL CHARACTERIZATION OF ADULT TICK *RHIPICEPHALUS SANGUINEUS* (LATREILLE, 1806) ANTIGENS BY WESTERN BLOT ANALYSIS USING SERA FROM INFESTED OR VACCINATED DOGS AND GUINEA PIGS.

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SUMMARY: Previous results showed that feeding by adult *Rhipicephalus sanguineus* ticks as well as vaccination using unfed adult tick extract induced strong resistance in tick-bite naive guinea pigs but not in dogs, as evident by the analysis of some biological parameters of female ticks, in a challenge infestation. Sera obtained from both hosts, under these conditions were employed in Western blot analysis in order to correlate immunity to antigens of unfed adult tick extract, which had been resolved by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). Differences in antigen recognition capacity among sera of infested or vaccinated guinea pigs and dogs were observed suggesting that some antigenic tick fractions are possibly involved in the induction of protection against ticks in guinea pigs. However, further studies, employing more purified tick extracts are required.

KEY WORDS: Ixodid ticks, *Rhipicephalus sanguineus*, Western blot, dog, guinea pig, immunology.

INTRODUCTION

It is known that although many hosts acquire resistance to ticks following repeated feeding (HEWETSON, 1971; WIKEL *et alii*, 1978; GEORGE *et alii*, 1985; ABDUL AMIR, 1987), some remain susceptible to re-infection (GARIN & GRABAREV, 1972; RANDOLPH, 1979). Bearing this in mind, immunological control of ticks such as vaccination, seems to be a favored alternative. A reduced demand on anti-parasite drugs would minimize possible adverse environmental effects and decrease the selective pressure for drug resistance, thus prolonging the useful life-span of the drugs currently in use. For this purpose a better understanding of tick x host relationships at the molecular level would be useful. Moreover, the evaluation and understanding of patterns of parasite evasion together with the elucidation of host resistance mechanisms, which may arise, could provide insight into other similar studies. An important step in this direction would be the isolation and characterization of immunogenic antigens from ticks, and to these ends Western blot analysis of antigens in tick extracts has been adopted in this investigation.

Previous works showed that the tick *Rhipicephalus sanguineus* was unable to induce resistance in dogs following repeated infestations or when inoculated as crude unfed adult extract meanwhile the same species of tick, under both conditions, induced a strong resistance in guinea pigs as demonstrated by challenge infestations (CHABAUD, 1950; SZABÓ *et alii*, 1995; BECHARA *et alii*, 1994).

Therefore the aim of this work was to investigate the factors responsible for the lack of resistance of dogs to this tick species by performing a preliminary characterization of the *Rhipicephalus sanguineus* tick antigens, with the aid of sera from resistant and non-resistant hosts (guinea pigs and dogs, respectively) which had previously been infested or vaccinated or obtained from tick-bite naive hosts. It should be stressed that dogs are the natural, non-resistant, hosts of *Rhipicephalus sanguineus* ticks and guinea pigs are a quite commonly used laboratory animal model in tick immunity assays.

MATERIALS AND METHODS

Ticks

A laboratory *Rhipicephalus sanguineus* tick colony was established to supply the experiments with unfed adult ticks. Initially engorged females were collected from dogs

at the Veterinary College Hospital in Jaboticabal, Brazil. Once identified they were maintained under constant temperature and relative humidity conditions of 29°C and 80%, respectively. Continuous tick supply was maintained by feeding each instar on tick-bite naive guinea pigs.

Hosts

Two animal species were used in these experiments: mongrel dogs (*Canis familiaris*): and guinea pigs (*Cavia cutleri*). All animals were tick-bite naive. Guinea pigs weighed about 500g at the beginning of the experiments. Dogs of about six kilograms and five month of age were used. Water and commercial ration (Purina) were given "ad libitum".

Infestations

Each animal species was subjected to three infestations using unfed adult ticks *Rhipicephalus sanguineus*. Every infestation, one month apart, consisted of four female and five male ticks in the case of rodents and 25 females and 30 males in the case of dogs. Ticks were placed inside a feeding chamber consisting of a plastic tube, 2.5 cm in diameter and 3 cm in length, glued on the previous day to the shaved back of the hosts. Chambers placed on dogs had twice this diameter. Neck collars were used to prevent grooming and the escape of ticks during the experiments was prevented by maintaining the animals in cages which were placed on trays surrounded by a gutter filled with a suspension of water and oil.

