EVALUATION OF TICK HEMOLYMPH AS A POTENTIAL MATERIAL FOR AN ANTITICK VACCINE

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PREFACE

This research project is a part of the worldwide effort to develop new methods for tick control. I hope that the results of this research will be instrumental in the development of a vaccine against the lone star tick and the American dog tick.

I am very grateful for the opportunity I had to study and work with the dedicated and enthusiastic group of tick researchers at Oklahoma State University.

I wish to express my sincere gratitude to all the people who assisted me in this work. I am especially indebted to my committee members, Dr. Robert W. Barker, Dr. John R. Sauer, Dr. Carl J. Fox, Dr. John T. Homer, and Dr. Mark R. Sanborn, for their guidance and valuable advice. Special thanks are due to our laboratory supervisor Ms. Thea Yellin and the staff of the Medical Entomology Laboratory. I am also thankful to the staff of the Entomology Department office and Mrs. Margaret Estes for their patience and care in typing this thesis.

Finally to my wife, Debra, I extend my deepest appreciation for her love and support.

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INTRODUCTION

Each part of this thesis is a separate and complete manuscript to be submitted for publication. Part I is being submitted to the Journal of Medical Entomology. Part II and IV are research notes being submitted to the Journal of Parasitology. Part III is being submitted as a chapter in the book Morphology, Physiology and Behavioral Biology of Ticks (Sauer, J. R. and J. A. Hair eds. Ellis-Horwood, Chichester, England).

Each part appears in this thesis in the format of the journal or book to which it is being submitted.

PART I

THE DEVELOPMENT OF *AMBLYOMMA AMERICANUM* AND *DERMACENTOR VARIABILIS* (ACARI: IXODOIDAE) FED ON RABBITS IMMUNIZED WITH THEIR HEMOLYMPH

Abstract. The hemolymph of Amblyomma americanum and Dermacentor variabilis was evaluated as a potential material for an antitick vaccine. Hemolymph was collected from replete females and centrifuged to remove the hemocytes. Cell-free hemolymph was used to immunize rabbits. Immunized rabbits developed high antihemolymph antibody titers (ca. 1.0×10^6) and had no ill side effects. Rabbits were infested with larvae, nymphs and adults at the same time. The biological performance of ticks fed on immunized rabbits was virtually identical to that of ticks fed on nonimmunized rabbits. Usually, the mean engorgments weights of nymphs and females and the weights of the egg-masses of both species were slightly higher for ticks fed on the nonimmunized rabbits but no significant differences were established due to a large standard deviation. The hemolymph electrophoretic patterns of ticks fed on immunized and nonimmunized rabbits were the same. The possibility of deactivating a single hemolymph component with specific antibodies is discussed.

INTRODUCTION

Immunization of mammals with antigens derived from tissues and secretions of bloodfeeding arthropods is being studied as a potential new method for control of ectoparasites. The host immune response to these antigens often reduced the parasites feeding success and caused internal damage to the parasite by immune components imbibed with the bloodmeal (Bankir 1982, Brown et al. 1984, McGowan et al. 1980 & 1981, Nogge 1978, reviews by Willadsen 1980 & Wikel 1982). Several investigators have demonstrated that tissue-specific antibodies cross the gut epithelium of hematophagous arthropods and attach to the internal organs that had served as sensetizing antigens (Schlein et al. 1976, Schlein & Lewis 1976, Ackerman et al. 1981). Nogge & Giannetti (1980) fed Tsetse flies an antisera against an osmoregulatory protein in their hemolymph and they all died. Recently, intact rabbit hemolysin antibodies were found in the hemolymph of Ixodes ricinus fed on rabbits immunized with sheep red blood cells (Brossard & Rais 1984). Bovine immunoglobulin G (IgG) against Theileria sergenti was found in the hemolymph of Ornithodoras moubata and Haemaphysalis longicornis fed on calf infected with the parasite (Fujisaki et al. 1984). These reports support previous work in which host IgG was found in the hemolymph of Dermacentor variabilis (Ackerman et al. 1981). Some investigators proposed that this phenomenon may be used to interfer with the normal function of hemolymph components in the tick hemocoel using specific antibodies taken in with the bloodmeal (Roberts 1968, Galun 1978,

Allen & Humphreys 1979, Ackerman et al. 1980, McGowan et al. 1980).

Most of the research toward an antitick vaccine has focused on the use of salivary gland antigen (SGA). Immunization with SGA mimics the natural acquisition of immunological resistance that occurs in many hosts (Wikel & Allen 1982). Augmentation of natural resistance by artificial immunization with SGA may lead to an intense hypersensitivity reaction harmful to the host. Conversely, hosts may become immunologically tolerant to SGA following repeted exposure to tick saliva (Berdyeu and Khudainazarova 1976). The saliva of some tick species contain toxins which when injected into the host can cause paralysis and death (Gothe et al. 1979).

We propose to investigate tick hemolymph as a potential material for antitick vaccine. Hosts are not exposed naturally to hemolymph proteins because they do not appear in the tick saliva (Dolp and Hamady 1971, Tatchell 1971). Therefore, the problems with hypersensitivity and immunotolarence are eliminated. Thus far, tick hemolymph has been used as an immunogen in rabbits without any harmful effects (Steparchenok-Rudnik et al. 1969, Tatchell 1971). Hemolymph antigens will be readily accessible to the specific antibodies that will pass into the hemocoel.

This study is designed to determine the effects of feeding Amblyomma americanum (L.) and Dermacentor variabilis (Say) on rabbits immunized with their hemolymph. Cell-free hemolymph was used because the antibodies in the tick hemocoel are unlikely to harm hemocytes without the cellular components of the host immune system. Electrophoretic studies of tick hemolymph (Dolp & Hamady 1971) revealed that nymphs and females share common proteins. This is probably true, to some extent, for larvae. Therefore, all three life stages will be fed

on the rabbits immunized with hemolymph of females. The feeding success, developmental rate and fecundity of these ticks will be studied. Changes in the size and charge of hemolymph proteins following a bloodmeal on the immunized hosts will be studied using electrophoresis.

MATERIAL AND METHODS

Ticks

The A. americanum used in this study originated from Eastern Oklahoma and were maintained in the medical entomology laboratory for the last 7 years. Dermacentor variabilis ticks were collected in Central Oklahoma and maintained in the laboratory for the last 5 years. Ticks were reared on rabbits and sheep as described by Patrick and Hair (1975). Adult ticks 3-5 month post molt were used in this study. Offspring of one or two females were used in each experiment.

Rabbits

White New Zealand rabbits 4-8 months old were used. None of these rabbits had ever been infested with ticks or mites before they were used in this study. Three sibling rabbits of both sexes formed a group. Two rabbits in each group were immunized with hemolymph and the third rabbit served as the nonimmunized control. Each tick species was fed on two groups of rabbits.

Immunization

Replete female ticks fed on sheep were rinsed for 1 minute with cold water to wash away serum exudate, blood, or tick feces from their exterior. Ticks were cooled for 30-60 minutes at 4°C. Hemolymph was collected into capillary tubes after amputating the distal portion of one or more legs with a fine tip forceps while ticks were held on a cold table (5-10°C). Approximately 5 µl of hemolymph were collected from

each tick. Hemolymph was transferred into 250 µl polyethylene micro-centrifuge tubes and centrifuged for 4-6 minutes at 12,000 xg (Coleman Instruments, Maywood, IL). The supernatant was removed and kept frozen (- 45°C) until used to immunize the rabbits. A Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) was used to determine the protein content of the hemolymph. Hemolymph was diluted with 0.01 M phosphate buffered saline at pH 7.2 to give a protein concentration of 30 mg/ml. Each immunized rabbit received 3 injections of 0.5 ml diluted hemolymph mixed with 0.5 ml of adjuvant. Freund's complete adjuvant (DIFCO Laboratories, Detroit, MI) was used for the first injection given intra-muscularly in both hind legs. Freund's incomplete adjuvant was used for the following two injections given subcutaneously at the neck area, at 3-week intervals. Serum samples were collected from immunized rabbits about 2 weeks after the last injection. An enzyme-linked immunosorbent assay (ELISA) was used to determine the antihemolymph antibody titers in the immunized rabbits using a similar procedure used for measuring antiovalbumin antibodies (Ben-Yakir 1985). Preimmunization serum samples of these rabbits were used as negative controls. The titer was defined as the reciprocal of the serum dilution which gave an absorbance value of 0.5 (on a scale of 0.0-2.0).

Tick Feeding

Two weeks after the last injection rabbits were infested with ticks of all three life-stages. Larvae and nymphs were fed on the ears in envelopes made of fine mesh polyester cloth held together with 1/2" Sticky-Back VELCRO® tape around the neck of the rabbit. About 400 larvae were placed on one ear and about 300 nymphs on the other ear. Adults were fed in six cells made of polystyrene sample vials affixed

with adhesive to the shaved backs of the rabbits (Ben-Yakir 1985). Seven pairs of ticks (males and females) were placed in each cell. Plastic collars prevented the rabbits from disturbing the feeding ticks (Watts et al. 1972).

