



Immunization of rabbits with *Amblyomma hebraeum* nymphal homogenates and implications for the host amplification system

S.D. TEMBO

Medical Microbiology Department, Medical University of Southern Africa
Box 211, Medunsa, 0204 South Africa

ABSTRACT

TEMBO, S.D. 1988. Immunization of rabbits with *Amblyomma hebraeum* nymphal homogenates and implications for the host amplification system. *Onderstepoort Journal of Veterinary Research*, 65:97–103

Immunochemical mechanisms involved in tick rejection by a host are not well documented. The role of serum globulins, and that played by the amplification system's humoral products (thrombin from the coagulation, plasmin from the fibrinolytic, and kallikrein from the kinin systems) in tick-resistant animal hosts have not yet been demonstrated. It is known, however, that factors C1, C3 and C5 of the complement system play a role in tick rejection, and that factors C3a and C5a are anaphylatoxins capable of degranulating leukocytes, thereby releasing pharmacologically active vasoamines which are involved in tick rejection.

In this study, levels of kininogen increased by 56% and those of fibrinogen by 19% in rabbits immunized with nymphal antigens. A highly significant ($P < 0,001$) number of nymphs that fed on the immunized rabbits failed to moult into adult stages.

It is reported for the first time, that increased levels of two glycoproteins, fibrinogen and kininogen occurred in rabbits immunized with homogenates of *Amblyomma hebraeum* ticks. The role played by the amplification system in tick rejection in resistant animals is clarified.

Keywords: Amplification system, bradykinin, coagulation, complement, fibrinogen, fibrinolytic, inflammation, immunization, kinin, kininogen, ticks, vasoamines

INTRODUCTION

Trager (1939a) observed that the acquisition of resistance in guinea pigs to *Ixodes scapularis* ticks could be effected through repeated exposure to this ectoparasite, and by passive transfer of serum from primed to naïve guinea pigs. Hence, he established for the first time that the immune system could defend vertebrate hosts against ectoparasites. He also pioneered immunoprophylaxis in laboratory animals against tick infestations (1939b). Guinea pigs were protected against *Dermacentor variabilis* larval challenge by intracutaneous injection of extracts prepared from whole larvae. Trager's work (1939a, 1939b)

initiated research in anti-tick vaccines by various groups of scientists.

Gregson (1941) injected guinea pigs with extracts prepared from semi-engorged *Dermacentor andersoni* nymphs and observed that a significant number of nymphs from immunized animals failed to moult. Further research on an anti-tick vaccine was based on salivary gland extracts (Binnington & Kemp 1980). Although such a vaccine performed well in laboratory models such as guinea pigs, using *Amblyomma americanum*, it did not have much impact against *Boophilus* ticks in cattle hosts (Ribeiro 1995). Much work on host immune responses to tick infestation, both at humoral and cellular levels have been reported (Wikel & Allen 1982; Willadsen 1980). Later,

Australian researchers moved to the concept of using antigens that are not normally exposed to the vertebrate host, such as midgut antigens, hence referred to as "concealed antigen" to produce an anti-tick vaccine (Willadsen & Kemp 1988).

Allen (1973) put forward the most plausible hypothesis which, until the present time, forms the biochemical and immunological basis for the theory of tick rejection by resistant animal hosts.

Factors involved in overcoming a host's haemostasis in resistant animals by haematophagus parasites, such as ticks, in order to locate blood and maintain its flow during ingestion, are well documented (Ribeiro, Makoul, Levine, Robinson & Spielman 1985; Ribeiro 1995).

MATERIALS AND METHODS

Ticks

Amblyomma hebraeum Koch 1844 nymphs were used for the production of the homogenates, and for challenge infestations. All the developmental stages of the tick were reared on Himalayan rabbits and in kept at $25 \pm 1^\circ\text{C}$, $75,0 \pm 5,0\%$ RH (Winston & Bates 1960) with a photo period of 14 h in summer and 12 h in winter.

Rabbits

Six-month-old tick naive Himalayan rabbits of both sexes, weighing $3,3 \pm 0,97$ kg, were reared at the Animal Production Unit, Medical University of Southern Africa. Rabbits were placed singly in cages (56 x 56 x 22 cm), and fed *ad libitum* on commercial pellets (Tembo & Rechav 1992).