Tick extract

Approximately 500 unfed adult ticks *Rhipicephalus sanguineus* were obtained from the colony. They were killed by immersion in liquid nitrogen, homogenized with a ground glass homogenizer in Phosphate Buffered Saline (PBS-pH 7.4) and then sonicated for 10 seconds 3 times and once for 60 seconds (intensity of 20 MHZ). This extract was centrifuged at 4°C for one hour at 12,000 g, the supernatant filtered through a 0.22 mm Millex-GV (Millipore) filter and stored at -40°C until use. The protein concentration determined according to Lowry *et alii* (1951) was of 2.5 mg/ml.

Vaccination of hosts

The immunization was similar for both dogs and guinea pigs. Briefly, inoculation with the unfed adult extract (UAE) was performed by subcutaneous route, three times at 15 day intervals. Guinea pigs were given a suspension containing 125mg of UAE and 50mg of saponin as adjuvant (Quil A, Superfos Biosector A/S, Denmark) diluted in PBS to a final volume of 1.0 ml per animal. Dogs were immunized with a double dose of protein.

Challenge infestation

Fifteen days after the last inoculation all vaccinated hosts were submitted to a challenge infestation. A control, tick-bite naive and non-vaccinated group of animals was also included. Infestations were performed as previously described.

Immune serum collection

Blood samples were collected from two dogs and four guinea pigs a week after the detachment of the last female tick in each infestation or, from the same number of hosts, a week after each immunization with the UAE. Blood was allowed to clot at 40°C for 1 h, refrigerated overnight and serum collected and stored at -40°C until required.

Western blot analysis

Initially 400 ml of UAE (2.5 mg of protein/ ml) was resolved by 1% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) according to LAEMMLI (1970). The concentration of the stacking and running gel was of 5% and 10%, respectively. The following protein standards (HMWSM - Sigma) were run at the same time: Miosin 205 KDa, Betagalactosidase 116 KDa, Fosforilase B 97 KDa, bovine Albumin 66KDa, egg albumin 45 KDa and Carbonic Anidrase 29 KDa. A mini gel system was adopted.

Western blot analysis was then performed according to the procedure of TOWBIN *et alii* (1979). Briefly, separated proteins were electrophorectically transferred to nitrocellulose paper which was then cut in 20 strips. Each strip, except for the standard, was incubated with a test serum for 2 hours. For the development of the blot, nitrocellulose strips were then incubated for 2 hours either with an anti-dog or anti-guinea pig IgG conjugated to alkaline phosphatase (diluted 1:20) and finally in its substrate (naphtol 2 mg, dimetilphormamide 0.2 ml, Tris 0.1 M pH 8.2, 9.8 ml, fast red 10 mg) for 5 minutes. Positive results were characterized by the appearance of red bands on the strips. The following test serum and respective dilutions were employed:

i) non-infested dog serum (1:20 and 1:80);
ii) three times infested dog serum (1:20, 1:40 and 1:80);
iii) vaccinated dog serum (1:20, 1:40 and 1:80);
iv) vaccinated and challenged dog serum (1:20 and 1:80);

v) non-infested guinea-pig serum (1:20 and 1:80);
vi) three times infested guinea pig serum (1:20, 1:40 and 1:80);
vii) vaccinated guinea pig serum (1:20, 1:40 and 1:80);
viii) vaccinated and challenged guinea pig serum (1:20 and 1:80).

RESULTS

Results displaying biological parameters of female ticks in challenge infestations following vaccinations or previous infestations are published elsewhere (BECHARA *et alii*, 1994; SZABÓ *et alii*, 1995).

The antigen-profile recognized by the sera of the hosts is illustrated in the Fig. 1. Many antigens of *Rhipicephalus sanguineus* were recognized by most of the sera and showed a wide range of molecular weights (45 to 205 KDa).

Antigens were not detected by sera from tick-bite naive and non-vaccinated dogs. Sera from naive and non-vaccinated guinea pigs, however, identified three faintly staining antigen groups (40, 66 and 205 KDa).

Following three infestations, sera from dogs recognized groups of faintly staining antigens of molecular weights 42, 66, 97, 116 and 205 KDa. Following three infestations, guinea pig serum recognized antigens of molecular weights 35, 40, 66, 116 and 205 KDa.