Evaluation of the Effect of Feeding Ticks on Immunized Rabbits

Replete and detached ticks were collected daily and the time it took them to feed was recorded. Two hundred replete larvae from each rabbit were placed in groups of 20 into 10 petri-dishes. Fifty randomly selected replete nymphs from each rabbit were weighed individually. Two hundred replete nymphs from each rabbit were placed in petri-dishes as above. All replete females were weighed and the total yield (the percent of ticks that fed successfully) per rabbit was noted. Twenty-five randomly selected females were placed individually in clear polystyrene jelly cups (30 ml capacity, Premium Plastic Inc., Chicago, IL). Small holes were made in the paper lids to inhance movement of air. All ticks were kept at 22 ± 3°C, 14:10 light:dark photoperiod, and 90% relative humidity. Ticks were examined daily. The molting time (number of days from repletion to molt) and the percent molted were recorded for larvae and nymphs. Preoviposition time, egg incubation time and percent eclosion were recorded. About 2 months after the onset of eclosion all newly hatched larvae were killed by placing them in a freezer (- 45°C) overnight. The dead larvae were separated from the leftovers of the egg-mass and the female and weighed together. The larvae weight was divided by the fraction of successful eclosion and then used as an estimate for the size of the original egg-mass. All results were analyzed using a one way analysis of variance (ANOVA).

Electrophoresis of Hemolymph

Hemolymph was collected from five repleat females from each rabbit as described above. Fresh samples from individual ticks were compared in a discontinuous polyacrylamide gel electrophoresis (PAGE) using a Protean™ dual slab cell apparatus and chemicals (Bio-Rad Laboratories, Richmond, CA). An aliquot of hemolymph containing approximately 80 µg protein was mixed with equal volume of sample buffer (pH 6.8). The samples were applied to a 0.75 mm slab gel. The staking gel and the running gel were 3.5% and 7.5% acrylamide, respectively. Electrophoresis was carried out with electrode Tris buffer at pH 8.1. The current was 15 mA per gel for the first 30 minutes and 10 mA per gel until the bromophenol blue tracking dye reached the bottom of the gel. Gels were fixed in 25% isopropanol and 10% acetic acid for 15 minutes at 60°C, stained with 0.025% Coomassie blue R-250 for 15 minutes at 60°C, and destained in 10% acetic acid overnight at room temperature. Destained gels were read using an Auto Scanner (Helena Laboratories, Beaumont, TX) set at a wave length of 545 nm. The protein bands of hemolymph from female ticks fed on either immunized or nonimmunized rabbits were compared.

RESULTS

The protein concentrations in the hemolymph collected was about 85 mg/ml for A. americanum and about 100 mg/ml for D. variabilis. Immunization with hemolymph induced the production of a high anti-hemolymph antibody titer without apparent side effects to the rabbits. The ELISA antihemolymph titer for D. variabilis was approximately $1.5x10^6$ and for A. americanum $8.0x10^5$. About 50-60% immunological cross-reactivity was found between the two types of antisera.

No differences were noted between the biological performance of ticks fed on immunized and nonimmunized rabbits (Tables 1 & 2). Larvae and nymphs of both species dropped off about 4-5 days post infestation and their yields ranged from 80-90%. Dermacentor variabilis females dropped off between days 7-12 post infestation and their yield ranged from 80-85%. Amblyomma americanum females dropped off between days 11-15 post infestation and their yield ranged from 75-80%. The mean engorgment weights of nymphs and females and the weights of the egg-masses of both species were usually higher for ticks fed on the nonimmunized rabbits but no significant differences ($P > 0.05$) were established due to a large standard deviation. No difference was detected in the hemolymph electrophoretic patterns of ticks fed on immunized and nonimmunized rabbits. All protein bands were the same as those described for rabbit-fed females of these species (Ben-Yakir 1985).

DISCUSSION

Despite the high antihemolymph antibody titers in the rabbits virtually no deleterious effects were noted in the ticks that fed on them. No detectible changes were noted in any of the major hemolymph proteins as a result of the antihemolymph antibodies that passed into the hemocoel. The protein stain (Comassie blue) used in the electrophoretic study was not very sensitive to small quantities of proteins. Therefore, a more sensitive protein stain may reveal changes in proteins that are of low concentration in the hemolymph.

Results indicate that immunization with cell-free hemolymph does not protect the hosts against ticks. Several investigators induced host resistance to ticks by immunizing with extracts of whole tick body or of internal organs which contained hemolymph components (McGowan et al. 1980 & 1981, and review by Wikel 1982). The protection provided by these extracts was probably due to SGA or its homologs (Whelan et al. 1984).

The results of this study suggest that only a small fraction of the antibodies taken in with the bloodmeal were able to pass into the hemocoel. Therefore, the production of polyclonal antibodies in the host against a large number of antigenic determinants, as was the case when hemolymph was used, will have little or no effect on the tick. However, antibodies passing into the hemocoel may still be used to deactivate a particular component that is present in low concentration in the hemolymph. The antibodies in the hemolymph may be used to inhibit enzymes, neutralize hormones or interfer with the function of some

molecules by agglutinating them. Once identified, the hemolymph component should be used in pure form as an immunogen so that a large quantity of specific antibodies will be produced against it. A hemolymph immunogen may provide the host with protection against several tick species at the same time as indicated by the immunological cross-reactivity between the antihemolymph antisera of the two tick genera used in this study. Research is under way in our laboratory to determine the concentration of host antibodies in the tick hemolymph. This information will be used to evaluate the potential of specific antibodies to deactivate a hemolymph component.

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Table 1. The biological performance of A. americanum* fed on rabbits immunized with their hemolymph (#1, 2, 4, 5) and nonimmunized rabbits (#3, 6).

	Rabbits					
	#1	#2	#3	#4	#5	#6
Larvae						
Mean Molting Time (days±SD)	20.1±1.8	18.9±2.7	19.1±3.5	18.6±2.2	19.6±1.2	19.4±1.9
% Molted	64	88	86	82	77	83
Nymphs						
Mean Engorgment Weights† (mg±SD)	11.9±3.3	12.2±3.3	11.9±3.1	11.7±2.9	12.1±3.4	12.2±3.1
Mean Molting Time (days±SD)	28.7±1.8	28.2±2.4	27.9±2.7	28.4±2.3	27.8±2.3	28.2±1.7
% Molted	92	95	96	93	96	94
Females						
Mean Engorgment Weights (mg±SD)	652±224	601±175	749±232	633±212	676±195	715±211
Mean Preoviposition Time (days±SD)	7.4±1.7	9.9±1.5	9.5±2.9	8.7±2.1	9.3±2.6	8.2±1.8
Egg Masses						
Mean Weights†† (mg±SD)	272±85	243±130	312±85	291±113	253±96	305±103
Mean Incubation Time (days±SD)	52.2±5.1	53.1±6.3	53.4±5.3	52.6±5.7	53.3±6.0	52.4±5.9
Mean Eclosion (%±SD)	80±19	86±18	83±16	81±20	79±19	87±18

*N = 200/rabbit for larvae and nymphs; 25/rabbit for females and egg masses.

†N = 50/rabbit.

††Estimated using the weight of the larvae divided by the fraction of successful eclosion.

Table 2. The biological performance of D. variabilis* fed on rabbits immunized with their hemolymph (#7, 8, 10, 11) and nonimmunized rabbits (#9, 12).

	Rabbits					
	#7	#8	#9	#10	#11	#12
Larvae						
Mean Molting Time (days±SD)	12.5±1.8	12.3±1.5	12.7±1.3	12.2±1.6	12.5±1.3	12.3±1.7
% Molted	95	95	96	94	95	94
Nymphs						
Mean Engorgment Weights (mg±SD)†	11.8±3.7	11.3±3.3	12.2±3.3	11.5±3.5	11.7±2.9	11.9±3.1
Mean Molting Time (days±SD)	29.5±2.0	30.3±1.8	30.7±1.5	30.4±1.9	29.7±2.4	30.1±1.9
% Molting	88	81	56	86	77	87
Females						
Mean Engorgment Weights (mg±SD)	444±130	454±123	491±145	437±140	462±121	480±127
Mean Preoviposition Time (days±SD)	7.9±1.3	8.3±1.4	8.0±1.2	8.2±1.3	8.3±1.5	7.9±1.4
Egg Masses						
Mean Weights (mg±SD)††	146±41	137±36	163±45	140±44	135±41	151±46
Mean Incubation Time (days±SD)	31.7±1.7	31.0±1.1	32.4±2.4	31.6±1.8	32.2±2.1	31.2±1.8
Mean Eclosion (%±SD)	93±2	88±3	87±3	85±4	88±4	86±3

*N = 200/rabbit for larvae and nymphs; 25/rabbit for females and egg masses.

†N = 50/rabbit.

††Estimated using the weight of the larvae divided by the fraction of successful eclosion.

PART II

AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR HOST IMMUNOGLOBULIN G IN THE HEMOLYMPH OF TICKS

Recently, several investigators reported that immunoglobulin G (IgG) of mammalian hosts crosses the gut epithelium of ticks and can be detected in the hemolymph. Nonspecific IgG of man and rabbit were demonstrated in the hemolymph of Dermacentor variabilis (Say) by Ouchterlony double diffusion and immunoelectrophoresis (Ackerman et al., 1981, J. Parasitol. 67:737-740). Rabbit antibodies against sheep red blood cells were detected in the hemolymph of Ixodes ricinus (L.) by the hemolysin test (Brossard and Rais, 1984, Experientia 40:561-563). Specific bovine IgG against Theileria sergenti was found in the hemolymph of Ornithodoros moubata Sambon and Haemphysalis longicornis Neumann using indirect fluorescent antibody tests (Fujisaki et al., 1984, Ann. Trop. Med. Parasitol. 78:449-450). The above reports did not establish that antibodies pass into the hemocoel of all ticks in the experimental population. Hemolysin antibodies were found only in a fraction of the ticks tested, whereas in the other studies investigators used hemolymph that had been pooled from several ticks. No information about the actual concentrations of host IgG in hemolymph was reported.

