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FINAL REPORT TO THE U.S. AGENCY FOR INTERNATIONAL DEVELOPMENT

PROGRAM IN SCIENCE AND TECHNOLOGY COOPERATION

Project: Control of ticks and tick-transmitted diseases
by vaccination of the host (DPE-5542-G-SS-4032-00)

May 1984 to November 1988

by Omar O. Barriga, Principal Investigator.

This project had two major objectives:

1. To initiate studies toward the production of a vaccine against ticks that infest ruminants (Research component), and

2. To train a Dominican scientist in the corresponding biotechnology and promote the establishment of a competent research research group in the Dominican Republic (Training component).

This report summarizes the progress made during the tenure of the grant

1. RESEARCH COMPONENT

1.1. Establishment of a valid laboratory model

Until we initiated this project, most of the research on anti-tick immunity had been done with laboratory rodents. We explained in our original proposal why these models are inadequate to predict immunity against ticks in ruminants. Studies with cattle have been done only recently but the difficulties of keeping bovines under well defined conditions and the cost involved make this approach an unlikely solution for long-term or detailed studies. We decided that only work with ruminants could authentically represent ruminant immunity, and that sheep or goats were the most convenient hosts for these studies. We selected sheep because the cost of the research was almost the same as with goats but sheep are economically more important.

Production of adult ticks, conducting 4-5 successive infections in sheep, performing polyacrylamide gel electrophoresis and Western blots, and analyzing the results commonly takes about a year. Restrictions in the budget prevented us from performing more than one experiment at a time. Also, in April 1986 Dr. Glen Needham, co-investigator in the project who was in charge of producing ticks and conducting sheep infestations in the Department of Entomology, resigned from the project. This forced us to move tick production, and sheep housing and infestation to the veterinary college.

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1.2. Setup of tick production and sheep infestations in the PI's laboratory

Between May and July 1986 we moved the tick rearing and sheep maintenance and infestation to the veterinary college. Since we did not have the incubators required for tick production nor funds to purchase one, we adapted a cell incubator in the PI's laboratory to regulate humidity and hours of light and darkness, and initiated tick rearing in it. We are happy to report that this adaptation worked very well and that we produced as many ticks as needed with no difficult.

We also ordered the construction of a metabolic cage to keep sheep during infestation (to avoid smashing of the ticks and contamination of the premises). Although not very comfortable for the researchers, it worked very well for the sheep. The animals were maintained at constant temperature and light-darkness cycles in the animal rooms of the veterinary college. Although a bit primitive, from the research point of view our tick production and sheep infestation operations worked impeccably.

1.3. Determination of tick parameters effected by host's immunity

A primary consideration in the study of anti-tick immunity is to be able to measure it accurately. Since no studies had been done on immunity of sheep to tick infestations, we had no previous studies to guide us. On the other hand, the reported effects of host's immunity with rodents or cattle subjected to infestations by different species of ticks were so inconsistent among the different host-parasite associations that provided little help. We decided, therefore, to establish the parameters of our sheep-*Amblyomma* model by studying the modifications of the tick population in actual successive infestations. In each infection we recorded the time that took for the ticks to complete their meal (time to detachment), number of ticks alive at detachment (survival to detachment), weight of ticks at detachment, time that took for the engorged ticks from detachment to the beginning of laying eggs (time to oviposition), number of ticks alive at the beginning of oviposition (survival to oviposition), weight of the egg mass at the end of oviposition, time that took for the first larva to appear (time to first larva), and percentage of egg batches that actually hatched (egg survival). These values were recorded for each tick, analyzed statistically, and compared among infestations.

1.4. Manifestations of resistance of sheep to *Amblyomma ticks*

For verification of the production of resistance of sheep to *Amblyomma* infestations, we produced five succesive infestations in 3 sheep and analyzed the characteristics of the ticks after each infestation. It was found that virtually all the recorded characteristics of the tick populations were affected by immunity. On average, the times to detachment and to oviposition increased significantly by the fourth infestation, the weight at detachment decreased by the fourth infestation, and the weight of the egg mass decreased regularly by the third and subsequent infestations (Figure 1). The conversion of engorged tick mass to egg mass also diminished regularly from the third infestation (Figure 2). Survival to detachment decreased only slightly by the fourth infestation but survival of ticks to oviposition and survival of eggs decreased very markedly by the fourth infestation.

None of these changes was expected to be due to climatic conditions because ticks and sheep were kept in constant environments. Still we correlated the variations of the tick populations with the season of the years and could verify that indeed they could not be attributed to weather conditions (Figure 4).

The final conclusion was that sheep develop an immune resistance against *A. americanum* that inhibited the feeding, fertility, and survival of the arthropod. We also found that the resistance needed on average three or four infestations to become effective, and that it did not eliminate the infestation totally. These latter results were expected because ticks would not exist or would be only a minor problem if infestations were able to produced strong immune resistance. As with other parasitic diseases, the goal of an anti-vaccine is to improve on, and not only imitate, the resistance that occurs in nature.

1.5. Determination of potential antigens

Most of the early work on anti-tick immunity focused on salivary antigens because the way in which ticks feed suggested that these should be immunologically active materials. Because it is known that tick salivary glands enlarge and acquire secretion granules during the first three days of feeding, it is likely that the unfed gland has different antigens than the glands of ticks that have fed for three days. Also, on a few occasions researchers had reported successful immunization with extracts of tick intestine. For these reasons we decided to compare the protein composition of extracts of unfed and three-day fed salivary glands and intestinal tract. Polyacrylamide gel electrophoresis (that shows proteins in general) revealed so many components in each extracts that it was difficult to judge differences. We did not insist on this line of work, however, because we were interested only in those proteins that were antigenic during normal infestations.

1.6. Determination of antigens reactive in successive infestations

Our original hypothesis was that protection-inducing antigens should be recognized only when protection becomes effective. Because the study of tick parameters in successive infestations had shown that anti-tick protection appeared only after two or three infestations, we expected that the sheep reacted to the protection-producing antigens only at this time. To verify our hypothesis, we reacted extracts of unfed and fed salivary glands and intestinal tract with serum taken before and after evidences of protection appeared, and compared the antigens revealed by each serum. The antigens related to protection are reviewed below (see 1.8.). We also prepared a graphic with all the *Amblyomma americanum* antigens recognized by our sheep (Figure 10).

