STUDIES ON RESISTANCE ACQUIRED BY RABBITS EXPERIMENTALLY INFESTED WITH *RHIPICEPHALUS EVERTSI EVERTSI* (NEUMANN, 1897) (ACARINA: IXODIDAE)

By

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I BARNABAS CHARLES NJAU, hereby declare to the Senate of the University of Dar es Salaam that this thesis is a result of my own work except where acknowledged in the text. It has not been submitted nor is it being concurrently submitted for a similar qualification in any other University.

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ABSTRACT

A study was conducted in order to provide information on the immunological responses of hosts to ticks utilizing the rabbit - *Rhipicephalus evertsi evertsi* model. Three groups of three rabbits each were allotted randomly to three adult tick doses comprising 20, 50 and 100 ticks and infested for four successive times in an attempt to make them acquire resistance. A similar study lasting for 5 infestations was conducted using 100, 500 and 1500 larvae of the same tick species. Resistance was assessed using various parameters including mean percentage of ticks engorged, mean engorgement weight, mean feeding period, mean egg mass laid, mean percentage hatch rate, mean percentage moult and mean premoult period. Hosts sera were examined for specific antibodies to tick salivary gland antigens (SGA) and immunopathological studies on the lesions caused by the ticks were done.

Rabbits acquired resistance to different life stages of the tick after one infestation and it was maintained during subsequent challenges. This resistance resulted in decreased tick feeding success, reduced reproductive potential and reduced numbers feeding normally. There was a significant mortality rate which affected immature stages more than adults. Tick instars that survived on resistant hosts manifested a combination of one and three host cycles in addition to the normal two host cycle shown by rabbits feeding on naive hosts. Small and large nymphs frequently matured on the hosts and moulted in favour of males and females

respectively. Moulting periods were shorter for small nymphs. After successful establishment on the host, nymphs were not significantly influenced by the hosts' immune response. Generally, egg hatch rate and nymphal moults were the parameters least influenced by host resistance.

Infestation to R. e. evertsi generated significant cross resistance against a challenge with the 3 life stages of R. appendiculatus and larvae and adults of Amblyomma variegatum. Cross resistance was measured in terms of reduction in numbers successfully engorging, and reduced weight of those ticks that engorged. The most significant inter-species cross-resistance was in R. e. evertsi infested rabbits challenged with R. appendiculatus. It was weak for A. variegatum. Immuneprecipitation using rabbit-anti R. e. evertsi sera and antigens isolated from the three tick species suggested that they share common antigens.

During primary infestation with adult R. e. evertsi, most rabbits developed paralysis. Rabbits which did not develop paralysis during the primary infestation could not be paralysed by subsequent tick challenges, an indication that they had acquired immunity to the toxin. Adult ticks which emerged from nymphs of weights between 5 and 24.9 mg were able to paralyse tick-naive rabbits. Paralysis was not induced by the tick instars.

Cutaneous cellular responses involving primarily lymphocytes developed at the tick feeding sites of resistant rabbits. Similar

delayed reactions were elicited by intradermal inoculation of R. e. evertsi extracts from SGA in the hosts. The magnitude of these reactions varied according to the dose of adult ticks used to sensitize hosts. It was poor for rabbits challenged with high numbers of ticks and in particular those paralysed. A common feature to these studies was that significant rejection and death occurred during secondary tick exposure, which continued to the 3rd and 5th infestations in hosts challenged with 20 adults and larvae respectively. During the primary tick challenges, antibodies to tick SGA were demonstrated in the first week using the Enzyme-Linked Immunosorbent Assay (ELISA) technique. With the agar gel double diffusion technique, antibodies could not be demonstrated until the fourth week. Peak antibody titres attained after secondary challenge could not be raised by increasing the frequency of tick infestations. However, hosts challenged with low numbers of adult ticks and those which suffered paralysis lost precipitating antibodies. This resulted in enhanced tick feeding.

Delayed type hypersensitivity (DTH), a T cell-mediated immune response, occurred at tick feeding sites on a resistant host. Suppression of T cells by administration of goat anti-rabbit thymocyte serum (ATS) to rabbits before tick challenge partially blocked acquisition of resistance. Consequently, tick engorged weights, feeding duration and fecundity were enhanced compared to the ATS untreated controls. In addition, tick mortality was reduced and rejection abolished. Both antibodies to tick SGA and inflammatory reactions at tick bite sites on ATS-treated hosts were

suppressed. Ticks fed on naive hosts significantly suppressed DTH response to sheep red blood cells (SRBC). A similar but poor suppression was observed on tick resistant hosts. The intensity of DTH was inversely related to tick engorgement weights.

The capacity of tick-infested resistant and naive rabbits to mount an immune response to a concurrent or subsequent challenge was investigated using SRBC and bovine serum albumin (BSA). Rabbits which completed four successive infestations with the different numbers of adult ticks described, like others prechallenged once for 5 days with 200 adults responded poorly to subsequent inoculation with SRBC. Both the magnitude and persistence of the antibody response to SRBC were reduced in hosts that were repeatedly challenged with 20 ticks. However, it was reduced and persistent in hosts exposed to the other challenge regimens. Rabbits that suffered tick paralysis had the lowest antibody response. The primary antibody response of naive rabbits to SRBC and BSA inoculated to coincide with tick infestation was severely suppressed, particularly response to BSA. Most rabbits thus treated died from paralysis. Of nine rabbits inoculated with SRBC at the time when ticks were observed to have started feeding (usually on day 3 of tick application), 5 were paralysed and died while in a group of 6 rabbits inoculated with BSA and similarly infested, one rabbit was paralysed and recovered. When SRBC and BSA were inoculated together in three naive rabbits while ticks had commenced feeding, two were paralysed and died. Conversely, when 3 naive rabbits previously inoculated with SRBC and BSA were boosted 14 days

later with the 2 antigens as a single inoculum to coincide with tick feeding, their secondary antibody response was only transiently suppressed. These hosts acquired rapid resistance to the ticks and one rabbit was paralysed and died. The characteristics of these responses would suggest that the inability of R. e. evertsi-infested rabbits to respond well to a simultaneous challenge with other antigens may be due to antigenic competition. Such competition is immunosuppressive and may also occur among different tick antigens inoculated in the host.

Elution of tick SGA in column chromatography showed that the antigen comprised two immunogenic fractions. By using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), one of the fractions resolved into 8 protein bands while the other fraction showed only one band. The latter band and 2 others in the previous fraction were of comparable molecular weight (M.W.) of 67,000 daltons. The remaining protein bands of fraction 2 had M.W. below this value.

This study has demonstrated that acquired resistance in rabbits affected feeding and egg laying of R. e. evertsi. Humoral and cellular factors participated in the expression of resistance. However, ticks were able to suppress these two components of host immune system, a feature suggested to facilitate ixodid feeding on resistant hosts. Since in this study antigens derived from R. e. evertsi salivary gland extract reacted with sera from rabbits infested with this tick, it suggests that they may be used to investigate host-tick relationship.

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LITERATURE REVIEW

Classification of ticks

Ticks belong to the phylum Arthoropoda in which insects are included. They are obligate haematophagous parasites of man, domestic and wild animals. Walker (1970) divided ticks into two main families, the Ixodidae (hard ticks) and Argasidae (soft ticks). The family Ixodidae includes about 650 species, while about 150 species are included in the family Argasidae. Members of the Ixodidae have a dorsal plate (scutum) which covers the entire dorsal surface in the males and a relatively small area just behind the capitulum in females. Argasid ticks lack the scutum, but possess leatherly integuments. The majority of ticks of veterinary importance belong to the family Ixodidae of which seven genera are of great economic importance (Arthur, 1962). These include: Haemaphysalis, Rhipicephalus, Dermacentor, Hyalomma, Boophilus, Ixodes and Amblyomma. Soft ticks are mainly parasites of man, bats and birds.

Life cycle of ticks

Ticks have complex life cycles. Eggs are laid by engorged females after dropping from the hosts. Ixodid females lay single large egg masses and die while Argasid females lay relatively smaller egg masses, feed, and repeat the process. Eggs hatch into six legged larvae which climb on to grass where they wait for a passing host. Upon successful attachment to

the host, the larvae engorge to repletion, drop off the host, and moult into 8-legged nymphs on the ground. The nymphs find a host and feed as described for the larvae, and then moult into 8-legged adults. Members of the Argasidae differ from those of Ixodidae by having 2 - 6 nymphal moults before an adult emerges. The adults feed and mate on the host after which the females drop to the ground to lay eggs. In general, ticks have been successful partly due to their high rate of multiplication and also by having few predators or diseases.

According to their life cycles, ticks can be divided into four categories:

a) One host ticks, for example Boophilus species, which remain on the host throughout the feeding and moulting phases of their life cycles. In this case, larvae are the only stages involved in host location.

b) In two host ticks, larvae find a host, feed and moult into nymphs on the same host. The nymphs feed, drop to moult on the ground and the emerging adult has to find a host. This life cycle is typical of Rhipicephalus evertsi evertsi.

c) In the three host ticks, for example R. appendiculatus each instar (larvae, nymphs, and adults) must find its own host. This feature is common with the family Ixodidae.

d) Multi-host ticks, like all Argasids undergo irregular feedings for each developmental stage whenever their hosts are available.

Economic importance of ticks

Ticks have been known and recognized since biblical times (Shaw 1970). However, their importance both as vectors of livestock disease agents and parasites of cattle was not realized until the second half of the 19th century when there was an urgent need to increase the world livestock products to feed the human population of the great industrial centres. Cattle had to be moved into tick infested areas, where many contracted and died of tickborne diseases. One of the tick-borne diseases, bovine babesiosis (Red water), was recognized in the U.S.A. in 1814 and was associated with Boophilus annulatus. In 1889 Smith and Kilborne (Shaw, 1970) recognized the aetiological agent of Red water and linked its transmission with B. annulatus.

At present East Coast fever (ECF) is the most important among the tick-borne diseases in East Africa. This is reflected by the fact that about 40% of calves born in enzoootic areas die of infection with Theileria parva parva and T.p. lawrencei (Anon, 1974). Rhipicephalus appendiculatus and R.e. evertsi are the main vectors of ECF (Neitz, 1955, Walker, 1974, Lourens and Tatchell, 1979). Neitz (1957) observed that the distribution of R.e. evertsi in East Africa frequently overlapped with that of R. appendiculatus and extended widely into areas where the latter was apparently unable to survive. A similar finding was reported by Pergram et al. (1981) who further described R.e evertsi as an ecologically facultative tick and R. appendiculatus as an obligate hygrophile tick. It

would therefore appear that in areas where R. appendiculatus is absent, R.e. evertsi could maintain transmission of ECF.

Ticks supersede all other arthropods in the maintenance and dissemination of many diseases affecting domestic animals (Steelman, 1976) and rank second to mosquitoes as vectors of human diseases. In tropical countries, ticks and tick-borne diseases are among the major factors contributing to the slow economic development of the livestock industry (Bram, 1975). For example, R.e. evertsi, a tick which has been used in the present study, is a vector of the causative agents of bovine babesiosis (Theiler, 1906), bovine theileriosis caused by T. mutans (Theiler, 1907), ovine theileriosis (Jansen and Neitz, 1956) and bovine spirochaetosis (Theiler, 1909). The tick also causes sheep paralysis (Walker, 1960; Gothe, 1981) and is a vector of Rickettsia conorii, which causes typhus in man (Gear, 1954).

Tick control

Campaigns of various types including use of acaricides and pasture spelling have been mounted to control ticks on and off the hosts respectively. Dipping or spraying domestic stock in acaricide solutions in order to kill ticks on the hosts has been the most popular method of tick control and in many situations it appears that no alternative control measures can be predicted in the near future. Apart from being costly and unable to provide a stable long term solution to tick problems, resistance to acaricides develops in the tick population

(Wharton, 1974; Whaton and Norris, 1980). Such resistance has been recorded for many tick species of economic importance in various countries (Mathewson and Baker, 1975; Wharton, 1976; Baker et al., 1977; Drummond, 1977) and has considerably frustrated efforts to control ixodid ticks by chemicals (Drummond, 1970; Walker, 1974; Londt et al., 1977). Durand (1976) suggested that the cost of developing new acaricides in relation to the economic return expected from their use before resistance occurs might discourage research leading to the development of new compounds.

The increasing world population and diminishing food production (notably animal proteins) due to vector and vector-borne diseases and also pressure to expand livestock production into parasite and disease prevalent areas (Odhiambo, 1967) is a driving force towards the search for new methods of tick control of lasting value, less dependent on chemicals, e.g. tick resistant animal species or the development of artificial resistance in cattle.

Rotational grazing as a means of interfering with host finding by ticks so that they die of starvation has been used with encouraging results (Sutherst et al., 1979). However, the method has the disadvantage of being inefficient in pasture utilization and therefore becomes unpopular to farmers. In other parts of the world where game animals play a significant role in the epidemiology of ticks and tick-borne diseases (Burridge, 1975), such a control method would be economically unjustifiable. Habitat modification to destroy tick breeding

sites, for example by heavy grazing or burning grass, has been used with promising results (Milne, 1944) but has some economic and environmental constraints.

Biological control of ticks has been suggested as one of the most promising prospects for the future (Nolan, 1981). However the use of microbes and parasitic wasps to control ticks has been attempted previously but without success (Cole, 1965; Lipa, 1971). Galun (1978) suggested an approach based on immunizing hosts to produce specific antibodies against the tick's own hormones or target antigens within the tick.

Recently, the use of tick resistant hosts, either as a result of tick infestation or artificial immunization to prevent or discourage ectoparasite feeding has received much attention. Wikel and Allen (1982) showed that the ability of such hosts to inhibit tick feeding was immunological and specific. Basic information regarding the structure and function of tick salivary glands, the feeding mechanism and associated secretions has been provided in detail by Kemp et al. (1982). Although evidence from a number of studies indicate that tick resistant cattle may be used as the backbone of tick control programmes (Francis and Little, 1964; Campbell, 1978; Willadsen, 1980; Wikel, 1980; Sutherst and Utech, 1980), there is paucity of fundamental knowledge regarding the mechanisms of this control process (Allen and Nelson, 1982). In addition, the fragmentary information available is restricted to only a few tick species (Willadsen, 1980).

Immune resistance of animals to tick infestation

The course of tick feeding is accompanied by the synthesis of various proteins (which act as antigens) which are secreted with saliva into the host (Krolak et al., 1983). Subsequently homocytotropic and skin sensitizing antibodies are synthesized by the host against these antigens (Allen, 1973; Wikel and Allen, 1976a,b). For example, antibodies acting as antienzymes against phosphomonoesterase (a tick digestive enzyme secreted during feeding) have been demonstrated in the blood of tick-resistant cattle (Reich and Zorzopulos, 1980). The possible effects of this host immune response to subsequent tick challenge have been discussed by Willadsen (1980) and collectively referred to as acquired resistance (Wharton and Norris, 1980), a phenomenon which limits the number of ticks which survive to maturity on the host.

The acquisition of resistance in both laboratory and domestic animals after tick infestation has been documented by several authors (Trager, 1939a; Roberts, 1968a; Musatov, 1970; Brossard, 1976; Fujisaki, 1978; Brown and Askenase, 1983). Studies by these workers showed that when hosts were infested with ticks, they acquired resistance after a single infestation and this resistance was maintained during subsequent exposures. Resistance was measured by a number of parameters, for example, weights of engorged ticks, the duration of the engorgement periods, the number and percentage of ticks engorging successfully, the fecundity of adult females and the hatchability of eggs following each infestation.

Conflicting results have been obtained with some of the parameters used to assess resistance. Engorgement weights of R. appendiculatus (Mongi, 1982) and Ixodes ricinus (Bowessidjaou et al., 1977) were reported to decrease progressively with the advance in the frequency of host challenge by ticks. Working with I. ricinus, Brossard et al. (1982) observed that the engorged weights decreased gradually up to third infestation, in a series of challenges but increased thereafter. Similar enhanced feeding ability was observed in Dermacentor variabilis on mice in secondary tick infestations (Den-Hollander and Allen, 1985).

Prolongation of feeding period has been demonstrated in hosts resistant to several tick species (Branagan, 1974; Bowessidjaou et al., 1977; Brown and Knapp, 1981; Brown, 1982; Brossard et al., 1982; Mongi, 1982). Recently it has been shown by de Castro et al. (1985) that the feeding period of R. appendiculatus on resistant hosts was shortened. There is inconsistent information on the proportion of different tick species maturing on the same or different hosts resistant to ticks (Trager, 1939b; Boese, 1974; Wikel and Allen, 1976a; Fujisaki, 1978; Mongi, 1982). It is apparent that various host species respond differently to the same or different tick infestation. Hence results obtained from a particular host-tick association may not apply to other such combinations. A considerable amount of work reported above concerned other tick species of economic importance. Rhipicephalus e. evertsi has been regarded as an economically

important tick (Walker, 1974). There is paucity of data on the host-parasite relationship regarding this tick.

Mechanisms of immune resistance

Many investigators have reported that host develops resistance to tick infestations but the underlying mechanisms have remained largely obscure. Studies on the mechanisms of immunological interactions between ticks and their hosts have indicated that humoral and cellular factors are likely to disrupt tick feeding on resistant hosts (Wikel, 1982a; Wikel and Allen, 1982).

Antibodies of various types have been demonstrated in the sera of hosts resistant to ticks (Brossard, 1976; Fujisaki, 1978; Willadsen et al., 1978; Brown, 1982). It has been shown that antibodies to the salivary gland antigens injected in the course of tick feeding, develop rapidly, attain threshold levels following secondary and subsequent challenge. Further increases are minimal, though the hosts become progressively more immune (Bowessidjaou et al., 1977). Some of the antibodies are specific against tick antigens, for example, digestive enzymes (Reich and Zorzopulos, 1980). Antibodies to tick salivary gland antigens (SGA) with their immunological properties were also detected in ticks that fed on resistant hosts (Ackerman et al., 1981). These antibodies have been shown to be associated with partial resistance against ticks (Brossard and Girardin, 1979).

Brossard et al. (1982) detected circulating antibodies as early as the sixth day in rabbits exposed to I. ricinus. Irvin et al. (1973) working with R. appendiculatus and Allen (1973) using B. microplus detected them in less than two weeks. Fisher (1983) working with mite-infested rabbits detected antibodies in the host at day 7 and 21 of primary infestation by Enzyme Linked Immunosorbent Assay (ELISA) and immunodiffussion tests. Fujisaki et al. (1980) reported that the period necessary for the acquisition of resistance in naive hosts infested with Haemaphysalis longicornis and the production of humoral antibodies was similar and suggested that the antibodies do play a major role in acquisition of resistance.

Continuous tick challenge has been shown to be necessary for the maintenance of antibody titres in the host (Brossard, 1977). Dineen (1963) suggested the existence of a balance between the host's immune response and tick numbers whereby the host tolerates a certain parasite load and rejects excess loads immunologically. Under these conditions, the number of parasites tolerated is not high and hosts never acquire strong resistance (Randolph, 1979; Samuel and Baker, 1979). Sprent (1969) and Hudson (1973) suggested that for such a coexistence to occur to the benefit of both partners, it is likely that the host exerts selective pressure on the parasite in order to modify its antigenic properties.

Attempts to correlate antibody titre with the degree of host resistance attained have given contradictory results.

Brossard (1976) and Fujisaki (1978) claimed that there was a positive relationship between indirect fluorescent and precipitating antibody titres and host resistance. Willadsen et al. (1978) disputed this and demonstrated a significant negative correlation between resistance and agglutinating antibody titres. He also reported that cattle with a low degree of resistance had high antibody titres. Riek (1962) did not observe any relationship between humoral antibody levels and the degree of host resistance to B. microplus. Fujisaki et al. (1980) observed that some hosts with circulating antibodies showed no resistance to tick infestations. These inconsistencies probably reflect the measurement of different types of antibody by a variety of techniques. However, O'Kelly and Seifert (1969) and Wharton et al. (1970) have suggested that animal breeds, their nutritional and physiological status are among the possible sources of variation in results. The position is further complicated by observations that in some hosts repeatedly challenged with ticks there is loss of circulating antibodies (Berdyev and Khudainazarova, 1976; Wikel and Osburn, 1982), a feature commonly observed in farm animals. Recent findings seem to suggest that this loss in host immunity may be due to tick-induced immunosuppression (Whelem et al., 1984; Ribeiro et al., 1985).

Possible release by parasites of mitogenic substances which cause polyclonal activation of suppressor T cells has been suggested as a cause of failure by hosts to eliminate helminths (Lelchuk and Playfair, 1980). Suppressor T cells

(T_S) have been described (Gershon and Kondo, 1979; Levich et al., 1984a, b) and shown to regulate delayed type hypersensitivity (DTH) (Claman et al., 1980) and proliferation of antigen primed T cells (Baxevanis et al., 1982; Levich et al., 1984a). A prostaglandin able to suppress T cell activity was demonstrated in I. dammini saliva (Ribeiro et al., 1985). This finding was consistent with previous reports of reduced response of T cell populations from tick infested hosts due to mitogenic stimulation (Wikel and Osburn, 1982; Wikel and Allen, 1982; Wikel 1982). The T cells play an important role in the expression of antibody (McConnell, 1978) and cell mediated immune responses (Valdimarsson, 1978) in parasitic infections. The vast majority of antigens require T lymphocyte interaction for the generation of an immune response (Miller and Mitchell, 1968). It is apparent that by affecting T cells, ticks may abolish, reduce or delay host response to antigens which may as a result lead to poor resistance to tick infestation. This may lead to a permanent association of ticks with their hosts.

Rhipicephalus e. evertsi secretes a paralysing toxin with an unknown role to success of tick feeding and host acquisition of resistance. Similar effects have been reported with other paralysing ticks (Stone and Wright, 1981). Consequently, data obtained for host resistance to other tick species may not apply to R.e. evertsi.

Coombs and Gel (1968) divided the hypersensitivity reactions into two classes, antibody dependent (immediate) and cell mediated (delayed). These hypersensitivity reactions are

intimately involved in the expression of resistance by hosts to tick infestation (Willadsen, 1980; Brossard et al., 1982) and have been extensively discussed by many authors (Eyre, 1980; Brown and Knapp, 1981; Johnston and Brown, 1985). During host resistance to ticks, there is an increased cellular response (hypersensitivity) to tick antigens at the feeding site which prevents excessive infestations.

In immediate hypersensitivity, tick antigens introduced into resistant hosts react with a specific class of homocytotropic antibody bound to mast cells and circulating basophils. This antigen-antibody complex reacts with immunoglobulin Fc receptors to cause degranulation of cells with the release of vasoactive amines (Brown and Askenase, 1985a) and they can also saturate the receptors such that they become inert (Shear et al., 1979; Pappars et al., 1981). The amines (especially histamine) are thought to be responsible for much of the rejection of ticks at the feeding site (Kemp, 1978). Willadsen et al. (1978) showed that B. microplus feeding on resistant hosts elicited an immediate hypersensitivity response whose intensity was related to the degree of inflammatory reaction at the feeding site. Earlier, Tatchell and Moorhouse (1968) had demonstrated that immediate hypersensitivity interfered with successful tick feeding, causing detachment within two hours and desertion of the area by 24 hours as the surface became encrusted with exudate. Histamine is probably one of the main effectors of immediate

hypersensitivity reactions in tick resistant hosts (Willadsen et al., 1979; Kemp and Bourne, 1980; Wikel, 1982b; Paine et al., 1983). It is thought to stimulate host grooming with subsequent loss of about 50% of tick instars from the infestation (Koudstaal et al., 1978). Tick rejection and death in situ have been associated with ingestion of the host inflammatory cells or lethal factors released after they degranulate (Allen, 1973; Bagnall, 1978; Kemp, 1978; Brown and Askenase, 1981; 1982). From these observations it appears that histamine and other mediators released at tick feeding sites interfere with tick feeding success.

Studies by Roberts (1968b), Wagland (1979) and Brown and Askenase (1985b) have shown that the resistance mechanism affected ticks soon after they attached and yet the ticks suffered little permanent damage since they continued to seek alternative feeding sites. Chinery and Ayitey - Smith (1977) suggested that ticks have a histamine - blocking factor which they possibly use to regulate histamine levels at the feeding site thus avoiding damage. Consistent with this finding is the suggestion by Tatchell and Moorhouse (1968) and Tatchell and Bennett (1969) that histamine was advantageous to newly attached larvae and therefore had a secondary role in the mediation of immunological reactions. This histamine blocking factor may be important for survival of ticks on resistant hosts (Willadsen et al., 1979).

Delayed type hypersensitivities are protracted T cell mediated immune reactions encountered in hosts repeatedly

attacked by arthropods (Mitchell, 1980). Such cell mediated cutaneous reactions have been shown to exert lethal effects and rejection of ticks by affected hosts (Wikel, 1979; Brown and Knapp, 1981; Brown and Askenase, 1983). This phenomenon has been demonstrated in hosts resistant to B. microplus (Roberts, 1968 a,b), D. variabilis (Allen, 1973), I. holocyclus (Bagnall, 1975b) and R. appendiculatus (Binta and Cunningham, 1984). Willadsen (1980) reviewing DTH indicated that peak cellular infiltration at the tick feeding site was achieved with ticks in situ but significant lymphocyte blastogenesis occurred after ticks had detached replete. He attributed the delayed lymphocyte blastogenesis to tick induced immunosuppression. This may indicate that some ticks avoid possible harmful effects of DTH inflammatory reactions contrary to the notion that the death of ticks is attributed to the predominance of the inflammatory cells in the feeding lesion (Nelson et al., 1977). It is possible that the accumulation of the inflammatory cells at the tick feeding site which do not undergo blastogenic transformation may not exert lethal effects on the ticks. Eling (1982) considered DTH in parasitic infections as a side effect of the failure of the host response to eliminate parasites.

Host DTH responses to tick infestations are characterized by various functional cell types. Lymphocytes and basophils have been shown to dominate the reactions depending on the animal species (Allen, 1973; Dvorak, 1976; Wikel et al., 1978; Brown and Knapp, 1981; Mbemba, 1983; Brown

et al., 1984). Eosinophils (Brown and Knapp, 1981) and Langerhans cells (Allen et al., 1979; Nithiuthai and Allen, 1984) have also been reported. Langerhans cells have characteristics in common with macrophages (Stingl et al., 1977; 1978; Rowden et al., 1977; Stingl, 1980) and like macrophages are thought to present tick antigens to lymphocytes. T-lymphocytes secrete lymphokines which recruit the bulk of circulating monocytes to form a granulomatous lesion at the tick feeding site and cause the skin to release histamine (Dumonde et al., 1982). Askenase (1977) described the function of basophils as the release of histamine. The role of eosinophils in the immune response to ticks has not been elucidated. In other host-parasite systems, two highly cationic proteins have been described in eosinophils. They are the major protein (Gleich et al., 1976) and eosinophil cationic protein (Olsson et al., 1977), with activity against nematodes have been demonstrated.

Mast cells like basophils have receptors for anaphylactic antibodies and therefore play a significant role in immediate hypersensitivity reactions (Ishizaka and Ishizaka, 1971). The finding by Nabel et al., (1981) that T lymphocytes induce mast cells to proliferate suggests that this cell type may be involved in DTH as well. The frequency of mast cells in ectoparasitic skin reactions in various animal species is inversely related to that of blood basophils (Schleger et al., 1976; Dvorak et al., 1979). The existence of histamine receptors on most cell types involved in immune response makes them amenable to central control by mast cells (Diaz et al.,

1979; Schwartz et al., 1981). A protein able to inhibit degranulation of mast cells has been identified in tick saliva (Bach, 1982). It is apparent that tick infestation may modulate cutaneous inflammatory responses by suppressing a single target cell.

From the present evidence it would seem that tick infestations exert profound effects (mostly negative) on a variety of host cell types involved in mediating immune resistance. The signals involved in the host-parasite interplay are unknown thus making it difficult to identify the regulatory circuits directly. There is a need to design easily manipulated experimental systems to understand mechanisms of host resistance to ticks. Studies related to the search of a potent immunogen to control ticks must be accompanied by research on the host immune response to tick infestations. A knowledge of the parameters of the immune response is instrumental in the development of protective immunity and a prerequisite to the development of rational immunoprophylactic measures.

The present study was designed to develop a model of tick resistance using rabbits infested with R.e. evertsi, and to investigate the mechanisms of resistance in order to understand how they can be manipulated to control tick infestations and associated vector-borne diseases. An attempt was made to gain insight into immunological cross resistance between R.e. evertsi and two other ixodid ticks, R. appendiculatus and Amblyomma variegatum and also to investigate the antigenic potential of tick salivary gland constituents.