The inhibition of ticks enzymes and neutralization of their hormones with specific antibodies have been considered as a possible method to debilitate ticks (Roberts, 1968, J. Parasitol. 54:657-662; Galun, 1978, Control of livestock pests by interference in their development, Bogata, Colombia, UC/AID Pest Management and Relative Environmental Protection Progress Report; Allen and Humphreys, 1979, Nature 280:491-493; Ackerman et al., 1980, J. Med. Entomol. 17:393-399; McGowan et al., 1980, J. Parasitol. 66:42-48). The aim of this study was to evaluate the potential for deactivating hemolymph components with specific antibodies by determining the prevalence and the concentration of host IgG in the

hemolymph. An assay for measuring the concentrations of host IgG in hemolymph of individual ticks was developed.

Adult Amblyomma americanum (L.) and D. variabilis, about 4-months post molt, were fed on a pair of rabbits, sheep, and calves. None of the hosts had been used to feed ticks prior to this study. Both species of ticks were fed on each host. Special care was taken to prevent mechanical damage to the feeding ticks. Ticks were fed in cells made of polystyrene sample vials affixed with adhesive to the shaved backs of the rabbits (Ben-Yakir, 1985, Ph.D. Thesis, Oklahoma State Univ. 68 p.). Plastic collars prevented the rabbits from disturbing the cells (Watts et al., 1972, J. Parasitol. 58:1105). On sheep and calves ticks were fed in modified stockinette cells (Ben-Yakir, 1985, loc. cit.). Replete females were collected within 12 hours of detachment. Serum was collected from the hosts at that same time. Ticks were weighed and then rinsed for 1 minute with cold water to wash away serum exudate, blood, or tick feces from their exterior surface. Ticks were cooled for 30-60 minutes at 4°C. Hemolymph was collected into precalibrated capillary tubes after amputating the distal portion of one or more legs with fine tip forceps while the ticks were held on a cold table (5-10°C). Approximately 5 µl of hemolymph were collected from each tick. Hemolymph with reddish tint, indicating it had been contaminated with gut content, was not used. The female ticks were kept and observed daily to determine the effect of hemolymph collection on their biological performance. Hemolymph samples were immediately diluted 1:50 in cold 0.01 M phosphate buffered saline at pH 7.4 with 0.05% tween 20 (PBS-tween 20). The diluted hemolymph was kept frozen in 250 µl polyethylene microcentrifuge tubes until they were assayed for host IgG content.

An enzyme-linked immunosorbent assay (ELISA) was used to detect host IgG in the hemolymph. The double antibody sandwich method was found to be the most sensitive and gave the most consistent measurements. The procedure outlined by Voller et al. (1979, The Enzyme Linked Immunosorbent Assay, Dynatech Lab., Alexandria, VA) was followed. NUNC 96F Immunoplate I (Irvine Scientific, Santa Anna, CA) was used. The working volume was 100 µl per well. Absorbance was measured with an EIA Reader (Model EL 307, BIO-TEK Instruments, Burlington, VT). Briefly, the steps of the procedure were as follows: 1) Antihost IgG antibodies (the IgG fraction of antihost IgG serum. Cooper Biomedical, Malvern, PA) at 500 ng/ml of carbonate-bicarbonate buffer pH 9.6 were adsorbed to the plate; 2) Diluted hemolymph samples (final dilution 1:1,000 to 1:10,000 in PBS-Tween 20) were added. Reference solutions of host IgG were used as positive controls, and a hemolymph sample from a tick fed on a different host was used as a negative control. All samples were assayed in duplicate wells; 3) Antihost IgG antibodies conjugated with alkaline phosphatase enzyme at 1:125 (v/v in PBS-Tween 20) was added. Following each of the above steps, plates were incubated in a humidity chamber for 3 hours at 23 ± 3°C or overnight at 4°C. At the end of incubation, wells were rinsed 3 times with PBS-Tween 20. 4) Alkaline phosphatase substrate at 1 mg/ml of 10% diethanolamin buffer pH 9.8 was added and plates were incubated for 30 minutes at 24 ± 2°C. The enzyme reaction was stopped by adding 50 µl of 3.0 M NaOH. Absorbance was read at a wave length of 405 nm.

The absorbance readings for the control samples were all below 0.2 (on a scale of 0.0-2.0). The absorbance reading for the reference solutions of host IgG at concentrations of 0.5-5.0 ng/ml were clearly

positive (>0.3). A linear correlation (r>0.96) existed between the absorbance readings and the IgG concentrations. Due to interference by nontarget hemolymph components the sensitivity of the assay decreased. Attempts to reduce the interference by centrifugation of samples at 12,000 xg for 4 minutes, or by "blocking" with bovine serum albumin, were unsuccessful. In order to determine the reduction in absorbance due to interference, reference solutions of rabbit IgG were assayed with various concentrations of hemolymph from sheep fed tick, which was negative for rabbit IgG. Ovine and bovine IgG reference solutions were assayed with hemolymph of rabbit fed ticks for the same reason. Ten separate pools of hemolymph were used for these assays. The mean absorbance values of the above assays were compared to the absorbance values of the reference solutions without hemolymph and the percent reduction in absorbance due to interference was calculated (Table 1). The absorbance readings of the hemolymph samples were corrected for the effect of interference by dividing them by (100-% reduction) $x10^{-2}$. The corrected absorbance readings were compared to a linear regression curve (the least squares method) of the absorbance values for the reference IgG solutions included on the same plate. The serum IgG concentrations were determined in a similar manner, but no interference was observed at the high dilution they were assayed (approx. $1:10^6$). The fraction of host serum IgG in the hemolymph was determined by dividing the mean concentration of host IgG in the hemolymph by the concentration of IgG in the serum.

Table 2 contains a summary of the results. All samples tested had measurable quantities of host IgG. No significant correlation existed between the engorgment weight of the ticks and the IgG concentration in

their hemolymph. The serum concentration of IgG had a direct effect on the concentration of IgG in the hemolymph. The fractions of host serum IgG found in the hemolymph of D. variabilis were consistantly greater then the fractions in A. americanum hemolymph. This may be due to the ability of D. variabilis to concentrate the bloodmeal about 3-fold compared with only 2-fold concentration by A. americanum (Koch and Sauer, 1984, Ann. Entomol. Soc. Am. 77:142-146). Slightly larger fraction of rabbit serum IgG was found in the tick hemolymph compared with the fraction of serum IgG of sheep and calves.

The antisera used in this assay contained antibodies against both heavy and light chains of the IgG molecule. Therefore, it was not determined whether intact IgG or only the antigen binding fragment (Fab') was present in the hemolymph. The hemolysin activity in the hemolymph of I. ricinus (Brossard and Rais, 1984, loc. cit.), which required an intact antibody molecule, suggests that some intact IgG can pass through the tick gut wall.

The main advantage of using ELISA was the small volume of hemolymph that it required. This permitted detection of host IgG in virtually every replete female tick used in this study. The collection of hemolymph from the ticks had no effect on their biological performance. Thus, ticks may be sampled at several different times in their life with a minimal effect on their normal function. Under ideal conditions samples from 45 ticks could be assayed on one plate and results could be obtained in 10 hours. This assay could also be used to measure the concentrations of other hemolymph components present in similar or higher concentrations to which specific antibodies can be produced. This assay may also be useful for identification of the source of bloodmeal in recently fed ticks.

Table 1. The percent reduction (mean ± SD) in absorbance values of reference IgG due to interference by nontarget hemolymph proteins.

Hemolymph Dilution	Percent*	
	A. americanum	D. variabilis
1:250	50 ± 13	80 ± 15
1:500	40 ± 10	65 ± 12
1:1,000	30 ± 7	50 ± 10
1:2,000	20 ± 4	40 ± 8
1:4,000	15 ± 2	25 ± 4
1:8,000	10 ± 1	15 ± 2

*Based on assays with 10 different pools of hemolymph.

Table 2. The concentration of host IgG in the hemolymph of A. americanum and D. variabilis replete females.

Host		Host IgG (µg/ml)				
		Serum ($x10^3$)	A. americanum*		D. variabilis*	
			(mean ± SD)	HF†	(mean ± SD)	HF†
Rabbit	#1	8.0	4.3 ± 2.1	1:1,852	6.4 ± 3.0	1:1,250
	#2	12.0	4.4 ± 1.8	1:2,703	10.9 ± 4.2	1:1,099
Sheep	#1	24.0	7.7 ± 3.9	1:3,125	12.5 ± 3.6	1:1,923
	#2	32.0	10.9 ± 4.3	1:2,941	20.5 ± 7.3	1:1,563
Calf	#1	22.0	7.0 ± 2.4	1:3,125	10.8 ± 3.8	1:2,041
	#2	14.0	5.5 ± 1.6	1:2,564	9.8 ± 3.9	1:1,429

*N = 20 ticks/host.

†HF = Hemolymph Fraction = $\frac{\text{Mean concentration of host IgG in the hemolymph}}{\text{concentration of host IgG in the serum}}$.