Preparation of nymphal homogenates

Nymphal homogenates were prepared from batches of three-week-old unfed laboratory bred *A. hebraeum* nymphs as previously described (Tembo & Rechav 1992).

Blood sampling

Blood samples for haematological and plasma protein analyses were collected immediately before the commencement of the experiment, and thereafter at weekly intervals from ear veins, as previously described (Tembo & Rechav 1992).

Plasma globulin analysis

Plasma globulins were analyzed to determine changes in their levels resulting from inoculations with nymphal homogenates (Tembo & Kiwanuka 1997). Processed plasma proteins were analyzed for albumin,

alpha-1, alpha-2, beta and gamma globulin levels by electrophoresis using the method of Laurell (1972).

Blood cell counts

Blood cell counts were done using differential methods. Thin blood smears on glass slides were stained with May-Grunwald and Giemsa stains, leukocytes were counted under a microscope under oil immersion, and the percentages of the various types calculated (Medway, Prier & Wilkinson 1969).

Immunization of rabbits

Four naive rabbits were each immunized with 1,5 mg of an *A. hebraeum* nymphal homogenate in 0,5 ml PBS to which a similar volume of Freund's incomplete adjuvant (FIA) (Sigma Chemical Company, St. Louis, Ma., USA) had been added. The homogenates were mixed with the adjuvant 3 h before the rabbits were inoculated. A second, similar inoculation was administered 14 d later. Twenty-eight days after the first inoculation, each rabbit was given a third injection consisting of 1,5 mg of homogenate in 0,5 ml PBS but without adjuvant. Another group of four naive rabbits was used as controls. Each of these received an injection of a solution containing 0,5 ml FIA in 0,5 ml PBS on days 0, 14 and 28 simultaneously with the immunized group. Immunizations were done as previously described (Tembo & Rechav 1992).

Fractionation of fibrinogen

Ten millilitres of rabbit plasma kept at 0°C from immunized and control rabbits was thawed at room temperature and centrifuged at $10000 \times g$ before precipitation with 8% ethanol at pH 7,5. Ethanol was removed by reverse osmosis in distilled water, and the fibrinogen lyophilized. The amount of purified fibrinogen was determined on an analytical balance (Blomback 1972; Blomback & Blomback 1972).

Purification of kininogen

Two and a half millilitres of rabbit plasma from each of the immunized rabbits were pooled in the presence of the anticoagulant, citrate-dextrose-phosphate. Similarly, plasma samples from each of the control animals were pooled. The resulting aliquots were analyzed separately (Turpeinen, Syvänen & Hamberg 1981). Gel chromatography (36,5 x 1,6 cm) on Sepharose 4B activated CNBr coupled with anti- α_2 human serum (anti- α_2 -HS) (Behring, K35 BL6) standardized with Tris buffer, pH 8, was done to remove other serum proteins (Syvänen, Turpeinen, Siimesmää & Hamberg 1981). Blood kininogen was recovered in Tris buffer and desalted on activated Sephadex G25 columns (36,5 x 1,6 cm) coupled to anti-kininogen (Behring, K16 BL12), and eluted with distilled water at a flow rate of 1 ml/min and lyophilized.

The amount of kininogen was estimated on an analytical balance and the molecular mass determined by polyacrylamide gel electrophoresis (8%) in the presence of sodium dodecyl sulphate (SDS), according to a standard method practised at Helsinki University, Finland (Syvänen *et al.* 1981; Kärkkäinen, Syvänen, Terpeinen & Hamberg 1982).

Challenge nymphal infestation

One hundred *A. hebraeum* nymphs were used in challenge infestations on each rabbit in the control and immunized groups. On the same day that the last injection was administered, the nymphs destined for each animal were released into a cotton bag (15 x 15 cm) glued (Bostik, Formex Industries, trade mark of Bostik Ltd, England) to the shaven back of the animal (Tembo & Rechav 1992). They were allowed 24 h to attach after which engorged nymphs from each animal were collected and weighed individually so that the group mean engorgement mass (MEM) could be calculated. The moulting success was determined by comparing the percentages of nymphs from control animals that moulted to those that did not moult from the immunized group.