1.7. Manifestations of immunosuppression in *Amblyomma* infestations

Our results showed that infestation of sheep with *A. americanum* depresses the ability of the host to mount an immune response. The ticks of sheep 1 and 3 actually completed their feeding and laid eggs sooner during the second and third infestations that during the first. They also took more blood and laid more eggs during the second and third infestations that during the first (Figure 1). In all sheep the conversion of engorged tick mass to egg mass was more favorable in the second infestation than in the first (Figure 2). A phenomenon of immunosuppression would be so important to the production of a vaccine that we wanted to verify this even further. Sera taken after each infestation were studied by ELISA against salivary gland extract. We found that the titers of specific antibodies increased after the first infestation but decreased regularly from the

second infestation in all sheep. Since one would expect that, in the absence of immunosuppression, successive infestations would induce secondary responses with an important increase of titers, this finding indicates that the infestations interfered with the production of specific antibodies.

The verification of immunosuppression in tick infestations changed considerably the possible approaches to an anti-tick vaccine. Now we must be concerned not only with the tick materials that produce immune protection but also with those that oppose the production of immunity.

1.8. Identification of protective and suppressive material

As explained above, we had reasons to believe that unfed salivary glands, 3-day fed glands, and intestinal tract might contain different tick antigens. Since there was not reliable information about what antigens could be protective, we decided to compared the protein composition of these three sources to see whether large differences were evident. We performed polyacrylamide gel electrophoresis of the respective extracts and found only minor differences (Figure 6). We then performed Western blots (that reveal only antigens rather than all proteins) with the sera taken after each infestation, and compared the antigens that were recognized by the host on each occasion. Careful comparison of all sera showed that four antigens (115,000, 38,000, 36,500, and 34,000 MW) reacted very strongly with the post-first infestation sera but became weaker or disappeared in later infestations (Figures 7a and 7b). Because they follow very closely the pattern of immunosuppression as revealed by antibody titers, we believe that they are related to suppression (suppression-associated antigens).

We also found that two antigens (35,500 and 29,000 MW) were recognized only when resistance appeared (as revealed by ticks parameters): with the post-third infestation sera in sheep 1 and 3, and with the post-second infestation serum in sheep 2 (Figure 7b). Because of the consistent association between resistance and presence of these antigens, we believe that they are related to resistance (protection-associated antigens).

1.9. Purification of selected antigens

We believe that the identification of suppression- and protection-associated antigens is a major breakthrough in anti-tick immunity and a crucial step toward the production of a vaccine. The obvious next step is to purify these materials and assay their biological activity. Because the protective and three of the suppressive antigens are too close in molecular weight, purification based on molecular size exclusion can be used only as a preparative step. We expect, however, that ion-exchange, or isoelectric point separations, or their combinations, will achieve satisfactory separation.

We did not have the equipment, budget, or time to assay these methods but we decided to try elution from electrophoresis gels. Because we cannot rely on attainment of antigens from ticks for the formulation of a commercial vaccine, at the end small quantities of the pertinent antigen will have to be obtained very pure to replicate it by chemical synthesis or DNA recombination. Elution from gels would be a perfectly adequate method for these purposes. To test this procedure we performed an electrophoresis of salivary gland extract and cut off the portion of gel between 48,500 and 36,500 MW. We homogenized, incubated in saline overnight, and centrifuged this portion, and used the supernate to run Western blots against post-four infestation serum in parallel with the crude extract. Figure 8 shows that the procedure worked very well.

Although methods with a better yield may be more convenient for attaining antigens for preliminary vaccinations, we believe that we have solved the problem of final purification of the protective antigen.

1.10. Cross reactivity between *Amblyomma* and *Boophilus* antigens

One of the original hypothéses in our project was that different species of ticks may have antigens in common and that these materials might be protective. *Boophilus* ticks were collected from infected cattle in the Dominican Republic and their salivary glands were obtained. Sera from a heavily infected cow (presumably highly susceptible to ticks) and an older cow with few ticks (presumably resistant to tick infestation) were also collected. The glands and the serum were shipped to the United States for further study. Extracts of the glands were examined by Western blot against post-first or fourth *Amblyomma* infestation sera in parallel with *Amblyomma* extracts. Unfortunately the *Boophilus* extract produced unsatisfactory results, probably because of contamination of the glands with host's blood or degradation during shipment. We will have to obtain more Boophilus glands. The results, nevertheless, showed cross-reactivity of a number of antigens which included the protection-associate antigen of 29,000 MW.

We also performed Western blots with extracts of *Amblyomma* and *Boophilus* glands against the sera of *Boophilus*-infected cows. The serum of the susceptible cow recognized three specific antigens, and that of the resistant cow five, in the *Amblyomma* extract. Although the results suffered because of the low quality of *Boophilus* glands, the serum of the susceptible cow recognized two antigens (130,000 and 20,000 MW) in the *Boophilus* extract that were not recognized by the serum of the resistant cow. These may be suppression-associated materials. Conversely, the serum of the resistant cow recognized three antigens (29,000, 50,000, and 53,000 MW) in the *Boophilus* extract that were not recognized by the serum of the susceptible cow. These may be protection-associated antigens. It is particularly exciting that one of these antigens (29,000 MW) appears to be the same protective antigen found in *Amblyomma*. This experiment must be repeated with more sera and better quality *Boophilus* extracts, however.

In summary, we believe that the research component of the project has produced excellent results that are tremendously encouraging to continue work toward the production of an anti-tick vaccine.

We have enclosed a copy of the manuscript submitted for publication to the Journal of Parasitology that reports some of our findings.

2. TRAINING COMPONENT

The Dominican trainee, Dr. Francisco Andujar, completed his training in English as a Second Language (which was required by the Ohio State University Graduate School for admission), applied and was accepted to the Graduate School, and did very well in his courses.

As his training in the laboratory progressed, he gradually took over the performance of the experiments and he became fully proficient in the theory and practice of the techniques used before graduation.