PART III

QUANTITATIVE STUDIES OF HOST IMMUNOGLOBULIN G PASSAGE INTO THE HEMOCOEL OF THE TICKS *AMBLYOMMA AMERICANUM* AND *DERMACENTOR VARIABILIS*

INTRODUCTION

The main method for tick control has been the treatment of hosts and their environment with chemical acaricides. Recent emphasis on environmental protection and pure food laws, as well as the emergence of tick strains resistant to acaricides, indicate a need to develop alternative methods for tick control. Vaccination of hosts with antigenic tissues and secretions of ticks may provide a new method to control ticks. Extracts of whole tick body and of various organs have been tested as to their ability to induce tick resistance in the host (McGowan et al. 1980 and 1981, Brown et al. 1984, and reviews by Willadsen 1980 and Wikel 1982). Antigens from tick salivary glands have been studied most extensively. For some tick species the antigenic component responsible for the induction of resistance has been identified (Brown et al. 1984). Many interactions between the salivary antigens and the host immune system have been described (Wikel and Allen 1982). In contrast, little is known about the composition of immunogens from other internal tissues of the tick. It is not known how these antigens induce host resistance to tick. Some investigators suggested that specific antibodies may be able to inhibit an enzyme or neutralize a hormone in the tick hemocoel (Roberts 1968, Galun 1978, Allen and Humphreys 1979, Ackerman et al. 1980, McGowan et al. 1980), but no evidence for such reactions have been found.

Recently, several investigators reported that immunoglobulin G (IgG)

of mammalian hosts crosses the gut epithelium of ticks without losing its ability to bind the antigen. When Dermacentor variabilis was fed on rabbits immunized with their salivary glands and ovaries the specific IgG crossed into the hemocoel and attached specifically to these organs (Ackerman et al. 1981). Intact rabbit hemolysin antibodies were found in the hemolymph of Ixodes ricinus fed on rabbits immunized with sheep red blood cells (Brossard and Rais 1984). Bovine IgG against Theileria sergenti was found in the hemolymph of Ornithodoras moubata and Haemaphysalis longicornis fed on a calf infected with the parasite (Fujisaki et al. 1984). The above reports did not establish that the passage of antibodies occurred in all individuals of the experimental population. Hemolysin antibodies were found only in a fraction of the ticks tested, whereas other investigators used hemolymph pooled from several ticks. No information about the actual concentration of IgG in the hemolymph was given.

Knowledge of the concentration of specific IgG in hemolymph could be used to evaluate the capacity of antibodies to deactivate hemolymph components. Both the rate and extent of antigen reaction with antibodies are greatly dependent on the concentrations of these molecules in the reaction medium. This study is designed to determine the concentrations of specific and total host IgG in the hemolymph of female Amblyomma americanum and D. variabilis. Specific antibodies were produced in hosts against ovalbumin, a simple immunogenic proten (MW ca. 40,000), which will simulate a hemolymph component to be deactivated by antibodies. The variations in the concentration of host IgG in the hemolymph of the two tick species and between individuals of the same species were investigated. The changes in the concentrations of host IgG in the

hemolymph during the bloodmeal were studied. Because the serum antibody concentrations of the hosts are often used to evaluate their immunological status, the IgG in the tick hemolymph were also expressed as a fraction of their host serum IgG concentration. The variations in these fractions in ticks fed on rabbits, sheep and calves were noted.

MATERIALS AND METHODS

The A. americanum used in this study originated from Eastern Oklahoma and were maintained in the medical entomology laboratory at Oklahoma State University for the last 7 years. Dermacentor variabilis ticks were collected in Central Oklahoma and maintained in the laboratory for the last 5 years. Ticks were reared on rabbits and sheep as described by Patrick and Hair (1975). Adult ticks 3-5 months post molt were used in this study. Offspring of one or two females were used in each experiment.

New Zealand White rabbits were the principal hosts in this study. None of the rabbits had been infested with ticks or mites before they were used in this study. The rabbits were 4-8 months old and weighed 2-3 Kg. Three sibling rabbits of both sexes formed an experimental group. Each tick species was fed on two groups of rabbits. Two rabbits in each group were hyperimmunized with ovalbumin and the third rabbit served as the nonimmunized control. Three mg of ovalbumin (grade VIII, Sigma Chemical Co., St. Louis, MO) were used per injection. Ovalbumin was dissolved in 1 ml saline and mixed with 1 ml of adjuvant. Freund's complete adjuvant (DIFCO Laboratories, Detroit, MI) was used for the first injection given intramuscularly in both hind legs. Freund's incomplete adjuvant was used for the following two injections given subcutaneously at the neck area, at 3-week intervals. Immunized rabbits were infested with ticks 2 weeks after the last injection. Special care was taken to prevent mechanical damage of the ticks. Ticks were fed in

cells made of polystyrene sample vials affixed with adhesive to the shaved backs of the rabbits (Ben-Yakir 1985). Plastic collars prevented the rabbits from disturbing the cells (Watts et al. 1972). Two cells were infested on day 1 and one cell was infested on days 3, 5, 7 and 9. Seven pairs of ticks (males and females) were placed in each cell. When 50% of the adult females had engorged and detached in the cells infested on day 1, the rabbit was bled by heart-puncture and then sacrificed and skinned. The skins with attached ticks were left for 24 hours at 23°C and 90% RH to allow the ticks to freely detach themselves and thus prevent potential damage from forced detachment of the partially engorged ticks. About 90% of the ticks detached within 24 hours. Those ticks that did not detach were removed by excising a small piece of skin around their mouthparts.

Two 6-month-old Hereford calves weighing about 100 Kg and two 8-month-old sibling Suffolk sheep weighing about 40 Kg were used as hosts. None of these animals had been used to rear ticks prior to this study. In each pair one animal was immunized with ovalbumin (15 mg per injection) as described above for the rabbits. Each animal was fitted with eight cells made from tubular orthopedic stockinette affixed with adhesive to the shorn upper side of the animal (Ben-Yakir 1985). The animals were held in headgate stanchions when ticks fed on them. Both species of ticks were fed on each animal. Thirty pairs of ticks were placed in each cell on days 1, 3, 5 and 7. When 50% of the females engorged and detached in the cells infested on day 1, all ticks were carefully removed with a fine-tipped forceps. Animals were bled by venapuncture at that time.

All detached ticks were weighed and assigned to a weight-group as

described in Table 1. Ticks were rinsed for 1 minute with cold water to wash away serum exudate, blood, or tick feces from their exterior. Ticks were cooled for 30-60 minutes at 4°C. Hemolymph was collected into precalibrated capillary tubes after amputating the distal portion of one or more legs with a fine-tipped forceps while ticks were held on a cold table (5-10°C). Hemolymph with reddish tint, indicating it had been contaminated with gut content, was not used. The proteins in the hemolymph and the gut content were compared using polyacrylamide gel electrophoresis (Ben-Yakir 1985) to confirm the purity of the hemolymph samples. Table 1 shows the mean quantities of hemolymph collected from each weight-group. Because it was difficult to measure quantities less than 1 µl, samples from weight-groups III and IV were often pools of several ticks. Hemolymph samples were immediately diluted 1:50 in cold 0.01 M phosphate buffered saline at pH 7.4 with 0.05% Tween 20 (PBS-Tween 20). The diluted hemolymph was kept frozen in 250 µl polyethylene microcentrifuge tubes until assayed for host IgG content.

An enzyme-linked immunosorbent assay (ELISA) was used to measure the amount of host IgG in the hemolymph. A double antibody sandwich method as outlined by Voller et al. (1979) was found to be the most sensitive and consistent. NUNC 96F Immunoplate I (Irvine Scientific, Santa Anna, CA) plates were used to bind the IgG fraction of antihost IgG serum at 500 ng/ml (Cooper Biomedical, Malvern, PA) or Ovalbumin at 50 µg/ml. Alkaline phosphatase was conjugated to the antihost IgG antibodies. All assayed materials were diluted with PBS-Tween 20 and were run in duplicate wells. Working volume for all reagents was 100 µl per well except for the NaOH solution (50 µl/well). Absorbance was measured with a Bio-Tek ETA Reader (Model EL 307 Bio-Tek Instruments,

Burlington, VT). The absorbance readings for host IgG at concentrations of 0.5-5.0 ng/ml were clearly positive compared with the negative controls. The detection range for rabbit antiovalbumin IgG were 1-10 ng/ml. These ranges were determined by using reference solutions of host IgG and of rabbit antiovalbumin IgG, respectively. A linear correlation ($r>0.96$) existed between the absorbance values and the IgG concentration at this range. During preliminary studies (Ben-Yakir 1985) it was found that interference by nontarget hemolymph components reduced the sensitivity of the assay. Centrifugation of the diluted hemolymph at 12,000 xg for 3 minutes did not eliminate the interference. Attempts to "block" the interfering components with various concentrations of bovine serum albumin were unsuccessful. Therefore, reference solutions of rabbit IgG were assayed with various concentrations of hemolymph from sheep fed ticks, which was negative for rabbit IgG. Ovine and bovine IgG reference solutions were assayed with hemolymph of rabbit fed ticks for the same reason. Ten separate pools of hemolymph were used for these assays. The mean absorbance values of these assays were used to determine the percent reduction in absorbance due to interference (Table 2). Reference IgG solutions and negative control hemolymph were assayed with every plate. Hemolymph from sheep fed ticks was used as a negative control in the rabbit IgG studies, and hemolymph from rabbit fed ticks served as the negative control for the bovine and ovine IgG studies. For the antiovalbumin IgG assay, hemolymph from ticks fed on nonimmunized hosts of the same species was used as a negative control. For consistency of results the enzyme reaction was stopped when the reference IgG solution of 2.0 ng/ml reached the absorbance value of 1.0 (on a scale of 0.0-2.0). This took about 30 minutes at room temperature.