CHAPTER I

STUDIES ON RESISTANCE IN RABBITS INFESTED WITH ADULT *RHIPICEPHALUS EVERTSI EVERTSI*

INTRODUCTION

The red-legged tick, *Rhipicephalus evertsi evertsi* is a tick with some natural capacity to transmit East Coast fever (Neitz, 1955; Walker, 1974; Lourens and Tatchell, 1979), a tick-borne disease of economic importance in Eastern and Central Africa. It also causes paralysis, a toxicosis in which the toxin has not been identified (Neitz, *et al.*, 1981; Stone and Wright, 1981). Toxins have been shown to be present in salivary glands of some ticks known to cause paralysis (Howell *et al.*, 1975). Trager (1939 a, b) showed that ixodid ticks induced resistance to future attacks by these ticks as a result of antigenic components in the saliva injected into the host. However, the effects of the paralysing toxin on the overall development of resistance is obscure since quantitative studies on toxic agents of most of ticks inducing toxicosis have not been done. Studies with the Australian paralysis tick *Ixodes holocyclus* have shown that some cattle developed resistance of uncertain duration (Doube and Kemp, 1975). Similar studies in dogs indicated development of resistance adequate to protect against lethal challenge (Stone *et al.*, 1982, 1983; Wright, *et al.*, 1983). Murnagham and O'Rourke (1978) working with *Dermacentor andersoni*, an American paralysis tick, failed to induce resistance although this was disputed later (Gregson, 1973).

The present study was designed to investigate the immunological interaction between ticks and their hosts using the R.e. evertsi - rabbit model.

MATERIALS AND METHODS

Rabbits

Twenty nine male and female naive New Zealand White rabbits weighing about 2 kg each were used. They were obtained from the Veterinary Research Department, Muguga, Kenya. In the laboratory, they were maintained individually in separate metal cages, offered standard rabbit cubes and water ad libitum for two weeks. At the end of this period, they were alloted to three groups each with animals of approximately equal weights. Two groups contained three rabbits each and one group five rabbits. The remaining 18 rabbits were paired and served as controls in the subsequent series of successive tick challenges.

Tick Colony

The colony of R. e. evertsi used in the study consisted of a tick strain which had been reared and maintained by feeding on rabbits at the ICIPE laboratory of the Livestock Ticks Programme, since 1972. All stages of ticks used were kept in an incubator at 25^{o}C and 85% relative humidity (RH). All ticks used in the experiment aged between 30-40 days from the date of moulting in order to eliminate the effect of tick age on feeding duration (Fujisaki,1978). Ticks used in the study had moulted from nymphs of approximately uniform weight. Ticks belonging to R. appendiculatus and Amblyomma variegatum species were similarly colonized.

Infestation

Three adult tick doses comprising 20, 50, and 100 ticks (sex ratio 50:50) each were prepared. They were alloted randomly to the three rabbit groups and applied on the rabbit ears following the method of Bailey (1960), with a slight modification. The modification involved leaving the tube containing ticks fixed to the rabbit's ears until all the ticks attached. The feeding duration was assessed from this period. Ticks were applied to the left ear of each rabbit for the first infestation. For the second infestation ticks were applied on the right ear and for the third and fourth infestations by alternating the ears. All challenge infestations except the second, were done seven days after completion of the previous exposure. The second infestation was done 14 days after completion of the first. Controls were included for each challenge. After completion of R. e. evertsi infestations, hosts were challenged once in turn with 20 and 8 adult R. appendiculatus and A. variegatum respectively.

Observations made on the hosts

Rabbits were examined daily from the sixth day of tick application and ticks that detached from the hosts were collected, weighed individually in glass tubes (2 x 6.5 cm) and placed in an incubator (25^{o}C 85% RH) to oviposit.

Resistance was assessed using a number of parameters which included, mean percentage engorged, mean engorgement weight, mean feeding period, mean preoviposition period, percent females laying

eggs, mean egg mass laid, mean egg incubation period and mean percent hatch rate.

Tick feeding period was taken as the time when all ticks were observed to have attached and started feeding, to the time of detachment as replete ticks. As an aid in the determination of egg incubation period, a hand lens was used to examine the egg masses daily from the time they showed white spots. Percent hatch rate was determined from day 21 to day 28 after initiation of hatching in two steps. In the first step, the total number of eggs in each egg mass laid were determined as follows: samples of 50 eggs each were removed from 6 egg masses laid by different female R. e. evertsi. These eggs were weighed individually on Cahn-21 Automatic electrobalance (Cahn-Ventron, USA) and a mean value calculated. This mean was used to estimate the total number of eggs laid by each tick. Ideally each tick egg would hatch into a larva.

In the second step, the larvae emerging from eggs laid by ticks in the study was determined as follows: The degree of hatching was initially assessed by visual inspection; comparing proportion of empty egg shells or hatched larvae with that of unhatched eggs for each egg mass. Egg masses observed to manifest similar hatching pattern were grouped together. From each group, 10% of the total samples was frozen at $-20^{o}C$ for 30 minutes and the larvae were then counted on a white background. The number of larvae counted, expressed as a fraction of the number of eggs estimated for the egg mass was the egg hatch which multiplied by 100, gave a percent hatch rate for each egg mass. The mean hatch

rates for the different groups reported above were determined. These group means were appropriately alloted as hatch rates, to all egg batches laid in this study.

Serum preparation

Ten millilitres of blood was collected from the marginal ear vein of each rabbit before ticks were applied. Subsequent bleedings were done at weekly intervals for 23 weeks. Sera were separated and stored at -20^{o}C until required for test.

Antigen preparation

Antigen was prepared from 800 pairs of salivary glands from both male and female R. e. evertsi fed on naive rabbits for 5 days. Ticks were dissected and the glands were removed and washed five times in phosphate buffered saline (PBS), pH 7.2. The glands were pelleted at 1000 g for 15 min. and then in twice their volume of the buffer, homogenized in a Tenbrok grinder (B. Braum, W. Germany). The homogenized glands were ultrasonicated in an ultrasonic transducer (Dawe Instruments, London, UK), at a frequency of 41.23 KC/S for 30 seconds. Ten microlitres of phenylmethylsulfonylfluoride (PMSF) (Sigma Chemical Company, St. Louis, MO. USA) was added to the preparation as a protease inhibitor. The sonicated homogenate was centrifuged at 15,000 g for 30 mins. at 4^{o}C. Protein concentration of the supernatant fluid was determined according to Lowry et al. (1951) and the supernate was stored at -20^{o}C as salivary gland antigens (SGA) in 4 aliquots of 1 ml each sufficient for an experiment.

Serological tests

The agar gel double diffusion test (Ouchterlony, 1949) was used to detect circulating precipitating antibodies against antigens inoculated into the rabbits by R. e. evertsi while feeding . Test sera were diluted in doubling dilutions in PBS.

The passive haemagglutination (PHA) test using tannic acid (Boyden, 1951) to couple antigen protein to red blood cells was used to detect haemagglutinating antibodies to tick SGA. Salivary gland antigens were coupled to washed sheep red blood cells (SRBC) in PBS. The sensitized cells were used as a 1% suspension in PBS, containing 1% normal rabbit serum. The test rabbit sera were heat-inactivated (56^{o}C, 30 mins.) and then diluted in doubling dilutions in Cooke U-bottom microtitre plates (Selby Scientific Ltd., Sydney, Australia). An equal volume (50 µl) of the 1% sensitized SRBC was added to the serum dilutions and the plates shaken and incubated at 37^{o}C for 1 hour. The plates were then incubated at 4^{o}C for 30 mins. The end titre of the serum was taken as the highest dilution showing complete agglutination of the cells at the end of 30 minutes.

The enzyme-linked immunosorbent assay (ELISA) was used to detect rabbit IgG specific for R. e. evertsi SGA. Optimal reactant concentration for antigens and conjugates were predetermined by titrations. Fifty microlitres of SGA diluted in carbonate-bicarbonate buffer (pH 9.6) was added to each well of a sterile Titertek microtitre plate. The dilution was such that each well had 0.75 µg of protein. The plates were left overnight at

4°C. The antigen-coated plates were washed the following day with PBS containing 0.05% polyoxyethylenesorbitan monolaurate (Tween-20) as the wash solution. Fifty microlitres of test sera diluted 1/10 (using PBS) was added to each well and incubated at 37°C for 30 minutes. After this period the plates were washed five times with distilled water followed by the wash solution to remove unbound antibodies. Fifty microlitres of goat anti-rabbit IgG (Miles Laboratories, Inc, UK) conjugated to horse-radish peroxidase was added to each well and similarly incubated at 37°C. The plates were similarly washed thereafter and 50 µl of the substrate, Orthophenylene-diamine (OPD) (BDH, Chemicals Ltd., Poole, England) in citrate buffer pH 5.0 with H_2O_2 (30% w/v) was added. The plates were kept in the dark for 30 minutes, after which the optical density, a measure of colour development and a semiquantitative measure of antibody concentration was determined with Titertek Multiskan-MC(Flow Laboratories, UK) at 492 nm.

Analysis of variance (Steel and Torrie, 1960) was performed on all data.

RESULTS

Mean engorgement weights of female R. e. evertsi fed repeatedly on the same rabbits

The mean engorged weights of the ticks detached from the hosts after the first infestation were 951.62, 794.70 and 706.60 mg respectively for rabbit groups challenged with 20, 50 and 100 ticks (Table 1). At the second infestation these weights were 223.3,

TABLE 1

MEAN ENGORGEMENT WEIGHTS (mg) OF FEMALE R.E. EVERTSI FED ON SENSITIZED AND NAIVE RABBITS

Rabbit groups	A	Control	B	Control	C	Control
Treatments	20 ticks		50 ticks		100 ticks	
Infestation 1	951.62 ± 261.60 (25)	-	794.70 ± 192.50 (68)	-	706.60 ± 239.30** (145)	-
Infestation 2	223.30 ± 186.04* (16)	784.88 ± 129.50 (20)	294.80 ± 243.90 (58)	915.15 ± 105.14 (47)	314.68 ± 205.70 (128)	824.92** ± 199.24 (88)
Infestation 3	65.97 ± 44.37 (12)	895.37 ± 142.70 (20)	218.49 ± 168.00 (50)	821.49 ± 158.16 (50)	139.22 ± 105.41 (115)	821.11** ± 203.88 (95)
Infestation 4	255.60 ± 178.76 (12)	905.56 ± 113.29 (20)	242.68 ± 168.63 (61)	816.50* ± 195.28 (49)	122.92 ± 101.46 (116)	793.21* 187.30 (95)
Group mean ± S.D.	374.12[a] ± 393.58	861.94[a] ± 719.10	387.56[a] ± 273.3	867.71[b] ± 49.43	320.86[a] ± 271.42	813.08[a] ± 562.76

Each asterisk represents a paralysed, dead rabbit
± mean ± standard deviation
( ) Total number of female ticks recovered for each host group
Group means not followed by a common letter are statistically different ($P < 0.05$)

294.8 and 314.68 mg respectively on hosts challenged with 20, 50 and 100 ticks and also below 50% of the initial and control infestations. The mean engorged tick weights for the control rabbits were approximately 800 mg. The weights and tick body sizes (Fig. 1) decreased during subsequent infestations. The decrease was progressive for the 100 but not for the 20 and 50 tick challenge regimens. In the 20 and 50 tick challenge regimens, the weights decreased up to the third infestation and improved thereafter. Very low engorgement weights were observed during the third infestation in the 20-tick challenge hosts. There was no statistical difference ($P > 0.05$) in the engorgement weights among the three treatment groups for the four infestations examined. However, the 50-tick challenged hosts yielded significantly lighter ticks ($P < 0.05$) than their controls. There was no difference in engorged weights ($P > 0.05$) between ticks replete on the 20 and 100-tick challenged hosts and the controls. Most of the ticks which failed to engorge and dropped prematurely from rabbits previously exposed to ticks, fed adequately on naive hosts. The feeding performance was better for ticks which dropped with low than high engorged weights, if they were applied to a host within 24 hours of detachment (Table 2).

Feeding period of female R. e. evertsi fed repeatedly on the same rabbits

At the end of the primary infestation, the mean feeding period of the ticks from the different treatments was similar, about 9 days (range 8.72 - 9.02 days) (Table 3). There was a gradual decrease in the mean feeding period, for all the rabbit groups, with increased frequency of tick challenge. However, mean feeding period

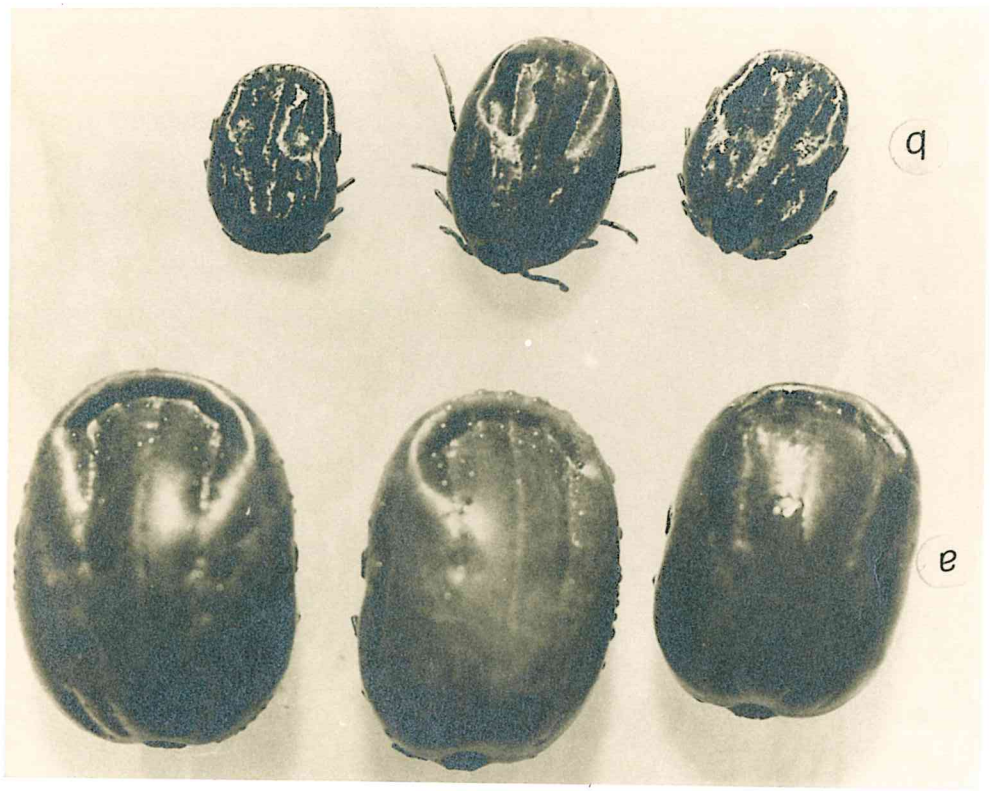
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FIGURE 1

Comparison between body sizes of R.e. evertsi females fed to repletion on rabbits which are naive (row a) and resistant to the same tick species (row b).

TABLE 2

FEEDING AND EGG LAYING POTENTIAL OF FEMALE R. E. EVERTSI REJECTED FROM RESISTANT AND SUBSEQUENTLY APPLIED ON NAIVE HOSTS

Time lag before refeeding to naive host (hr)	No. of ticks applied	% engorging	Mean feeding period (days)	Engorgement Weight (mg)		% fecund	Mean egg wt (mg)	% fertility	% hatchability
				before*	after**				
1	18	100	4.00 ±2.14	42.09 ±23.92	264.62 ±187.38	72.2	115.32 ±101.00	92.31	64.58 ±37.63
24	13	46.15	4.33 ±1.03	14.68 ±8.87	376.79 ±218.14	50	206.03 ±138.32	67	52.08 ±35.38
48	10	10	4	17.21 ±12.50	98.5	100	12.9	100	48.56
72	15	0	-	38.68 ±29.78	-	-	-	-	-

* Mean weight after feeding on a resistant rabbit

** Mean weight of the same ticks after feeding on a naive animal

TABLE 3

MEAN FEEDING PERIOD OF FEMALE *R. E. EVERTSI* ON NAIVE AND RESISTANT RABBITS (DAYS ± S.D.)

Rabbit groups	A	Control	B	Control	C	Control
Treatments	20 ticks		50 ticks		100 ticks	
Infestation 1	8.72 ±1.43		8.78 ±1.24		9.02 ±1.71	
Infestation 2	9.00 ±1.94	7.45 ±0.94	7.30 ±1.50	8.92 ±1.22	7.74 ±1.51	8.79 ±1.30
Infestation 3	7.17 ±1.10	7.96 ±1.12	7.94 ±1.27	8.59 ±1.16	8.07 ±2.02	8.87 ±0.98
Infestation 4	7.06 ±1.78	8.18 ±0.47	7.06 ±1.78	8.01 ±1.26	6.94 ±1.19	8.63 ±0.77

during the second infestation in the 20 tick challenge group was longer, i.e. 9 days. After the fourth infestation, the feeding period was 7.06, 7.06 and 6.94 days for the 20, 50 and 100 tick challenge rabbits respectively. These values were not significantly different. Ticks which had their feeding periods interrupted on previously challenged hosts fed for an extra 4 days when applied immediately on naive hosts (Table 2).

Percent yield of engorged female R. e. evertsi fed repeatedly on the same rabbits

Percent yield of engorged ticks from the three treatment groups varied from 60 to 96.7 during the experiment (Table 4). Lowest yields were observed during the third infestation, being 60, 66.7 and 76.67 percent respectively for the 20, 50 and 100 tick challenge regimens. Subsequent changes were insignificant among the three treatments.

Percent fecund female R. e. evertsi observed during each progressive sensitization of rabbits

Almost all of the female ticks fed on naive rabbits during the first infestation laid eggs (Table 5). The numbers of such females decreased with the subsequent infestations. The lowest numbers were observed during the second infestation for the rabbit groups challenged with 50 and 100 ticks and the third infestation for the 20 tick challenge rabbit group. After this, more fecund females were noticed with the 20 and 50 tick challenge regimens and a constant number was maintained for the 100 tick challenge group. Nearly all the females which fed on the controls were fecund.

TABLE 4

PERCENT FEMALE *R. E. EVERTSI* ENGORGING ON REPEATEDLY SENSITIZED AND NAIVE RABBITS

Rabbit groups	A	Control	B	Control	C	Control
Treatments	20 ticks		50 ticks		100 ticks	
Infestation 1	83.30 (25) (30)	-	90.70 (68) (75)	-	96.70 (145) (150)	-
Infestation 2	80.00 (16) (20)	100.00 (20) (20)	73.30 (58) (75)	94.00 (47) (50)	81.13 (128) (150)	88.00 (88) (100)
Infestation 3	60.00 (12) (20)	100.00 (20) (20)	66.70 (50) (75)	100.00 (50) (50)	76.67 (115) (150)	95.00 (95) (100)
Infestation 4	60.00 (12) (20)	100.00 (20) (20)	81.30 (61) (75)	98.00 (49) (50)	77.30 (116) (150)	95.00 (95) (100)

( ) Shows number of female ticks completing engorgement/total females applied

TABLE 5

PERCENT FECUND FEMALE R. E. EVERTSI FED ON NAIVE AND TICK RESISTANT RABBITS

Rabbit groups	A	Control	B	Control	C	Control
Treatments	20 ticks		50 ticks		100 ticks	
Infestation 1	100 (25) (25)	-	95.59 (65) (68)	-	93.79 (136) (145)	-
Infestation 2	81.25 (13) (16)	100 (20) (20)	48.28 (28) (58)	100 (47) (47)	46.10 (59) (128)	98.8 (87) (88)
Infestation 3	50 (6) (12)	100 (20) (20)	58 (29) (50)	100 (50) (50)	66.09 (76) (115)	100 (95) (95)
Infestation 4	75 (9) (12)	95 (19) (20)	90.15 (55) (61)	92.8 (45) (49)	66.38 (77) (116)	100 (95) (95)

( ) Shows number of fecund ticks/total completing engorgment

Agglutinating antibodies appeared in rabbits challenged with 100 ticks as reported for the other rabbit groups. The primary antibody response increased progressively to a peak titre of ($\log_2$) 3.25 by day 21 (Fig. 5). Between day 21 and 28 and also day 49 and 70 the host antibody response was marked by wide variations. With this degree of variation the decline is not significant. The fourth challenge performed on day 70 stimulated a strong response; ($\log_2$) 4.5. This response started to decline by day 91 and was not affected by challenge with either R. appendiculatus or A. variegatum.

Humoral antibody response in rabbits infested with R. e. evertsi as detected by the ELISA test

Initial infestations of rabbits with 20 R. e. evertsi elicited antibodies to SGA detectable by the ELISA test at day 7 of tick feeding (Fig. 6). A peak antibody activity corresponding with the first infestation was attained on day 14 and declined during the subsequent tick free period of 14 days. The second tick challenge performed on day 28 after the first challenge stimulated a significant secondary immune response which was maximum at day 35. The third tick infestation, applied on day 49, stimulated an increased response until the fourth infestation which again raised antibody levels. There was a marked variation in antibody responses followed by a gradual decline. The decline was not affected by cross challenge with adult R. appendiculatus. Subsequent infestation with A. variegatum raised antibody levels again. Antibodies developed a fortnight later in the absence of tick challenge.

TABLE 6

MEAN PREOVIPOSITION PERIODS OF FEMALE *R. E. EVERTSI* FED ON NAIVE AND SENSITIZED RABBITS (DAYS ± S.D.)

Rabbit groups	A	Control	B	Control	C	Control
Treatments	20 ticks		50 ticks		100 ticks	
Infestation 1	4.36 ±0.49	-	4.85 ±0.57	-	4.56 ±0.62	-
Infestation 2	5.15 ±1.77	4.75 ±0.55	4.90 ±1.62	4.02 ±0.79	4.34 ±1.10	4.49 ±0.61
Infestation 3	5.50 ±1.52	4.60 ±0.68	4.68 ±1.49	4.08 ±0.42	5.87 ±1.58	4.13 ±0.57
Infestation 4	5.00 ±1.32	4.56 ±0.63	5.02 ±1.19	5.04 ±0.62	6.08 ±1.68	4.61 ±0.63
Grand mean ± s.d.	5.00 ±0.48	4.64 ±0.10	4.86 ±0.14	4.38 ±0.57	5.21 ±0.89	4.41 ±0.25

TABLE 7

MEAN WEIGHTS (MG ± S.D) OF EGG MASSES OVIPOSITED BY FEMALE R.E. EVERTSI FED ON NAIVE AND SENSITIZED RABBITS

Rabbits groups	A	Control	B	Control	C	Control
Treatments	20 ticks		50 ticks		100 ticks	
Infestation 1	518.68 ± 140.60 (25)	-	435.90 ± 150.40 (65)	-	375.42 ± 162.70 (136)	-
Infestation 2	102.54 ± 110.40 (13)	467.68 131.20 (20)	161.70 ± 131.20 (28)	505.70 ± 161.10 (47)	147.73 ± 106.23 (59)	436.16 ± 157.05 (87)
Infestation 3	31.92 ± 19.65 (6)	489.68 ± 138.20 (20)	106.60 ± 104.59 (29)	475.96 ± 118.83 (50)	54.46 ± 50.42 (76)	432.50 ± 172.63 (95)
Infestation 4	102.70 ± 88.70 (9)	494.31 ± 117.16 (20)	94.17 ± 96.79 (55)	448.50 ± 123.27 (45)	53.10 ± 54.67 (77)	445.62 ± 165.07 (95)
Group mean	188.96ab	483.89b	199.55a	476.72bc	157.68a	438.09bd
± S.D.	± 222.33	± 14.23	± 160.25	± 28.63	± 151.77	± 6.77

( ) Number of fecund females sampled.
± Mean ± standard deviation
Group means not followed by a common letter are statistically different ($P < 0.05$)

TABLE 8

MEAN INCUBATION PERIOD OF R.E. EVERTSI EGGS FROM FEMALES FED REPEATEDLY ON SENSITIZED RABBITS (DAYS ± S.D.)

Rabbit groups	A	B	C
Treatments	20 ticks	50 ticks	100 ticks
Infestation 1	25.68 ± 0.84	26.08 ± 1.38	26.73 ± 1.36
Infestation 2	28.00 ± 1.55	27.17 ± 0.99	27.28 ± 1.41
Infestation 3	26.50 ± 1.76	26.96 ± 2.05	26.23 ± 1.63
Infestation 4	25.89 ± 1.62	26.79 ± 1.84	25.24 ± 1.86
Group mean ± s.d.	26.52 ± 1.05	26.50 ± 0.67	26.37 ± 0.87
Control	26.12 ± 1.05	26.32 ± 1.26	25.98 ± 1.52

Percent egg hatch rate

The mean percent hatch rates were 64.83, 73.77 and 65.6 after the first infestation of rabbits with 20, 50 and 100 tick regimens respectively (Table 9). A percent hatch rate of about 60% was observed during the second infestation for all the treatment groups and was maintained with slight variation during subsequent challenge in the 50 and 100 tick challenge groups. The hatch rate was poor (31.46%) during the third challenge of hosts with 20 ticks and showed a tendency to improve with the fourth infestation. Ticks which otherwise would not have laid eggs as a result of their feeding being interrupted on previously exposed hosts, oviposited smaller egg masses with about 60% hatch rate when fed on naive hosts (Table 2).

Development of precipitating antibodies in rabbits repeatedly infested with adult R. e. evertsi

Precipitating antibodies were detected in the sera of all tick infested rabbits. They were detected as early as the second week of primary infestation in some rabbits in the 100 tick challenge group (Fig. 2). Rabbits in the 20 and 50 tick challenge regimens developed antibodies detectable by the third and fifth week of primary challenge respectively. All the three rabbit groups except the 50-tick group exhibited anamnestic responses in the subsequent infestations, peak responses were attained after secondary tick exposure.