In 1987, he completed his graduate work at the Ohio State University, wrote his Master dissertation, defended it, and obtained his Master of Science degree in Immunoparasitology.

When he returned to the Dominican Republic, he was completely proficient in the techniques we used in this project, and had been properly trained in the design, analysis, and interpretation of research in animal health. I believe that he is fully capable to continue his research in the Dominican Republic and expand to other areas of animal health if he so desires.

Dr. Andujar is currently working with the Central Veterinary Laboratory of the Dominican Secretary of Agriculture as a tick specialist, and teaching parasitology in two of the four veterinary schools in the Dominican Republic: He has formed a research team with two colleages of in the Dominican Secretary of Agriculture: Dr. Julio Concepcion, pathologist, and Dr. Joselyn Quirico, parasitologist. Dr. Concepcion and Quirico are former graduate students of mine. The last I knew was that they had built a special facility at the Veterinary Central Laboratory to work with tick infested cattle and were actively looking for support to continue tick research with *Boophilus* ticks and cattle.

For what concerns the American participation, the training of Dr. Andujar was an unqualified success.

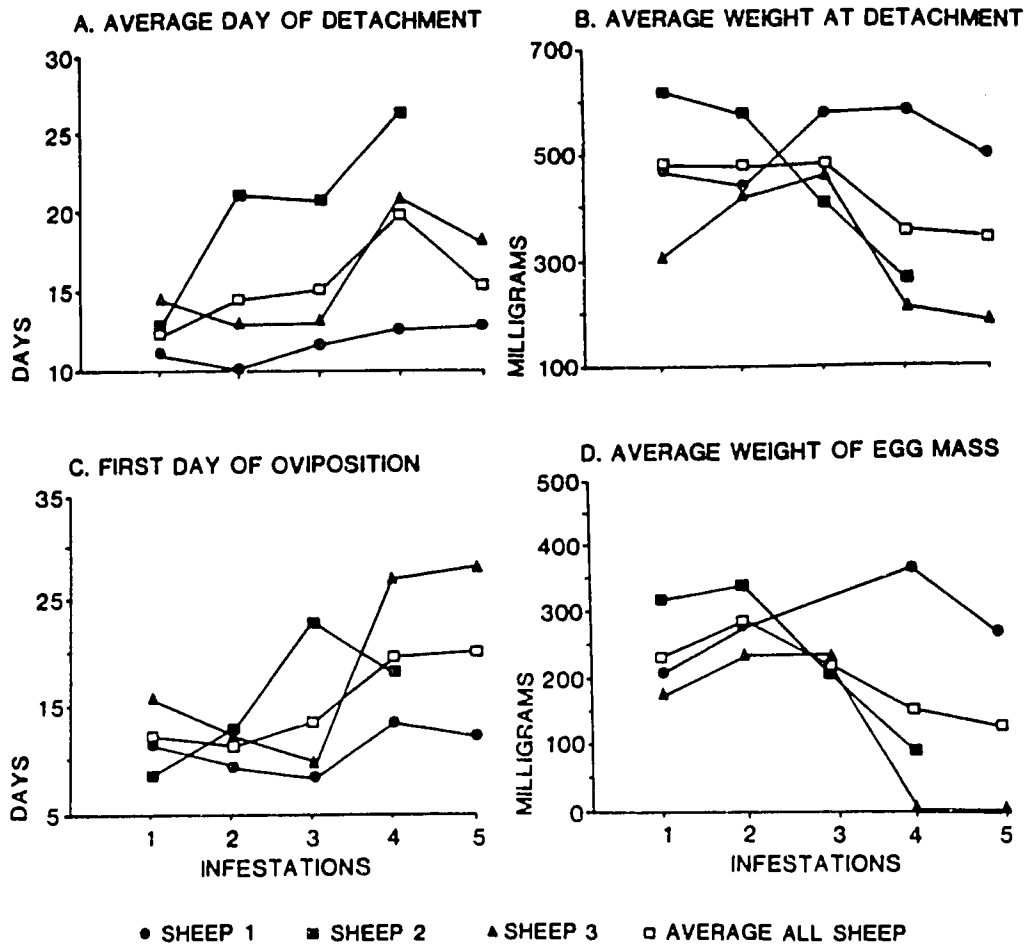

Figure 1. Feeding and oviposition characteristics of ticks during successive infestations of sheep with Amblyomma americanum.