Hemolymph samples were diluted to give absorbance values of 0.4-1.8 and these values were corrected for the effect of interference by dividing them by (100-% reduction) $x10^{-2}$. The concentration of host IgG in the samples were determined by comparing the corrected absorbance values to a linear regression curve (the least squares method) calculated from the absorbance values for the reference IgG solutions tested on the same plate. The fraction of host IgG that was found in the hemolymph was determined by dividing the mean concentration of host IgG in the hemolymph by the serum concentration of IgG. The fraction of anti-ovalbumin IgG found in the hemolymph of sheep or calf fed ticks was estimated by comparing the corrected mean absorbance values of the hemolymph samples with the absorbance values of the serum.

RESULTS

The sites of tick feeding on most hosts in this study became intensely irritated about one week after the initial infestation. The special feeding cells were very effective in protecting the ticks from the host grooming activity.

Most hemolymph collected had a brown-green tint, that became lighter as feeding progressed. In weight-groups I and II about 10-20% of the ticks had colorless hemolymph that coagulated very slowly and allowed collection of large volumes. The protein patterns of the hemolymph samples and the gut content were clearly different (Ben-Yakir 1985). When hemolymph was diluted with PBS-Tween 20 a brown precipitate formed near the tip of the capillary tube and it disappeared upon mixing. This precipitate also formed in 0.05 M Tris buffer solution (pH 7.2) and distilled water, and it was not affected by the addition of phenyl-thiourea (prevents melanization of insect hemolymph) to the diluent solutions.

The absorbance values for the control samples were all below 0.2. Hemolymph was diluted 1:250-1:1,000 to measure antiovalbumin IgG and 1:1,000 to 1:8,000 for measuring the total host IgG. The lowest detectible concentrations of IgG in the hemolymph were 0.5 µg/ml for antiovalbumin IgG and 0.3 µg/ml for total IgG.

Tables 3 through 6 summarize the concentrations of total host IgG in the hemolymph. Tables 7 and 8 summarize the concentrations of

antiovalbumin rabbit IgG in the hemolymph. Several samples in group III and all samples in group IV were pooled hemolymph from 2-5 ticks, therefore, a lower number of samples are shown for these groups. Within each group there was no apparent relations between the tick body weights and the IgG concentration in the hemolymph. Ticks with colorless hemolymph usually had the lowest IgG concentrations. These ticks greatly increased the variability of the results in weight-groups I and II.

The serum IgG concentrations, as established by the ELISA, were slightly higher than the reported values for rabbits (Kosma et al. 1967) and sheep, and slightly lower than the reported values for calves (Tizard 1977). In the hyperimmunized rabbits the specific antiovalbumin IgG was about 20-40% of the total serum IgG. A large increase (about 50%) in serum IgG occurred in both sheep from the time of D. variabilis removal to the time of A. americanum removal, 4 days later. Tables 9 and 10 summarize the fractions of total and antiovalbumin host IgG that was found in the hemolymph.

DISCUSSION

This is the first report of host IgG in the hemolymph of female A. americanum. The lack of similarity between the protein patterns of the hemolymph samples and the tick gut content, and the consistency of the results of this study, both indicate that the IgG detected in the hemolymph had passed naturally into the hemocoel of these ticks. Based on this study and previous reports (Ackerman et al. 1981, Brossard and Rais 1984, Fujisaki 1984) it appears that the crossing of serum proteins, including antibodies, into the hemocoel is a widespread phenomenon among ticks. The host IgG in the hemocoel maintains its ability to bind specifically to the antigen it was raised against. It was not determined in this study whether intact IgG molecules or only the antigen binding fragments (Fab') were present in the hemolymph. This information has little bearing on our goal to inhibit or neutralize an antigen in the hemolymph. The hemolysin activity in the hemolymph of I. ricinus (Brossard and Rais 1984), which requires an intact antibody molecule, indicates that at least some intact antibodies are able to pass through the gut wall of ticks. It is not known how these large molecules (MW 180,000 daltons) are able to avoid digestion and pass through an intact layer of gut epithelium. According to Akov (1982) the digestive enzymes of ticks act rapidly on hemoglobin but slowly upon other blood proteins. There is a slight possibility that some of the undigested blood proteins are transported into the hemocoel to aid in osmoregulation,

as was reported for host albumin in the hemolymph of Tsetse flies (Nogge and Giannetti 1980).

A gradual increase in host IgG concentration was generally observed as feeding progressed. The concentration of host IgG in the hemolymph was the highest during the rapid feeding phase and around detachment time (weight-group I). This may be due to the accumulation of IgG in the hemocoel or to an increase in the gut permeability as it stretches to accomodate the bloodmeal. All ticks in weight-group I had a detectable concentration of IgG in their hemolymph. The concentration of IgG in the host serum had a direct relationship to the IgG concentration in the hemolymph. Among ticks that fed on the same host the standard deviation was usually about 40% of the mean. These variations may be due in part to differences in the fluid content of the hemolymph at the time of collection. For example, the ticks with colorless hemolymph and relatively low IgG concentrations probably failed to excrete the excess fluid via their salivary glands. The findings of greater fractions of host IgG in the hemolymph of D. variabilis may be due to their ability to concentrate the bloodmeal about 3-fold compared with only 2-fold concentration by A. americanum (Koch and Sauer 1984). Slightly larger fractions of rabbit IgG were found in the tick hemolymph compared with the fractions of serum IgG of sheep and calves. This may be due to an actual difference between these hosts or to an experimental artifact, since each host was tested with a different set of reagents.

Based on these results it might be expected that 0.5 to 3.0 µg/ml of specific IgG will be available for reaction with a hemolymph antigen in ticks feeding on a vaccinated host. This reaction may lead to inhibition of an enzyme, neutralization of a hormone or agglutination

and precipitation of hemolymph components which would interfere with their normal function. Only hemolymph components at low concentration are expected to be affected by specific antibodies to a significant extent. Although the total concentration of soluble proteins in the hemolymph of replete female ticks is 85 mg/ml for A. americanum and 100 mg/ml for D. variabilis (Ben-Yakir 1985), enzymes and hormones in the hemolymph are likely to be present at low concentrations. The results of this study also provide a possible explanation of why ticks fed on rabbits hyperimmunized with cell-free hemolymph suffered only minor deleterious effects (Ben-Yakir 1985). Rather than immunizing a host with an entire tissue, like hemolymph, there is a need to identify a single antigenic molecule or a particular antigenic determinant in the hemolymph that its deactivation with specific antibodies will produce deleterious changes in ticks.

Most of the research toward developing an antitick vaccine has focused on the use of salivary gland antigen. This approach is based on the detailed study of the immunological mechanisms responsible for the natural development of tick resistance in the host. These studies have led to relatively successful induction of resistance in naive animals by immunization with antigens from salivary glands (Wikel and Allen 1982, Brown et al. 1984). When extracts of whole tick homogenates or specific internal organs were used as immunogens (McGowan et al. 1980 and 1981 and review by Wikel 1982) the resistance that was induced was probably due to salivary antigens or their homologs in other tick tissues (Wikel-personal communications). Resistant hosts usually exhibit intense cutaneous basophilic hypersensitivity reaction to tick saliva which is the apparent mechanism that prevents ticks from feeding successfully on them. This

form of resistance is most effective against larvae because of their short mouthparts. Usually, only the nymphs and adults of the two tick species used in this study feed on domesticated animals. Several problems may be caused by hyperimmunization with salivary antigens. Exaggerated hypersensitivity often results in harmful effects in the host (Riek 1958). Intense cutaneous inflammation may produce skin lesions that affect the hide quality of cattle and in some instances may be exploited by secondary pathogens. On the other hand, hosts may become immunologically tolerant to salivary antigens following repetitive exposure to tick saliva (Berdyev and Khudainazarova, 1976).

Immunization with a hemolymph component that the host never encounters naturally eliminated the risk of hypersensitivity or immunotolerance. This type of immunization would use tick hemocoel as the site where the host immune system harms the tick. In addition, natural resistance will continue to play its role without being affected by the artificial immunization. It was established in this study that female ticks might be particularly susceptible to this type of immunization providing an appropriate antigen can be found in the hemolymph.

A study of the proteins in the hemolymph of A. americanum and D. variabilis has been initiated with the aim of identifying proteins that are present at low concentrations in the hemolymph of female ticks during the rapid-feeding phase. When identified the effects of deactivating these proteins with specific IgG will be investigated with the hope that these studies will provide material for an antitick vaccine.

SUMMARY

The concentration of specific (antiovalbumin) and total host immunoglobulin G (IgG) were measured in the hemolymph of female Amblyomma americanum and Dermacentor variabilis fed on rabbits, sheep and calves. An enzyme-linked immunosorbent assay (ELISA) was used to determine the concentration of IgG in the hemolymph of individual females every 2 days during their bloodmeal. Results were compared with the IgG concentrations in the host serum to determine the fraction of serum IgG that was found in the hemolymph. In most ticks the IgG concentration in the hemolymph increased as feeding progressed and reached a maximum level around the time of repletion. The fraction of host serum IgG that was found in the hemolymph at repletion time was 1:1,000 to 1:2,000 for D. variabilis and 1:2,000 to 1:4,000 for A. americanum. The concentration of antiovalbumin specific IgG in the hemolymph was about 2 μg/ml at repletion. Results were similar for ticks fed on all host species used in this study. The information from this study is being used currently for selecting an appropriate hemolymph component for an antitick vaccine.