Presentation of data

Blood parameters are presented in (g/l) of various blood components at various intervals (cf. Table 1). For statistical analyses, the trapezoidal area rule (\pm S.D.) (Franklin & Newman 1973) was applied in determining levels of significance in the increases of concentrations of blood components over a given time and the two sample *t*-test employed.

A student *t*-test was used to determine mean feeding periods and mean engorgement mass differences, and the chi-square test for a 2 x 2 contingency table was employed in the statistical analyses of the levels of significance in moulting percentages.

RESULTS

The results are presented in Tables 1–5.

Immune response

No significant ($t = 0,87$; $P < 0,41$) increases in beta globulins were observed in the immunized rabbits, but the gamma globulin levels did increase significantly ($t = 3,81$; $P < 0,009$). Non-significant ($P > 0,05$) increases in leukocyte numbers were observed (Table 1).

Effects on feeding success

Nymphs from immunized animals fed for significantly ($t = 2,26$; $P < 0,05$) shorter periods, weighed significantly less ($t = 7,12$; $P < 0,0025$), and highly signifi-

cant ($Ch^2 = 14,9$; $P < 0,001$) numbers failed to moult into adult stages (Table 2).

Fibrinogen

The mean concentration of the glycoprotein fibrinogen increased by 19% in rabbits immunized with *A. hebraeum* nymphal homogenates, in comparison to that of the control group of rabbits injected with FIA (Table 3).

Kininogen

The mean plasma concentration of the glycoprotein kininogen increased by 56% in rabbits immunized with *A. hebraeum* nymphal homogenates, in comparison to that of the control group of rabbits (Table 4).

Estimation of kininogen molecular mass

The molecular mass of rabbit kininogen was estimated at 60 kilodaltons using standards in Polyacrylamide gel electrophoresis in the presence of SDS (Table 5).

DISCUSSION

Expression of resistance in animal hosts is dependent on the antigenicity and immunogenicity of the secretory components from the tick injected during tick feeding, or the homogenates used in immunization (Tembo & Kiwanuka 1997). Environmental, genetic and nutritional factors have also been observed to influence resistance in animals (Sutherst, Kerr, Mayward & Stegeman 1983). However, the following are regarded as dependable parameters for evaluating host resistance to ticks:

- Reduced number of ticks that feed to repletion
- Reduction in mean engorgement mass
- Reduction in fecundity
- Changes in feeding duration
- At times, also death of ticks (Heller-Haupt, Varma, Rechav, Langi & Trinder 1987; Clarke, Els, Heller-Haupt, Rechav & Varma 1989)

Increases in plasma globulins have been observed in animal hosts that have been exposed to tick infestations (Rechav & Dauth 1987; Clarke *et al.* 1989), as well as in animals that had been immunized with various tick antigen (Njau & Nyindo 1987; Varma 1990; Tembo & Rechav 1992; Tembo & Kiwanuka 1997).

Plasma globulins are glycoproteins, and their elevated levels in resistant animal hosts have been mistakenly considered to have a direct influence on feeding ectoparasites, such as ticks. However, the enzyme, apyrase, which is found in the saliva of most insects that have been examined to date is capable

TABLE 1 Comparison of the blood components immunized group (1) of rabbits with those in the control groups (2)

| Blood component | Group | Mean area under curve (\pm S.D.) | <i>t</i> | <i>P</i> |
|------------------------------|--------|--|----------|----------|
| Beta ^a globulins | 1 2 | 138,2 \pm 10,9 0,41 131,9 \pm 9,7 | 0,87 | 0,41 |
| Gamma ^a globulins | 1 2 | 167,5 \pm 6,4 148,3 \pm 7,7 | 3,81 | 0,009 |
| Heterophils ^b | 1 2 | 1473,5 \pm 39,9 1474,5 \pm 52,3 | 0,03 | 0,97 |
| Lymphocytes ^b | 1 2 | 1560,3 \pm 34,0 1547,6 \pm 22,2 | 0,62 | 0,55 |
| Monocytes ^b | 1 2 | 46,9 \pm 1,3 45,7 \pm 0,6 | 1,70 | 0,14 |