Vaccination of hosts induced antibodies that recognized tick antigens of the following molecular weights: 40, 43, 57, 66, 105, 116 and 205 KDa (dogs) and 30, 35, 57, 66, 97, 105, 116 e 205 KDa (guinea pigs).

Sera from dogs initially vaccinated and challenged by infestation recognized the same antigens as dogs that had only been vaccinated but also recognized an antigen of 35 KDa . Similarly guinea pig vaccinated then challenged by infestations, again recognized the same antigen-profile

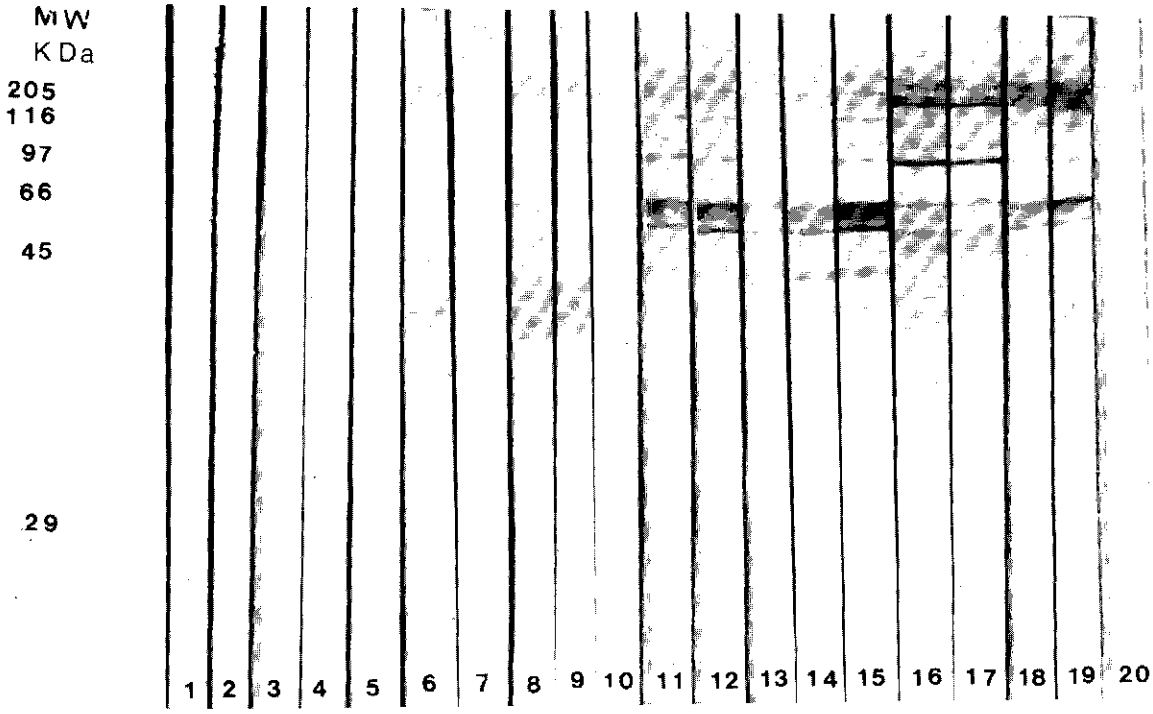

Fig. 1 - Immunoblots of unfed adult *Rhipicephalus sanguineus* extract separated by SDS-PAGE. Immunological reactions were obtained with the following serum:

1-noninfested dog serum (1:20)
2-noninfested dog serum (1:80)
3-three times infested dog serum (1:20)
4-three times infested dog serum (1:40)
5-three times infested dog serum (1:80)
6-noninfested guinea pig serum (1:20)
7-noninfested guinea pig serum (1:80)
8-three times infested guinea pig serum (1:20)
9-three times infested guinea pig serum (1:40)
10-three times infested guinea pig serum (1:80)
11-vaccinated and challenged dog serum (1:20)
12-vaccinated and challenged dog serum (1:80)
13-vaccinated dog serum (1:20)
14-vaccinated dog serum (1:40)
15-vaccinated dog serum (1:80)
16-vaccinated and challenged guinea pig serum (1:20)
17-vaccinated and challenged guinea pig serum (1:80)
18-vaccinated guinea pig serum (1:20)
19-vaccinated guinea pig serum (1:40)
20-vaccinated guinea pig serum (1:80)

identified by sera from guinea pigs receiving vaccination alone. The recognition seemed to be much more intense in challenged hosts.