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Table 1. Weight-groups of feeding ticks and quantities of hemolymph collected*

Group Number	Days Post Infestation	Weight Range (mg)		Hemolymph Collect/Tick(µl)	
		A. americanum	D. variabilis	Mean	Range
I	8-12	400-1200	300-800	5.0	0.5-60
II	6	60-400	80-300	3.0	0.5-20
III	4	10-60	15-80	1.0	0.2-2.0
IV	2	4-10	5-15	0.3	0.1-0.5

*Ticks were fed on rabbits, sheep and calves. There were no significant weight differences between ticks fed on the different host species.

Table 2. The percent reduction (mean ± SD) in absorbance values of reference IgG due to interference by nontarget hemolymph proteins.

Hemolymph Dilution	Percent*	
	A. americanum	D. variabilis
1:250	50 ± 13	80 ± 15
1:500	40 ± 10	65 ± 12
1:1,000	30 ± 7	50 ± 10
1:2,000	20 ± 4	40 ± 8
1:4,000	15 ± 2	25 ± 4
1:8,000	10 ± 1	15 ± 2

*Based on assays with 10 different pools of hemolymph.

Table 3. The concentration (mean ± SD) of total rabbit IgG in the hemolymph of A. americanum females fed on rabbits.

Weight-Group*	Hemolymph IgG in µg/ml (Number of Samples)					
	Rabbit-1	Rabbit-2	Rabbit-3	Rabbit-4	Rabbit-5	Rabbit-6
I	7.8 ± 4.2 (11)	3.4 ± 1.5 (10)	12.1 ± 2.7 (10)	3.9 ± 1.6 (10)	9.0 ± 3.0 (11)	5.6 ± 1.2 (10)
II	1.3 ± 1.0 (9)	0.7 ± 0.3 (9)	0.8 ± 0.3 (10)	1.0 ± 0.4 (7)	2.6 ± 1.6 (8)	1.3 ± 0.5 (10)
III	0.6 ± 0.2 (5)	0.4 ± 0.1 (6)	0.4 ± 0.1 (6)	0.5 ± 0.3 (6)	0.8 ± 0.2 (5)	0.6 ± 0.3 (6)
IV	0.7 ± 0.4 (5)	0.5 ± 0.1 (6)	0.4 1 0.1 (4)	0.4 ± 0.2 (4)	1.3 ± 0.5 (5)	0.5 ± 0.3 (4)
Serum IgG (µg/ml)	15.0×10^3	9.0×10^3	17.0×10^3	8.0×10^3	18.0×10^3	16.0×10^3

*See Table 1.

Table 4. The concentration (mean ± SD) of total rabbit IgG in the hemolymph of D. variabilis females fed on rabbits.

Tick Weight-Group*	Hemolymph IgG in µg/ml (Number of samples)					
	Rabbit-7	Rabbit-8	Rabbit-9	Rabbit-10	Rabbit-11	Rabbit-12
I	13.7 ± 7.3 (11)	16.4 ± 6.7 (10)	8.2 ± 4.3 (11)	10.4 ± 4.2 (11)	6.9 ± 2.9 (11)	9.7 ± 3.7 (11)
II	7.1 ± 3.2 (9)	13.4 ± 8.2 (9)	6.2 ± 4.2 (8)	7.2 ± 3.6 (10)	5.0 ± 2.8 (9)	4.4 ± 2.3 (9)
III	1.2 ± 0.5 (7)	2.5 ± 1.7 (8)	1.2 ± 0.5 (7)	1.0 ± 0.4 (8)	1.6 ± 0.7 (7)	1.8 ± 0.8 (6)
IV	0.9 ± 0.7 (6)	1.7 ± 1.0 (4)	1.7 ± 0.9 (4)	0.8 ± 0.4 (5)	0.8 1 0.5 (4)	1.0 ± 0.3 (3)
Serum IgG (µg/ml)	12.0×10^3	20.0×10^3	8.0×10^3	10.0×10^3	12.0×10^3	9.0×10^3

*See Table 1.

Table 5. The concentration (mean ± SD) of total ovine and bovine IgG in the hemolymph of A. americanum females fed on sheep and calves.

Weight-Group*	Hemolymph IgG in µg/ml (Number of Samples)			
	Sheep-1	Sheep-2	Calf 1	Calf-2
I	10.0 ± 4.3 (17)	9.8 ± 4.1 (16)	5.8 ± 4.4 (16)	4.7 ± 2.6 (13)
II	3.6 ± 2.5 (9)	4.2 ± 1.4 (10)	4.0 ± 3.7 (12)	2.6 ± 1.8 (11)
III	1.2 ± 0.9 (6)	1.5 ± 1.1 (5)	1.5 ± 1.4 (7)	0.7 ± 0.6 (12)
IV	2.8 ± 1.2 (4)	1.4 ± 0.6 (4)	3.5 ± 1.1 (4)	2.6 ± 0.8 (4)
Serum IgG (µg/ml)	36.0×10^3	34.0×10^3	18.0×10^3	12.0×10^3

*See Table 1.

Table 6. The concentration (mean ± SD) of total ovine and bovine IgG in the hemolymph of D. variabilis females fed on sheep and calves.

Tick Weight-Group*	Hemolymph IgG in µg/ml (Number of Samples)			
	Sheep-1	Sheep-2	Calf-1	Calf-2
I	9.8 ± 3.4 (15)	11.3 ± 3.5 (15)	8.4 ± 5.1 (20)	7.6 ± 3.8 (15)
II	1.0 ± 0.5 (9)	4.8 ± 3.1 (10)	1.3 ± 1.0 (9)	4.5 ± 3.6 (9)
III	0.8 ± 0.6 (7)	1.8 ± 1.3 (7)	1.3 ± 0.5 (6)	2.2 ± 1.4 (8)
IV	2.1 ± 1.6 (4)	2.5 ± 1.5 (4)	3.7 ± 2.7 (5)	2.7 ± 1.1 (4)
Serum IgG (µg/ml)	23.0×10^3	24.0×10^3	18.0×10^3	12.0×10^3

*See Table 1.

Table 7. The concentration (mean ± SD) of antiovalbumin rabbit IgG in the hemolymph of A. americanum females fed on rabbits hyperimmunized with ovalbumin.

Tick Weight-Group*	Hemolymph IgG (µg/ml) (Number of Samples)			
	Rabbit-1	Rabbit-2†	Rabbit-4	Rabbit-5†
I	2.8 ± 1.7 (9)	0.8 ± 0.6 (10)	0.9 ± 0.5 (8)	2.6 ± 1.3 (9)
II-IV	<0.5	<0.5	<0.5	<0.5
Serum IgG (µg/ml)	5.0×10^3	4.0×10^3	3.0×10^3	4.5×10^3

*See Table 1.

†Serum and hemolymph of the control rabbits #3 and #6 were negative.

Table 8. The concentration (mean ± SD) of antiovalbumin rabbit IgG in the hemolymph of D. variabilis females fed on rabbits hyperimmunized with ovalbumin.

Tick Weight-Group*	Hemolymph IgG (µg/ml) (Number of Samples)			
	Rabbit-7	Rabbit-8†	Rabbit-10	Rabbit-11†
I	3.1 ± 0.5 (10)	4.6 ± 0.9 (8)	3.4 ± 1.2 (9)	4.3 ± 0.9 (9)
II	1.9 ± 1.5 (8)	3.5 ± 0.7 (7)	2.5 ± 1.5 (8)	3.0 ± 0.6 (8)
III	0.9 ± 0.2 (5)	1.6 ± 0.6 (6)	1.3 ± 0.5 (6)	1.4 ± 0.5 (5)
IV	1.3 ± 1.0 (3)	0.8 ± 0.2 (3)	0.8 ± 0.3 (3)	1.7 ± 1.1 (3)
Serum IgG (µg/ml)	4.0×10^3	6.0×10^3	4.5×10^3	5.0×10^3

*See Table 1.

†Serum and hemolymph of the control rabbits #9 and #12 were negative.

Table 9. The fraction* of total host serum IgG that was found in the hemolymph of female ticks of weight-group I.†

Host††	No. of Samples	A. americanum	No. of Samples	D. variabilis
Rabbit-1;7	11	1:1,887	11	1:909
Rabbit-2;8	10	1:2,632	10	1:1,250
Rabbit-3;9	10	1:1,408	11	1:1,000
Rabbit-4;10	10	1:2,083	11	1:1,000
Rabbit-5;11	11	1:2,000	11	1:1,724
Rabbit-6;12	10	1:2,341	12	1:909
Sheep-1	17	1:3,571	15	1:2,326
Sheep-2	16	1:3,448	15	1:2,128
Calf-1	16	1:3,125	20	1:2,128
Calf-2	13	1:2,564	15	1:1,587

* = $\frac{\text{Mean concentration of host IgG in the hemolymph}}{\text{concentration of host IgG in the serum}}$.

† See Table 1.

†† See Tables 3-6.

Table 10. The fraction* of antiovalbumin host serum IgG that was found in the hemolymph of female ticks of weight-group I.†

Host††	No. of Samples	A. americanum	No. of Samples	D. variabilis
Rabbit-1;7	9	1:1,786	10	1:1,282
Rabbit-2;8	10	1:5,000	8	1:1,299
Rabbit-4;10	8	1:3,333	9	1:1,316
Rabbit-5;11	9	1:1,695	9	1:1,163
Sheep-1	12	1:3,030	11	1:1,493
Calf-2	16	1:4,000	20	1:2,128

*For rabbits:

$$= \frac{\text{Mean concentration of antiovalbumin in the hemolymph}}{\text{concentration of antiovalbumin IgG in the serum}}$$

For sheep and calf:
Estimated by comparing the corrected mean absorbance values of the hemolymph to the absorbance values of the sera.