^a = Mean mg.day/ml

^b = Mean numbers.day/ml

TABLE 2 Comparison in mean feeding days of nymphs from immunized rabbits and control rabbits

| | Immunized rabbits | Control rabbits | <i>t</i> | <i>P</i> |
|-----------------------|-------------------|------------------|----------|----------|
| Nymphs recovered | 259 | 351 | — | — |
| Mean feeding days | 6,4 \pm 2,12 | 7,3 \pm 2,74 | 2,26 | 0,05 |
| Engorgement mass (mg) | 62,4 \pm 7,45 | 66,3 \pm 11,04 | 7,12 | 0,0025 |
| Number moulting | 120 | 219 | 14,9 | 0,001 |
| Number not moulting | 139 | 132 | — | — |
| Moulting percentages | 46,2 | 62,6 | — | — |

TABLE 3 The amounts of fibrinogen (g/l) recovered from immunized and control rabbits by fractionation with 8 % ethanol

| | Original plasma sample (ml) | Volume ethanol (ml) | Mass precipitate (mg) | Estimated fibrinogen entration in plasma (g/l) |
|-----------|-----------------------------|---------------------|-----------------------|--|
| Immunized | 10 | 4 | 0,025 | 2,5 |
| Control | 10 | 4 | 0,021 | 2,1 |

TABLE 4 The amount of kininogen (g/l) recovered from immunized and control rabbits by gel and affinity chromatography

| | Original plasma sample (ml) | Volume recovered (sepharose column) (ml) | Volume recovered (sephadex column) (ml) | Lyophilised amount in plasma (g/l) | Estimated conc. in plasma (g/l) |
|-----------|-----------------------------|--|---|------------------------------------|---------------------------------|
| Immunized | 10 | 31 | 22 | 0,0792 | 3,6 |
| Control | 10 | 28 | 26 | 0,0623 | 2,3 |

TABLE 5 Estimation of molecular mass of kininogen by polyacrylamide gel electrophoresis (8 %) in the presence of sodium dodecyl sulphate

| Standards | Distance moved (cm) | Front (cm) | Relative mobility (cm) | Molecular mass (Daltons) |
|------------------|---------------------|------------|------------------------|--------------------------|
| Transferrin | 6 | 13,3 | 0,448 | 78,000 |
| Albumin | 7 | 13,3 | 0,522 | 68,000 |
| Oval albumin | 10 | 13,3 | 0,750 | 43,000 |
| Kininogen sample | 9 | 13,3 | 0,686 | 60,000 |

of hydrolyzing serum and plasma glycoproteins such as globulins (Champagne 1994; Ribeiro 1995). Increased globulin levels in animals that have been exposed to tick infestations can therefore only serve to indicate the antigenic properties of the tick secretory antigens injected during feeding, or tick homogenates used in the inoculations (Tembo & Kiwanuka 1997). Recent studies have shown increased levels of gamma globulins in sensitized animals (Clark *et al.* 1989; Rechav, Clark, Els & Dauth 1991; Tembo 1996). These gamma globulins comprise immunoglobulins G and M (IgG & IgM) which are known mediators of type III hypersensitivity, and, together with fibrinogen, can initiate the complement system (Andersson, Einarson & Lunden 1979).

In this study, although no correlation could be made between the increases in the serum globulins and the degree of resistance acquired by the immunized animals, there was a non-significant increase in the β -globulins, this band of globulin comprising of C3 and IgA, amongst other components, which demonstrates that the amplification system of the immune system was initiated (Andersson *et al.* 1979).

The direct role of phagocytic leukocytes against tick antigens secreted while feeding is not well documented, but it is known that vasoamines, such as histamine, released from degranulated cells play an important role in tick rejection (Allen 1973; 1989; Willadsen 1980; Tembo & Rechav 1992).

Theoretically, there seem to be three stages in the rejection of ticks in sensitised animals.