It is noteworthy that there were differences in reaction intensity and recognition of antigens if sera of vaccinated or infested hosts were used. Sera of dogs, in particular, recognized more antigens and reacted much more intensely following vaccination.

DISCUSSION

One of the reasons for the immunological characterization of the antigens of the tick *Rhipicephalus sanguineus*, using the Western blot analysis was to generate information about immunogenicity and antigenicity of these tick antigens when inoculated into the host through natural contact (infestation) or vaccination. BROWN (1988) and RUTTI & BROSSARD (1989) used similar analysis to determine which antigens were capable of inducing resistance in the hosts. Furthermore, WHELEN *et alii* (1984) also considered this assay to be of importance because it can provide preliminary data about molecular aspects of tick x host relationships. However, the main goal of the present analysis was to attempt to identify parameters that may be important in understanding the lack of resistance inducement by this tick species in its natural host, the dog. Guinea pigs were used because they could provide information about host resistance mechanisms that may be suppressed in the dog due to specific evasion and suppression mechanisms of the parasite, developed throughout the long coexistence of this tick species and its natural host (HOOGSTRAAL, 1956; THEIS & BUDWISER, 1956).

Unfortunately comparisons of the results are rather difficult to perform due to the intense reactivity of serum from the hosts with many tick antigens mainly between proteins ranging from 45 to 205 KDa, nevertheless some preliminary observations can be made.

The results suggested that there are many different patterns of antibody production following vaccination and infestation. Sera from tick-bite naive dogs did not recognize tick antigens whereas sera from guinea pigs did recognize some tick antigens suggesting cross reactivity between tick and unrelated antigens in this rodent. In a previous study SZABÓ *et alii* (1995) observed that ticks *Rhipicephalus sanguineus* laid smaller egg masses when fed on tick-bite naive guinea pigs if compared to those obtained from ticks fed on tick-bite naive dogs. Tick antigen cross reactivity in guinea pigs may account for this difference.

Although Western blot analysis is not quantitative, it was interesting to observe a much more intense reactivity of sera from animals - both dogs and guinea pigs - vaccinated with UAE than that of sera from animals infested on three occasions. Vaccination also led to the recognition of a greater number of tick antigens by both dogs and guinea pigs. This may be explained on that when ticks were sonicated before inoculation, this would expose animals to antigens not presented to host tissues following infestation of the skin.

Although use of the mini gel proved to be difficult due to detection of antigens of similar molecular weights, four main conclusions could be drawn from this study; i)-*Rhipicephalus sanguineus* antigens are highly immunogenic in guinea pigs irrespective of the inoculation route; ii)-*Rhipicephalus sanguineus* antigens, although antigenic to dogs seem to be less immunogenic in this species as although they are recognized by sera following vaccination they are unable to induce resistance to re infestation; iii)-vaccination presents more antigens to the host than infestation, as seen by a more intense reaction and detection of more antigens and iv)-dogs responded to tick antigens following vaccination but not following infestation. However, even then they did not develop resistance implying a high degree of tolerance of this species to the presence of *Rhipicephalus sanguineus* antigens. Further studies are required to understand the lack of resistance induced by the tick *Rhipicephalus sanguineus* following dog infestation.

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SUMÁRIO

O parasitismo pelo carrapato adulto *Rhipicephalus sanguineus*, assim como a imunização utilizando extrato de carrapato adulto não-alimentado induzem forte resistência em cobaias, mas não em cães sem contato anterior com o

carrapato, como fica evidente pela análise de alguns parâmetros biológicos de carrapatas em infestação desafio. Soro obtido destes hospedeiros, vacinados ou infestados, foi empregado em ensaio "Western blot" para se correlacionar imunidade a antígenos do extrato de carrapato adulto não-alimentado, separados eletroforeticamente em gel de poliacrilamida (SDS-PAGE). Diferenças foram observadas quanto à capacidade de reconhecimento dos antígenos de carrapatos entre soros de cobaias e cães vacinados ou infestados sugerindo que algumas das frações antigênicas possam estar envolvidas na indução de resistência contra carrapatos em cobaias. Entretanto, estudos adicionais com extratos mais purificados de carrapatos se fazem necessários.

PALAVRAS-CHAVE: carrapatos ixodídeos, *Rhipicephalus sanguineus*, Western blot, cão, cobaia, imunologia.

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