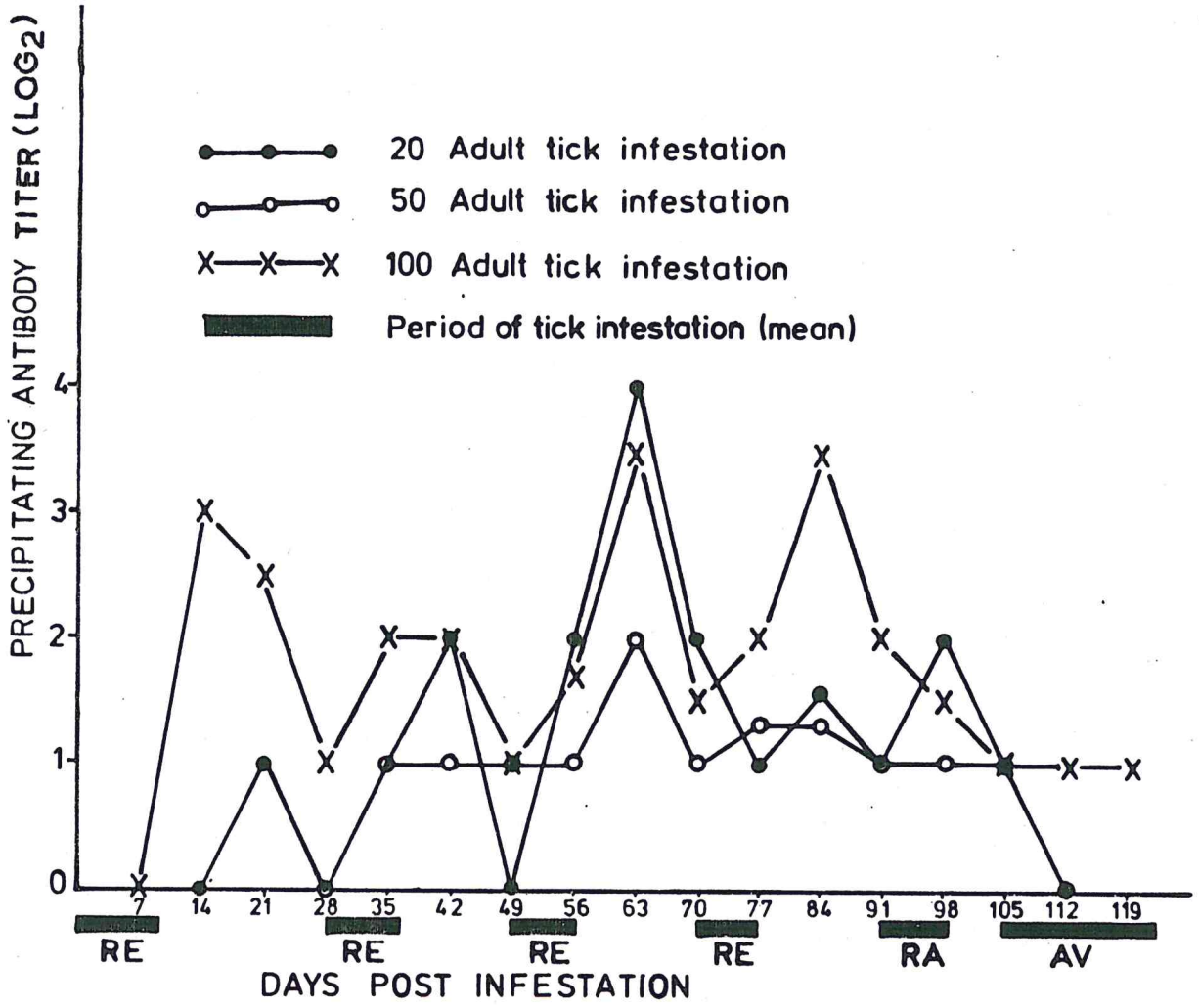
Cross-challenge with 20 adult R. appendiculatus elicited an antibody response in the rabbit group sensitized with 20 adult

TABLE 9

THE MEAN HATCHING PERCENT OF EGGS LAID BY FEMALE *R. E. EVERTSI* DURING REPEATED INFESTATION OF RABBITS

Rabbit groups	A	B	C
Treatments	20 ticks	50 ticks	100 ticks
Infestation 1	64.83 ±29.60 (25)	73.77 ±22.60 (65)	65.60 ±27.67 (136)
Infestation 2	60.80 ±29.03 (13)	58.17 ±30.18 (28)	54.42 ±24.14 (59)
Infestation 3	31.46 ±35.66 (6)	50.44 ±26.79 (29)	62.22 ±23.64 (76)
Infestation 4	48.00 ±32.04 (9)	62.45 ±25.12 (55)	54.02 ±27.89 (77)

( ) Number of egg batches examined is shown in parenthesis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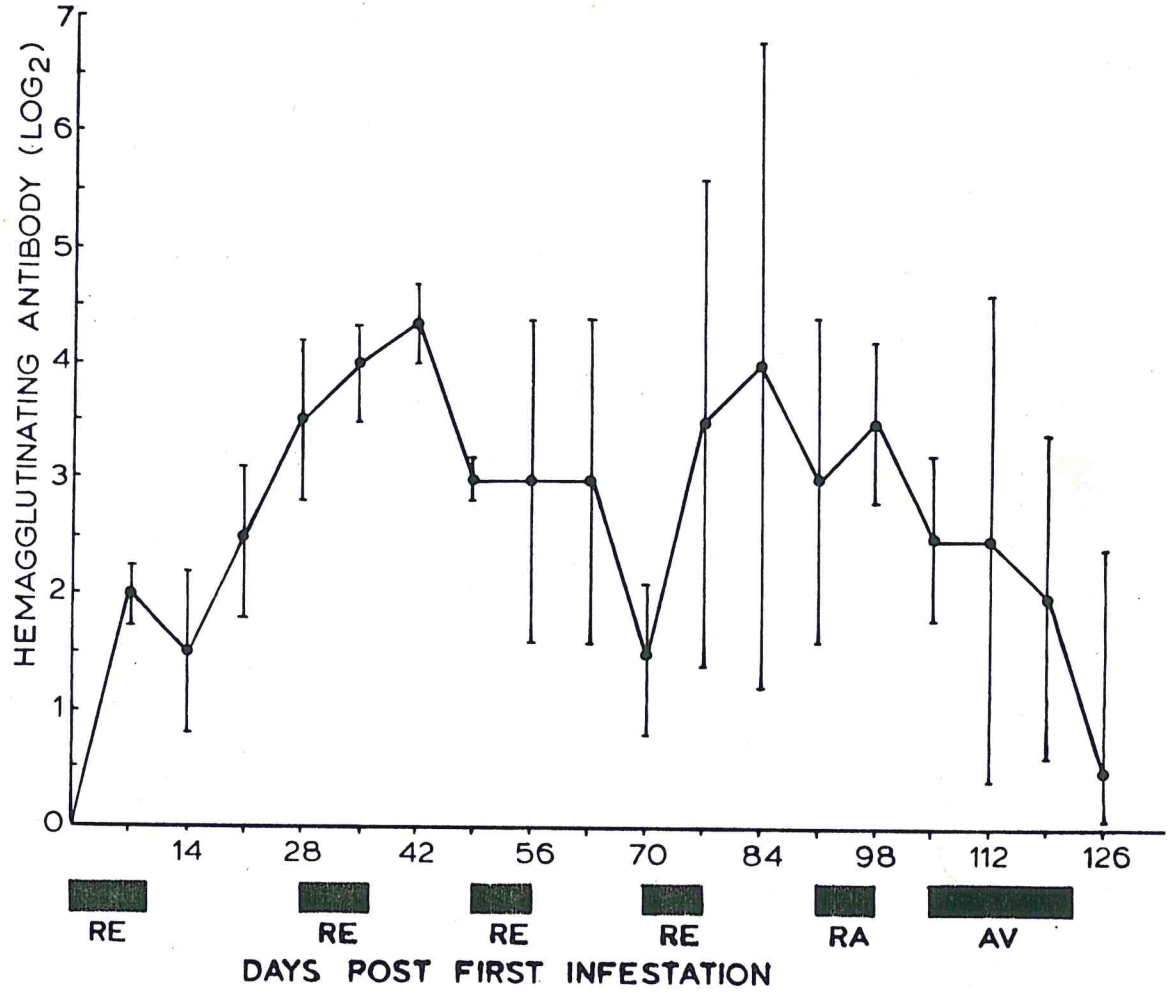
DAYS POST INFESTATION

R. e. evertsi only. A similar challenge with 8 A. variegatum did not elicit antibody response in the three treatment groups.

Development of agglutinating antibodies in rabbits repeatedly infested with adult R. e. evertsi

Agglutinating antibodies were detected in rabbits in each of the different tick challenge regimens by day 7 of initial infestation. A progressively increasing antibody response, attaining peak value on day 42, was observed in the 20 tick challenge group (Fig. 3) which then declined and was not revived by the third infestation. Although the host immune response recovered after the fourth tick challenge, the mean titres showed wide standard deviations. Challenge with either 20 adult R. appendiculatus or 8 adult A. variegatum did not improve on the declining immune response.

Agglutinating antibody titres of about ($\log_2$) 2 were observed in hosts challenged with 50 ticks by the end of first week of initial infestation (Fig. 4). A decline of the titres was noted in the second week of tick feeding, followed by an increase which was maximum on day 28 when the second tick challenge was applied. The tick challenge caused a sharp decline in the titres to ($\log_2$) 1.5 by day 35. After this, gradual recovery which was augmented by the third infestation occurred. At the end of this infestation, the titres dropped until the 4th tick challenge. Significant recovery occurred after the fourth infestation was completed. Heterologous challenge with R. appendiculatus and A. variegatum did not affect the decline in antibody titres.


HEMAGGLUTINATING ANTIBODY (LOG_2)
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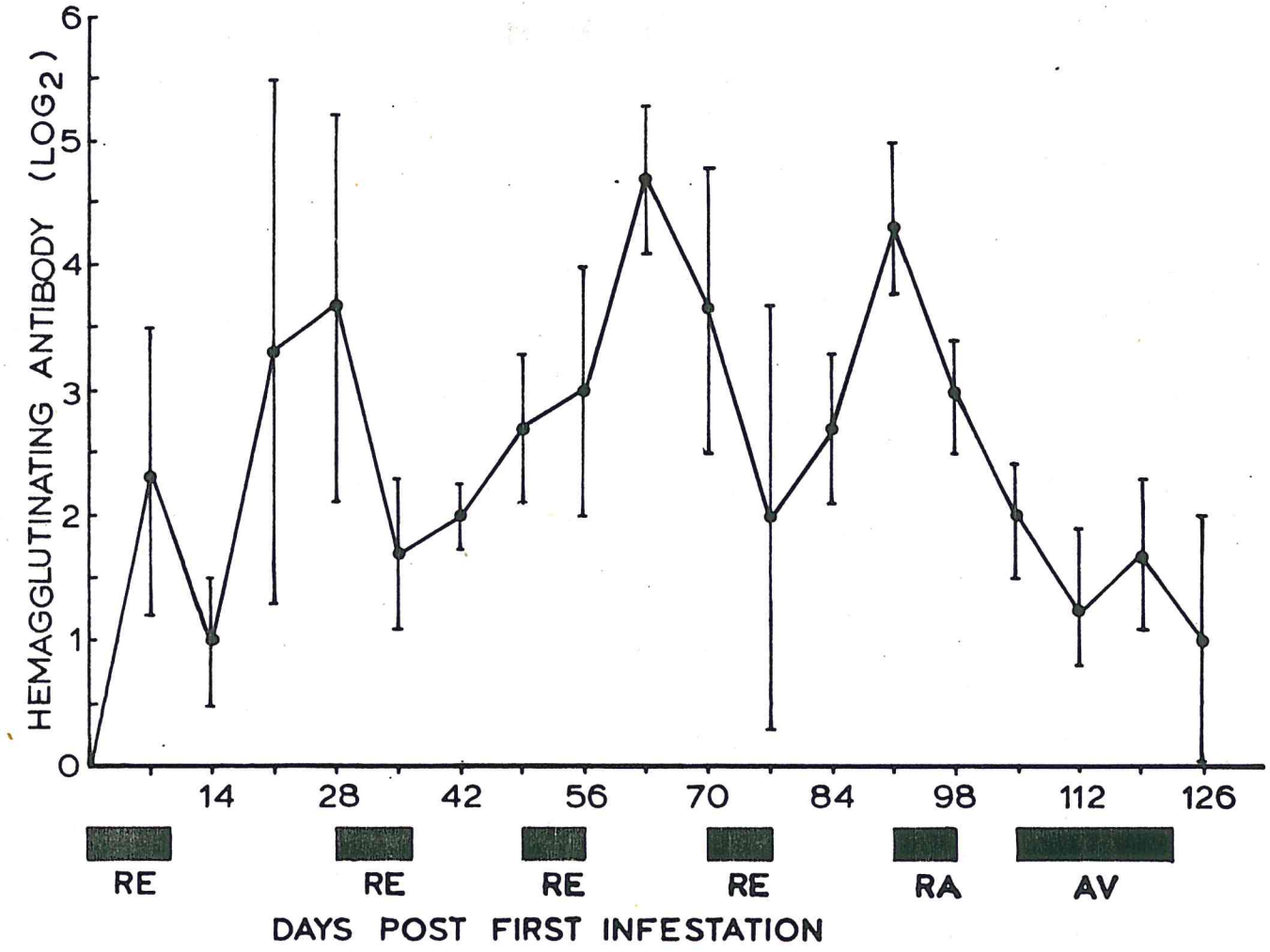
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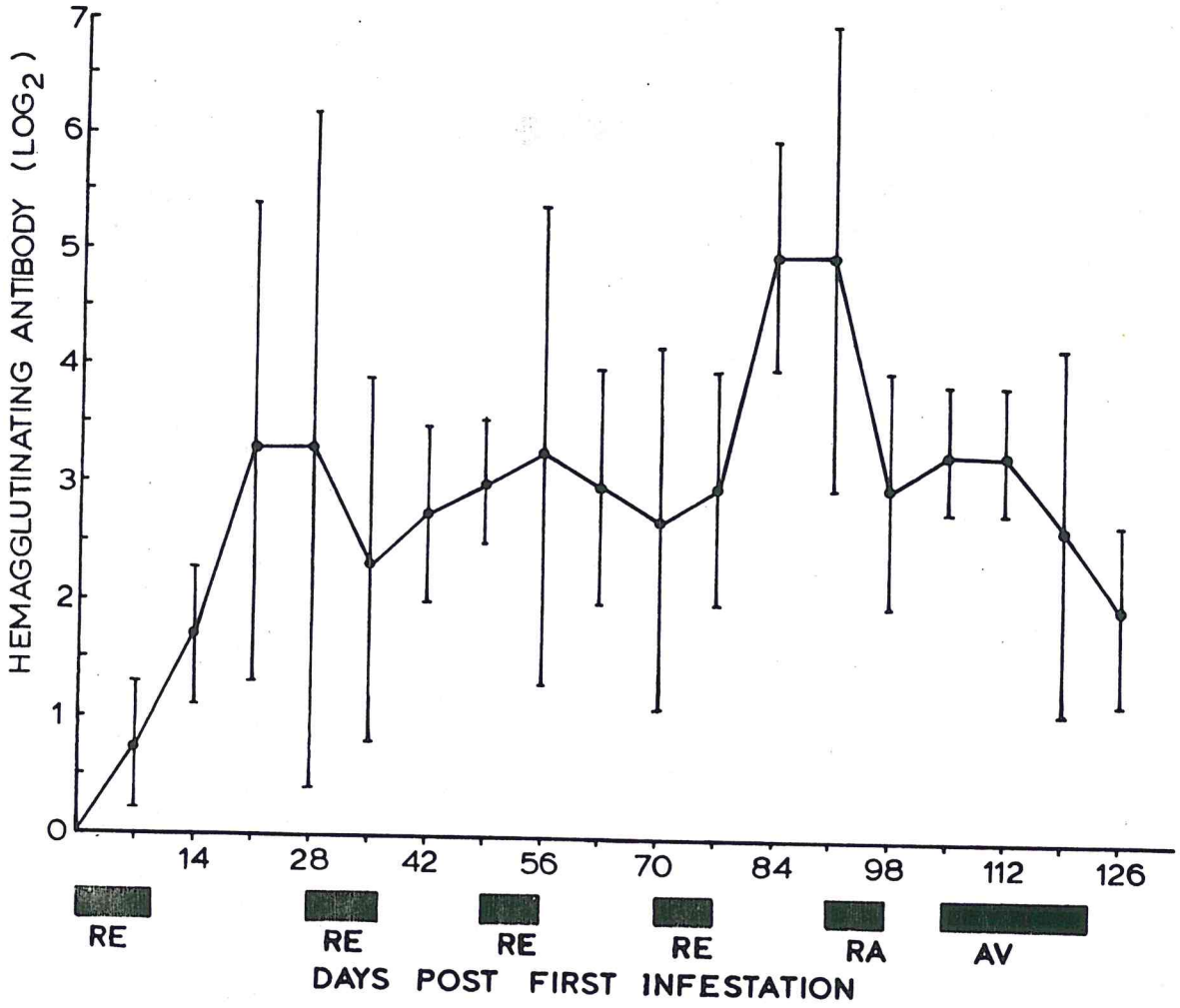
DAYS POST FIRST INFESTATION

Agglutinating antibodies appeared in rabbits challenged with 100 ticks as reported for the other rabbit groups. The primary antibody response increased progressively to a peak titre of ($\log_2$) 3.25 by day 21 (Fig. 5). Between day 21 and 28 and also day 49 and 70 the host antibody response was marked by wide variations. With this degree of variation the decline is not significant. The fourth challenge performed on day 70 stimulated a strong response; ($\log_2$) 4.5. This response started to decline by day 91 and was not affected by challenge with either R. appendiculatus or A. variegatum.

Humoral antibody response in rabbits infested with R. e. evertsi as detected by the ELISA test

Initial infestations of rabbits with 20 R. e. evertsi elicited antibodies to SGA detectable by the ELISA test at day 7 of tick feeding (Fig. 6). A peak antibody activity corresponding with the first infestation was attained on day 14 and declined during the subsequent tick free period of 14 days. The second tick challenge performed on day 28 after the first challenge stimulated a significant secondary immune response which was maximum at day 35. The third tick infestation, applied on day 49, stimulated an increased response until the fourth infestation which again raised antibody levels. There was a marked variation in antibody responses followed by a gradual decline. The decline was not affected by cross challenge with adult R. appendiculatus. Subsequent infestation with A. variegatum raised antibody levels again. Antibodies developed a fortnight later in the absence of tick challenge.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3
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5
6
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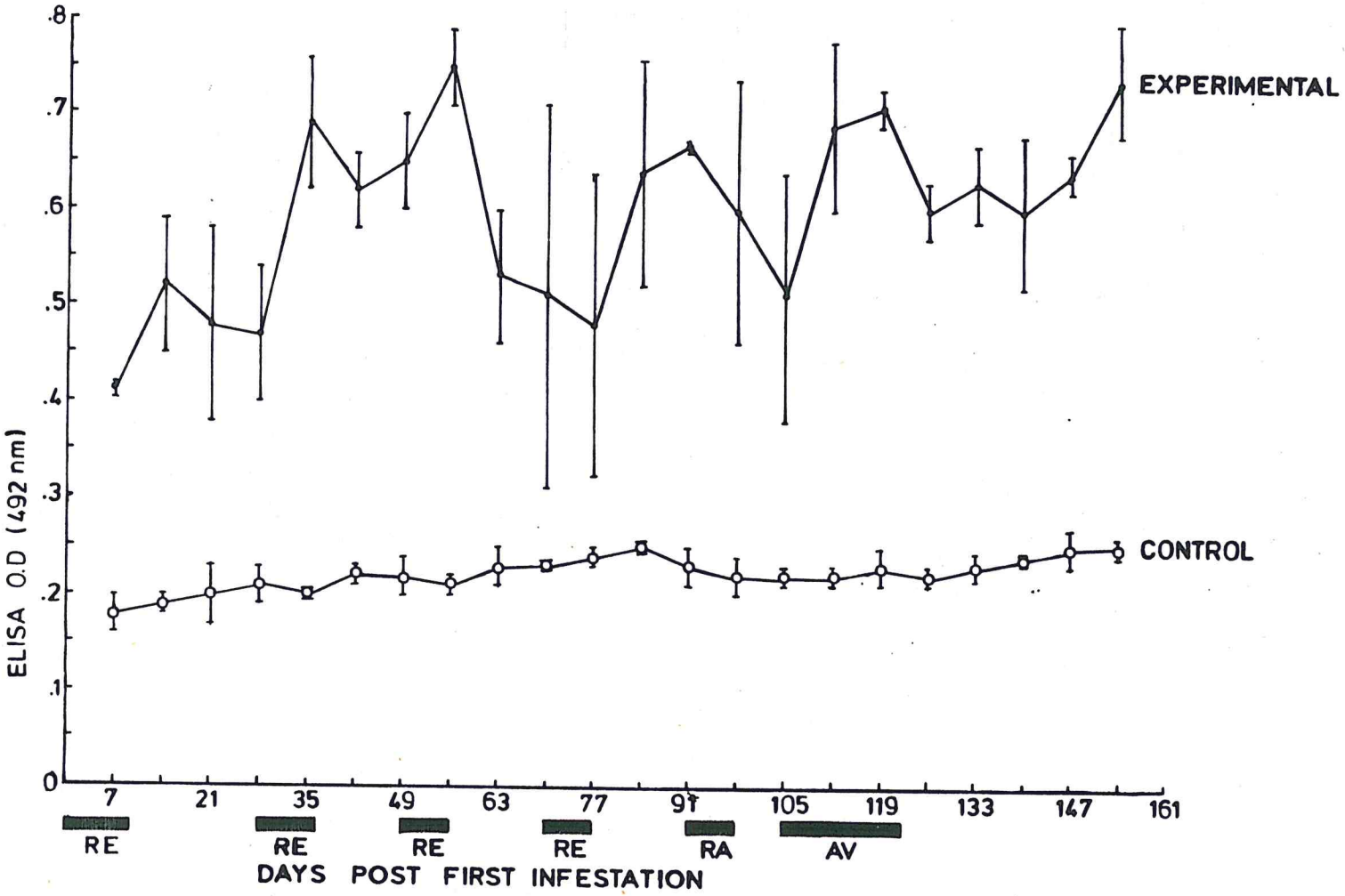
DAYS POST FIRST INFESTATION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49
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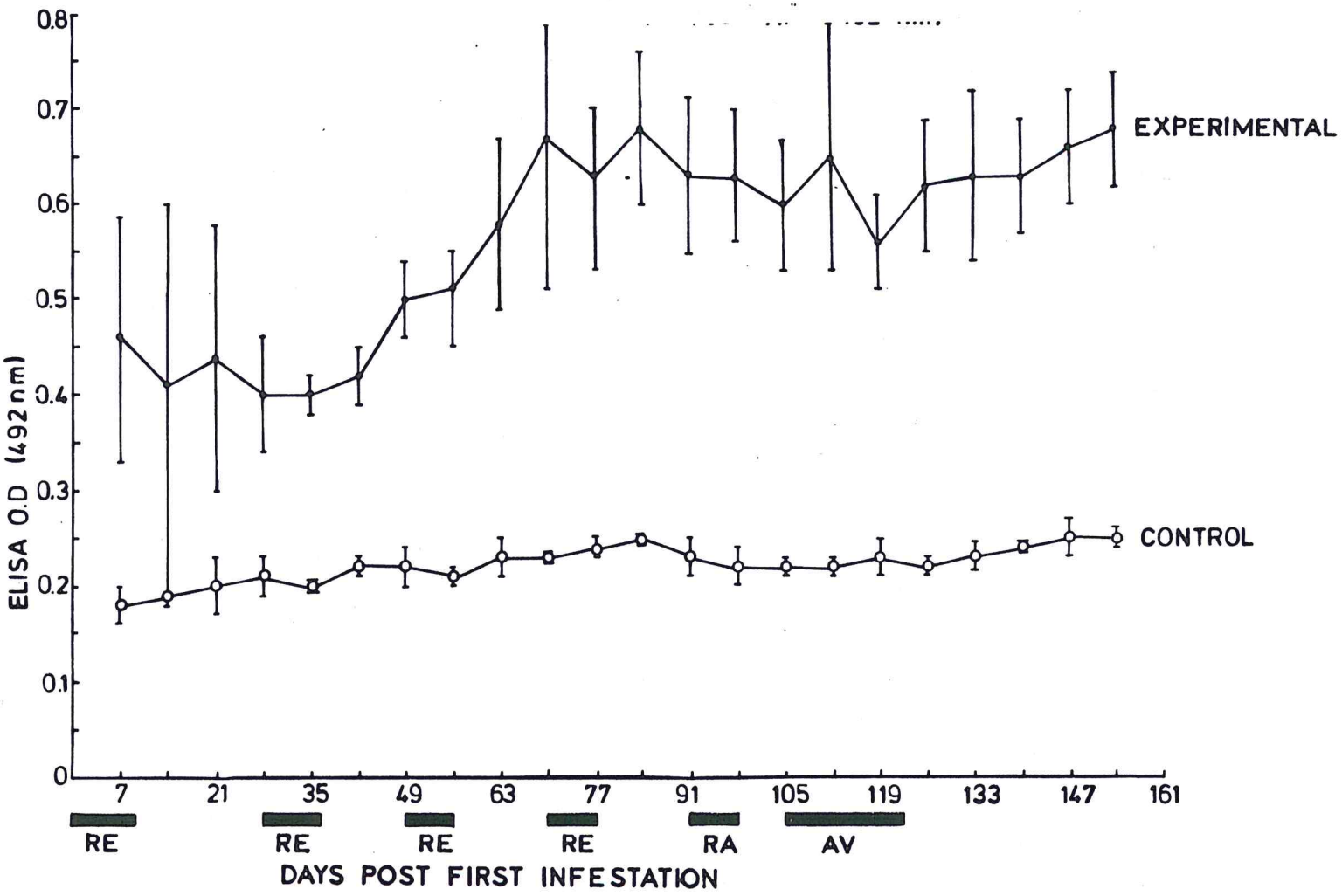
77
91
105
119
133
147
161
RE
RE
RE
RE
RA
AV
DAYS POST FIRST INFESTATION

Initial antibody activity was detected in host sera challenged by 50 ticks by day 7 of primary infestation (Fig. 7). Between day 7 and 49 after the first tick challenge, rabbits did not develop immune responses superior to that observed on day 7, despite secondary infestation. A significant progressive increase in antibody response occurred after the second infestation. A mean maximum ELISA extinction value of 0.6 units was recorded at day 70 and maintained with minor fluctuations throughout the study.

Rabbits challenged with 100 ticks developed initial antibody activity at day 7 of primary challenge (Fig. 8) which increased significantly at day 14. Thereafter, the antibody response declined drastically up to day 49. The titres increased progressively after the third challenge and plateaued as described for the 50 tick challenge hosts. Challenge with R. appendiculatus and A. variegatum did not increase the antibody titres established by R.e. evertsi infestation.

Development of tick paralysis

Of 11 rabbits infested with 100 ticks, 8 became paralysed and died. Seven of the 9 rabbits challenged with 50 ticks were paralysed and 5 died. Most cases of paralysis occurred between day 6-9 of primary infestation. However, one rabbit in a group of 9 challenged with 20 ticks was paralysed and died during the second infestation (Table 10).


0.8
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ELISA O.D (492 nm)
EXPERIMENTAL
CONTROL
7
21
35
49
63
77
91
105
119
133
147
161
RE
RE
RE
RE
RA
AV
DAYS POST FIRST INFESTATION

FIGURE 8

Development of serum antibodies to SGA in three rabbits repeatedly infested with 100 adult R. e. evertsi (RE) and later challenged with R. appendiculatus (RA) and A. variegatum (AV), as determined by the ELISA test. Each point on the graph represents a mean of 18 replicates; six replicates for each rabbit serum sample diluted 1:10 in PBS. Antigen concentration was 0.75 µg protein per well. Goat anti-rabbit IgG was diluted 1:1500.

TABLE 10

INCIDENCE OF PARALYSIS IN RABBITS INFESTED BY 3 DIFFERENT TICK LOADS OF ADULT *R. E. EVERTSI*

Rabbit group	No. of rabbits	No. of ticks applied per rabbit	Time to onset of paralysis (day)**	No. of rabbits developing paralysis	No. of rabbits dying after developing paralysis
1	11	100	6 - 7	8	8
2	9	50	6 - 9	7	5
3	9	20	7	1*	1
Total	29	NA	NA	16(55%)	14(88%)

* Developed during secondary tick challenge

** Observed from the time all ticks applied had attached on the host

NA Not applicable

DISCUSSION

This study has shown that rabbits acquired resistance to adult R. e. evertsi after a single infestation. Resistance was manifested during the second infestation, as evidenced by reduced numbers of adult fed ticks, decreased weights of ticks after feeding,shortened tick feeding periods and smaller egg masses laid. This pattern of host resistance is typical of that already reported for other tick species (Trager, 1939 a, b; Brossard, 1976; Allen and Humphrey, 1976; Bowessidjaou et al., 1977, Fujisaki, 1978).

Engorgement weights have been reported to decline progressively when I. ricinus (Bowessidjaou et al., 1977), Haemaphysalis longicornis (Fujisaki, 1978) and R. appendiculatus (Mongi, 1982) were fed repeatedly on the same hosts. Such a pattern of decline in engorgement weights was observed in this study up to the third infestation for the three treatment groups. It was more pronounced at the third infestation on hosts challenged with 20 and 100 ticks than with 50 ticks (Table 1). After the third infestation, engorged weights increased for ticks fed on hosts challenged with 20 and 50 ticks and progressively declined on 100 tick challenge hosts. The enhanced feeding of R. e. evertsi after third challenge reported above, would indicate loss of resistance. Similar results have been reported with D. variabilis on deer mice (Trager, 1939a), I. trianguliceps with CBA mice (Randolph, 1979), I. ricinus on rabbits (Brossard et al., 1982) and D. variabilis in BALB/c mice (Den-Hollander and Allen, 1985). This phenomenon is interpreted to indicate loss of resistance by hosts repeatedly challenged with low numbers of R. e. evertsi. Loss of antibodies

was evident after the third tick exposure (Figs. 2 and 6). This supports earlier observations by Wikel and Osburn (1982) and Whelen et al., (1984) that calves repeatedly challenged with low numbers of D. andersoni lost precipitating antibodies.

Ticks fed better on hosts challenged with 50 than with either 20 or 100 ticks. Experimental hosts in the 50 tick challenge group in which most became paralysed and recovered manifested lower precipitating antibodies than the other two groups (Fig. 2). The role of these antibodies in the expression of resistance in tick—paralysed rabbits is unclear.

It has been reported that R. appendiculatus (Branagan, 1974; Mongi, 1982) and I. ricinus (Bowessidjaou et al., 1977; Brossard et al., 1982) feed longer on resistant than non-resistant hosts. Investigations on this parameter using R. appendiculatus (de Castro et al., 1985), H. longicornis (Fujisaki, 1978) and R. e. evertsi in the present study indicated shortened feeding period on resistant hosts.

Rhipicephalus e. evertsi fed on resistant hosts had longer preoviposition periods than those on naive controls. This is in agreement with observations made by Brossard et al., (1982) in I. ricinus and Fujisaki (1978) in H. longicornis . It is possible as suggested by Londt and Van der Bijl (1977) that the prolonged preoviposition period may be due to the long time interval taken for the few eggs to traverse the long oviduct of such ticks.

Results presented in this study have demonstrated that large egg masses with high hatch rates were laid by ticks fed on naive rabbits in the primary infestation (Table 7, 9) while smaller egg masses were laid as hosts acquired resistance. This may be due to the possibility that antibodies to the tick SGA, as demonstrated in the resistant hosts (Fig. 1) were ingested by the ticks. These antibodies have been shown to cross the digestive tracts of ticks and to exert their potency against salivary glands (Ackerman et al., 1981; Brossard and Rais 1984). Therefore antibodies developed against this tick may have interferred with the ixodid salivary gland with the result that the blood meal ingested could not suffice for maintenance and egg production.

The incubation period of eggs laid in the course of the study was approximately 26 days (Table 8) and did not differ from the controls. However, the tick preoviposition periods were prolonged. In similar studies with R. appendiculatus (Mongi, 1982) and I. ricinus (Brossard et al., 1982), it was reported that incubation periods were longer for eggs laid by ticks fed on resistant as compared to naive hosts.

Resistance induced by 20 tick challenge significantly altered egg hatch rate which decreased from 65% (first infestation) to 31% (third infestation) (Table 9). Such an effect was not observed for eggs laid by ticks fed on hosts challenged either with 50 or 100 ticks. Although resistance reduced fecundity (Table 7), fertility was not abolished. Resistance induced by other tick species has been reported to effectively block hatch rate (Bowessidjaou et al.,

1977; Allen and Humphrey, 1979; Mongi, 1982). However, the observations made in this study, like those by Riek (1962) and Fujisaki (1978) suggest that resistance allow a few ticks to complete life cycle successfully.