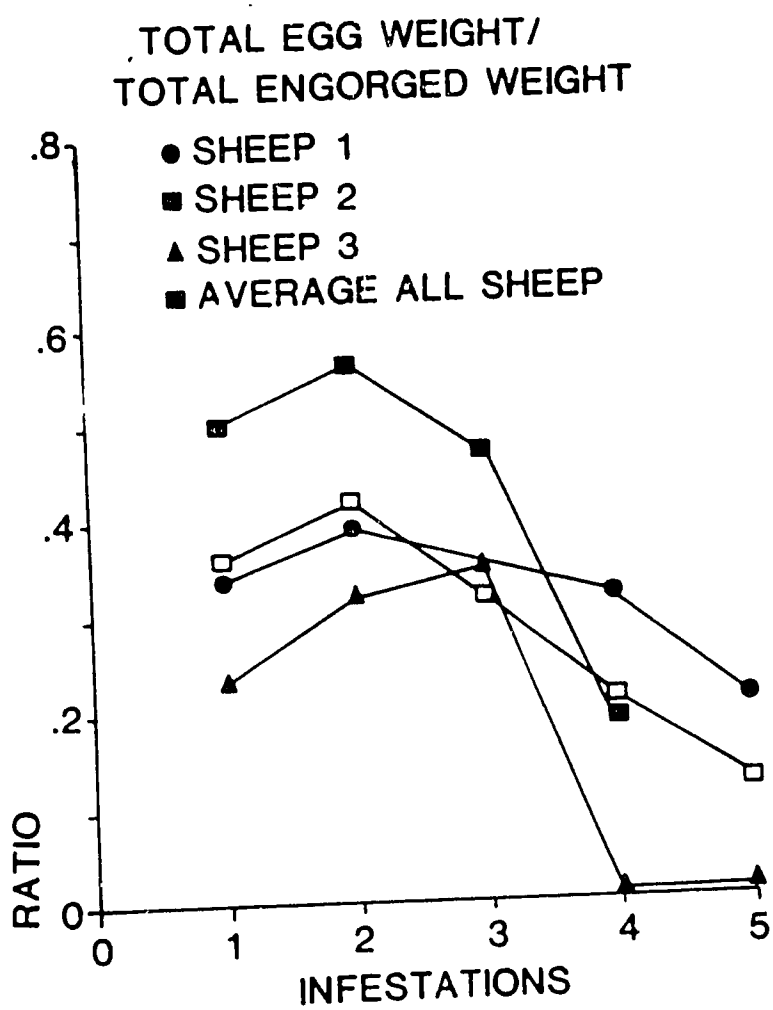

Figure 2. Total weight of engorged ticks that converted into egg mass during successive infestations of sheep with Amblyomma americanum.

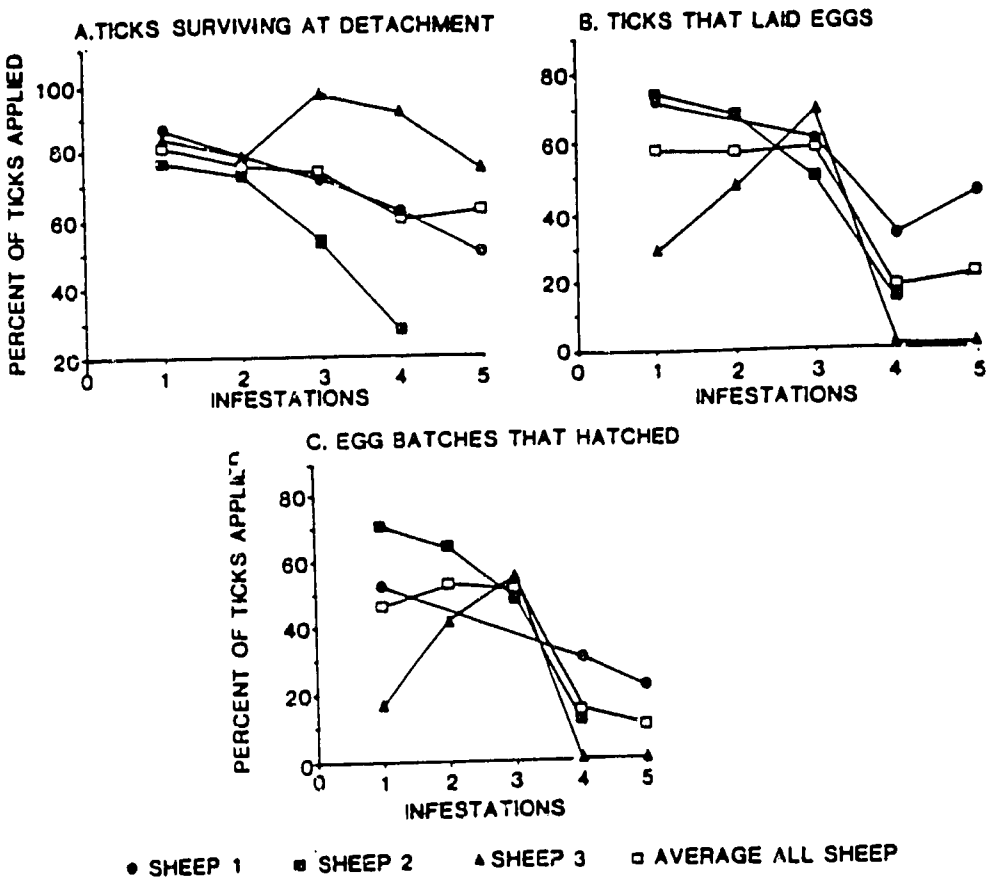

Figure 3. Survival of ticks and eggs at different times during successive infestations of sheep with Amblyomma americanum.

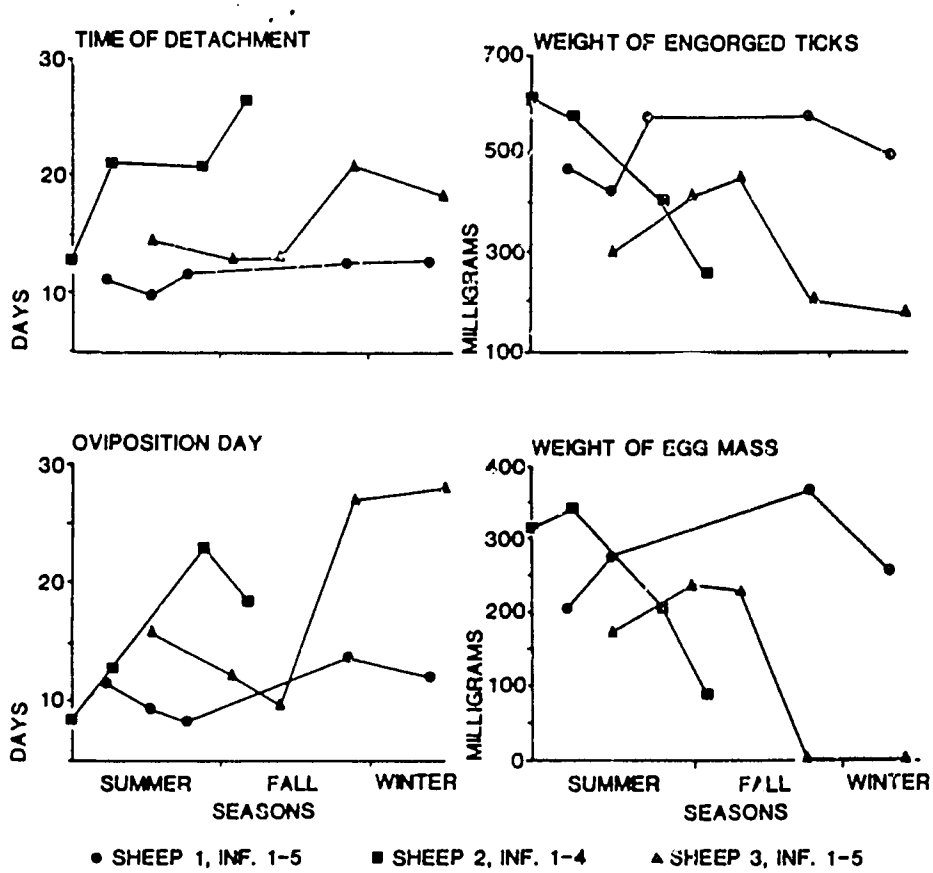

Figure 4. Feeding and oviposition characteristics of ticks during successive infestations of sheep with Amblyomma americanum related to seasons.