†See Table 1.

††See Tables 7 and 8.

PART IV

ELECTROPHORETIC STUDIES OF HEMOLYMPH FROM FEEDING *AMBLYOMMA AMERICANUM* (L.) AND *DERMACENTOR VARIABILIS* (SAY)

Several investigators reported that antibodies injested by ticks during feeding crossed the gut epithelium of the tick without losing the affinity for their specific antigens (Ackerman et al., 1981, J. Parasitol. 67:737-740; Brossard and Rais, 1984, Experientia 40:561-563; Fujisaki et al., 1984, Ann. Trop. Med. Parasitol. 78:449-450). Thus, specific antibodies may be used to inhibit an enzyme or neutralize a hormone of the tick hemolymph which could debilitate tick. Studies showed that only a small fraction of the host serum antibodies can be found in the hemolymph of Dermacentor variabilis (Say) and Amblyomma americanum (L.) (Ben-Yakir, 1985, Ph.D. Thesis, Oklahoma State Univ. 68 p.). Therefore, hemolymph components that are at low concentration are more likely to be inhibited or neutralized to a significant extent. In addition, a single antigenic component of the hemolymph might be used for immunizing the host in order to produce a sufficiently high and specific antibody titer.

This study was designed to provide preliminary information about the proteins in the hemolymph of D. variabilis and A. americanum. Hemolymph samples of feeding and replete females were studied. Each tick species was fed on two rabbits. Rabbits were 6-months-old and weighed 2-3 Kg, none of them was used to feed ticks prior to this study. Adult ticks were fed in six cells made of polystyrene sample vials affixed with adhesive to the shaved back of the rabbits (Ben-Yakir, 1985, loc. cit.). Plastic collars prevented the rabbits from disturbing the cells. (Watt et al., 1972, J. Parasitol. 58:1105). Two cells were infested on day 1 and one cell was infested on days 3, 5, 7 and 9. Seven pairs of ticks (males and females) were placed in each cell. When 50% of the adult females had engorged and detached in the cells infested on day 1,

rabbits were bled by cardiac-puncture, then sacrificed and skinned. The skins with attached ticks were left for 24 hours at 23 ± 2°C and 90% RH. This procedure allowed the ticks to freely detach themselves and prevented the potential damage from forced detachment of the partially engorged ticks.

The detached ticks were weighed and assigned to weight-groups as described in Table 1. Ticks were rinsed for 1 minute with cold water to wash away serum exudate, blood, or tick feces from their exterior surfaces. Ticks were cooled for 30-60 minutes at 4°C. Hemolymph was collected into precalibrated capillary tubes after amputating the distal portion of one or more legs with a fine-tipped forceps, while ticks were held on a cold-table (5-10°C). Hemolymph with reddish tint, indicating it had been contaminated with gut content, was not used. Table 1 lists the mean quantities of hemolymph collected from each weight-group. Whenever it was possible, samples were collected from individual ticks. Because it was difficult to measure quantities less than 1 µl, several samples in weight-groups III and IV were pooled hemolymph from several ticks. Fresh hemolymph samples were assayed by discontinuous polyacrylamide gel electrophoresis (PAGE) using a Protean™ dual slab cell apparatus and chemicals (Bio-Rad Lab., Richmond, CA). An aliquot containing approximately 80 µg proteins was mixed with equal volume of sample buffer (pH 6.8). The samples were applied to a 0.75 mm slab gel. The staking gel and the running gel were 3.5% and 7.5% acrylamide, respectively. Electrophoresis was carried out with electrode Tris buffer at pH 8.1. The current was 15 mA per gel for the first 30 minutes and 10 mA per gel until the bromophenol blue tracking dye reached the bottom of the gel. Because most protein bands were crowded at the upper

portion of the gel, samples were run again for double the original time to obtain better separation. Gels were fixed in 25% isopropanol and 10% acetic acid for 15 minutes at 60°C, stained with 0.025% Coomassie blue R-250 for 15 minutes at 60°C, and destained in 10% acetic acid overnight at room temperature. In order to obtain information about the relative concentration of the proteins, gels were scanned with an Auto Scanner^{TM} (Helena Lab., Beaumont, TX) set at a wave length of 545 nm.

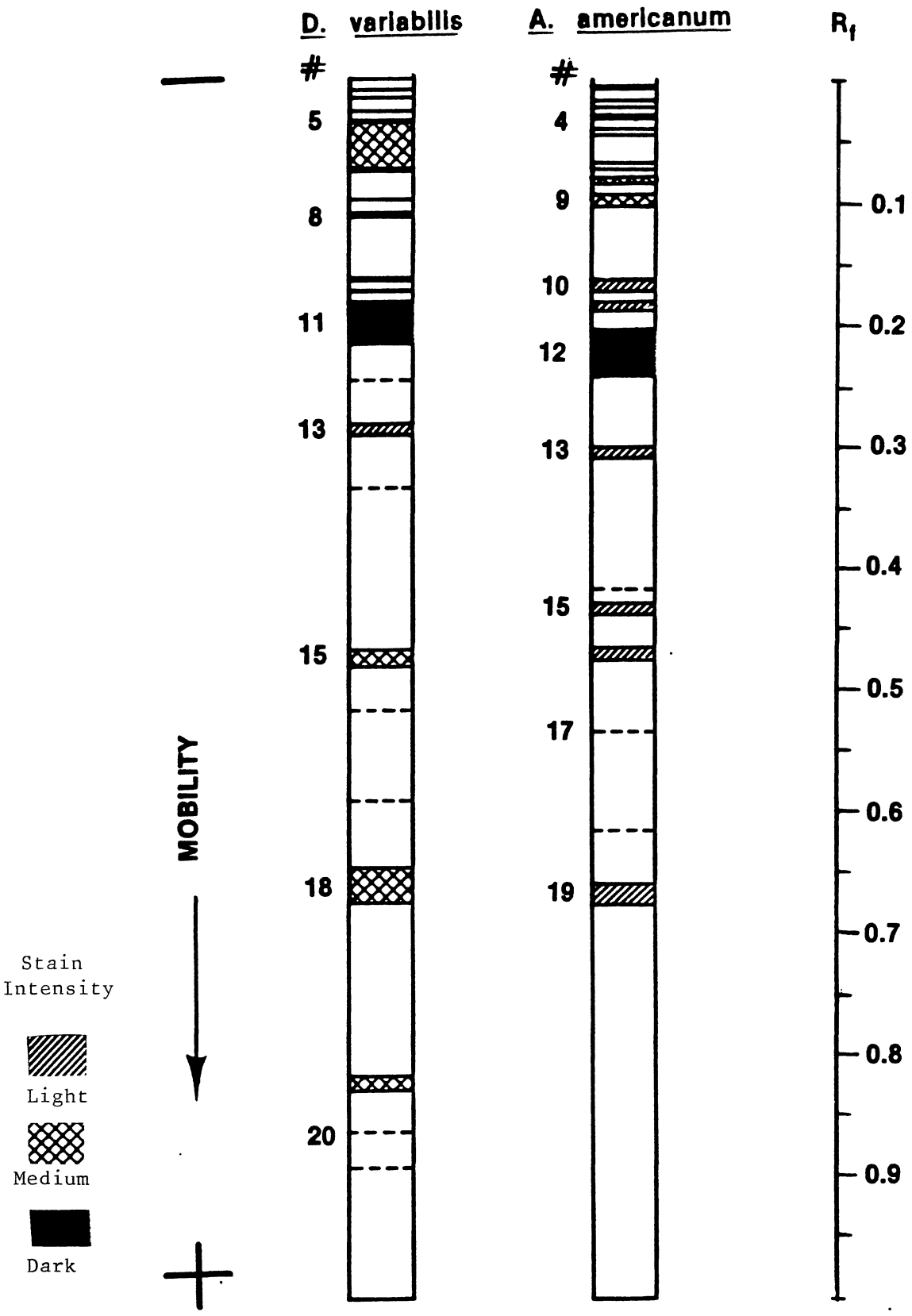
Figure 1 shows hemolymph proteins of feeding females detected by PAGE of 6 samples from each weight-group. A total of 19 bands were detected in A. americanum hemolymph and 21 bands in D. variabilis. Most protein bands were present throughout the feedings period. In A. americanum band #3 was absent in group I, #5 and #6 were absent in groups I and IV, #7 decreased in concentration as feeding progressed, #15 and #16 only one of these was usually present in groups I and II, #19 increased in concentration as feeding progressed. In D. variabilis bands #6, #8 and #10 increased in concentration as feeding progressed, #7 was present only in group I, #12 and #13 decreased in concentration as feeding progressed, #20 and #21 were present in some individuals in all four groups. The number of protein bands and their pattern were similar to previous electrophoretic studies reported for Ixodid ticks (Araman, 1979, Recent Advances in Acarology, Academic Press, N.Y. vol. I:385-95; Dolp and Hamady, 1971, J. Med. Entomol. 8:632-642; Tatchell, 1971, Insect Biochem. 1:47-55). Further studies are needed to identify and isolate hemolymph proteins that occur in low concentrations, because these will be the most likely candidates for an antitick vaccine.