The effects of resistance on feeding and reproductive performance, observed during the second challenge (Table 1) when ticks were applied on a non-infested ear of a rabbit in which the other ear had been infested once, suggest the phenomenon to be immunological. As reported earlier, circulating antibodies were observed in the treatment groups throughout the study period. They were detected in most rabbits by first week of primary challenge using ELISA and PHA tests and as late as the 5th week by agar gel double diffusion test. Fisher (1983) detected precipitating antibodies to Psoroptes cuniculi in rabbits as late as 3rd week of primary challenge. These results concur with those of Fox et al. (1967) who suggested that immunodiffusion tests may not be sensitive enough for the diagnosis of ectoparasitic infestations. The results reported here suggest that the ELISA and PHA have practical values in early detection of anti-tick antibodies.

In this experiment, where sera were sequentially collected, a rise and fall of antibody response in repeatedly challenged hosts was detectable, notably with PHA and immunodiffusion tests (Figs. 2, 3, 4 and 5). Thus, a specific humoral antibody response to tick infestation could be inferred. Using the ELISA test on sera similarly collected from the 20 tick challenge hosts, it was shown (Fig. 6) that for the first 3 sets of repeated infestations, titres

were fluctuating following exposure. A significant decline occurred after the third challenge. At the fourth (homologous) and subsequent heterologous infestations, both the mean titres and their standard deviations, increased. The increase was less than observed in the second and third infestations. Such a pattern of response suggests loss of resistance when 20 ticks were used repeatedly to challenge hosts.

The results of this study have indicated that adult R. e. evertsi induced paralysis when fed on naive hosts (Table 10). In the 100-tick challenge regimen more rabbits became paralysed and died than in either 20 or 50 tick challenges. Subsequent tick challenges performed on the rabbits that survived the primary effects of paralysis failed to induce paralysis except in one rabbit challenged with 20 ticks. This indicates that apart from the development of resistance to ticks, immunity to tick paralysis developed in the rabbits after primary exposure. This phenomenon has also been reported for other tick species (Ross, 1935; Oxer and Ricardo, 1942; Stone and Wright, 1980). One rabbit in the 20 tick challenge regimen succumbed to paralysis during secondary tick infestation. It is possible that in this rabbit, there was inadequate immunity which could not prevent the development of subsequent paralysis as shown by Stone and Wright (1980, 1981) and Stone et al. (1982). Many animals challenged with 100 ticks developed paralysis and all paralyzed animals died, indicating the possibility that the amount of toxin injected induced paralysis-associated mortality before immunity to the toxin could develop. These results confirm those of Stone et al. (1982) that

once immunity to tick paralysis has been conferred, high circulating antibody titres are not necessary to prevent occurrence of paralysis.

SUMMARY

Twenty nine rabbits were exposed to infestations with different numbers of 20, 50 and 100 adult R. e. evertsi. Of these rabbits, nine were exposed to four infestations and the rest single challenges. At primary infestation, 16(55%) rabbits developed paralysis and of these 14(88%) died. More deaths occurred in rabbits challenged with 100 ticks than with either 20 or 50 ticks. Immunity to paralysis and subsequent resistance to tick feeding developed in the hosts that survived the initial challenge. Rabbits were also resistant to reinfestation as evidenced by reduced numbers of adult fed ticks, decreased weights of ticks after feeding and smaller egg masses. Antibodies to SGA were detected in sera of infested hosts by the passive haemagglutination test, agar gel double immuno- diffusion and enzyme-linked immmunosorbent assay. Antibody titres (threshold) were attained after secondary challenge with R.e. evertsi and were not elevated by infestations with R. appendiculatus and A. variegatum. Rabbits challenged with 20 ticks displayed declining titres with advanced frequency of challenge and a few rabbits in this group developed paralysis. There was no decline in antibody titres in groups of rabbits exposed to 50 and 100 tick loads. Ticks which fed poorly on resistant hosts, engorged to better weights on naive hosts. There was no evidence that host resistance totally blocked the tick life cycle.

CHAPTER 2

STUDIES ON ACQUIRED RESISTANCE IN RABBITS TO INSTARS OF RHIPICEPHALUS EVERTSI EVERTSI

Introduction

Development of resistance in animals after adult ticks have fed on them has been investigated by several workers including Trager (1939a), Garin and Grabarev (1972), Brossard (1977), Bowessidjaou et al.(1977), Brossard and Girardin (1979) and Brossard et al. (1982). A number of parameters including reduction in tick numbers and reduction in engorged tick weights have been used to assess resistance. Allen and Wikel (1978) and Branagan (1974) reported that, resistance induced higher mortalities for the larvae than for either nymphs or adults of 3-host ticks. These studies assessed the effects of homologous resistance on the performance of the life stage of the tick which stimulated it.

Brown et al. (1984) observed that in a 3-host tick, Ambylyomma americanum resistance induced by one life stage was expressed against the other two stages in the life cycle. Sutherst et al. (1979) observed that in the 3-host tick, Heamaphysalis longicornis host resistance affected the survival of larvae more than that of nymphs and adults. Similar studies have shown that the population of Boophilus microplus, a one host tick was found to be adversely affected by loss of larvae (Roberts, 1968b; Utech et al., 1978). Wagland (1979) did not observe such resistance during primary infestation with B. microplus despite the 3 weeks duration of the tick life cycle.

Natural occurrence of intraspecific homologous resistance in two host ticks may have significant implications. However, this has not been investigated. This study was therefore designed in order to investigate the ability of different R.e. evertsi instars to induce host resistance and the possible effects of larval feeding on emerged nymphs during primary infestation.

MATERIALS AND METHODS

Rabbits

Thirty three naive, New Zealand White rabbits, of the same weight as described in Chapter 1 were used. Of these, nine rabbits were divided into three groups of three rabbits each and served as experimental units. The remaining twenty four rabbits were paired and served as controls in the subsequent series of successive tick challenges.

Ticks

Ticks used in this study, their source and maintenance has been mentioned in Chapter 1.

Host infestations

Three different tick loads composed of 100, 500 and 1500 larvae were counted as follows: A transluscent strip of polythene paper (0.5 x 4 cm) was placed into a tube containing the larvae. A few larvae at a time (less than 15) were allowed to crawl onto the paper strip, counted by means of a telecounter and placed in glass tubes (5 x 1 cm), surrounded by ice cubes in order to reduce their movement to a minimum. After the required number was attained, the

tubes were removed from the ice bath. These tick loads were alloted randomly to the three rabbit groups and applied on the rabbits ears following the method of Irvin and Brocklesby (1970). Five infestations were carried out on alternate rabbit ears. The time interval between two consecutive infestations was seven days. The full protocol used in the infestation is shown in Table 11.

Ticks were observed for larval-nymphal feeding periods which were assessed from the time most larvae had attached to detachment of replete nymphs. Detached, replete nymphs were counted to determine the percentage that moulted from larvae, weighed individually and alloted to the following weight categories: 0-4.9, 5-9.9, 10-14.9, 15-19.9 and 20-24.9 mg. The body length of each nymph was measured in the midline from posterior margin of basis capituli to the caudal margin using a pair of dividers in order to construct frequency distributions. Nymphs in the different weight categories were then incubated in glass tubes as described in Chapter I in order to moult. The time taken to initiate and complete moults by nymphs in each weight category was recorded and the mean taken as the moulting period. After moulting, the number of emerging adults were counted, sexed and expressed as a fraction of total nymphs incubated and a percent moult obtained. Percent moult failure was similarly calculated and any colour abnormality noted.

TABLE 11

THE NUMBER OF LARVAE APPLIED AT EACH INFESTATION FOR THE THREE TREATMENT GROUPS

Rabbit groups	A		B		C	
Treatments	Expt.	Control	Expt.	Control	Expt.	Control
Infestation 1	100	0	500	0	1500	0
Infestation 2	100	100	500	500	1500	1500
Infestation 3	100	100	500	500	1500	1500
Infestation 4	100	100	500	500	1500	1500
Infestation 5	100	100	500	500	1500	1500

Serum collection

Blood for separation of serum was collected from each rabbit as described in Chapter 1.

Preparation of antigens from engorged instars

Homogenates of fully fed larvae and nymphs to be used as antigens were prepared as follows: about 4000 larvae were allowed to feed on ears of two naive rabbits (about 2000 larvae per ear). Larval feeding was interrupted on the 5th day by manual removal of all larvae from one of the ears of each rabbit. The remaining larvae were allowed to feed and drop off as replete nymphs. The harvested larvae and detached nymphs were washed separately three times in cold PBS by centrifugation at 1000 g at 4^{o}C for 5 minutes. The larvae and nymphs were packed separately into Tenbrok grinders and ground in twice their volumes of cold PBS. The homogenates were transferred into sterile universal bottles and sonicated as described in Chapter 1. After this, the homogenates were centrifuged at 3000 g at 4^{o}C for 30 minutes and the supernatants were retained. The supernatants were further centrifuged at 15000 g at 4^{o}C for 30 minutes after which they were tested in agar gel double diffusion for possible reaction with immune serum against adult R. e. evertsi SGA. Since larval antigens recognized antibodies against adult tick SGA, the latter antigen was used in all serological work reported here.

Serological tests

Agar gel double diffusion, passive haemagglutination and ELISA tests were carried out as described in Chapter 1.

RESULTS

Larval-nymphal feeding period

At the end of primary larval challenge, the combined feeding period of the two tick instars for the three treatment groups was approximately similar i.e. 19 days (Table 12). A decrease in this parameter occurred during the second and third challenges in the three rabbit groups. It was gradual for nymphs fed on the 100 and 500 larval challenge rabbits and severe for 1500 larvae exposed group. The feeding period showed a tendency to decline after the third infestation for the three experimental groups. There was no significant difference ($P > 0.05$) observed in this parameter among the three treatments and also between each treatment and its control.

Percent nymphal yield

About 80% of the larvae applied initially to rabbit groups challenged with 100 and 500 larvae respectively emerged as nymphs (Table 13). Only 45% of those applied to rabbits for 1500 larvae challenge regimen matured into nymphs. In subsequent challenges with 100 larvae, between 30 and 50% of the total larvae applied on the hosts emerged as nymphs, whereas less than 50% was observed for the remaining two treatments. Partially engorged larvae (detached) were observed also among the replete nymphs. The percent mean

TABLE 12

LARVAL-NYMPHAL FEEDING PERIOD OF R. E. EVERTSI INSTARS ON NAIVE AND SENSITIZED RABBITS (DAYS ± S.D.)

Rabbit groups	A	Control	B	Control	C	Control
Treatments	100 Larvae		500 Larvae		1500 Larvae	
Infestation 1	20.09 ±2.80	-	19.69 ±3.63	-	19.08 ±0.51	-
Infestation 2	19.87 ±1.82	17.14 ±0.26	18.79 ±0.22	17.45 ±0.62	18.94 ±0.48	17.84 ±3.43
Infestation 3	17.04 ±1.67	17.86 ±0.50	18.21 ±0.81	17.47 ±0.77	15.90 ±0.37	18.04 ±1.31
Infestation 4	17.63 ±0.95	17.56 ±0.37	17.77 ±2.43	17.29 ±0.14	18.31 ±3.02	18.41 ±3.80
Infestation 5	18.05 ±0.47	18.53 ±0.63	16.36 ±0.43	18.01 ±0.31	15.60 ±0.69	17.92 ±1.17

TABLE 13

PERCENT REPLETE *R. E. EVERTSI* NYMPHS RECOVERED FROM RABBITS SEQUENTIALLY SENSITIZED WITH DIFFERENT NUMBERS OF INSTARS

Rabbit groups	A	Control	B	Control	C	Control
Treatments	100 Larvae		500 Larvae		1500 Larvae	
Infestation 1	89.00	-	74.27	-	45.00	-
Infestation 2	33.30	92.00	33.33	71.10	12.00	50.00
Infestation 3	49.00	82.50	29.33	69.70	22.02	41.40
Infestation 4	50.00	74.00	26.67	68.20	30.00	36.83
Infestation 5	28.00	91.00	31.00	68.80	17.30	44.00

nymphal yields among the three treatment groups were statistically similar. However, they were significantly different from their corresponding controls; being $P < 0.05$, $P < 0.025$, $P < 0.05$ for challenge with 100, 500 and 1500 larvae respectively.

Mean engorged weights of R. e. evertsi nymphs replete on rabbits reinfested with larvae

The mean engorged weights of nymphs after primary infestation of rabbits with 100 larvae was 14.8 mg (Table 14). A significant concomitant engorged weight reduction was not observed in the subsequent infestations. Nymphs at the first and fifth infestations had approximately similar engorged weights (14.8 and 14.78 mg).

Replete nymphs from hosts initially challenged with 500 and 1500 larvae respectively had mean weights comparable to that reported above. The mean weights at the end of second infestation were 11.43 and 10.84 mg respectively for the 500 and 1500 larvae challenge hosts. The weight of nymphs from the remaining three infestations did not differ significantly from these values.

The group means of nymphal weights for the 100, 500 and 1500 larvae infested hosts were 14.14, 12.18 and 11.91 mg respectively (Table 15); and did not differ significantly among each other. The similar means for the corresponding controls were 15.04, 14.02 and 14.42 mg. They differed significantly from the respective treatments, being $P < 0.1$, $P < 0.025$ and $P < 0.01$ for the 100, 500 and 1500 larvae challenge regimens respectively.

TABLE 14

MEAN ENGORGEMENT WEIGHTS OF NYMPHS FED ON RABBITS WHICH WERE REPEATEDLY EXPOSED TO DIFFERENT NUMBERS OF *R. E. EVERTSI* LARVAE (mg ± s.d.)

Rabbit groups	A	Control	B	Control	C	Control
Treatments	100 Larvae		500 Larvae		1500 Larvae	
Infestation 1	14.80 ± 3.98 (266)	-	14.00 ± 3.23 (1114)	-	13.63 ± 3.91 (2020)	-
Infestation 2	13.43 ± 4.10 (100)	14.93 ± 3.27 (184)	11.43 ± 3.69 (500)	14.01 ± 4.07 (711)	10.84 ± 3.88 (530)	13.52 ± 3.62 (1503)
Infestation 3	13.27 ± 3.82 (147)	14.87 ± 3.79 (165)	11.81 ± 3.52 (440)	13.99 ± 3.21 (697)	11.60 ± 3.61 (991)	14.90 ± 3.55 (1242)
Infestation 4	14.46 ± 3.73 (149)	15.43 ± 3.66 (147)	12.35 ± 3.98 (400)	14.08 ± 3.17 (682)	11.11 ± 3.61 (1386)	15.18 ± 3.65 (1105)
Infestation 5	14.74 ± 3.35 (83)	14.91 ± 3.82 (150)	11.29 ± 3.24 (462)	14.00 ± 3.15 (688)	12.36 ± 3.92 (888)	14.06 ± 3.81 (1312)
Group mean ± s.d	14.14 ± 0.73	15.04 ± 0.26	12.18 ± 1.10	14.02 ± 0.04	11.91 ± 1.12	14.42 ± 0.76

Number of nymphs examined for each group is shown in parenthesis

TABLE 15

COMPARISON OF GROUP MEAN ENGORGED WEIGHTS OF NYMPHS FED ON RABBITS CHALLENGED WITH DIFFERENT LOADS OF R. E. EVERTSI LARVAE

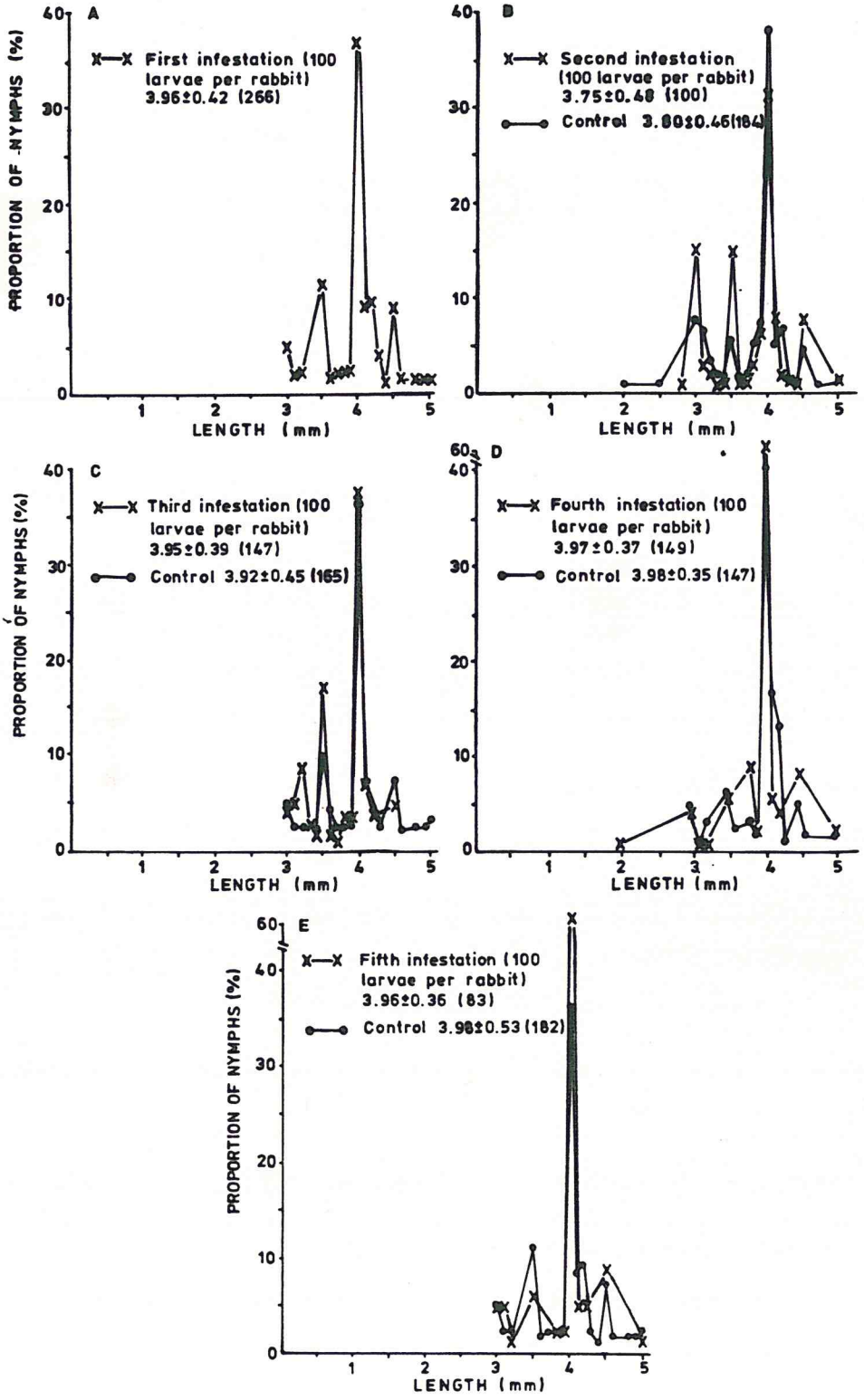
Tick challenge load	Group mean for Treatment	Control	$F_{1,7}$	p
100	14.14	15.04	4.99	0.1
500	12.18	14.02	10.92	0.025
1500	11.91	14.42	14.21	0.01

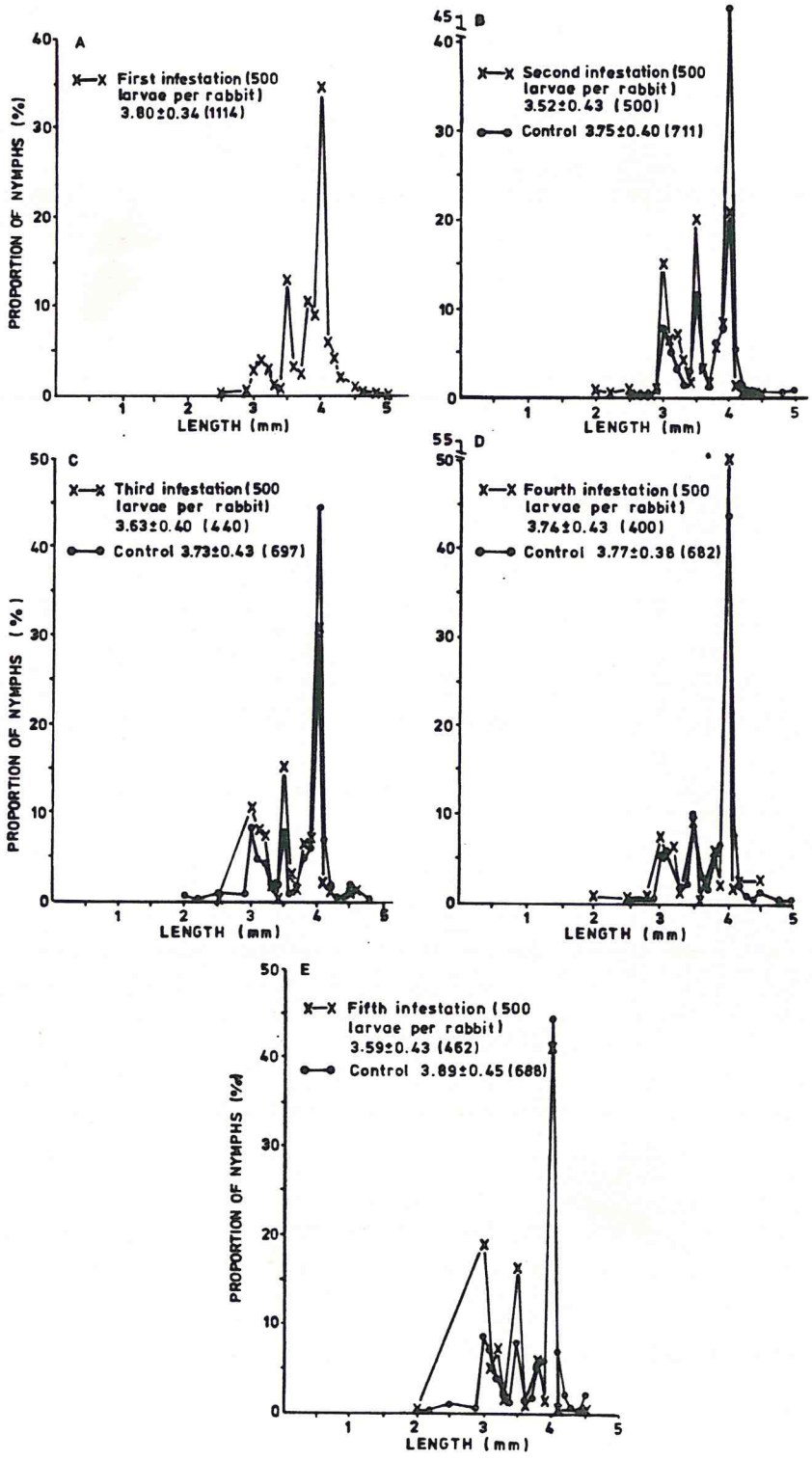
Frequency distribution and mean body length of nymphs fed on host sensitized with different numbers of R.e. evertsi larvae.

The frequency distribution of the body length of nymphs engorged on rabbits exposed to the different treatments with larvae are shown in Figures 9, 10 and 11. Most nymphs, maturing on hosts in the three treatments, after primary infestation, had lengths between 3.5 and 4 mm. During subsequent challenge, small sized nymphs appeared. Irrespective of the challenge frequency, a high proportion of nymphs of length 4 mm were observed. The proportion of the 4 mm nymphs maturing on the host seemed to decline as the frequency of tick challenge was advanced. Nymphs which matured on previously exposed hosts had wrinkled dorsal integument and were slightly smaller than those from naive hosts. A few of these nymphs were abnormally coloured (red, blue, mottled or even white).

Nymphal moulting periods

Replete nymphs from the three rabbit groups during initial challenge moulted in about 15 days after dropping (Table 16). The range of moulting periods increased slightly with increased frequency of tick challenge. Nymphs moulted as early as day 7 of detachment from hosts challenged with 500 larvae at 3rd infestation (Table 16). Nymphs weighing less than 5 mg had shorter ($P < 0.05$) moulting periods than those above 10 mg (Table 17).


A
First infestation (100 larvae per rabbit) 3.96±0.42 (266)
B
Second infestation (100 larvae per rabbit) 3.75±0.48 (100)
Control 3.80±0.46(184)
C
Third infestation (100 larvae per rabbit) 3.95±0.39 (147)
Control 3.92±0.45 (165)
D
Fourth infestation (100 larvae per rabbit) 3.97±0.37 (149)
Control 3.98±0.35 (147)
E
Fifth infestation (100 larvae per rabbit) 3.96±0.36 (83)
Control 3.98±0.53 (182)
PROPORTION OF NYMPHS (%)
LENGTH (mm)


A
First infestation (500 larvae per rabbit) 3.80±0.34 (1114)
PROPORTION OF NYMPHS (%)
LENGTH (mm)
B
Second infestation (500 larvae per rabbit) 3.52±0.43 (500)
Control 3.75±0.40 (711)
LENGTH (mm)
C
Third infestation (500 larvae per rabbit) 3.63±0.40 (440)
Control 3.73±0.43 (697)
PROPORTION OF NYMPHS (%)
LENGTH (mm)
D
Fourth infestation (500 larvae per rabbit) 3.74±0.43 (400)
Control 3.77±0.38 (682)
LENGTH (mm)
E
Fifth infestation (500 larvae per rabbit) 3.59±0.43 (462)
Control 3.89±0.45 (688)
PROPORTION OF NYMPHS (%)
LENGTH (mm)

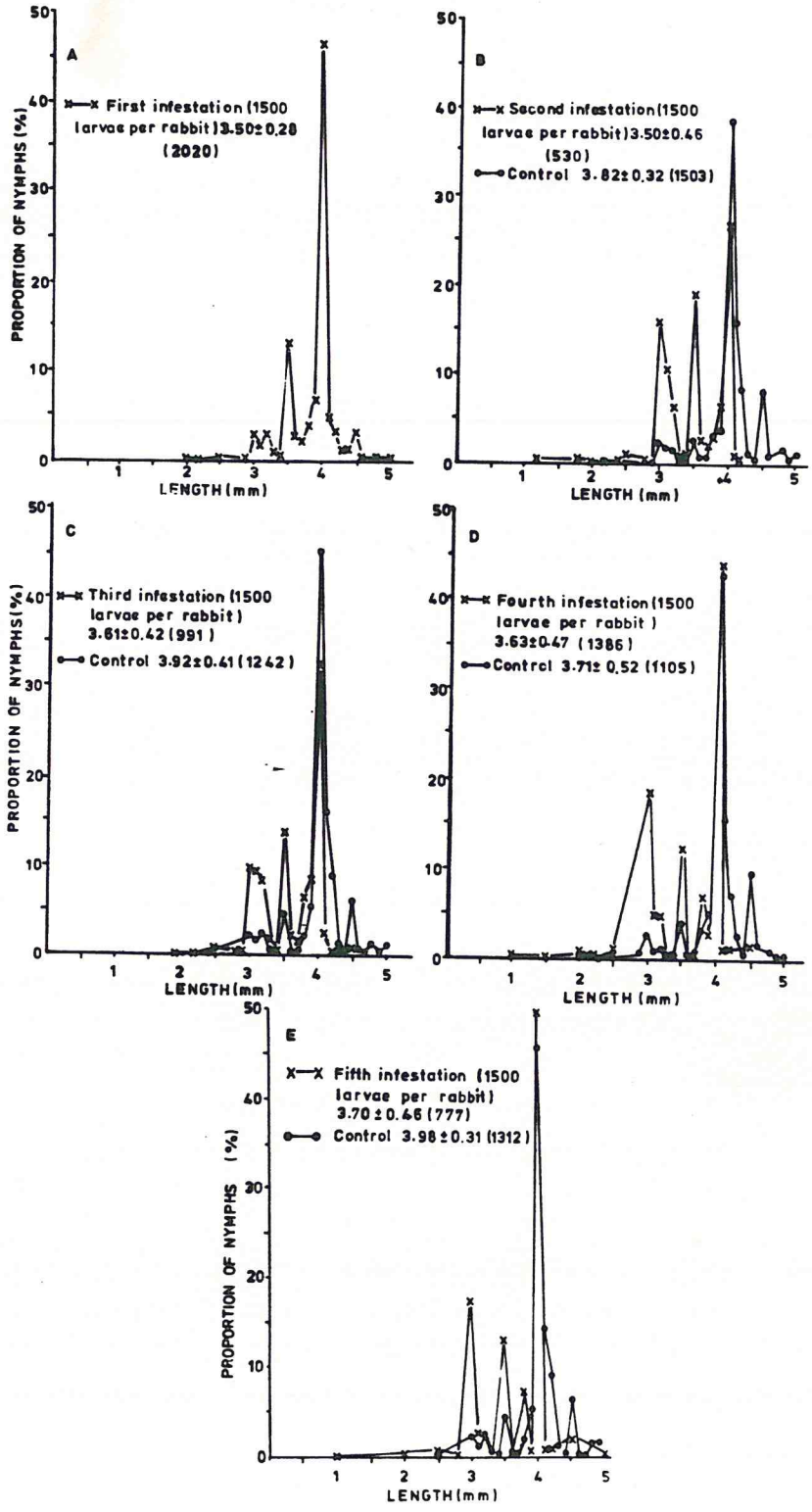

A
First infestation (1500 larvae per rabbit) 3.50±0.28 (2020)
B
Second infestation (1500 larvae per rabbit) 3.50±0.46 (530)
Control 3.82±0.32 (1503)
C
Third infestation (1500 larvae per rabbit) 3.61±0.42 (991)
Control 3.92±0.41 (1242)
D
Fourth infestation (1500 larvae per rabbit) 3.63±0.47 (1386)
Control 3.71±0.52 (1105)
E
Fifth infestation (1500 larvae per rabbit) 3.70±0.46 (777)
Control 3.98±0.31 (1312)
PROPORTION OF NYMPHS (%)
LENGTH (mm)

TABLE 16

MEAN MOULTING PERIODS OF *R. E. EVERTSI* NYMPHS FROM LARVAE REPEATEDLY FED ON THE SAME RABBIT (DAYS ± S.D.)