Primary rejection

The primary host tick rejection reaction occurs within hours after tick attachment due to an immediate hypersensitivity response stimulated by the secretion of salivary proteins of the tick (Schlenger, Lincoln, McKenna, Kemp & Roberts 1976; Willadsen 1980; Brown & Askenase 1981; De Castro & Crewe 1993). This stage of tick rejection was reported to occur within 48 h in cattle that had been infested with *B. microplus* larvae (Roberts 1968; Opdebeeck 1989). Prostaglandin E_2 (PGE_2), potentiates pain as a result of bradykinin, a vasoamine which mediates inflammation (Williams & Peck 1977; Kärkkäinen, Syvänen, Turpeinen & Hamberg 1982). Bradykinin, a nonapeptide, has a half life of 20s, is a product of kininogen cleaved by the enzyme kallikrein, and is activated in blood by kininases (Stites, Stobo, Fudenberg & Wells 1982). It has also been observed to be chemotactic to polymorphonuclear cells. Pain resulting from this reaction focuses attention of the host on the parasite's feeding site, and increases grooming behaviour. In this study kininogen levels and grooming increased more in immunized rabbits in comparison to those of the control group.

Secondary rejection

The secondary host's tick rejection reaction involves platelet aggregation which provides the main haemostatic obstacle at the feeding site (Mustard & Packham 1977). Ixodid ticks feed for an extended period to engorge, thereby providing ample time for inflammation to promote haemostasis at the feeding site (Hamberg, Turpeinen, Kärkkäinen & Syvänen 1980; Kemp, Stone & Binnington 1982; Wikel & Allen 1982). In this study, ticks fed for shorter periods on immunized rabbits and weighed less. The reduction in mean engorged mass could indicate that the platelet plug formed at the feeding sites prevented the ticks from ingesting an adequate amount of blood. These observations, and previous findings (Tembo & Rechav 1992), are similar to those of Varma, Heller-Haupt, Trinder & Langi (1990) on *Rhipicephalus appendiculatus* adult ticks fed on immunized guinea pigs, and bradykinin is a vasoamine and accounts for known biological functions of the kininogen-kinin system as one of the mediators in inflammation (Kärkkäinen *et al.* 1982).

Inflammatory reactions in the rabbits at the tick attachment sites were manifested by erythema, and oedema. The erythematous reaction increases the flow of blood to the feeding site (Kemp, Stone & Binnington 1982). Oedema, on the other hand, reduces blood flow, as has been observed in the skin of cattle resistant to *B. microplus* (Tatchell & Moorhouse 1968). Inflammation, therefore, both hinders and helps tick feeding.

Tertiary rejection

A host tick rejection is a complex mechanism involving the amplification systems, which comprise coagulation, kinin, fibrinolytic and complement systems (Stites *et al.* 1982).

The complement system seems to play a cardinal role in a host's defence against tick infestation. Factors released from lysed cells contain potent compounds which are largely responsible for inhibiting the feeding success of ticks.

Anaphylatoxins C3a and C5a of the complement system mediate inflammation by causing mast cell degranulation. Mast cells contain, *inter alia*, in their granules:

- The pharmacologically active heparin, histamine, serotonin, kinin protease and SRS-A
- Tissue degrading chymase
- Eosinophil and neutrophil chemotactic factors of anaphylaxis (ECF-A and NCF-A)
- Platelet aggregating factors (PAF) (Stites *et al.* 1982)

Basophils and neutrophils also contain histamine. Neutrophils and macrophages, which also participate

in inflammation, are a major source of SRS-A, prostaglandins and kinin (Cooper, 1982).

Thrombin, bradykinin and plasmin are known to activate C1 (Stites *et al.* 1982). Vargaftig, Chignard & Benveniste (1981) observed that adenosine diphosphate (ADP) from injured cells, collagen fibrils (exposed in subendothelial tissues), thrombin (produced after activation of the coagulation cascade) and PAF¹ (platelet aggregating factor, released by leukocytes) are the most important stimuli in inflammatory reactions in immunized animals.

Kemp, Koudstaal, Roberts & Kerr (1976) reported the failure of *B. microplus* larvae to gain mass on resistant animals, which lead to their subsequent death.