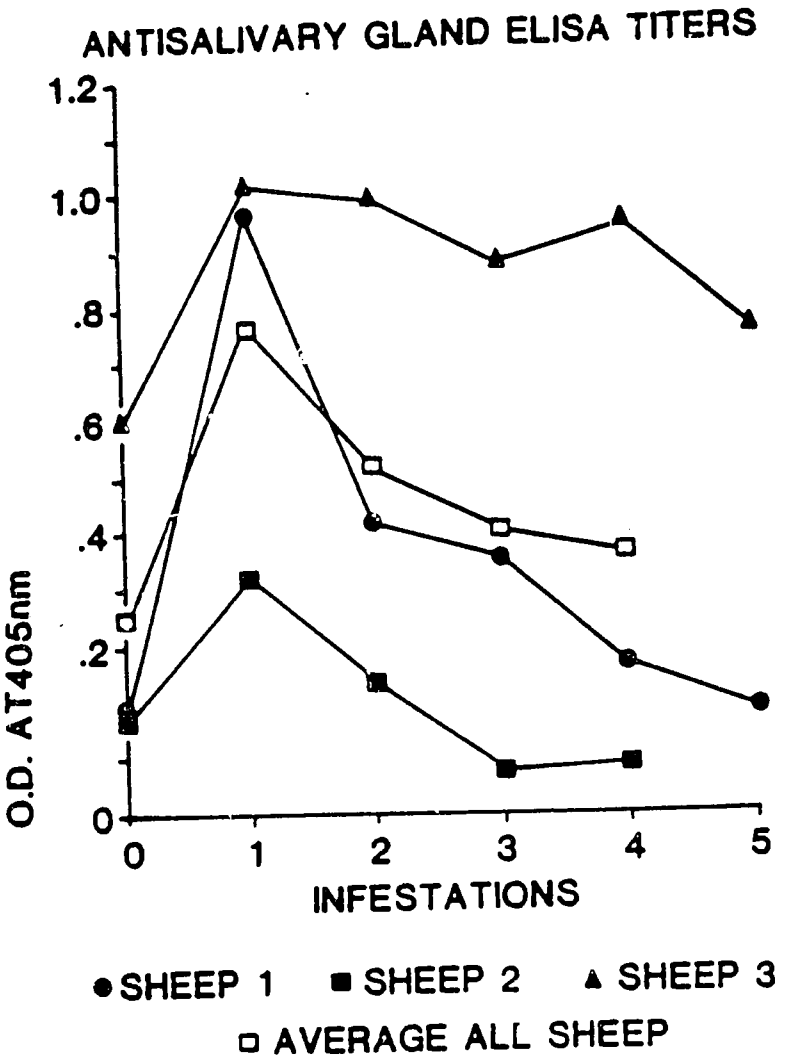

Figure 5. Antibody titers of sera of sheep infected repeatedly with Amblyomma americanum taken before infestation and after each infestation and studied by ELISA against an extract of unfed tick salivary glands.