Rabbit groups	A	Control	B	Control	C	Control
Treatments	100 Larvae		500 Larvae		1500 Larvae	
Infestation 1	15.53 ± 0.54 (14 - 20)*	-	15.05 ± 0.95 (12 - 19)	-	14.92 ± 0.55 (13 - 18)	-
Infestation 2	16.30 ± 0.96 (14 - 18)	15.13 ± 0.27 (13 - 18)	15.91 ± 0.39 (14 - 18)	15.48 ± 0.37 (13 - 18)	15.91 ± 0.56 (14 - 18)	15.05 ± 0.45 (13 - 19)
Infestation 3	15.80 ± 0.85 (14 - 18)	14.86 ± 0.81 (13 - 17)	15.02 ± 0.58 (7 - 18)	15.66 ± 0.36 (13 - 17)	15.14 ± 0.64 (11 - 18)	15.71 ± 0.70 13 - 18)
Infestation 4	14.80 ± 0.93 (13 - 17)	15.47 ± 0.66 (13 - 18)	14.80 ± 0.56 (11 - 18)	15.82 ± 0.27 (13 - 17)	14.57 ± 1.03 (10 - 17)	15.14 ± 0.53 (13 - 19)
Infestation 5	14.75 ± 0.53 (13 - 17)	14.92 ± 0.61 (14 - 18)	14.06 ± 1.09 (12 - 18)	15.14 ± 0.29 (14 - 19)	14.77 ± 1.24 (10 - 19)	15.23 ± 0.72 (14 - 18)
Group mean ± s.d.	15.44 ± 0.66	15.10 ± 0.28	14.99 ± 0.66	15.53 ± 0.29	15.06 ± 0.52	15.28 ± 0.29

* Moulting period range

TABLE 17

MEAN MOULTING PERIODS OF DIFFERENT WEIGHT CATEGORIES OF *R. E. EVERTSI* NYMPHS REPEATEDLY FED ON THE SAME RABBITS (DAYS)

Weight category (mg)	0 - 4.9	5 - 9.9	10 - 14.9	15 - 19.9	20 - 24.9
Infestation 1	14.10 (16)	14.62 (268)	15.03 (538)	15.67 (762)	15.84 (26)
Infestation 2	15.27 (68)	15.76 (567)	16.33 (511)	16.56 (376)	17.00 (13)
Infestation 3	14.39 (48)	14.62 (512)	15.25 (1035)	15.70 (560)	15.77 (13)
Infestation 4	13.69 (58)	14.56 (205)	14.94 (367)	15.44 (311)	15.17 (10)
Infestation 5	12.72 (26)	14.20 (295)	14.79 (415)	15.11 (237)	15.90 (7)
Group mean ± s.d.	14.03* ±0.94	14.75 ±0.59	15.27 ±0.62	15.70 ±0.54	15.94 ±0.66

* Significantly different from those above 10 mg of weight ($P<0.05$)

Numbers of nymphs in each weight category are shown in parentheses

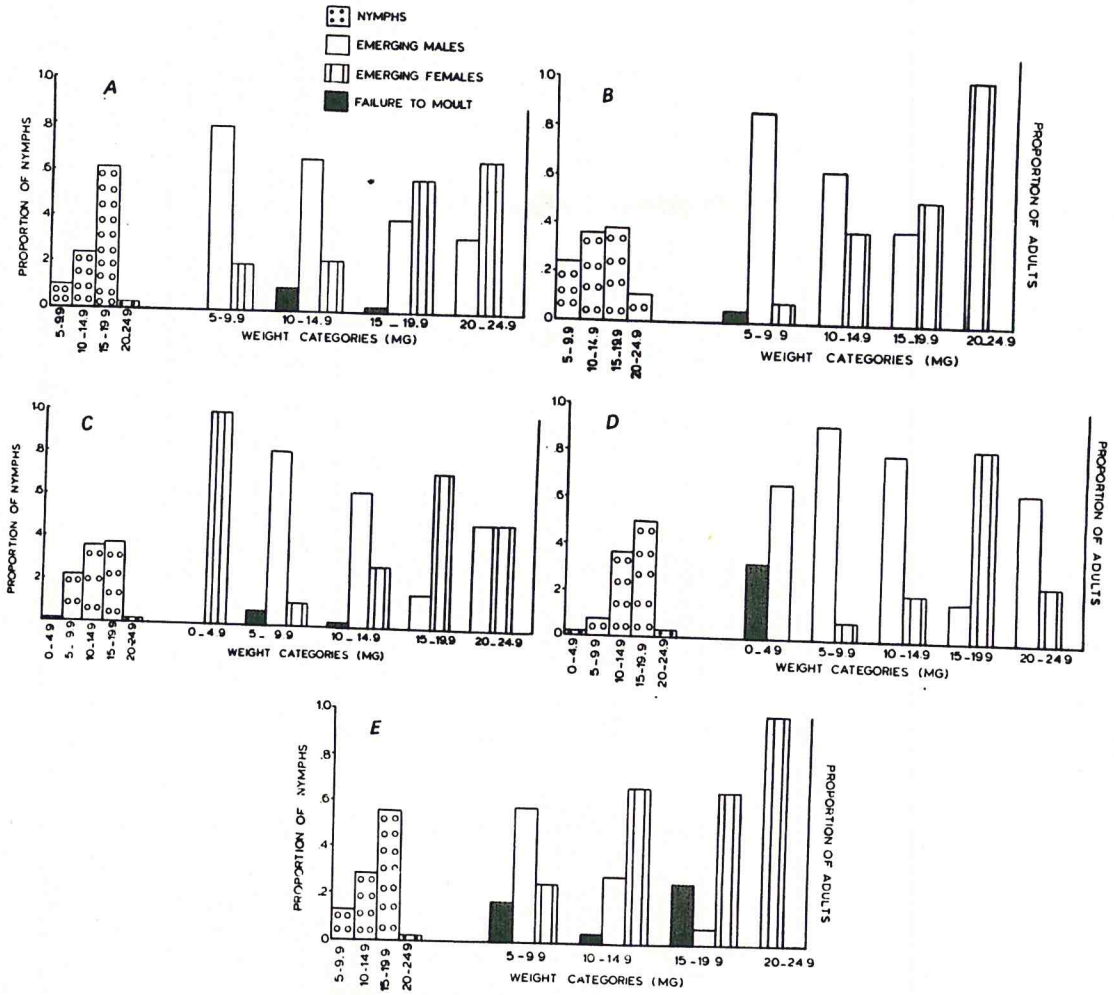
Weight categories and moulting performance of nymphs fed on hosts sensitized with different numbers of R. e. evertsi larvae

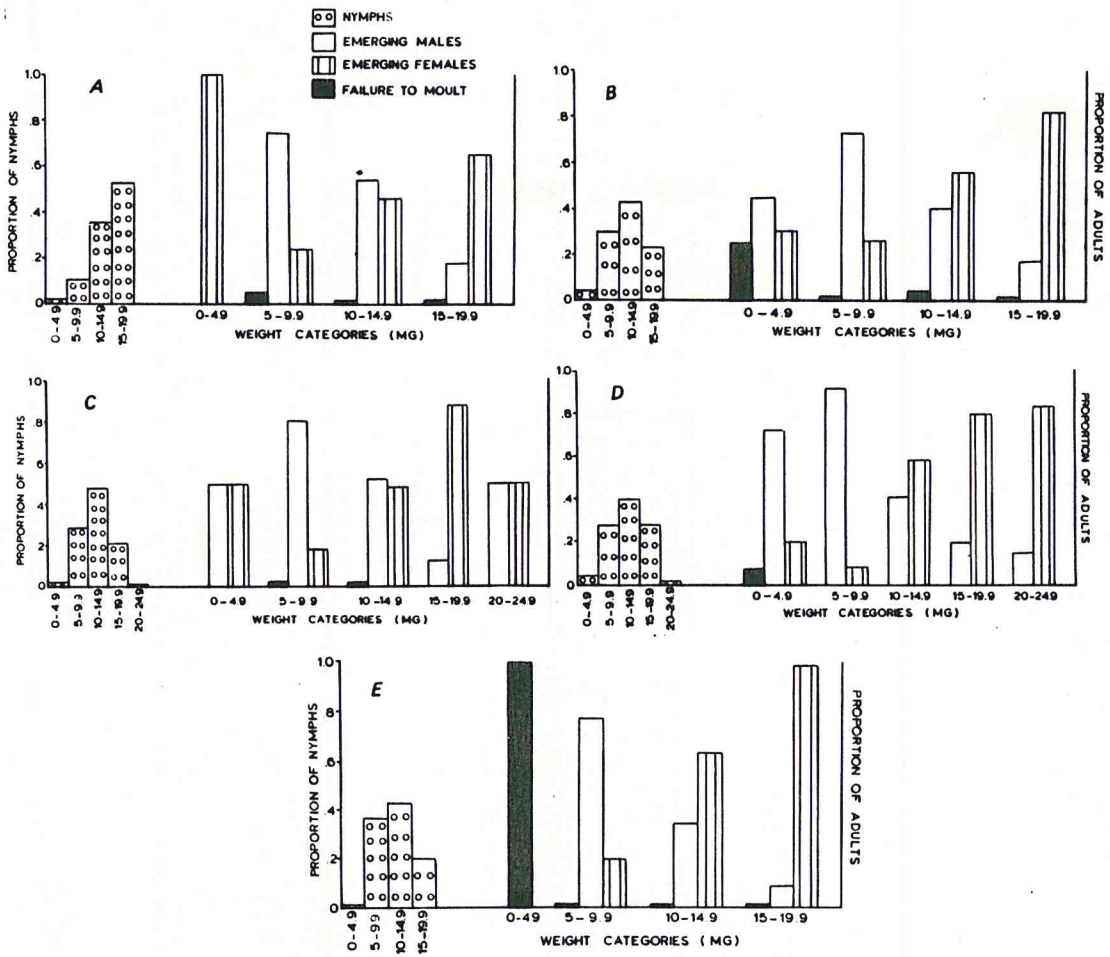
Nymphs which engorged on hosts infested with different numbers of larvae were allocated on weight basis to five categories; 0 - 4.9, 5 - 9.9, 10 - 14.9, 15 - 19.9 and 20 - 24.9 mg (Figs. 12, 13 and 14). Three of these categories; 5 - 9.9, 10 - 14.9 and 15 - 19.9 appeared in each infestation, irrespective of the tick challenge regimen used and contained most of the replete nymphs. Nymphs of weights less than 5 mg appeared occasionally, after primary infestation of hosts with 100 larvae, and regularly in the 500 and 1500 larvae challenge groups (Figs. 13 and 14). Nymphs which weighed more than 20 mg occurred in all infestations with 100 larvae. In the remaining two treatment groups, they appeared irregularly, particularly after the initial challenges (Figs. 10 C, D and 11 B, C, E). This weight category, like 0 - 4.9 mg contained lower proportion of ticks than the other three weight categories.

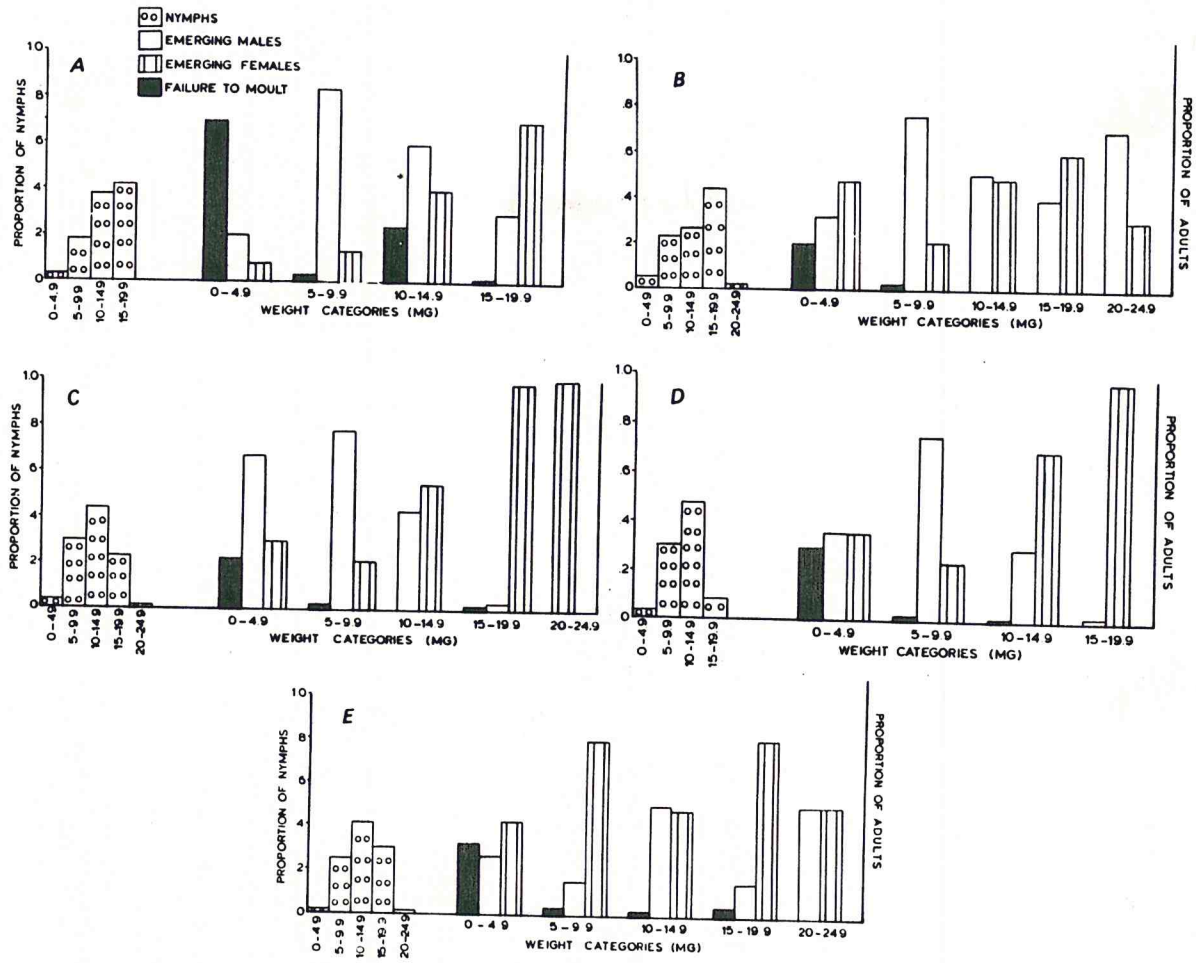
Nymphal moult failures were observed for all the weight categories except 20 - 24.9 mg. These failures were generally less than 20% for nymphs of weight above 5 mg (Figs. 12, 13 and 14). They were heavy, occasionally approaching 100%, for nymphs of weight below 5 mg (Figs 13 E and 14 A). In successful moulting, the trend was that with heavy nymphs there were more females at moulting than males while with light nymphs more males than females were observed.

Precipitating antibodies to larval antigen

Majority of rabbits infested with larvae, did not develop


NYMPHS
EMERGING MALES
EMERGING FEMALES
FAILURE TO MOULT
A
B
C
D
E
PROPORTION OF NYMPHS
PROPORTION OF ADULTS
WEIGHT CATEGORIES (MG)
5-9.9
10-14.9
15-19.9
20-24.9
0-4.9
5-9.9
10-14.9
15-19.9
20-24.9


NYMPHS
EMERGING MALES
EMERGING FEMALES
FAILURE TO MOULT
A
B
C
D
E
PROPORTION OF NYMPHS
PROPORTION OF ADULTS
WEIGHT CATEGORIES (MG)
0-4.9
5-9.9
10-14.9
15-19.9
20-24.9


NYMPHS
EMERGING MALES
EMERGING FEMALES
FAILURE TO MOULT
A
B
C
D
E
PROPORTION OF NYMPHS
PROPORTION OF ADULTS
WEIGHT CATEGORIES (MG)
0-4.9
5-9.9
10-14.9
15-19.9
20-24.9

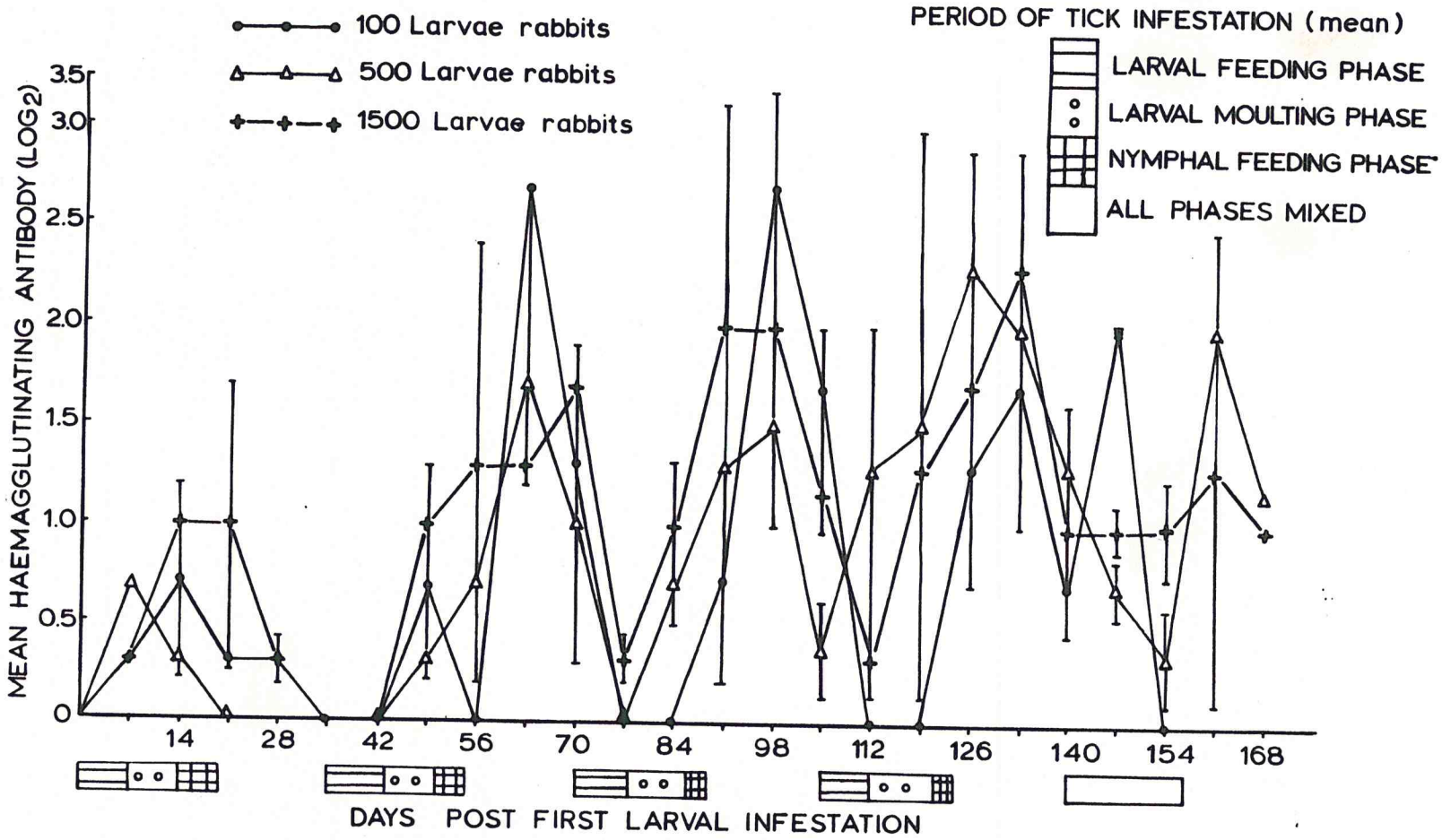
detectable precipitating antibodies throughout the study period. Low antibody titers were detected in two of the rabbits challenged with 1500 larvae by day 77 after primary challenge. During the same period, one of the three rabbits challenged with 500 larvae was positive. The highest titer attained by the three rabbits was $\log_2$, after the third challenge.

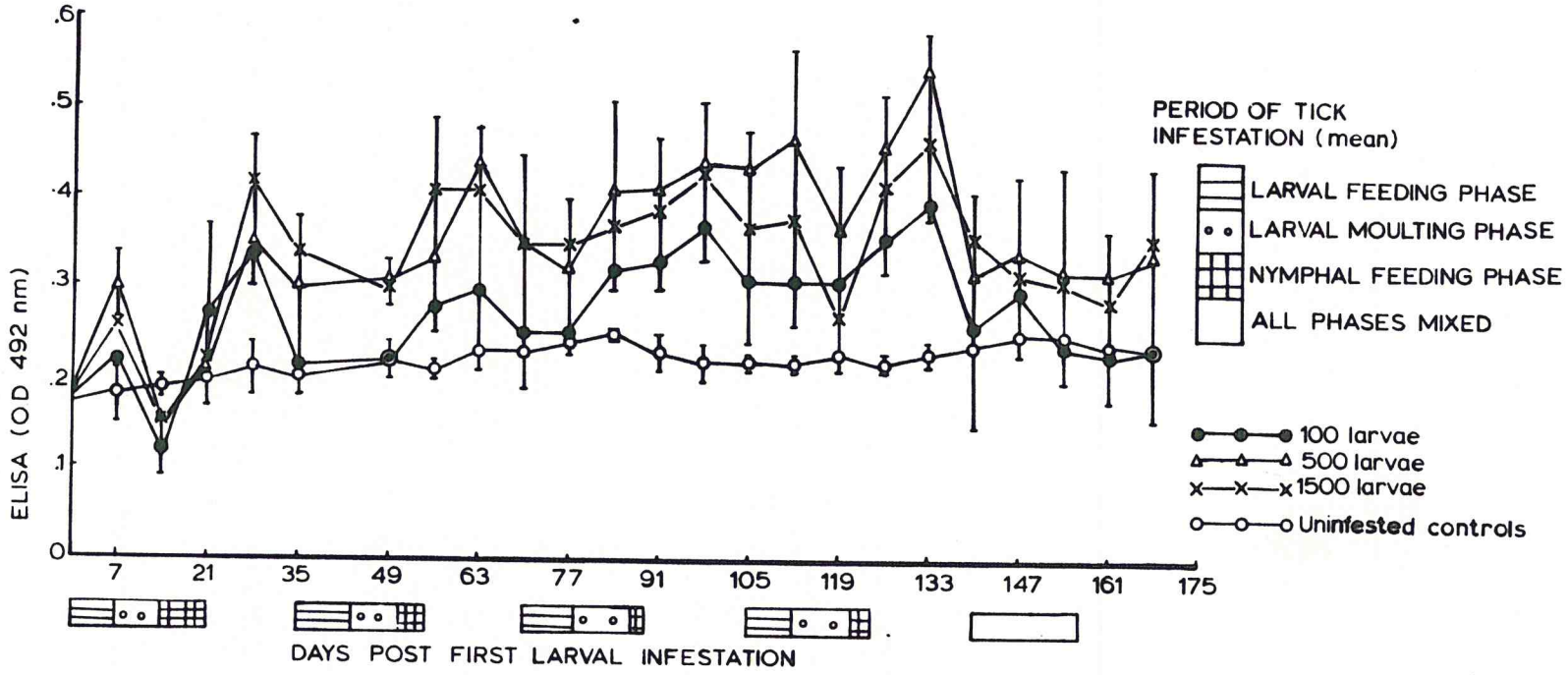
Agglutinating antibodies to larval antigens

Low agglutinating antibody activity was observed by day 7 after primary challenge in the three treatment groups (Fig. 15), and disappeared at the end of the infestation for hosts infested with 100 and 500 larvae. In hosts challenged with 1500 larvae, they were detected for as long as 14 days after completion of the primary infestation. Antibodies reappeared in all rabbits, 7 days after secondary challenge (Fig. 15, day 42). A decline occurred at the larval-nymphal transition period for the 100 larvae challenge group. The general trend in the infestations was that a decline and rise in antibodies coincided with the feeding of larvae and nymphs respectively. The observed titers did not exceed ($\log_2$) 3 and showed a tendency to decline in the absence of tick feeding.

Humoral antibody response to larval antigens as detected by the ELISA test

Antibody activity to antigens inoculated by ticks in the process of feeding in rabbits initially challenged with different numbers of R. e. evertsi larvae was detected at day 7 of tick feeding (Fig. 16). It was a of lower magnitude than reported for adults (Chapter 1). With advanced frequency of tick infestations,


100 Larvae rabbits
500 Larvae rabbits
1500 Larvae rabbits
PERIOD OF TICK INFESTATION (mean)
LARVAL FEEDING PHASE
LARVAL MOULTING PHASE
NYMPHAL FEEDING PHASE
ALL PHASES MIXED
MEAN HAEMAGGLUTINATING ANTIBODY (LOG2)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
14
28
42
56
70
84
98
112
126
140
154
168
DAYS POST FIRST LARVAL INFESTATION


.6
.5
.4
.3
.2
.1
0
ELISA (OD 492 nm)
7
21
35
49
63
77
91
105
119
133
147
161
175
DAYS POST FIRST LARVAL INFESTATION
PERIOD OF TICK INFESTATION (mean)
LARVAL FEEDING PHASE
LARVAL MOULTING PHASE
NYMPHAL FEEDING PHASE
ALL PHASES MIXED
100 larvae
500 larvae
1500 larvae
Uninfested controls

progressively higher ELISA values were observed up to the fourth challenge. The fifth infestations were manifested by a drop in the ELISA values to levels below those of previous challenges. For the 100 larval challenge hosts, the drop approached those of uninfested control rabbits. Significant antibody activity coincided with the feeding of nymphs. Sensitization regimen employing 100 larvae resulted in immune response, inferior to that stimulated by either 500 or 1500 larvae from day 35.

DISCUSSION

The response of two groups of rabbits to repeated challenges with 100 and 500 larvae showed that about 70% of the larvae matured as nymphs initially (Table 13) and about 45% were observed in rabbits similarly challenged with 1500 larvae. During subsequent infestations, in rabbit groups challenged with 500 and 1500 larvae less than 30% of the applied larvae matured as nymphs. On the other hand, on rabbits sensitized with 100 larvae, between 30 - 50% of applied larvae attained nymphal stage. Resistance to ticks has been defined as the ability of a host to limit the number of ticks which mature on it (Wharton et al., 1973). Animals of high resistance, therefore have a low percentage of parasitic ticks surviving on them. These data suggest that resistance to the tick instars was acquired by the rabbits. It was expressed better by hosts challenged with high rather than low numbers of the instars.

About 50% nymphs matured on hosts from the initial challenge of 1500 larvae. A percent reduction of such magnitude was not

observed with either 100 or 500 larvae challenges. Considering the cycle of R. e. evertsi to be 15 - 20 days (Table 12), resistance commenced during primary feeding. Wagland (1979), in a similar study with B. microplus, did not observe resistance at this early time, despite the cycle of 19 - 21 days. It is speculated that host resistance was larval dose dependent and through density dependent mortality factors, caused the varying loss of ticks by the three treatment groups. However, the numbers of each instar lost could not be estimated for a tick with such a life cycle.

Significant mortalities and detachments of larvae and pharate nymphs were observed during the first 24 - 48 hours after attachment in secondary and subsequent infestations, a phenomenon already reported in other tick species (Bennett, 1975; Roberts, 1968bc). Instars surviving this period, continued their life cycle with minimal interference from host resistance. However a few deaths and moult failures of partially and completely engorged nymphs were observed after successful detachment, a feature similarly noted with R. appendiculatus (Branagan, 1974) and B. microplus (Tatchell, 1969a). The significant parasite loss observed may be due to an immunologically antibody mediated response. Antibodies detected in the hosts by the ELISA test (Fig. 16) may have reacted with tick antigens inoculated while feeding, on surface of certain leucocytes, and caused histamine release. This product has been associated with mortality and premature detachment of larvae in resistant hosts (Kemp, 1978; Willadsen et al., 1979). Although several factors,

including histamine, influence hypersensitivity reactions (Willadsen, 1979), the amount of mast cell bound specific antibodies seems to play a major role (Willadsen et al., 1978). Thus, more instars lost by host challenged with 500 and 1500 larvae than with 100 larvae may be attributed to the corresponding superior immune response.