It would seem, therefore, that the coagulation, fibrinolytic, kinin and the complement systems do cooperate in limiting the blood intake by ectoparasites through inflammatory reactions, thereby allowing feeding ticks to ingest only the serum proteins and leukocytes, and not erythrocytes (Wells 1982). The involvement of the coagulation system in tick feeding has been reported by Waxman, Smith, Arcuri & Vlasuk (1990), and more recently by Joubert, Crause, Gaspar, Clarke, Spickett & Neitz (1994). The purified anticoagulants inhibit the clotting cascade at the junction of the extrinsic and intrinsic pathway (factor Xa).

Trager (1939a) reported that ticks that fed on resistant animals appeared to be pale in colour, which signified insufficient ingestion of erythrocytes. In this study, significant ($\text{Chi}^2 = 14.9$; $P < 0.001$) numbers of nymphs failed to moult into adult stages.

This study showed that both fibrinogen and kininogen levels increased in rabbits after immunization, and both these glycoprotein compounds lead to the initiation of the complement system (Syvänen *et al.* 1981). Therefore, more work needs to be done on the amplification systems in tick resistant animal hosts to determine the role these systems play during feeding on resistant hosts. The amplification system could hold the key to the understanding a host's resistance to tick infestations and the humoral mechanisms induced by tick vaccines. To achieve this goal it is important that both a biochemical and immunological approach be applied.

ACKNOWLEDGEMENTS

I thank Drs Tytti Kärkkäinen and Ulsulla Turpeinen, Biochemistry Department, University of Helsinki, Finland for advice and help in the purification of both kininogen and fibrinogen.

My sincere thanks to Prof. O. Mäkelä, Head of Bacteriology and Immunology Department, Faculty of Medicine, University of Helsinki, Finland for his advice and criticisms of this manuscript.

This work is dedicated to the late Professor Ulla Hamberg, University of Helsinki, former Head of Immunochimistry, who was one of the pioneers in kininogen studies. I was privileged to have been one of her post-graduate students.

REFERENCES

- ALLEN, J.R. 1973. Tick resistance: basophils in skin reactions of resistant guinea pigs. *International Journal of Parasitology*, 3: 195–200.
- ALLEN, J.R. 1989. Immunology of interactions between ticks and laboratory animals. *Experimental & Applied Acarology*, 7:5–13.
- ANDERSSON, L.-O., EINARSSON, M. & LUNDEN, R. 1979. Inhibitors of proteolysis in plasma, in *Plasma proteins*, edited by B. Blomback & L.A. Hanson. New York: J. Wiley & Sons: 336.
- BINNINGTON, K.C. & KEMP, D.H. 1980. Role of salivary glands in feeding and disease transmission. *Advances in Parasitology*, 18:315–339.
- BLOMBACK, B. 1972. Fibrinogen to fibrin transformation, in *Blood Clotting Enzymology*, edited by W.H. Seegers. New York: Academic Press: 143.
- BLOMBACK, B. & BLOMBACK, M. 1972. The molecular structure of fibrinogen. *Annals of the New York Academy of Science*, 202:77.
- BROWN, S.J. & ASKENASE, P.W. 1981. Cutaneous basophil responses and immune resistance of guinea pigs to ticks: passive transfer with peritoneal exudate cells or serum. *Journal of Immunology*, 127:2164–2167.
- CLARKE, F.C., ELS, D.A., HELLER-HAUPT, A., RECHAV, Y. & VARMA, M.G.R. 1989. Expression of acquired immunity to immature stages of the tick *Rhipicephalus evertsi evertsi* by rabbits and guinea pigs. *Medical Veterinary Entomology*, 3:35–39.
- CHAMPAGNE, D.E. 1994. The role of salivary vasodilators in blood feeding and parasite transmission. *Parasitology Today*: 431–433.
- COOPER, N.R. 1982. The complement system, *Basic and Clinical Immunology*, edited by D.P. Stites, J.D. Stobo, H.H. Fundenberg & J.V. Wells. Los Altos, California. Lange Medical Publication: 124–135.
- DE CASTRO, J.J. & CREWE, R.M. 1993. Host resistance in cattle tick control. *Parasitology Today*, 9:13–17.
- GREGSON, J.D. 1941. Host immunity to ticks. *Proceeding of Entomological Society*. British Columbia, 38:12–31.
- FRANKLIN, D.A. & NEWMAN, G.B. 1973. A guide to medical mathematics. Oxford: Blackwell Scientific Publications: 384–415.
- HAMBERG, U., TURPEINEN, U., KÄRKKÄINEN, T. & SYVÄNEN, A.-C. 1980, in *Enzymatic release of vasoactive peptides* (8th Workshop Conference Hoechst, June 18–20), edited by F. Gross & H.G. Vogel, New York: Raven Press: 235–246.
- HELLER-HAUPT, A., VARMA, M.G.R., RECHAV, Y., LANGI, A.O. & TRINDER, P.K.E. 1987. Immunization of laboratory animals against the tick, *Amblyomma variegatum* using homogenates from unfed larval ticks. *Medical Science Research*, 15:1371–1372.
- JOUBERT, A.M., CRAUSE, J.C., GASPAR, A.R.M.D., CLARKE, F.C., SPICKETT, A.M. & NEITZ, A.W.H. 1994. Isolation and characterization of an anticoagulant present in the salivary glands of the bont-legged tick, *Hyalomma truncatum*. *Experimental and Applied Acarology*, 19:79–92.
- KÄRKKÄINEN, T., SYVÄNEN, A.-C., TURPEINEN, U. & HAMBERG, U. 1982. Isolation and immunologic properties of a