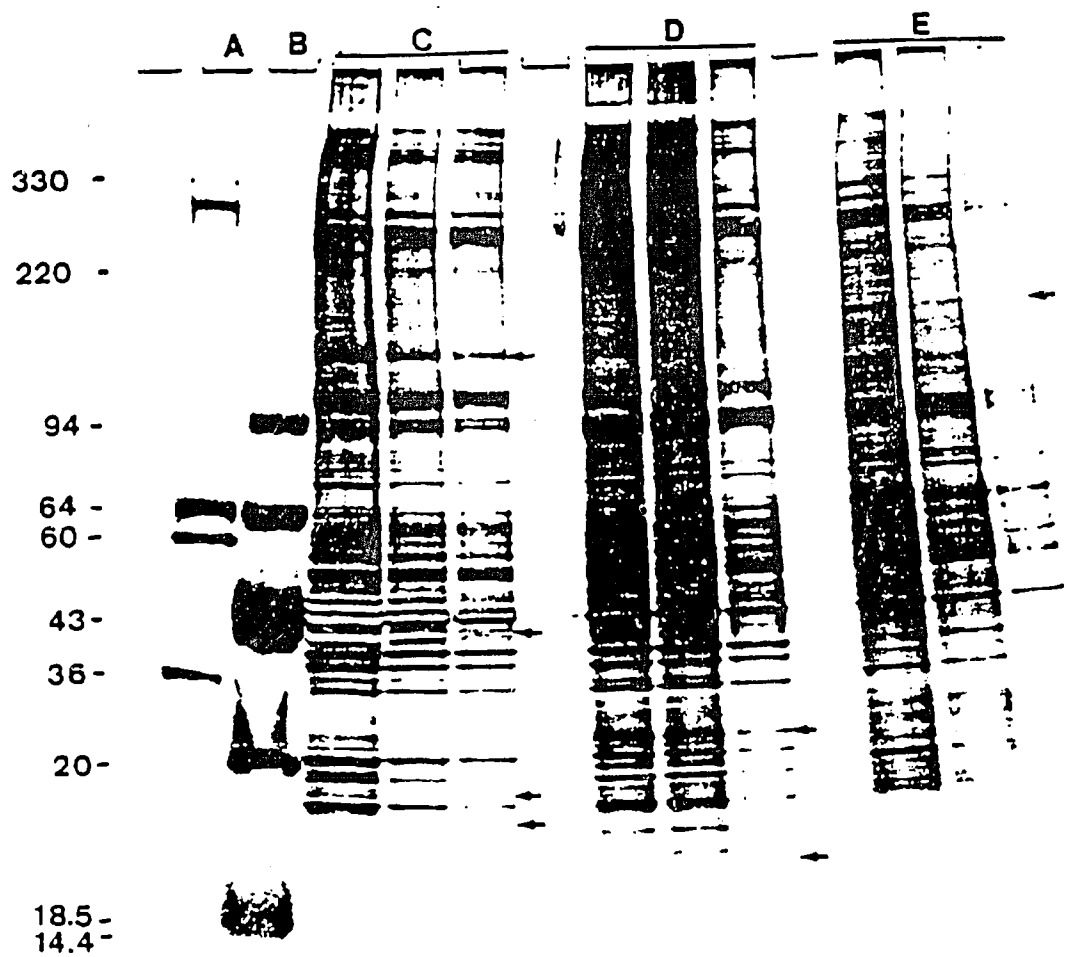

Figure 6. Polyacrylamide gel electrophoresis of extracts of Amblyomma americanum unfed salivary gland (C), salivary gland after three days of feeding (D), and unfed digestive tract (E). The concentration of the extracts used increases from left to right. Lanes A and B are high and low molecular weight markers, respectively. The numbers on the left correspond to molecular weights in thousands. The arrows show the proteins unique to the respective extract.

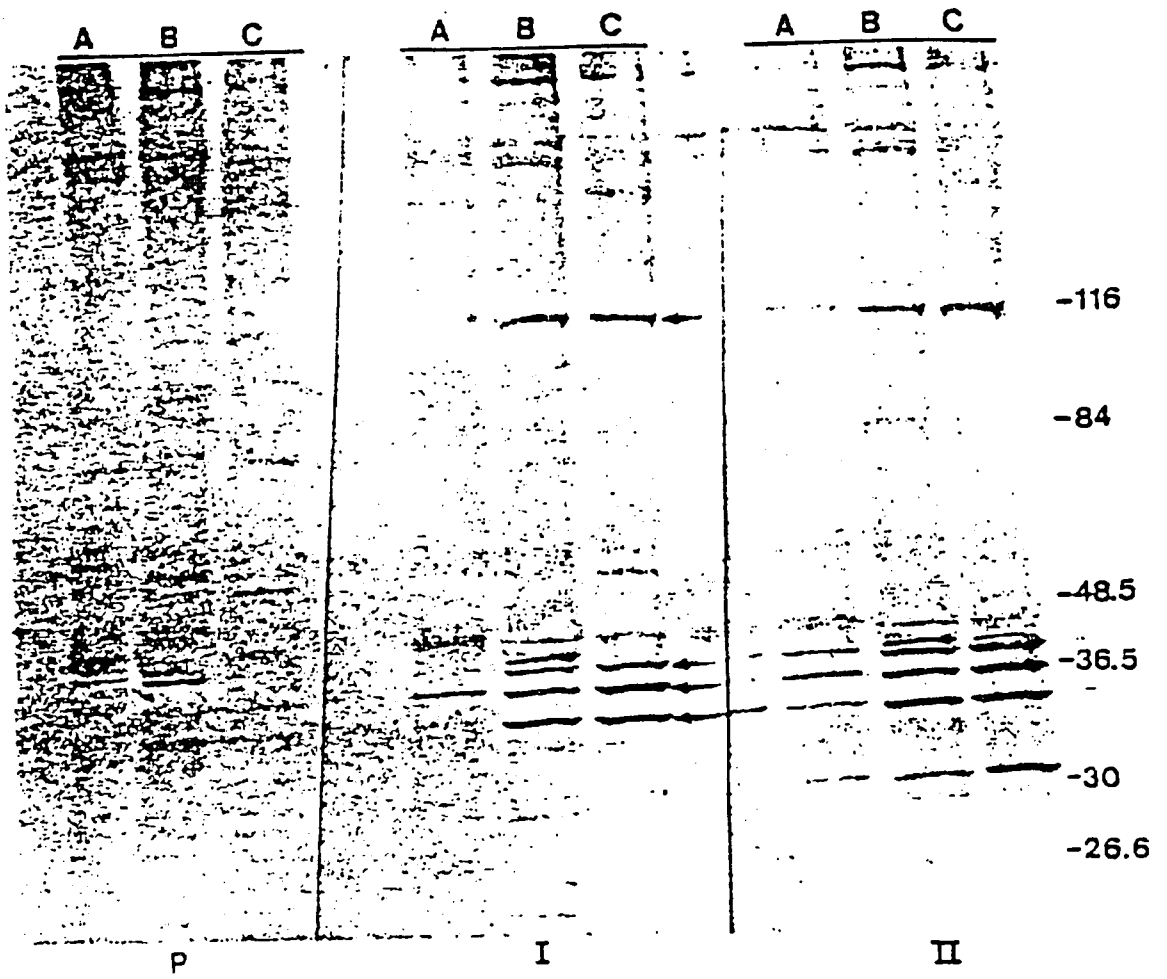

Figure 7a. Western blots of extracts of Amblyomma americanum digestive tract (A), unfed salivary glands (B), and 3-day fed salivary gland (C) against sera taken prior to (P) or after the first (I) or second (II) infestation of a sheep with A. americanum. The number on the right correspond to molecular weights in thousands. The arrows show the 115,000, 38,000, 36,500, and 34,000 MW antigens associated with suppression.