Replete nymphs detached from repeatedly infested rabbits were alloted into five weight categories, 0 - 4.9, 5 - 9.9, 10 - 14.9, 15 - 19.9 and 20 - 24.9 mg. A comparison of the results in Tables 13 and 14 indicated that a reduction in numbers of larvae which fed normally on the hosts, was not accompanied by a concomitant reduction in engorged weights, a pattern also reported with Dermacentor variabilis larvae (Den-Hollander and Allen, 1985). They suggested acquired resistance to be expressed in a two fold manner; firstly in total prevention of larval feeding and secondly interfere with feeding of those larvae already established. While the same phenomenon was observed in this study, it was reported further that light nymphs were frequently encountered as tick exposures were increased. Besides, nymphs in weight categories 5 - 9.9, 10 - 14.9 and 15 - 19.9 mg were constantly yielded by the hosts. It would appear that a significant reduction in larval engorged weights would upset the sex ratio to the detriment of the species. It seems advantageous for the parasite to maintain good engorged weights on resistant hosts.

In this study the feeding and moulting periods (Table 12, 16) of the instars on test and control hosts were also not significantly

different ($P > 0.05$). However, nymphs of weight less than 5 mg (Table 17) had shorter moulting periods ($P < 0.05$) than those above 10 mg and also higher rate of moult failure (Figs. 9D, 10E and 11A,E). Moult failures were rare among the heavy nymphs irrespective of the challenge frequency. Predominantly more males than females moulted from weight category 5 - 9.9 and the reverse was true for 15 - 19.9 mg category. These data suggest that, where the instars had established, host resistance did not significantly influence the feeding and moulting performance. In addition, the number of instars, successfully feeding to repletion on hosts infested repeatedly, is a better estimate of resistance than the other parameters described above.

Moult failures of underweight nymphs reported above, may have been due to poor feeding performance and inadequate synthesis of the moulting hormone, ecdysone (Solomon et al., 1982). Abnormally coloured nymphs like those described by Trager (1939b), Bagnall and Doube (1975) and Brown and Knapp (1981) were observed and died before moulting. Other category of nymphs which failed to moult had variegated, cuticles. Brown and Knapp (1981) suggested that ticks feeding on resistant hosts may have impaired cuticle formation and therefore unable to moult.

Nymphs fed repeatedly on the same rabbits were slightly shorter than those fed on naive hosts (Figs. 9, 10 and 11) and majority had wrinkled dorsal integuments. Brown and Knapp (1981) observed the same and attributed it to changed quality of blood meal

in resistant hosts. Balashov (1972) studied body growth of some ixodid ticks during feeding and reported body lengths to increase with weight gains, suggesting that fed nymphs are longer than unfed ones. Wagland et al. (1979) reported reduced growth rates of nymphs fed on resistant compared to naive hosts. The variation in size observed in the nymphs may be due to imperfect feeding on the resistant hosts.

Transient wrinkled integument, associated with reduced body size, has been attributed to water loss by ticks during preparatory stage of feeding (Balashov, 1972). The presence of wrinkled dorsal cuticle in most R. e. evertsi nymphs maturing on the resistant rabbits suggests that water loss is more pronounced during feeding on resistant than naive hosts.

Hoogstraal (1978) suggested that ticks with one host cycle and possibly two, are saved from many vicissitudes. However, these life cycles may be mutually disadvantageous. Brown et al. (1984) showed that resistance stimulated by one stage in the tick life cycle, may affect the other homologous stages. Consistent with this finding, some partially replete larvae were observed to detach from the hosts, after secondary challenge. These larvae moulted off the hosts, when incubated as described above. Such detachment, interrupting the life cycle, is possibly linked to host resistance. Mason and Norval (1981) reported that such ticks complete the interrupted feeding on other hosts with the result that they

potentiate spread of tick borne diseases. It is suggested that some R. e. evertsi life stages exhibit a 3-host cycle on resistant hosts, possibly to avoid the deleterious effects of resistance mechanisms. Nuttall (1913) reported interrupted feeding of R. appendiculatus as a major cause of the great variation in size of the genus. The same may apply to the field population of R. e. evertsi and attributed partially to host resistance.

In the sera collected at regular intervals, a rise and fall in antibody response in repeatedly tick infested rabbits was detected, suggesting the response to be systemic (Figs. 15 and 16). Antibody titers were high, approximately constant between second and fourth infestations and decreased significantly thereafter. During this period, a constant number of nymphs matured on each treatment group (Table 13). These proportions of ticks may be that described as antigenically compatible with the hosts by Roberts (1968a), and established by immunological rejection of excess loads (Dineen, 1963; Sprent, 1962; Seifert, 1971; Windon et al., 1984). The loss of titers during the fifth challenge may indicate either that the established parasite load was unable to improve on the established resistance or that the challenge regimens induced immunosuppresion or tolerance.

SUMMARY

Resistance to R. e. evertsi intars was acquired by rabbits after a single exposure to different numbers of 100, 500 and 1500 larvae. Resistance was manifested mainly by significant losses of instars, host systemic antibody response and 3-host cycle behaviour by the tick instars. It was higher, in terms of parasite loss, for hosts exposed to 1500 larvae than those exposed to the other 2 tick loads. Engorged weights of nymphs were not significantly altered in the course of infestations. Five nymphal engorged weight categories existed: 0 - 4.9, 5 - 9.9, 10 - 14.9, 15 - 19.9 and 20 - 24.9 mg. Nymphs of weights in 5 - 9.9, 10 - 14.9 and 15 - 19.9 were always observed while those in 0 - 4.9 mg appeared as the frequency of parasite challenge was increased. Nymphs which matured on resistant hosts were smaller than those from naive controls. Moulting efficiency was not significantly affected by resistance except for nymphs of weight 0 - 4.9 mg. The moulting trend was that light and heavy nymphs moulted into males and females respectively, and confirms results of Rechav et al. (1977) on the same tick species. It is suggested that where instars have established, host resistance does not significantly influence their feeding and subsequent performance.

CHAPTER 3

INDUCTION OF PARALYSIS IN RABBITS INFESTED WITH ADULT RHIPICEPHALUS EVERTSI EVERTSI

Introduction

Ticks are known mainly because of the diseases (protozoan, viral, bacterial and rickettsial) they transmit to man and domestic animals (Balashov, 1972). Many ticks can transmit more than one disease either vertically or horizontally. However, certain diseases can be propagated only or chiefly by specific ticks, for example East Coast fever which is transmitted primarily by R. appendiculatus. Conditions such as toxicoses are caused by a few tick species (Stone and Wright, 1981). Paralysis, one of the most important manifestation of these toxicoses, is an ascending condition which develops during the active phase of tick engorgement (Gothe, 1981; Stone et al., 1983). The paralysis is caused by a salivary gland toxin secreted into the host (Binnington and Stone, 1981) and the condition has been reported in cattle, sheep, bison, man and the fowl (Hadwen, 1913; Jellison et al., 1951; Doube and Kemp, 1975; Gothe and Verhalen, 1975 and Gothe et al., 1979) .

Toxins have been shown to be present in whole tick body extracts (Murnagham, 1958), salivary glands (Howell et al., 1975; Wright et al., 1983) and eggs of ixodid ticks (Riek, 1957; Neitz et al., 1981). Gregson (1973) established that the toxin is exhausted with increasing age.

Stone and Wright (1981) reviewed important ticks which cause

paralysis and cited Ixodes rubicundus, R. e. evertsi and Argas arboreas as relevant in Africa. Exhaustive work on the Australian paralysis tick, I. holocyclus has provided evidence of the magnitude of the problem and remedial measures were suggested (Doube et al., 1977; Stone et al., 1982; Stone et al., 1983; Wright et al., 1983). Information on paralysis caused by R. e. evertsi is scanty (Gothe, 1981).

Unlike most other arthropod-associated diseases, epidemics of tick paralysis, do not depend on the presence of large populations of ticks. It was shown that a single tick can cause paralysis in a naive dog while 10-20 ticks produced the same effect in 80-160 kg calves (Doube and Kemp, 1975; Doube et al., 1977; Stone and Wright, 1981). In studies of paralysis in sheep, Gothe (1981) concluded that only R. e. evertsi attaining initial weights of between 15 and 20 mg caused this syndrome. It was therefore desired to investigate the ability of R. e. evertsi of different weights to induce paralaysis in rabbits.

MATERIALS AND METHODS

Six naive outbred - New Zealand White rabbits about 6-8 weeks old were infested with 1000 R. e. evertsi larvae on one ear. The larvae were applied according to the method of Bailey (1960). Detached replete nymphs collected were weighed individually and grouped into 5 weight categories of 0 - 4.9, 5 - 9.9, 10 - 14.9, 15 - 19.9 and 20 - 24.9 mg. The nymphs in each weight category were incubated at 28^{o}C, 85% relative humidity and allowed to moult. The

adults which emerged were counted (50:50 sex ratio) and applied onto an ear of each of the 32 naive rabbits as shown in Table 18. A similar second challenge was made on the same rabbits two months later. Parameters studied were the feeding period, engorgement weights and the ability of the ticks to induce noticeable signs of paralysis in the rabbits. Paralysed rabbits that died were necropsied and postmortem diagnosis established.

RESULTS

The mean feeding period of the ticks was about 8 days (range 6.15 - 9.14 days)(Table 19). However, the heavier ticks fed slightly longer than the light ones. Adult ticks emerging from nymphs weighing between 5 and 24.9 mg induced paralysis when fed on naive rabbits (Table 19). No paralysis occurred in similar rabbits infested with adult ticks which moulted from nymphs weighing less than 5 mg. The number required to induce paralysis varied between light and heavy ticks. A minimum of 60 ticks in nymphal weight categories 5 - 9.9 and 10 - 14.9 mg was required to induce paralysis. For weight categories 15 - 19.9 and 20 - 24.9 mg, 20 adult ticks were enough to induce paralysis. Lighter ticks (5 - 9.9 mg nymphal weight) caused paralysis in one rabbit, without death. Paralysis caused by heavier ticks (moulted from nymphs of weight above 15 mg) was higher than that observed with ticks from lighter nymphs. Of the 32 rabbits used, 17 (53.13%) became paralyzed and 12 (71%) died (Table 20). It was observed that paralysis in rabbits occurred when ticks were actively sucking blood during mating.

TABLE 18

ADULT *R. E. EVERTSI* MOULTED FROM NYMPHS OF KNOWN WEIGHTS AND NUMBERS USED TO INFEST DIFFERENT GROUPS OF RABBITS

Group category	Number of rabbits used	Nymphal tick weight category (mg)	Number of adult ticks used
1	3	0 - 4.9	150
2	3	5 - 9.9	60
3 (a)	3	10 - 14.9	20
(b)	3	10 - 14.9	60
4 (a)	3	15 - 19.9	20
(b)	3	15 - 19.9	30
(c)	6	15 - 19.9	50
(d)	5	15 - 19.9	100
5	3	20 - 24.9	20

TABLE 19

INCIDENCE OF PARALYSIS INDUCED BY DIFFERENT WEIGHTS CATEGORIES OF R. E. EVERTSI FED ON NAIVE RABBITS

Rabbit group and No. of animals used (in brackets)	Nymphal weight category of ticks (mg)	No. of ticks used per rabbit	Adult mean feeding period (days)	Adult mean engorgement wt.(mg ± s.d.)	Time to onset of paralysis (days)	Paralysis incidence	
						No. of animals affected	No. of animals which died
1 (3)	0 - 4.9	150	6.15 ± 0.38	190.22 ± 104.25 (43)*	-	-	-
2 (3)	5 - 9.9	60	9.00 ± 2.12	560.69 ± 79.27 (75)	6	1	-
3 a(3)	10 - 14.9	20	7.52 ± 1.47	697.16 ± 130.05 (26)	-	-	-
b(3)	10 - 14.9	60	7.14 ± 1.25	777.94 ± 106.09 (87)	5-6	2	2
4 a(3)	15 - 19.9	20	8.72 ± 1.43	951.62 ± 261.60 (25)	0	0	0
b(3)	15 - 19.9	30	6.97 ± 1.63	953.82 ± 137.35 (49)	5-7	1	1
c(6)	15 - 19.9	50	8.85 ± 0.10	814.55 ± 28.07 (140)	5-7	5	2
d(5)	15 - 19.9	100	9.02 ± 1.71	708.63 ± 239.30 (145)	5-7	4	4
5 (3)	20 - 24.9	20	9.14 ± 1.94	991.98 ± 241.58 (27)	5-7	3	2

* Number of replete female ticks

TABLE 20

MEAN ENGORGED WEIGHTS OF ADULT R. E. EVERTSI OF DIFFERENT WEIGHT CATEGORIES AFTER THE SECOND INFESTATION ON THE SAME RABBITS 2 MONTHS LATER

Rabbit group and No. of surviving animals used	Nymphal weight category of ticks used (mg)	No. of ticks used per rabbit	Adult mean engorgement weight (mg)	No. of rabbits paralysed
1 (3)	0 - 4.9	Not tested	Not tested	-
2 (3)	5 - 9.9	60	185.62 ± 142.14 (37)*	-
3 a (3)	10 - 14.9	Not tested	Not tested	-
b (1)	10 - 14.9	60	246.12 ± 215.10 (18)	-
4 a (3)	15 - 19.9	20	233.5 ± 181.10** (14)	1
b (2)	15 - 19.9	30	276.70 ± 200.00 (20)	-
c (4)	15 - 19.9	50	301.5 ± 252.9 (67)	-
d (1)	15 - 19.9	100	289.72 ± 199.52 (44)	-
5 (1)	20 - 24.9	20	125.75 ± 109.11 (8)	-

* Number of female ticks observed

**One rabbit became paralysed and died before ticks were replete. In the two tick infestation, 17(53.13%) rabbits were paralysed and of these 12(71%) died.

Irrespective of weight category, the onset of paralysis occurred approximately on day 6 of feeding.

Paralysis in the rabbits was first noticed on the fore legs. It ascended slowly and the affected rabbits displayed signs of respiratory distress and developed aspiration pneumonia diagnosed at postmortem examination. There was irritation on the eye corresponding to the infested ear. Some of the rabbits died in about 2 days after the onset of clinical signs.

All rabbits except one that survived paralysis during primary tick infestation withstood a second paralysis on tick challenge two months later (Table 20). The development of paralysis and death in one rabbit occurred after the application of 20 ticks of nymphal weight 15 - 19.9 mg. The engorgement weights of the ticks were reduced significantly compared with those of the primary challenge (Table 19).

DISCUSSION

The results of this experiment have shown that adult *R. e. evertsi* emerging from nymphs which weighed from 5 - 24.9 mg induced paralysis when fed on naive rabbits. Paralysis developed when the ticks were actively sucking blood while mating. This period was between day 6 and 9 of feeding irrespective of the weight category of the ticks used. The ability of ticks to induce paralysis was related to numbers of ticks applied to the hosts. Generally the lighter the ticks, the more were their numbers required to induce paralysis. The

intensity and severity of the paralysis was related to tick engorged weights as reported by Gothe (1981).

Gothe (1981) examined the ability of R. e. evertsi to induce paralysis in sheep and reported that only ticks weighing between 15 and 20 mg could induce the syndrome. Results reported here differ from those reported by Gothe (1981) in that ticks outside the weight ranges suggested by him induced paralysis as shown in Table 19. This difference could be related to distribution of the toxic principle in the tick strain or to the ability of the tick to parasitize hosts. Among tick species causing paralysis, some strains are unable to induce the syndrome (Bezuidenhout and Malherbe, 1981). It is therefore possible that most ticks used in this study, contained the toxic principle which was restricted to a specific weight mass. Hence, paralysis does not develop where a few ticks or light weight ticks feed on hosts. This situation may result either in little toxin being introduced into the host or to protective immunity developing in the host in the course of the toxin being introduced.

Many of the rabbits developed paralysis during primary tick challenge. Most rabbits that withstood paralysis during the initial challenge did not succumb to paralysis at subsequent challenge with the same tick doses 2 months later. This indicates that immunity developed as a result of tick infestation and that this immunity persisted for some time. However, one of the three rabbits challenged with 20 ticks (15 - 19.9 mg nymphal weight) developed paralysis and died during the second infestation (Table 20).

Effective immunity possibly did not develop after the primary challenge with 20 ticks. This is in agreement with Stone and Wright (1980) and Stone et al. (1982), who showed that paralysis could develop in partially immune hosts. It is possible that in this study challenge with 20 ticks from nymphs of weights from 15 to 19.9 mg did not introduce as much antigen into the hosts as a comparable challenge with ticks weighing from nymphal weight 20 - 24.9 mg.

Studies of immunity to paralysis induced by I. rubicundus and I. holocyclus have also indicated development of resistance by hosts as a result of either natural or artificial infestations (Stone et al., 1982, 1983). Results presented here together with those of previous workers strongly suggest that such methods could be employed in protecting animals against tick paralysis.

SUMMARY

Adult R. e. evertsi which moulted from nymphs of known engorged weights (5 to 24.9 mg), were able to induce paralysis when fed on naive rabbits. Of 32 naive rabbits infested with adult ticks of various weight categories, 17(53.3%) became paralyzed; 12 of the 17(71%) died. Adults moulting from nymphs weighing between 5 - 9.9 mg induced and maintained paralysis only briefly. Heavier adult ticks induced and maintained paralysis in rabbits which resulted in many deaths and a few recoveries. A mean adult engorged weight of about 560 mg from 60 light ticks was apparently necessary for paralysis to develop. Heavier engorgement weights were attained at the expense of host mortality.

CHAPTER 4

THE IMMUNE RESPONSE OF RABBITS INFESTED WITH RHIPICEPHALUS EVERTSI EVERTSI AND CONCURRENTLY INOCULATED WITH NON-TICK ANTIGENS

INTRODUCTION

Specific immune responses of various hosts to different tick species have been demonstrated (Bowessidjaou *et al.*, 1977; Fujisaki, 1978 and McGowan *et al.*, 1980). However, effects of concurrent infestations or immunizations on animals resistant to ticks have received little attention. Evidence exists to show that repeated tick challenges may cause specific precipitating antibodies to wane (Wikel and Osburn, 1982), enhance tick feeding in subsequent challenges (Brossard *et al.*, 1982, Den-Hollander and Allen, 1985; Chapter 1 in the study) or recrudescence of hemoparasitic infection (Corrier *et al.*, 1979).

Immunosuppression as a phenomenon and its underlying mechanisms in endoparasitic infections has been reviewed by Terry and Hudson (1982). Some data have been reported for demodicosis in dogs (Scott *et al.*, 1974; Corbett *et al.*, 1975) and *D. andersoni* infestation in cattle and guinea pigs (Wikel *et al.*, 1978; Wikel and Osburn, 1982; Whelen *et al.*, 1984). On the other hand, data exist as regards hosts immunosuppression during trypanosomiasis (Greenwood *et al.*, 1973;Rurangirwa *et al.*, 1979). In view of the projected control of ticks by vaccination, tick induced immunosuppression and

paralysis may have special significance where immunization of livestock with different antigens is practiced. Therefore, this study was designed to investigate the antibody response in R. e. evertsi-infested hosts, concurrently immunized with heterologous antigens.

MATERIALS AND METHODS

Host antibody response to SRBC after repeated challenges with various tick loads as detected by direct haemagglutination test.

Twelve rabbits were used to assess the ability of hosts previously infested repeatedly with ticks to mount an immune response to SRBC. They were divided into four groups of three rabbits each. Three of the 4 groups were sensitized four times with 20, 50 and 100 adult R. e. evertsi as described in Chapter 1. The fourth group served as an uninfested control. Thirty days after completion of the last tick infestation, all rabbits in the four groups were inoculated subcutaneously (SC) with 0.5 ml of 1% SRBC suspended in PBS. All rabbits were bled through the marginal ear vein before inoculation with SRBC and at weekly intervals thereafter for four weeks. Sera were prepared and stored at -20°C till required for use in the direct haemagglutination (DH) test.

When required for the test, the rabbit antisera were heat-inactivated (56°C, 30 mins.) and then diluted in doubling dilutions using PBS in U-shaped wells of microtitre plates (Flow Laboratories, UK). An equal volume (50 µl) of 1% SRBC prepared as

described in Chapter 1, was added to the serum dilutions, the plates were shaken and incubated at 37°C for 1 hour. The plates were then kept at 4°C for another hour and thereafter the highest serum dilution giving 100% agglutination of the cells was read as the titre.

Antibody response to SRBC of a 50 tick and 200 tick challenged rabbits

Two groups of three naive rabbits each infested separately with 50 and 200 adult R. e. evertsi were used to study the immune response to SRBC. The infestations were carried out as described earlier. Rabbits challenged with 200 ticks had the infestation terminated on day 5 of feeding by manual removal of the ticks. Immunization with SRBC, bleeding schedule and determination of serum antibodies to SRBC were done as stated above. Data for the immunized, uninfested rabbit controls in the above experiment was also used here for comparison purposes.

Antibody response of 50 tick challenged rabbits concurrently inoculated with SRBC and bovine serum albumin (BSA)

Three naive rabbits infested once with 50 adult R. e. evertsi as described previously, were used to assess immune response to a simultaneous primary inoculation with SRBC and BSA. Each rabbit was inoculated intravenously (i.v) first with 2 ml, 1% SRBC and then with 12 mg BSA, (separately) after ticks had attached. These immunizations were repeated on day 10 of tick feeding. Three rabbits were not infested but were inoculated with BSA and SRBC and served as controls. The rabbits were bled on a 5 day interval basis after inoculations for three weeks. Serum antibodies to SRBC and BSA were respectively determined by the DH and the PHA methods respectively as

described previously. A slight modification involving coupling of BSA to bovine red blood cells instead of sheep was made in the technique.

Antibody response of 50 tick infested rabbits after inoculation with BSA, SRBC and SGA

The above experiment was repeated, using two groups of six rabbits each, inoculated separately with BSA and SRBC. A third group of 3 rabbits was introduced, inoculated with 2 ml, PBS, and served as infested, unimmunized control. Two additional groups of three rabbits each were immunized either with BSA or SRBC. These served as uninfested, immunized controls. Rabbits were bled every three days for two weeks. The ELISA test as described in Chapter 1, was utilized to detect host antibodies to antigens presented by ticks while feeding. Linear regression (Steel and Torrie, 1960) was used to correlate these host antibodies with tick infestation period. Engorged weights of replete ticks were used to indicate possible development of resistance by hosts.

Immune response of 20 tick infested hosts to secondary inoculation with BSA and SRBC

Secondary immune response to a combined immunization with BSA and SRBC of rabbits infested with 20 adult R. e. evertsi was studied using 2 groups of 3 rabbits each. One group was primed with simultaneous inoculation, i.v, of 12 mg BSA and 2 ml, 1% SRBC, followed by a secondary (booster) immunization on day 14 with 6 mg BSA and 1 ml, 1% SRBC. The booster was administered when 20 adult R. e. evertsi applied had started to engorge. The remaining group of

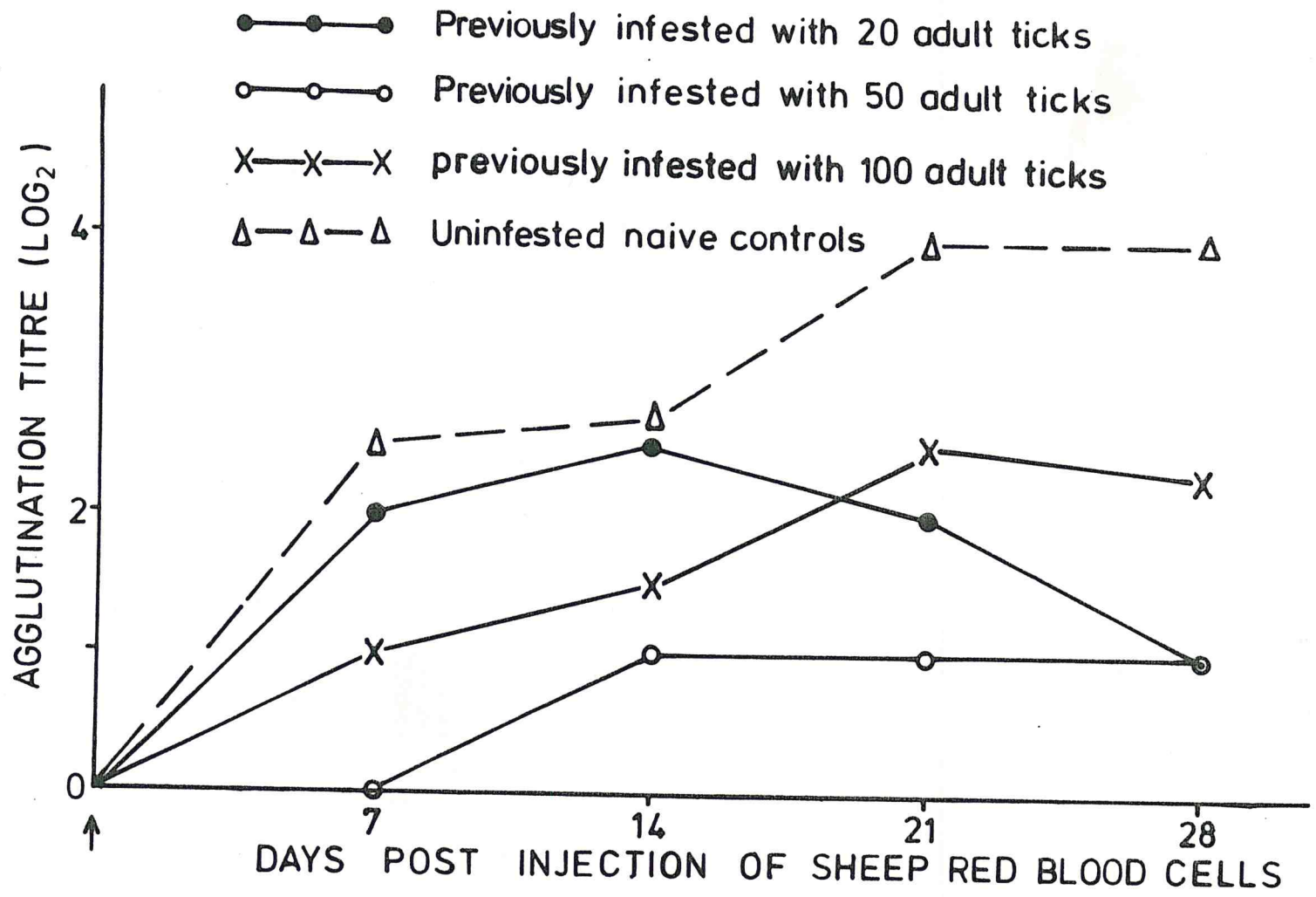
rabbits was similarly treated but were inoculated with PBS to serve as unimmunized and infested control.

The rabbits were bled twice weekly throughout the tick infestation period. Serum antibodies to SRBC and BSA were determined by the DH and the PHA methods respectively as described previously. Agar gel double diffusion and ELISA tests were used to detect development of antibodies to tick salivary gland antigens as outlined in Chapter 1. Sera obtained between day 2 and 4 of tick feeding from the infested rabbits was separated into IgM and IgG by Sephacryl S-300 column chromatography as described in the gel filtration technical manual (Pharmacia Fine Chemicals, Sweden). The proportions of the two classes of immunoglobulins were studied. Feeding performance of the ticks was also used to indicate possible acquisition of resistance by the hosts.

RESULTS

Host antibody response to SRBC after repeated challenges with various tick loads as detected by direct haemagglutination test

All rabbits which completed four successive infestations with different numbers of adult *R. e. evertsi* developed antibodies to SRBC which were lower than the uninfested controls (Fig. 17). The difference in the titres between the tick-infested and control rabbits was significant ($P < 0.01$) during the third week of observation. Antibodies were detected by day 7 of immunization in rabbit group sensitized with 20 and 100 adult ticks. There was, however, a 7 day delay period in the humoral response for the 50 tick


Previously infested with 20 adult ticks
Previously infested with 50 adult ticks
previously infested with 100 adult ticks
Uninfested naive controls
AGGLUTINATION TITRE (LOG2)
4
2
0
7
14
21
28
DAYS POST INJECTION OF SHEEP RED BLOOD CELLS

challenge group. This rabbit group manifested the poorest immune response to SRBC compared to the remaining two groups. A declining humoral response was observed in the rabbit group sensitized with 20 adult ticks from day 14.