- heterogenous antigen with the characteristics of the heavy chain of human plasma kininogen. *Molecular Immunology*, 19: 179–189.
- KEMP, D.H., STONE, B.F., & BINNINGTON, K.C. 1982. Tick attachment and feeding: Role of mouth parts, feeding apparatus, salivary gland secretions and host response, in *Physiology of ticks*, edited by F.D. Obenchain & R. Galun, Pergamon Press: 119–168.
- KEMP, D.H., KOUDESTAAL, D., ROBERTS, J.A. & KERR, J.D. 1976. *Boophilus microplus*: the effect of host resistance on larva attachments, and growth. *Parasitology*, 73:123–136.
- LAURELL, C.B. 1972. Composition and variation of the gel electrophoretic fractions of plasma, proteins cerebrospinal fluid and urine. *Scandinavian Journal of Clinical Laboratory Investigation*, 29, suppl. 124: 71.
- MEDWAY, W., PRIER, J.E. & WILKINSON, J.S. 1969. *A textbook of veterinary clinical pathology*. Baltimore. Williams & Wilkens: 522.
- MUSTARD, J.F. & PACKHAM, M.A. 1977. Normal and abnormal hemostasis. *British Medical Bulletin*, 33:187.
- NJAU, B.C. & NYINDO, M. 1987. Detection of immune response in rabbits infested with *Rhipicephalus appendiculatus* and *Rhipicephalus evertsi evertsi*. *Research in Veterinary Science*, 43:212–217.
- OPDEBEECK, J.P. 1989. Protective efficacy of antigens solubilised from gut membranes of the cattle tick *Boophilus microplus*. *Immunology*, 66:149–155.
- RECHAV Y. & J. DAUTH 1987. Development of resistance in rabbits to immature stages of the Ixodid tick *Rhipicephalus appendiculatus*. *Medical and Veterinary Entomology*, 1:177–183.
- RECHAV, Y., CLARKE, F.C., ELS, D.A. & DAUTH, J. 1991. Development of resistance in laboratory animals to adults of the tick *Rhipicephalus evertsi evertsi*. *Journal of Medical & Veterinary Entomology*, 5:29–34.
- RIBEIRO, J.M.C. 1995. How ticks make a living. *Parasitology Today*, 11:91–94.
- RIBEIRO, J.M.C., MAKOUL, G.T., LEVINE, J., ROBINSON, D.R. & SPIELMAN, A. 1985. Antihaemostatic, antiinflammatory, and immunosuppressive properties of the saliva of a tick *Ixodes dammini*. *Journal of Experimental Medicine* 161:332–344.
- ROBERTS, J.A. 1968. Resistance of cattle to the tick *Boophilus microplus* (Canestrini). II. Stages of the life cycle of the parasite against which resistance is manifest. *Journal of Parasitology*, 54:667–73.
- SCHLENGER, A.V., LINCOLN, D.T., MCKENNA, R.V., KEMP, G.H. & ROBERTS, J.A. 1976. *Boophilus microplus*: Cellular responses to larval attachments and their relationship to host resistance. *Australian Journal of Biological Sciences*, 29:499–512.
- STITES, D.P., STOBO, J.D., FUDENBERG, H.H. & WELLS, J.V. 1982. The historical background of Immunology, in *Basic and Clinical Immunology*. Los Altos, California. Lange Medical Publishers: 1–5.