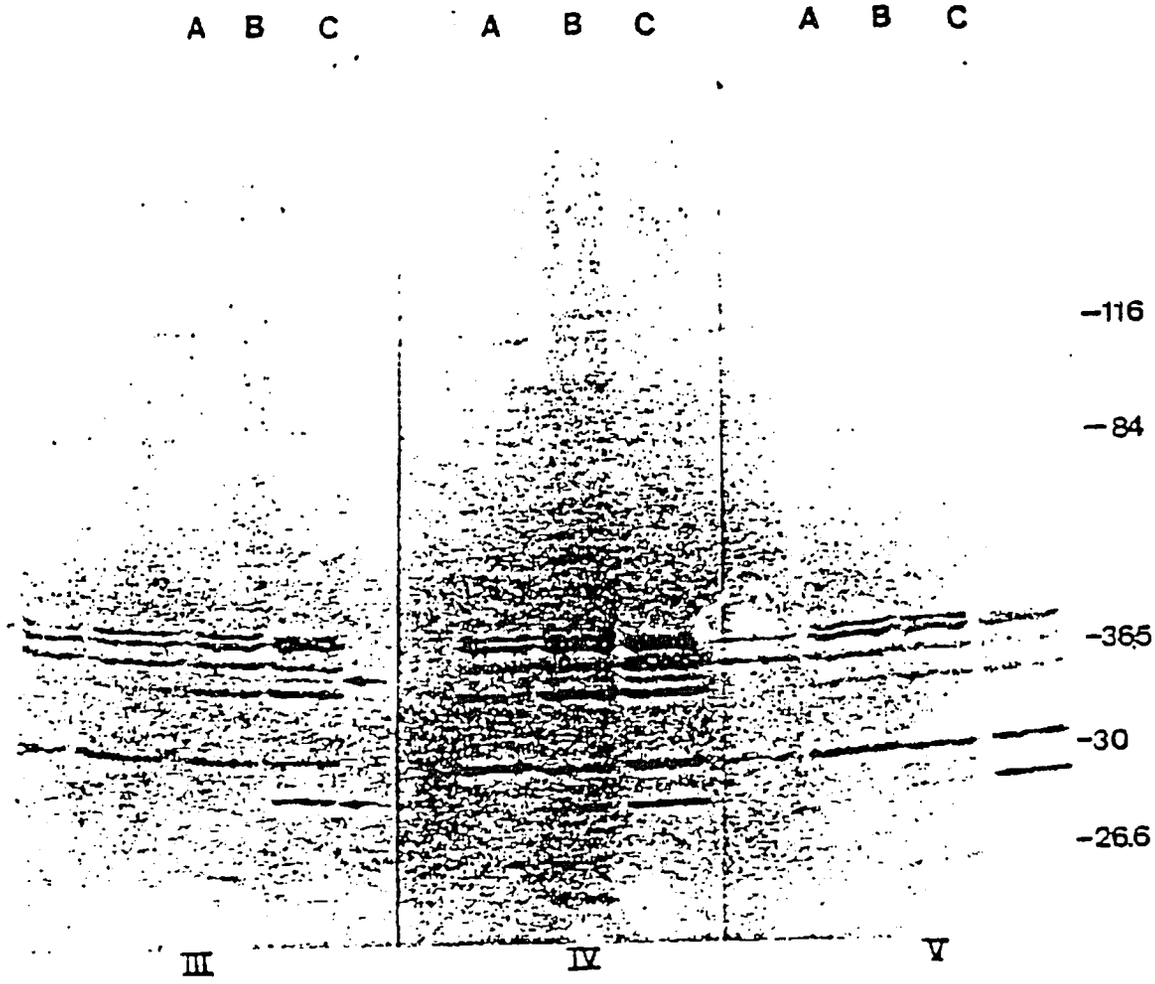

Figure 7b. Western blots of extracts of Amblyomma americanum digestive tract (A), unfed salivary glands (B), and 3-day fed salivary gland (C) against sera taken after the third to fifth (III to V) infestation of a sheep with A. americanum. The number on the right correspond to molecular weights in thousands. The arrows show the 35,500 and 29,000 MW antigens associated with protection.

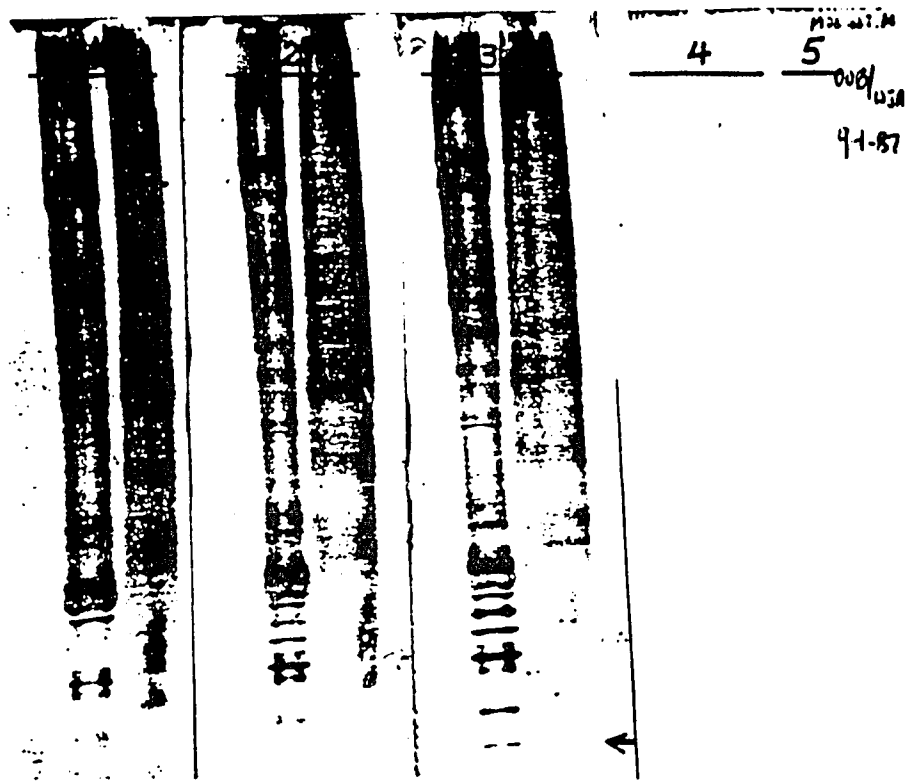

Figure 8. Western blots of Amblyomma americanum (left lanes) and Boophilus microplus (right lanes) salivary glands against sera of an noninfested sheep (1), a sheep infected once (2) or four times (3) with A. americanum, and a specific-pathogen free sheep (4). Lane 5 are molecular weight markers. The arrow shows a 29,000 MW antigen present in the extracts of both ticks.