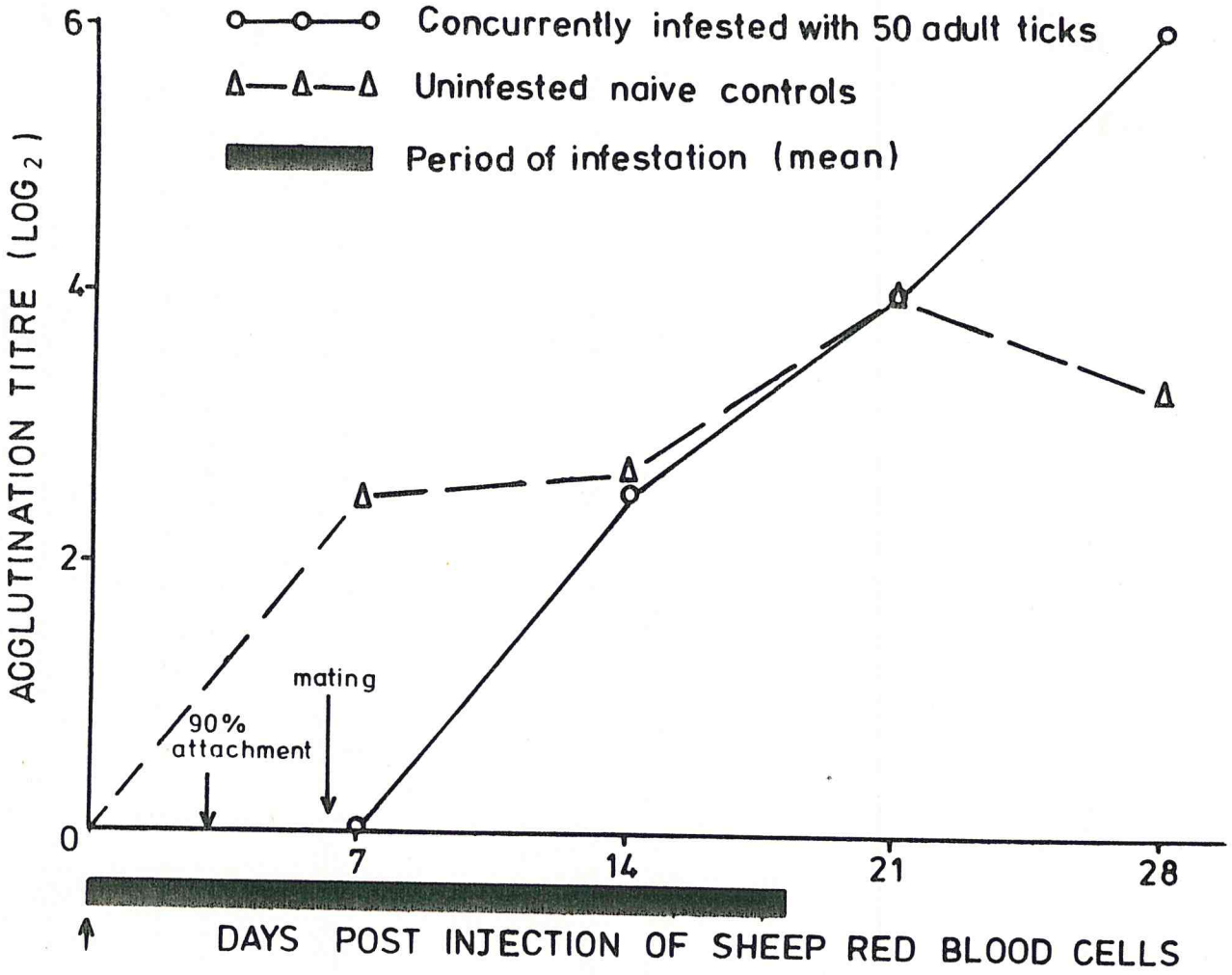
Antibody response to SRBC of rabbits infested with 50 and 200 adult R. e. evertsi

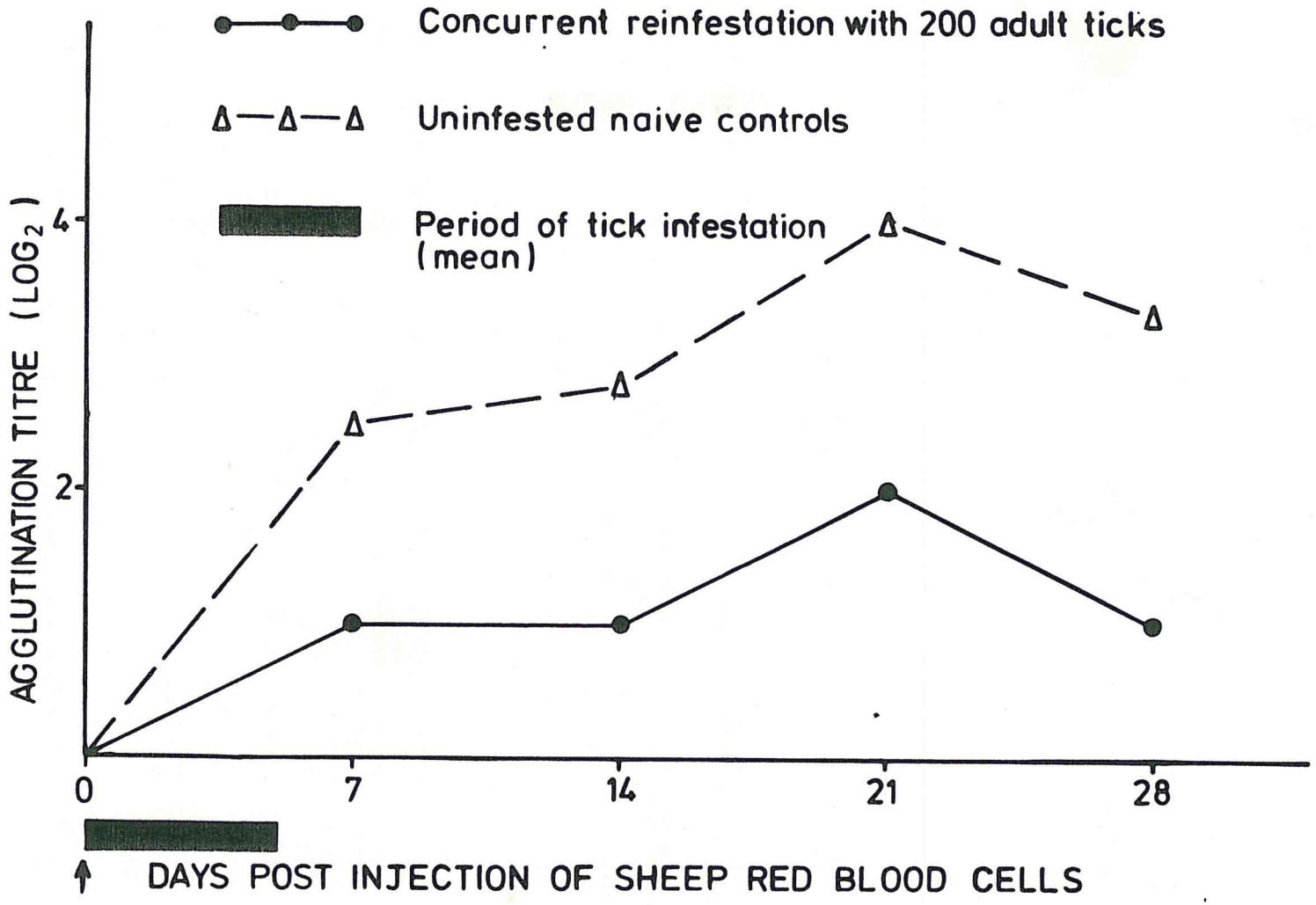
When rabbits were concurrently exposed to 50 adult R. e. evertsi and inoculated with SRBC, the animals' antibody response to SRBC was considerably reduced (Fig. 18). The antibody response to SRBC was not detected during the first 7 days of the tick feeding. Two of the three tick-infested rabbits developed paralysis and died by day 13 despite developing some antibodies to the SRBC. Rabbits that survived tick paralysis made a significant compensatory immune response to the SRBC after day 14 of tick challenge (Fig. 18).

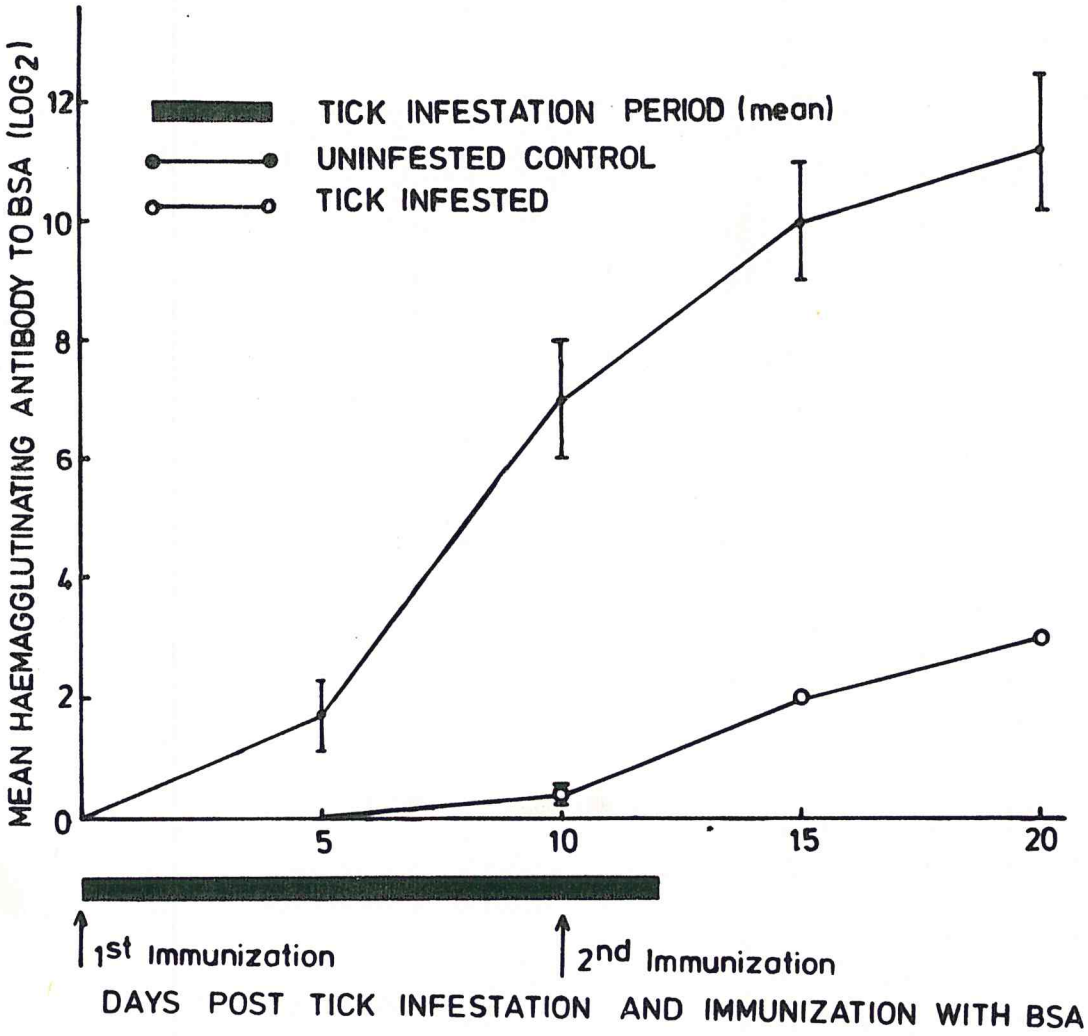
Two hundred adult R.e. evertsi parasitizing rabbits induced poor host antibody response ($P < 0.001$) to SRBC compared to uninfested controls inspite of premature (manual removal of ticks) termination of the infestation (Fig. 19). The peak antibody titre of the infested group was about 50% lower than the controls.

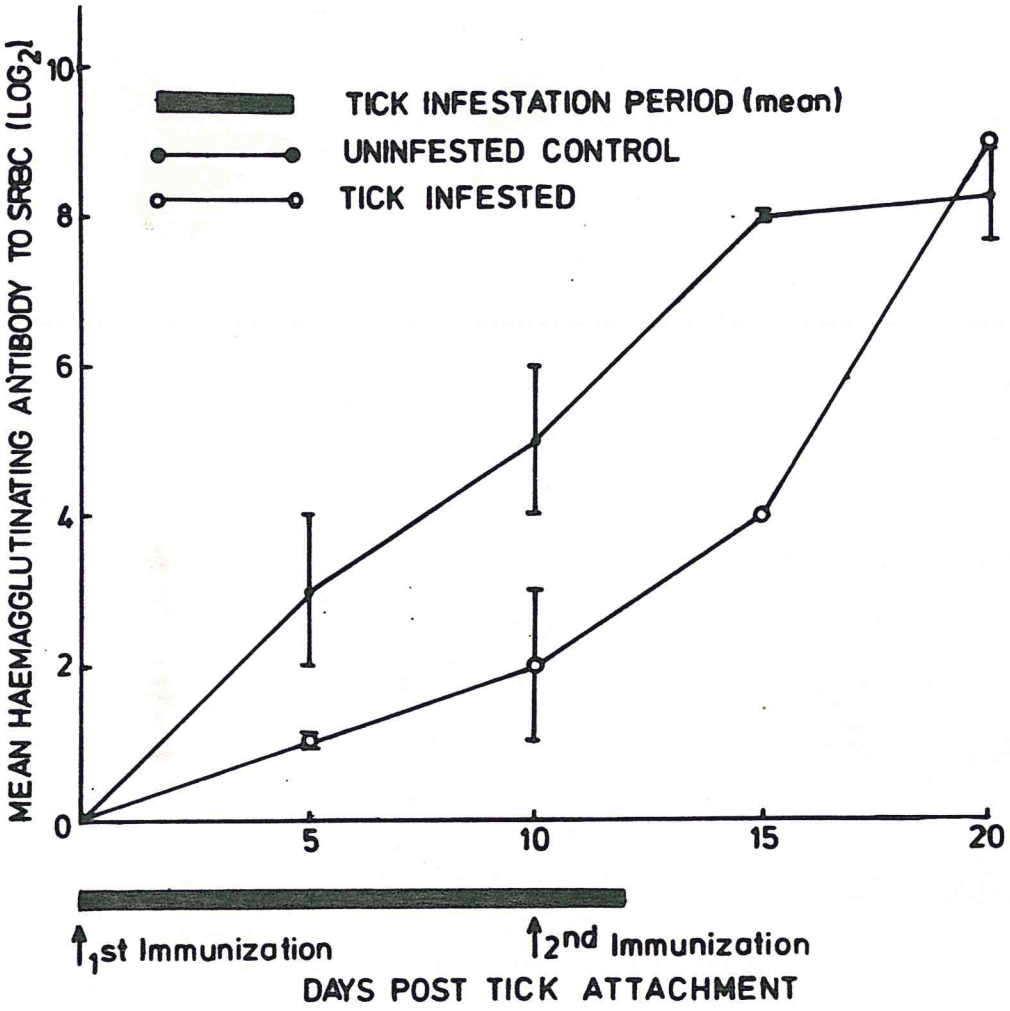
Antibody response of 50 tick challenged rabbits concurrently inoculated with BSA and SRBC

In the course of tick feeding, primary host antibody response to BSA was virtually blocked (Fig. 20). Host antibody response to SRBC (Fig 21) was superior to that seen in animals inoculated with


Concurrently infested with 50 adult ticks
Uninfested naive controls
Period of infestation (mean)
ACGLUTINATION TITRE (LOG2)
6
4
2
0
90% attachment
mating
7
14
21
28
DAYS POST INJECTION OF SHEEP RED BLOOD CELLS


Concurrent reinfestation with 200 adult ticks
Uninfested naive controls
Period of tick infestation (mean)
AGGLUTINATION TITRE (LOG2)
4
2
0
7
14
21
28
DAYS POST INJECTION OF SHEEP RED BLOOD CELLS


TICK INFESTATION PERIOD (mean)
UNINFESTED CONTROL
TICK INFESTED
MEAN HAEMAGGLUTINATING ANTIBODY TO BSA (LOG2)
0
2
4
6
8
10
12
5
10
15
20
1st Immunization
2nd Immunization
DAYS POST TICK INFESTATION AND IMMUNIZATION WITH BSA


TICK INFESTATION PERIOD (mean)
UNINFESTED CONTROL
TICK INFESTED
MEAN HAEMAGGLUTINATING ANTIBODY TO SRBC (LOG2)
0
2
4
6
8
10
5
10
15
20
1st Immunization
2nd Immunization
DAYS POST TICK ATTACHMENT

BSA. Two of the 3 tick-infested rabbits developed paralysis and died by day 13 of primary immunization with BSA and SRBC. However, the survivors made a compensatory response to SRBC but not BSA after day 15 of infestation (Fig. 21). Booster immunization performed on day 10 after the initial dose influenced the response of the survivors of tick paralysis. The uninfested control rabbits responded better to both BSA and SRBC compared to those infested with ticks.

Antibody response of a 50 tick infested rabbit groups to BSA, SRBC and SGA

The primary antibody response to BSA of tick-infested rabbits was not detectable during the first 9 days of tick feeding (Table 21). Agglutinating antibody titre (measured by PHA test) of about ($\log_2$) 2.5 was apparent by day 14, when tick feeding was completed. A significant primary antibody response was evident in the uninfested, immunized control rabbits by day 6 of antigen inoculation and was better than that of the infested animals, being ($\log_2$) 8 by day 14.

The primary antibody response of rabbits to SRBC was severely depressed but not abolished by the feeding ticks (Table 22). Three of the 6 tick-infested rabbits were paralysed and died between day 6 and 9 of the study. On the sixth day (Table 22) the mean antibody titre of surviving rabbits was ($\log_2$) 0.5, compared with a titre of 3 for the tick-free control rabbits. As the tick infestation approached completion, both tick-infested and uninfested rabbits manifested approximately similar antibody response.

TABLE 21

HAEMAGGLUTINATING ANTIBODY TITRES TO BSA IN RABBITS INFESTED WITH ADULT *R. E. EVERTSI* (Log_2)

Rabbit number	Number of ticks used	Days post tick infestation				
		0	3	6	9	14
R4	50	0	0	0	0	2
R5	50	0	0	0	0	1
R6	50	0	0	0	0	4
R7	50	0	0	0	0	3
R8	50	0	0	0	0	3
R9	50	0	0	0	0	2
mean ± s.d		0	0	0	0	2.5 ±1.05
C_4^*	0	0	<1.0	1	4	6
C_5	0	0	<1.0	1	3	8
C_6	0	0	<1.0	2	6	10
mean ± s.d		0	<1.0	1.33 ±0.58	4.33 ±1.53	8.00 ±2.00

* Uninfested but immunized rabbits

TABLE 22

DIRECT HAEMAGGLUTINATION ANTIBODY TITRES TO SRBC IN R. E. EVERTSI INFESTED RABBITS (Log_2)

Rabbit number	Tick number applied	Days after tick infestation				
		0	3	6	9	14
R10	50	0	0	0*	0	0
R11	50	0	0	0	3	3
R12	50	0	<1.0	2	4*	0
R13	50	0	<1.0	0*	0	0
R14	50	0	0	1	4	5
R15	50	0	0	1	4	5
mean ± s.d**		0	0	0.50 ±0.84	3.75 ±0.50	4.67 ±0.58
C_1	-	0	<1.0	3	4	6
C_2	-	0	<1.0	4	6	8
C_3	-	0	<1.0	2	5	5
mean ± s.d		0	0	3.00 ±1.00	5.00 ±1.00	6.33 ±1.53

* died of paralysis

** mean ± standard deviation

Letter C shows uninfested but immunized control rabbits

In the course of tick feeding, the three rabbit groups showed antibody activity to tick SGA demonstrable by the ELISA test (Fig. 22). Activity increased progressively as ticks engorged on rabbits inoculated with BSA and SRBC. By day 6 of tick feeding, the ELISA reading had increased by an average of 0.1 and 0.05 absorbance units for rabbits inoculated with BSA and SRBC respectively. During the same period, there was no significant activity detected in tick infested rabbits, inoculated with PBS. Significant elevated ELISA values were observed in all rabbits towards the end of second week of infestation. Significant correlations were observed between ELISA anti-tick antibody values and the tick feeding duration. The correlation values (r) and the levels of significance (P) were $r = 0.97$, $P < 0.1$; $r = 0.93$, $P < 0.05$; and $r = 0.86$, $P < 0.1$ respectively for rabbits immunized with BSA, SRBC and PBS.

The three slopes were not homogeneous ($P < 0.01$). When the slopes were pooled and tested further, it was found that the degree of heterogeneity was significant ($P < 0.05$), indicating that SRBC and BSA inoculated into tick-infested hosts affect the rate at which antibodies to SGA develop.

The feeding period of ticks on immunized and control rabbits was approximately 8 days (Table 23). However, ticks engorged to smaller weights on rabbits inoculated with BSA and PBS than with SRBC. The latter host group suffered tick paralysis involving four of the six rabbits and three died. Two of the six rabbits inoculated with BSA developed paralysis without mortality. Although rabbits

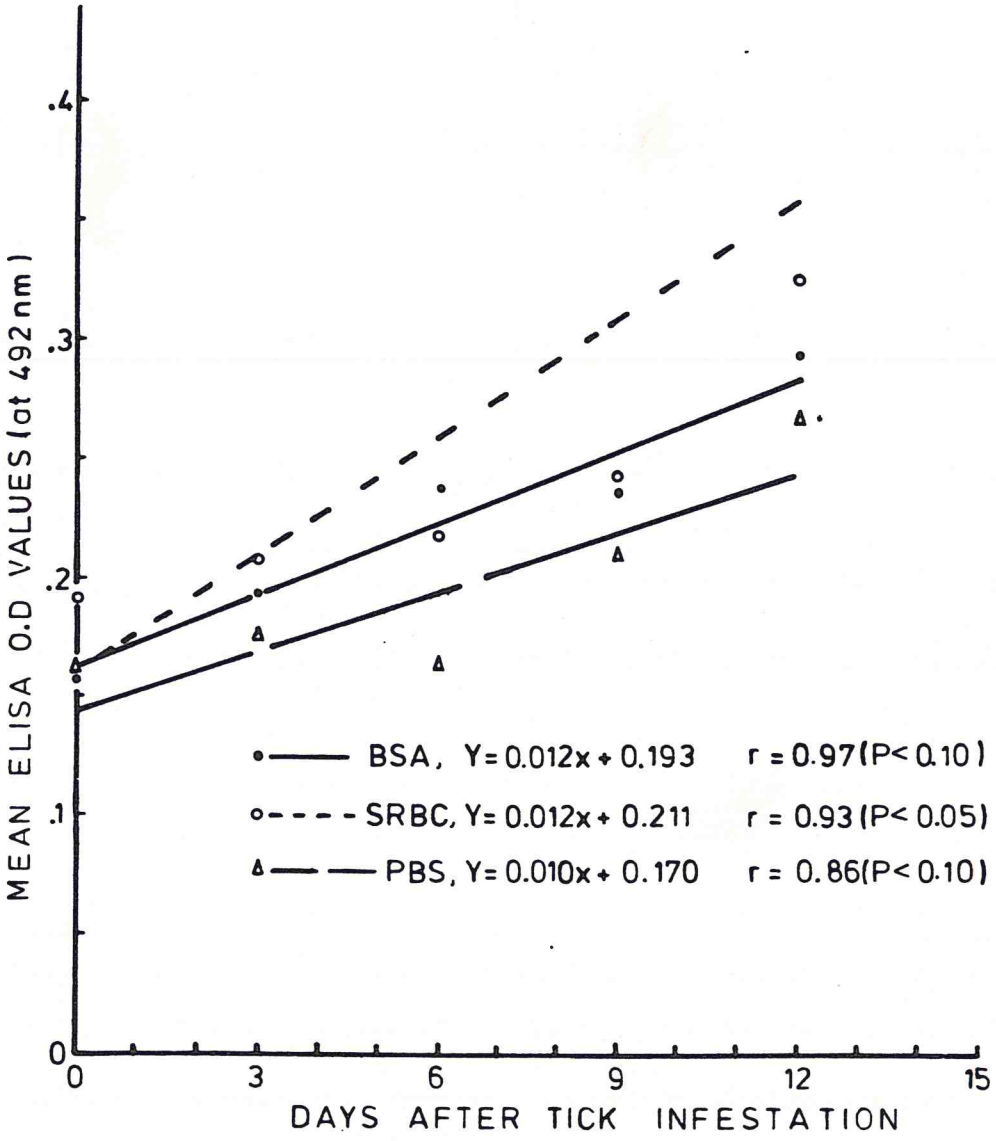

MEAN ELISA O.D VALUES (at 492 nm)
.4
.3
.2
.1
0
0
3
6
9
12
15
DAYS AFTER TICK INFESTATION
BSA, Y= 0.012x + 0.193 r = 0.97 (P< 0.10)
SRBC, Y= 0.012x + 0.211 r = 0.93 (P< 0.05)
PBS, Y= 0.010x + 0.170 r = 0.86 (P< 0.10)

TABLE 23

INFLUENCE OF SRBC AND BSA INOCULATED INTO RABBITS ON FEEDING PERFORMANCE OF 50 ADULT R.E. EVERTSI

Treatment (No. animals)	Mean feeding period (days ± s.d)	% repletion	Mean engorgement weight (mg ± s.d)	Rabbit paralysis**	
				morbidity %	mortality %
BSA (6)	8.91 ± 1.51	100	779.84 ± 31.10	33.33(2)	0
SRBC (6)	8.04 ± 0.85	52*	840.25 ± 52.01	67(4)	75(3)
PBS (3)	8.71 ± 1.13	92	799.65 ±183.10	67(2)	0

* 3 rabbits died before end of tick infestation

** Number of paralysed rabbits is shown in parenthesis

inoculated with PBS had paralysis morbidity comparable to those inoculated with SRBC, mortality was not observed.

Immune response of 20 tick infested rabbits to secondary inoculation with BSA and SRBC

Host secondary antibody response to both SRBC and BSA was suppressed but not abolished during the first week of tick feeding (Fig. 23 and 24). The lowest host antibody response for the two antigens occurred on day 3 of tick feeding (or booster immunization). The antibody response of hosts to BSA was more suppressed than to SRBC. Furthermore, it was characterized by marked variation between individual animals (Fig. 23).

Feeding performance of 20 adult R. e. evertsi on rabbits previously immunized with SRBC and BSA

All ticks applied on immunized and control rabbits fed to repletion (Table 24). The mean engorged weight of ticks fed on immunized hosts was 498.41 mg, compared to 957.82 mg on controls. Besides, the feeding period was shorter for ticks applied on rabbits inoculated with BSA and SRBC than those fed on control hosts.

Humoral antibodies against tick SGA, in infested rabbits during secondary immunization with BSA and SRBC

Antibodies against ticks parasitizing rabbits challenged with BSA and SRBC were detected by the ELISA and agar gel double diffusion tests as early as the third day of tick feeding (Table 25). Similar antibodies were detected after 2 weeks of tick infestation in the

FIGURE 23

Anamnestic antibody response to SRBC in rabbits inoculated with BSA and SRBC and cocurrently infested with 20 adult R. e. evertsi. Each point on the graph represents a mean of antibody titres in three rabbits. Antibody titres at the time of tick application were taken as the control.