- SUTHERST, R.W., KERR, J.D. MAYWARD, G.F. & STEGEMAN, D.A. 1983. The effect of season and nutrition on the resistance of cattle to the tick *Boophilus microplus*. *Australian Agricultural Research*, 34:329–339.
- SYVÄNEN, A.-C., TURPEINEN, U., SIIMESMÄÄ, T. & HAMBERG, U. 1981. A radioimmunoassay for the detection of molecular forms of human plasma kininogen. *FABS Letters*, 129:241–245.
- TATCHELL, R.J. & MOORHOUSE 1968. The feeding process of the cattle tick *Boophilus microplus*. II. The sequence of host tissue changes. *Parasitology*, 58:441.
- TEMBO, S.D. 1996. Studies on the immunochemical properties of *Amblyomma hebraeum* and *Amblyomma marmoreum* (Acari: Ixodidae) nymphal antigens. Ph.D. thesis. Medical University of Southern Africa, Medunsa, South Africa.
- TEMBO, S.D. & RECHAV, Y. 1992. Immunization of rabbits against nymphs of *Amblyomma hebraeum* and *A. marmoreum* (Acari: Ixodidae). *Journal of Medical Entomology*, 29:757–760.
- TEMBO, S.D. & KIWANUKA, A. 1997. Acquisition of protective immunity in *Geochelone pardalis* against *Amblyomma marmoreum* (Acari: Ixodidae) nymphal homogenates. *Onderstepoort Journal of Veterinary Research*, 64:1–4.
- TRAGER, W. 1939a. Acquired immunity to ticks. *Journal of Parasitology*, 25:57–81.
- TRAGER, W. 1939b. Further observations on acquired immunity to the tick *Dermacentor variabilis*. *Journal of Parasitology*, 25:137–139.
- TURPEINEN, U., SYVÄNEN, A.-C. & HAMBERG, U. (1981). Kininogen molecular heterogeneity by affinity chromatography on concovallin and *Riconus communis* lectins. *FEBS Letters*: 137–142.
- VARGAFTIG, B.B., CHIGNARD, M. & BENVENISTE, J. 1981. Present concepts on the mechanism of platelet aggregation. *Biochemistry and Pharmacology*, 30:263.
- VARMA, M.G.R., HELLER-HAUPT, A., TRINDER, P.K.E. & LANGI, A.O. 1990. Immunization of guinea pigs against *Rhipicephalus appendiculatus*. *Immunology*, 71:133–138.
- WAXMAN, L., SMITH, D.E., ARCURI, K.E., & VLASUK, G.P. 1990. Tick anticoagulant peptide factor is a novel inhibitor of blood coagulation factor Xa. *Science*, 248:593–596.
- WELLS, J.V. 1982. Immune mechanisms in tissue damage, in *Basic and clinical Immunology*, edited by D.P. Stites, J.D. Stobo, H.H. Fundenberg & J.V. Wells. Los Altos, California. Lange Medical Publications: 136–155.
- WIKEL, S.K. & ALLEN, J.R. 1982. Immunological basis of host resistance to ticks, edited by F.D. Obenchain & R. Galun, Oxford: Pergamon Press: 169–196.
- WILLADSEN, P. 1980. Immunity to ticks. *Advances in Parasitology*, 18:293–313.
- WILLADSEN, P. & KEMP, D.H. 1988. Vaccination with “concealed” antigens for tick control. *Parasitology Today*, 4:196–197.
- WILLIAMS, T.J. & PECK, M.J. 1977. Role of prostaglandins, aspirin-like drugs and analgesia. *Nature*, 270:530.
- WINSTON, P.W. & BATES, D.H. 1960. Saturated solutions for control of humidity in biological research. *Ecology*, 41:232–236.