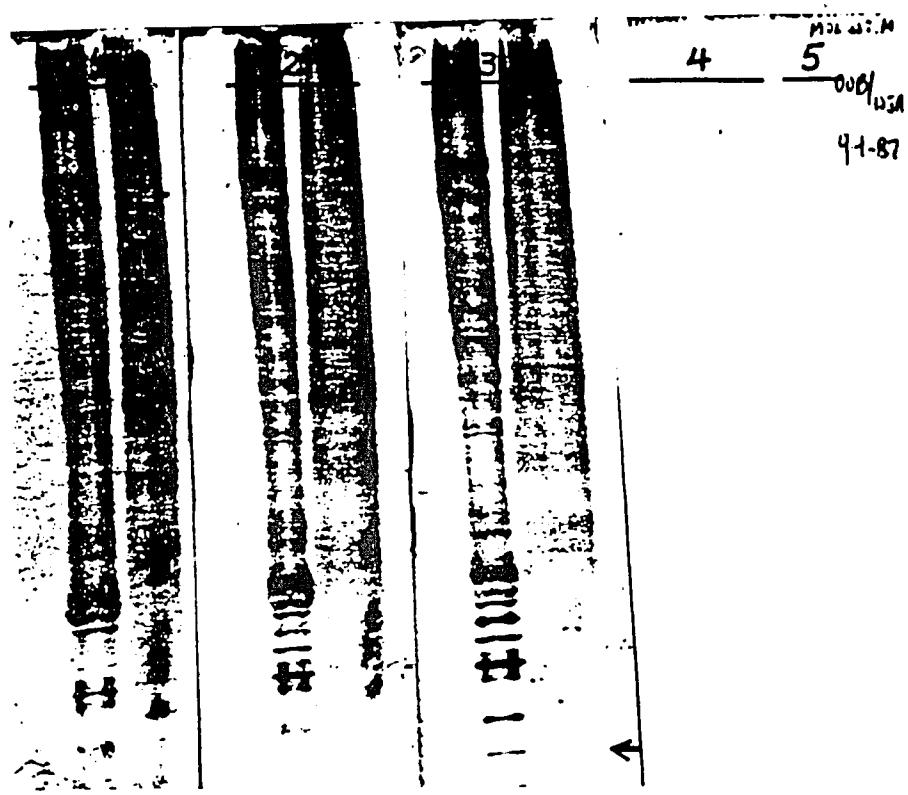

Figure 9. Western blots of Amblyomma americanum (left lanes) and Boophilus microplus (right lanes) salivary glands against sera of an noninfested sheep (1), a sheep infected once (2) or four times (3) with A. americanum, and a specific-pathogen free sheep (4). Lane 5 are molecular weight markers. The arrow shows a 29,000 MW antigen present in the extracts of both ticks.

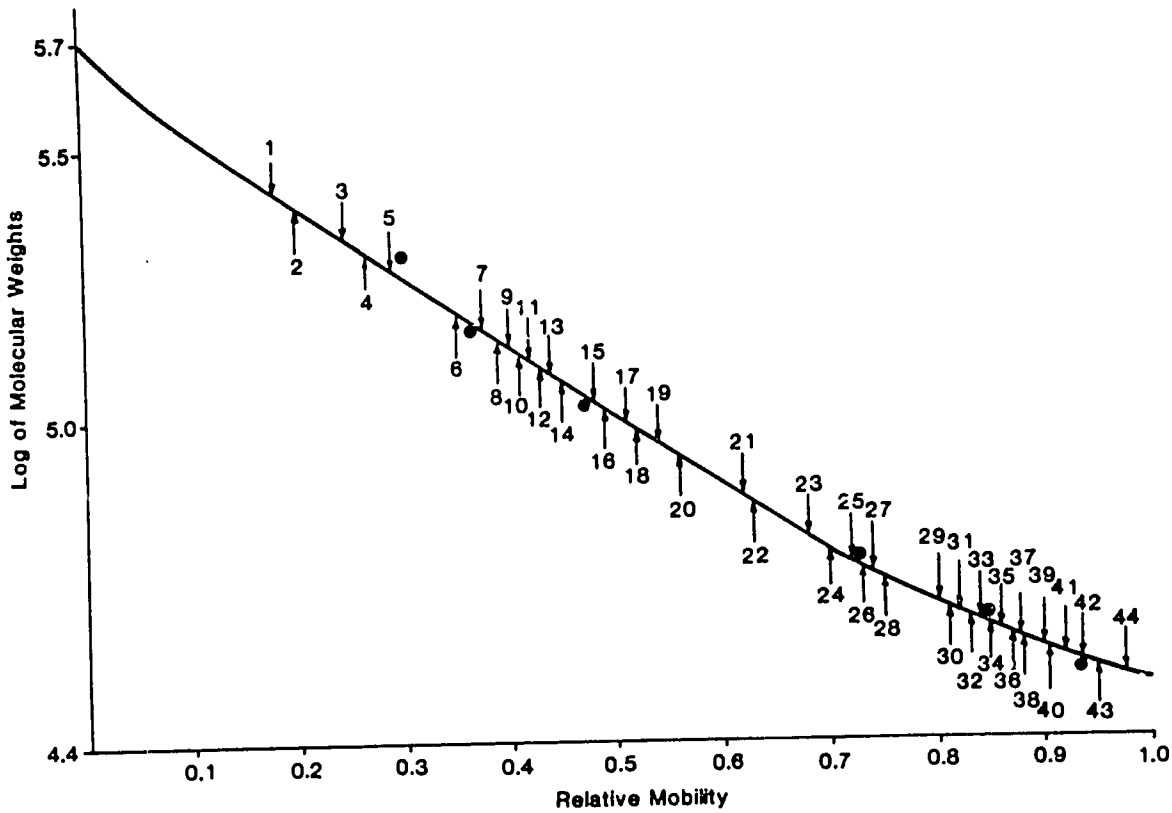

Figure 10. Graphics of the antigens of Amblyomma americanum recognized by three sheep subjected to 4-5 infestations. The arrows represent the antigens detected by Western blots of the sera of the sheep against extracts of tick salivary glands. The circles represent molecular weight markers.