Table 1. Weight-groups of feeding ticks and quantities of hemolymph collected.*

Group Number	Days Post Infestation	Weight Range (mg)		Hemolymph Collect/Tick (µl)	
		A. americanum	D. variabilis	Mean	Range
I	8-12	400-1200	300-800	5.0	0.5-60
II	6	60-400	80-300	3.0	0.5-20
III	4	10-60	15-80	1.0	0.2-2.0
IV	2	4-10	5-15	0.3	0.1-0.5

*Ticks were fed on rabbits.

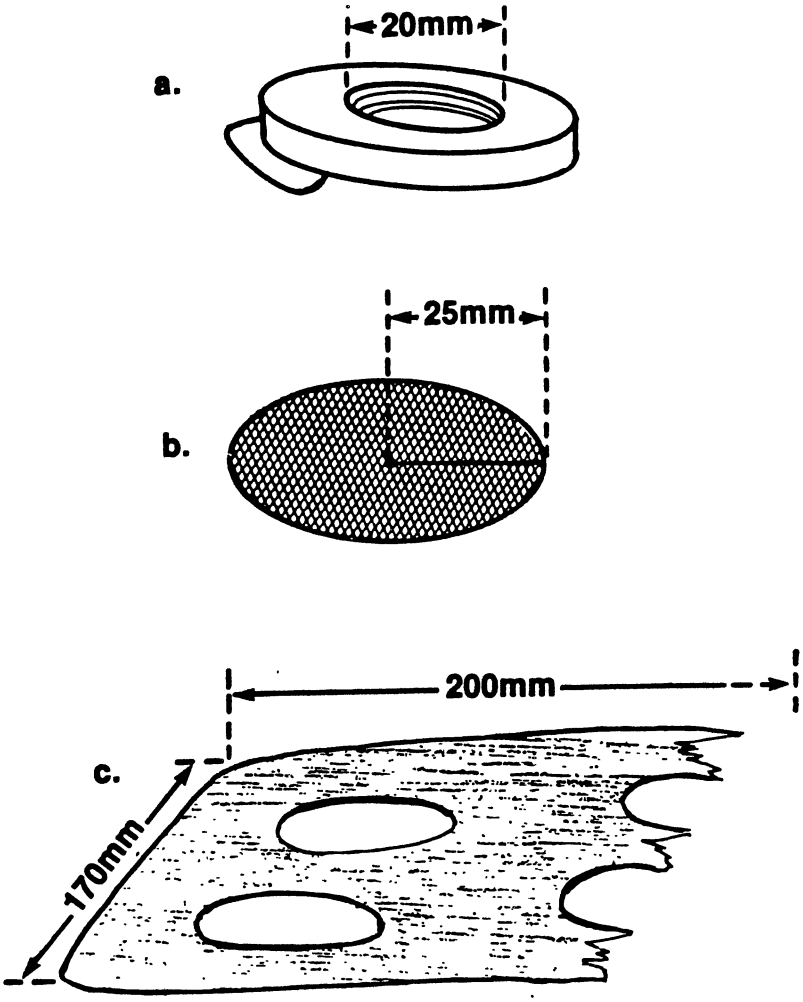
Figure 1. The pattern of all hemolymph proteins of feeding female ticks obtained by polyacrylamide gel electrophoresis


D. variabilis
A. americanum
R_f
#
5
8
11
13
15
18
20
#
4
9
10
12
13
15
17
19
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
MOBILITY
Stain Intensity
Light
Medium
Dark

APPENDIXES

APPENDIX A

CELLS USED TO FEED TICKS ON RABBITS

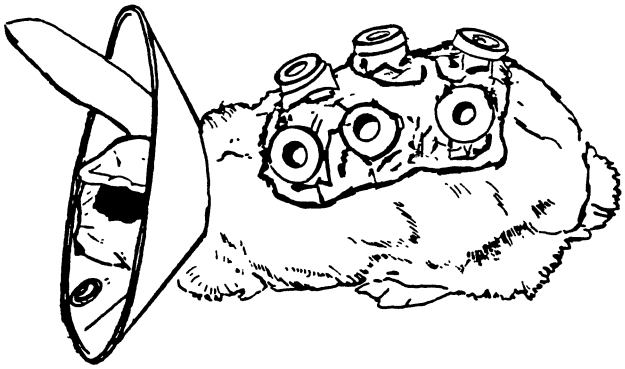


Materials:

a. Polyethylene snap-cap
b. Fine-mesh polyester cloth
c. Heavy duty denim cloth
d. 13 dram polystyrene sample vial
e. 2 mm thick plexiglass

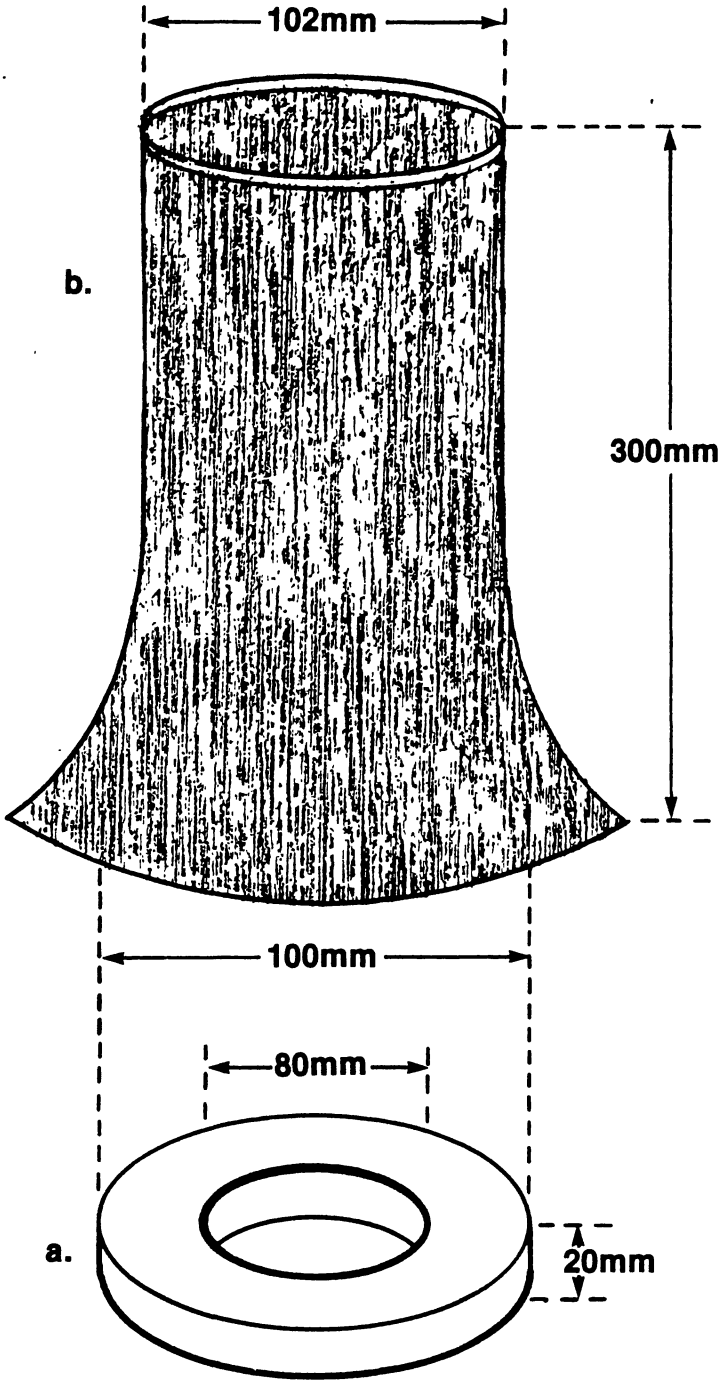
Construction Procedure:

1. Saw a ring of plexiglass
2. Saw the upper 25 mm of a sample vial
3. Glue plexiglass ring to the cut edge of vial
4. Cut denim and fit cells in the holes
5. Shave the rabbit back
6. Glue cells with cloth to the shaved back with industrial adhesive (#4499, 3M Co. St. Paul, MN)



APPENDIX B

CELLS USED TO FEED TICKS ON SHEEP AND CALVES

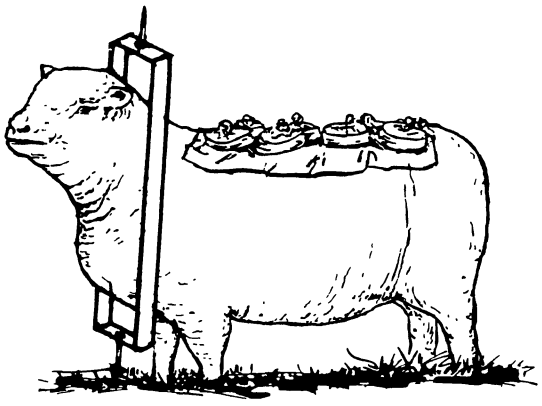


Materials:

a. Small cell polyurethane foam
b. Orthopedic stockinette

Construction Procedure:

1. Cut ring of foam rubber
2. Cut stockinette
3. Shear sheep
4. Glue rings of foam to the shorn back with industrial adhesive (#4499, 3 M Co., St. Paul, MN) and allow to dry for 20 minutes.
5. Apply industrial adhesive to the top, outside and around the ring, spread the bottom edge of the stockinette over the ring and glue them together.



VITA 2

David Ben-Yakir

Candidate for the Degree of

Doctor of Philosophy

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