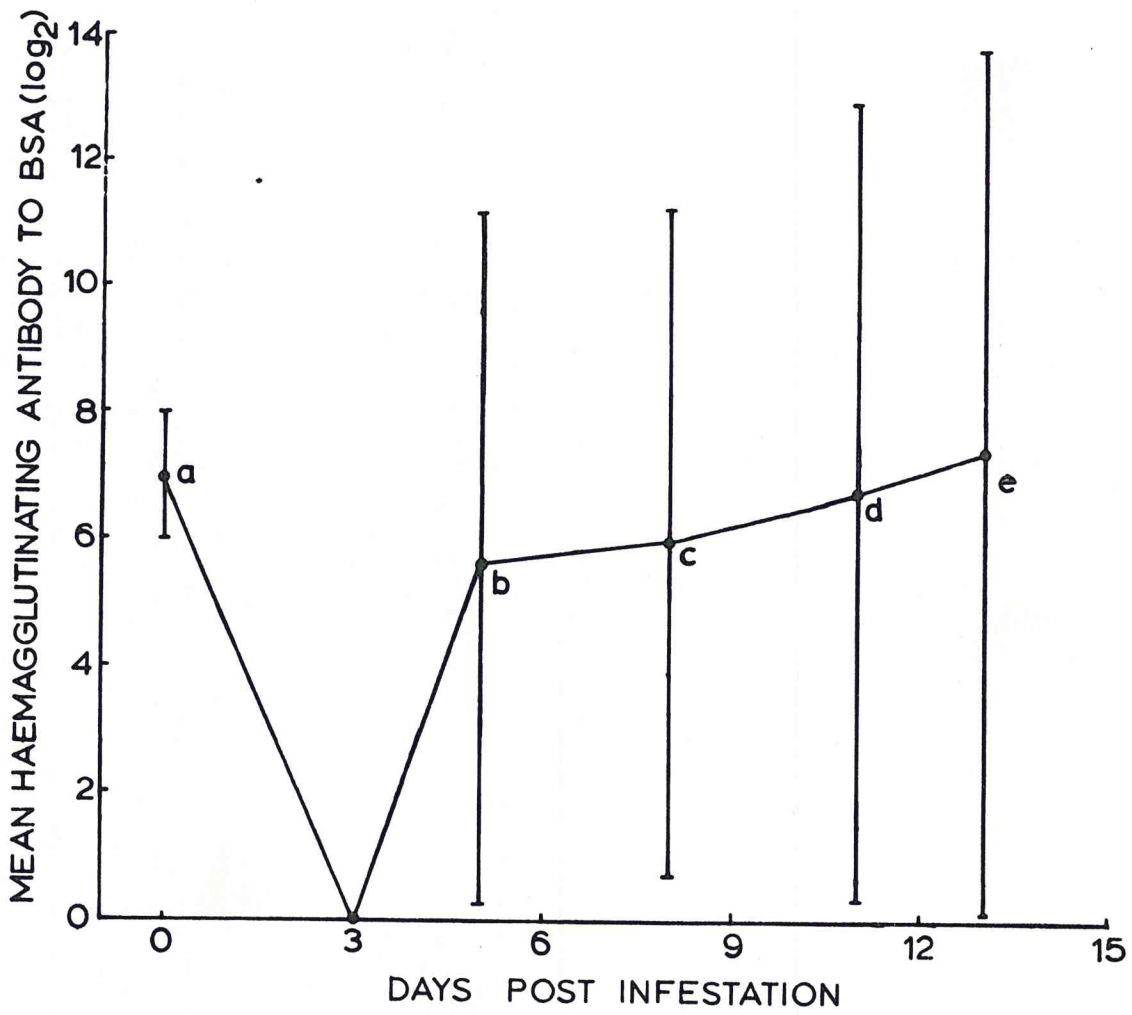

MEAN HAEMAGGLUTINATING ANTIBODY TO BSA (log_2)
14
12
10
8
6
4
2
0
a
b
c
d
e
0
3
6
9
12
15
DAYS POST INFESTATION

TABLE 24

EFFECTS OF SECONDARY IMMUNIZATION WITH BSA AND SRBC ON 10 FEMALE *R. E. EVERTSI* FEEDING ON NAIVE RABBITS

Rabbit group	Source of inoculum	% replete ticks	Mean feeding period (days)	Mean engorgement weights (mg)
1	BSA/SRBC	100	6.63 ± 1.33	498.41 ± 338.99
2	PBS	100	8.52 ± 1.39	957.82 ± 128.32

TABLE 25

SERUM ANTIBODY RESPONSE TO PRIMARY INFESTATION WITH R. E. EVERTSI OF RABBITS IMMUNIZED TWICE WITH BSA AND SRBC AS DETERMINED BY ELISA (OD AT 492 nm) AND IMMUNODIFFUSION TESTS

Days post infestation	Rabbit X *		Rabbit 81		Rabbit 82	
	ELISA OD 492*	Presence (+)/ absence (-) of precipitating antibody	ELISA OD 492*	Presence (+)/ absence (-) of precipitating antibody	ELISA OD 492*	Presence (+)/ absence (-) of precipitating antibody
0	0.229 ± 0.053**	-	0.255 ± 0.013	-	0.262 ± 0.011	-
3	0.395 ± 0.012	+	0.373 ± 0.012	-	0.307 ± 0.011	-
7	0.209 ± 0.022	-	0.225 ± 0.04	-	0.310 ± 0.033	+
9	0.377 ± 0.071	+	0.422 ± 0.008	+	0.355 ± 0.005	-
11	0.246 ± 0.02	-	0.435 ± 0.03	+	0.454 ± 0.03	-
13	0.299 ± 0.04	-	Died		0.432 ± 0.032	-
Positive control serum	0.611 ± 0.04	+				
Negative control serum	0.202 ± 0.03	-				

* Antigen concentration = 0.75 µg protein/well; goat anti-rabbit lgG conjugate dilution = 1:1500, serum dilution = 1:10; ** Mean ± standard deviation of 6 replicates per sample

control rabbits (Table 26). Chromatographic separation of these sera indicated proportionately high levels of IgM in addition to IgG (Figs. 25 and 26).

DISCUSSION

The antibody response to a primary challenge with SRBC of rabbits previously infested repeatedly with adult R. e. evertsi was reduced (Fig. 17). Depression of antibodies to SRBC has also been reported in hosts infested with trypanosomes (Goodwin et al., 1972) and helminths (Mota-Santos et al., 1976, 1977). Therefore, the reduced antibody response to SRBC observed in the present study indicates that the tick sensitization procedure adapted for the rabbits induced immunosuppression. These results are in agreement with those of Wikel and Osburn (1982) who showed that similar sensitization regimens of calves with D. andersoni caused immunosuppression.

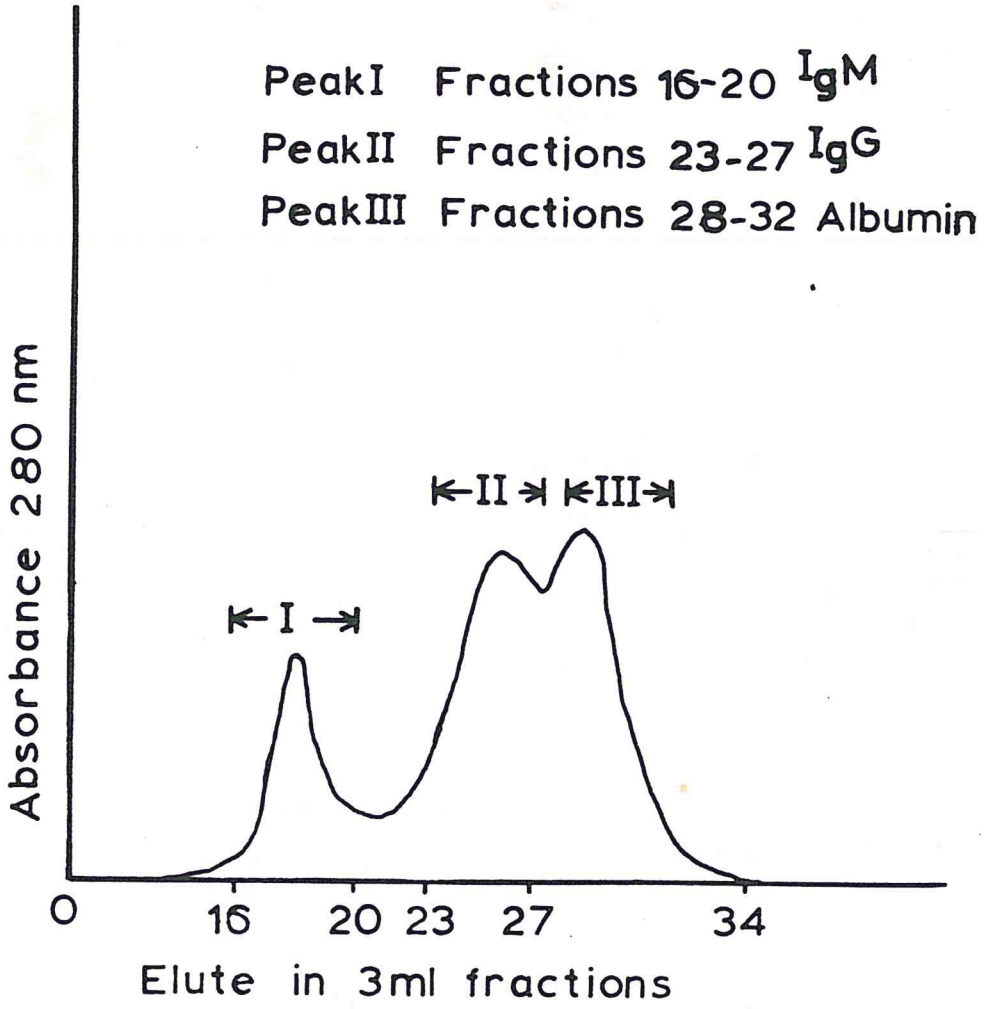
Immunosuppression was more severe in rabbits sensitized with 50 than with 20 and 100 adult ticks. The 50 tick rabbit group was paralysed during primary infestation and recovered, while paralyzed rabbits in the two other groups died. Paralysis toxin, therefore, seemed to exacerbate immunodepression. It has been shown that severe immunosuppression is only significant if it results in partial inhibition of certain potentially host protective antiparasite immune responses to natural infection and it is not favourable for the parasite to kill its host (Mitchell, 1980). It is suggested from the study that paralyzing toxin is an essential salivary component to immunosuppress hosts and might ensure survival of the tick species.

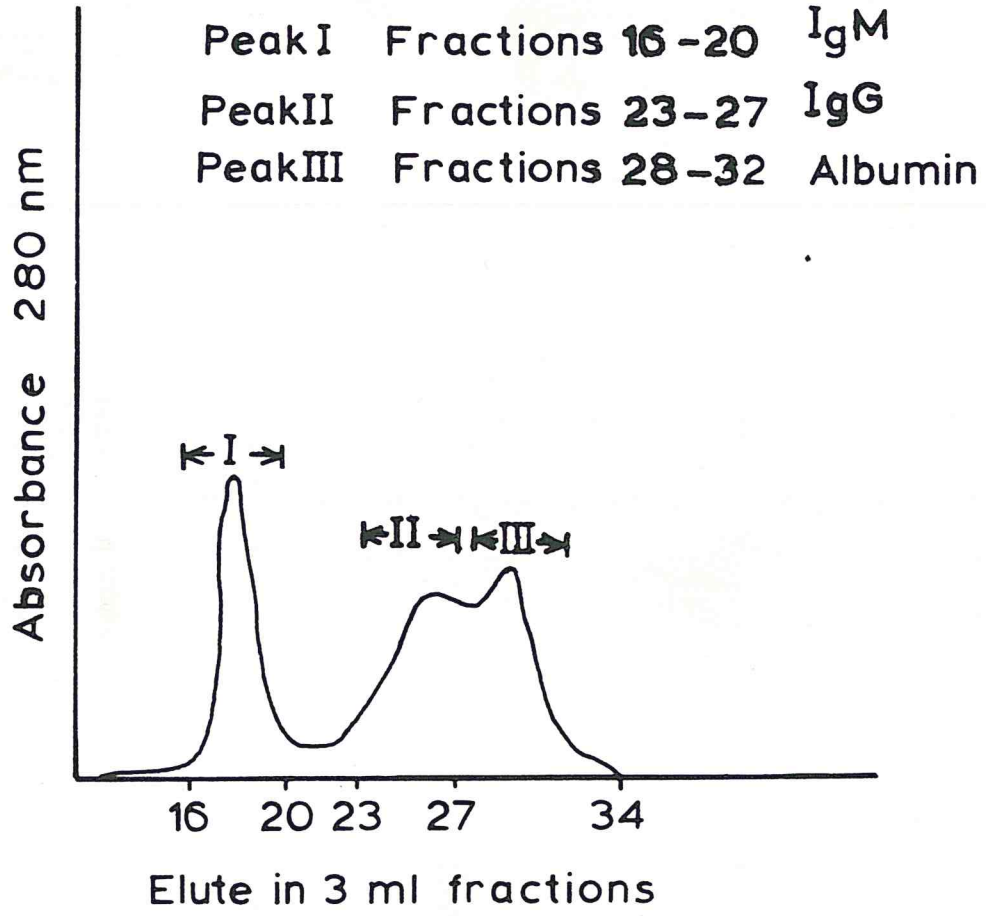
TABLE 26

SERUM ANTIBODY RESPONSE TO PRIMARY INFESTATION WITH ADULT *R.E. EVERTSI* OF RABBITS INOCULATED WITH PBS AS DETERMINED BY ELISA (OD AT 492 nm) AND IMMUNODIFFUSION TESTS

Days post infestation	ELISA OD 492	Presence (+)/ absence (-) of precipitating antibody
0	0.218 ± 0.051	-
3	0.251 ± 0.05	-
7	0.270 ± 0.06	-
9	0.293 ± 0.072	-
13	0.399 ± 0.10	+*
Postive control serum	0.611 ± 0.04	+
Negative control serum	0.202 ± 0.03	-

* One rabbit


PeakI Fractions 16-20 IgM
PeakII Fractions 23-27 IgG
PeakIII Fractions 28-32 Albumin
Absorbance 280 nm
I
II
III
0
16
20
23
27
34
Elute in 3ml fractions


Peak I Fractions 16-20 IgM
Peak II Fractions 23-27 IgG
Peak III Fractions 28-32 Albumin
Absorbance 280 nm
I
II
III
16
20
23
27
34
Elute in 3 ml fractions

Massive numbers of ticks have been used to sensitize hosts and subsequent immunosuppression was not investigated (Roberts, 1968a; Wagland, 1975; Willadsen et al. 1978). Eckbald et al. (1984) reported that calves vaccinated against anaplasmosis were not immunosuppressed by high challenge doses with B. microplus and D. albipictus. The present results (Fig. 19) have shown that challenge of rabbits with 200 adult R. e. evertsi caused immunosuppression, despite restricting parasite feeding duration. These data suggest that, at the height of tick population interruption of massive tick infestations on hosts by acaricides may not prevent onset of immunosuppression, and that not all tick species cause immunosuppression.

The present work has shown further that primary (Figs. 18, 20 and 21), unlike secondary (Figs. 23 and 24) antibody response to SRBC and BSA inoculated separately or in conjuction with groups of naive rabbits simultaneously infested with a load of 20 or 50 adult R. e. evertsi was severely suppressed compared to uninfested controls and mortalities (Table 23) occurred. Suppression was more severe for BSA than SRBC and that the primary antibody response to BSA was delayed. However, secondary antibody response to the two antigens was temporarily depressed. Such observations indicate that immunosuppression resulted from the tick infestation.

Paralysis accompanied by deaths occurred in tick-infested rabbits whenever immunizations with SRBC alone or mixed with BSA was attempted. Taussig (1978) observed that in animals immunized with a

mixture containing a variety of other antigens that the immune response to one can be severely depressed while the response to the other is unaffected, a phenomenon he called "antigenic competition". Therefore it appears from the present study that SRBC suppressed host immune response to tick SGA and BSA which resulted in the development of low level antibodies. Consequently, the high number of tick-infested rabbits, inoculated with SRBC that became paralysed and died could be ascribed to reduced antibody response to tick SGA.

The primary antibody response of tick-infested hosts to BSA and SRBC (Tables 21 and 22) was suppressed in the course of tick feeding, while that to the ectoparasite antigens was affected to varying degrees among the host groups studied (Fig. 22). ELISA absorbance anti-tick antibody values were highly correlated linearly with tick feeding period ($r = 0.97$) on hosts inoculated with BSA. Similar data for hosts inoculated with SRBC and PBS showed curvilinear relationship when plotted on an equal interval scale. The curvilinear data reported during tick feeding on hosts inoculated with PBS and SRBC support the hypothesis by Wikel and Allen (1982) and is suggestive of immunosuppression.

Tick loads applied onto naive rabbits inoculated with BSA caused a low incidence of paralysis without mortality (Table 23) while heavy mortalities affected rabbits inoculated with SRBC. Ticks engorged to smaller weights in a slightly longer period on hosts treated with BSA than with either SRBC or PBS. Host antibody response to tick salivary gland antigens of the rabbit group

inoculated with BSA was highly correlated with tick feeding period suggesting a steady acquisition of ixodid resistance.

Simultaneous booster immunization (with SRBC and BSA) and tick infestation of naive hosts, enabled them to acquire resistance to tick feeding rapidly. Resistance was manifested by reduction in engorged weights ($P < 0.005$) and feeding periods of ticks fed on immunized compared to unimmunized control hosts (Table 24). Specific antibodies to tick salivary gland antigens were detected by the ELISA and agar gel double diffusion tests by day 3 of tick feeding (Table 25), confirming acquisition of resistance to the ixodid tick. Sera taken at this period from the hosts had significant levels of IgM (Figs. 25 and 26), indicating an early immune response.

The major finding of this study was that tick feeding suppress host antibody response to homologous and heterologus antigens. Steroid hormones released by stressed hosts, have been shown to suppress induction of immune response too (Synder and Unanue, 1982). The delay in humoral response to BSA and SRBC observed in this study may have been partly associated with the stress of tick feeding.

SUMMARY

Investigations on the antibody response of rabbits under different challenge regimens with adult R. e. evertsi was conducted using sheep red blood cells (SRBC) and bovine serum albumin (BSA). Rabbits which completed four repeated challenges with different numbers of the tick responded poorly to a subsequent challenge with SRBC. Naive rabbits infested with ticks and simultaneously immunized

with SRBC had a depressed antibody response to both SRBC and tick salivary gland antigens and also experienced a high mortality rate due to tick paralysis. A similar effect was observed if BSA and SRBC were inoculated simultaneously but not when BSA was given alone. Naive rabbits primed with BSA and SRBC, boosted with the same antigens at the time of tick infestation, acquired resistance to the ectoparasite, demonstrable as early as third day of tick feeding. The host response to the two antigens was transiently depressed while ticks were feeding. The reduced antibody response to these heterologous antigens suggests tick infestations caused immunosuppression in the rabbits.

CHAPTER 5

STUDIES ON IMMUNOLOGICAL SPECIFICITY OF RESISTANCE ACQUIRED BY RABBITS TO INFESTATIONS WITH RHIPICEPHALUS EVERTSI EVERTSI

Introduction

Sera from animals sensitized with SGA from one tick species have been shown to cross-react with antigens from other tick species (Trager, 1939a; Brown and Askenase, 1981; McTier *et al.*, 1981; Whelan *et al.*, 1984). This finding suggests the possible existence of common antigens among ixodid ticks which may be important in the induction of resistance. Furthermore, immune response against ticks is not species specific. These features may render control of several tick species by a vaccine appropriately developed from one tick species. *Rhipicephalus appendiculatus*, *R. e. evertsi* and *A. variegatum* are ticks of economic importance in East Africa (Theiler, 1950; Hoogstraal, 1956; Neitz, 1957; Walker, 1970). Since the three species of ticks occur sympatrically, an experiment was designed to investigate the possibility that they share common antigen.

MATERIALS AND METHODS

Fifty one adult New Zealand White rabbits of the same age and weight as described in Chapter 1, were used. Of these, 28 had been sensitized to *R. e. evertsi* (adults and larvae) and the remaining 23 were naive controls. They were divided into 5 groups as

shown in the experimental protocol in Table 27. Except for the controls, rabbits used in experiment A were of the three treatment groups in Chapter 1. Animals were challenged initially with larvae of R. e. evertsi and subsequently with adult R. appendiculatus and A. variegatum. Rabbits in groups B, C, and D were similarly sensitized but with a slight modification. The modification involved the use of 20 adult ticks (50:50 sex ratio) to challenge hosts repeatedly three times. During each sensitization, tick infestation was allowed to run to completion and hosts were given 7 days tick-free period before a subsequent sensitizing load was applied. Rabbits in experiment E were those sensitized five times with 100, 500 and 1500 R. e. evertsi larvae in Chapter 2.

Appropriate numbers of homologous and heterologous tick species as shown in Table 27 were counted and applied to rabbit ears as described previously. Tick challenges were performed 7 days following completion of the last sensitization with R. e. evertsi. Only single infestations were performed.

As the engorged ticks dropped from the hosts, they were collected, counted and weighed. Adult ticks were weighed individually while larvae were weighed in groups. Parameters used to assess possible existence of cross resistance between R. e. evertsi and the other two tick species included percent engorged ticks, mean engorged weights, feeding duration, percent of instars moulting and fecundity of females. Agar gel double diffusion technique as described previously, was used to detect activity between antigens derived from the different tick species and immune serum from rabbits

TABLE 27

NUMBER OF HETEROLOGOUS OR HOMOLOGOUS TICKS APPLIED ON RABBITS EXPERIMENTALLY SENSITIZED WITH *R. E. EVERTSI* ADULTS

Group number	Number of rabbits	Immune status	Number of homologous or heterologous ticks applied per tick
Experiment A			
I	8	Resistant	100 *R.e. evertsi* larvae/ 20 *R. appendiculatus* adults/ 8 *A. variegatum* adults
II	3	Naive	100 *R.e. evertsi* larvae
III	3	Naive	20 *R. appendiculatus* adults
IV	3	Naive	8 *A. variegatum* adults
Experiment B			
I	5	Resistant	100 *R. appendiculatus* larvae
II	4	Naive	100 *R. appendiculatus* larvae
Experiment C			
I	4	Resistant	100 *R. appendiculatus* nymphs
II	4	Naive	100 *R. appendiculatus* nymphs
Experiment D			
I	3	Resistant	100 *A. variegatum* larvae
II	3	Naiva	100 *A. variegatum* larvae
Experiment E			
I	8	Resistant	20 *R.e. evertsi* adults
II	3	Naive	20 *R.e. evertsi* adults

infested with adult R. e. evertsi. The antigens were salivary gland extracts prepared from the glands of adult R. e. evertsi, and R. appendiculatus and larval homogenates of A. variegatum as previously described. Salivary gland antigens of both R. appendiculatus and R.e. evertsi were fractionated in Sephacryl S-300 column chromatography as described in the gel filtration technical manual (Pharmacia Fine Chemicals, Sweden). The eluates obtained were tested for antigenic activity as as in the agar gel doube diffusion technique.

RESULTS

Feeding and moulting performance of 100 R. e. evertsi instars on rabbits made resistant to the adults

Rabbits previously exposed to adult R. e. evertsi adversely affected both the number and engorged weights of nymphs which emerged from applied larvae (Table 28). The mean nymphal engorged weights was 4.92 mg and this was significantly different from those of the control animals (15.62 mg) ($P < 0.001$). These hosts allowed cumulatively 7.43% of the fed larvae to emerge as nymphs as compared to 59.33% nymphal yield observed in naive controls. Moulting percent of 75 and 92 was observed for nymphs engorged on resistant and naive hosts respectively.

Feeding performance of female R. appendiculatus on rabbits resistant to R. e. evertsi

Approximately all female R. appendiculatus applied on rabbits sensitized with different numbers of adult R. e. evertsi fed to

TABLE 28

FEEDING AND MOULTING PERFORMANCE OF 100 R. E. EVERTSI INSTARS ON RABBITS RESISTANT TO ADULTS OF THE SAME TICK

Rabbit number	Immune status	Number of replete nymphs	Mean engorgement weight (mg ± s.d)	Nymphs moulting (No.)	Sexes	
					Males	Females
R 10	Resistant	1	5.2	1	1	-
R 40	Resistant	14	3.12 ± 2.25	10	7	3
R 60	Resistant	26	5.18 ± 3.29	21	15	6
R 80	Resistant	11	6.19 ± 4.02	7	2	5
R 70	Resistant	0	-	-	-	-
R 30	Resistant	0	-	-	-	-
R 91	Resistant	0	-	-	-	-
Total		52(7.43%)	4.92 ± 1.29*(31.5%)**	39(75%)	25	14
C_1	Naive	80	14.36 ± 4.04	70	27	43
C_2	Naive	55	15.91 ± 3.71	55	27	28
C_3	Naive	43	16.60 ± 3.45	40	17	23
Total		178(59.33%)	15.62 ± 1.15*(100%)**	165(92%)	71	94

*Group mean ± standard deviation **Engorged weights expressed as percentage of control

repletion with mean engorged weights ranging from 197.91 to 268.48 mg compared to 394.17 mg for the controls (Table 29). Analysis of variance showed that ticks fed on resistant rabbits were lighter ($P < 0.005$) than those fed on naive hosts. However, ticks fed on rabbit group sensitized with 20 adult R. e. evertsi fed to slightly smaller weights than those observed for the remaining two groups sensitized with 50 and 100 R. e. evertsi. Although the percent fecund females observed between ticks fed on sensitized and naive hosts were similar, about 100% (Table 29), egg masses laid differed significantly ($P<0.005$). Mean egg weights laid by ticks fed on rabbits sensitized with 20, 50 and 100 R. e. evertsi were 103.43, 142.67 and 134.4 mg respectively while the controls laid eggs weighing 207.48 mg.

Feeding performance of female Amblyomma variegatum on rabbits resistant to R. e. evertsi

The group mean engorged weight of female A. variegatum fed on rabbits under 20, 50 and 100 adult R. e. evertsi sensitization regimens were 374.1, 874.6 and 987.38 mg respectively and significantly lower ($P<0.005$) than 2437 mg observed on susceptible controls (Table 30). Engorgement weights were lower for ticks fed on rabbit group sensitized with 20 adult R. e. evertsi than with either of the two remaining tick doses used ($P<0.05$). Mean engorged weights of ticks fed on rabbit group sensitized with 50 adult R. e. evertsi was smaller than standard deviation. The mean was approximately equal to standard deviation for ticks fed on rabbits sensitized with

TABLE 29

FEEDING AND REPRODUCTIVE PERFORMANCE OF FEMALE *R. APPENDICULATUS* ON RABBITS RESISTANT TO ADULT *R. E. EVERTSI*

Immune status (No. animals)	*R. e. evertsi* sensitizing dose/rabbit	Mean feeding period (days)	% repletion	Mean engorgement weight (mg)	% fecund females	egg weight (mg)
Resistant (2)	20	5.05 ± 0.85*	95	197.91 ± 93.43 (50%)**	100	103.43 ± 59 (50)**
Resistant (3)	50	6.24 ± 0.87	96.7	268.48 ± 120.19 (68%)	100	142.67 ± 17.60 (69%)
Resistant (3)	100	6.50 ± 1.38	100	247.44 ± 116.26 (63%)	93.3	134.40 ± 68.48 (65%)
Naive (3)	-	5.47 ± 1.31	96.7	394.17 ± 58.56 (100%)	96.60	207.48 ± 39.3 (100%)

* Mean ± standard deviation

** Weights expressed as percent of the control; 20 ticks applied per rabbit

TABLE 30

FEEDING AND REPRODUCTIVE PERFORMANCE OF FEMALE *A. VARIEGATUM* ON RABBITS RESISTANT TO *R. E. EVERTSI* ADULTS

Immune status (No. animals)	*R. e. evertsi* sensitizing dose/rabbit	Mean feeding period (days)	% repletion	Mean engorgement weight (mg)	% fecund females	egg weight (mg)
Resistant (2)	20	15.88 ± 3.52*	100	374. 1 ± 146.70 (15%)**	37.5	115.03 ± 10.31 (14%)**
Resistant (3)	50	19.50 ± 5.81	100	874. 6 ± 903.30 (36%)	33.3	601.33 ± 500.37 (77%)
Resistant 3	100	15.60 ± 3.13	83.80	987.38 ± 770.06 (41%)	50	407.22 ± 333.35 (52%)
Naive (3)	-	15.33 ± 2.10	100	2437 ± 917.8 (100%)	83.33	777.66 ± 313.10 (100%)

* Mean ± standard deviation

** weights expressed as percent of the control, eight ticks applied per rabbit

100 adult R. e. evertsi. The number of fecund females and their egg laying capacity were drastically reduced on resistant rabbits. Ticks fed on rabbits sensitized with 20, 50 and 100 R. e. evertsi laid mean egg masses of 115.03, 601.33 and and 407.22 mg respectively while those fed on controls laid 778 mg.

Feeding and moulting performance of 100 R. appendiculatus larvae parasitizing rabbits sensitized with adult R. e. evertsi

Table 31 shows that R. appendiculatus larvae fed on rabbits previously exposed to adult R. e. evertsi had a mean engorged weights of 0.37 mg, which was significantly smaller ($P < 0.05$) than 0.54 mg observed for larvae fed on naive controls. Percent mortalities of 43.4 and 16.5 respectively were observed in larvae fed on resistant and naive groups of rabbits. Rabbits previously exposed to R. e. evertsi induced moult failure of about 10.95% of engorged larvae. This contrasted with a similar figure of 2.4% on naive hosts. None of the larvae fed on resistant hosts completed moulting while 97.6% of larvae fed on naive control rabbits made successful moults.

Feeding performance of 100 R. appendiculatus nymphs on rabbits resistant to R. e. evertsi

Rabbits made resistant to R. e. evertsi allowed 60.3% R. appendiculatus nymphs to feed to repletion compared to 92.3% nymphs observed on controls (Table 32). The mean engorged weights of nymphs were smaller than 9.08 mg of nymphs fed on naive rabbits ($P < 0.005$).

TABLE 31

FEEDING AND MOULTING PERFORMANCE OF 100 R. APPENDICULATUS LARVAE ON RABBITS RESISTANT TO R. E. EVERTSI ADULTS AND NAIVE CONTROLS

Rabbit group (No. animals)	Immune status	Mean feeding period	% repletion	% mortality	Mean engorgement weight (mg ± s.d.)	% moulting *			
						Failure	Partial		Complete
							dead	live	
I (5)	Resistant	4.44 ±0.53	56.6 (283)/500	43.4 (217)/500	0.37 ±0.11 (68.5%)**	10.95 (31)/(283)	6.01 (17)/(283)	83.04 (235)/(283)	0
II (4)	Naive	5.00 ±1.26	83.5 (334)/(400)	16.5 (66)/(400)	0.54 ±0.02 (100%)**	2.4 (8)/(334)	0	0	97.6 (326)/(334)

( ) number of replete larvae/total larvae applied

* number of nymphs affected/total engorged larvae

** engorged weights expressed as percentage of control

TABLE 32

FEEDING PERFORMANCE OF 100 R. APPENDICULATUS NYMPHS ON RABBITS RESISTANT TO R. E. EVERTSI

Rabbit number	Immune status	Number of replete nymphs	Mean engorgement weight (mg)
R 10	Resistant	70	3.01
R 30	Resistant	20	1.93
R 40	Resistant	92	6.25
R 91	Resistant	50	3.76
Total		241(60.3%)	3.74 ± 1.84*
C_1	Naive	98	9.26
C_2	Naive	92	8.64
C_3	Naive	84	9.12
C_4	Naive	95	9.28
Total		369(92.3%)	9.08 ± 0.30*

* Group mean ± standard deviation

Feeding performance of 100 Amblyomma variegatum larvae on rabbits resistant to R. e. evertsi

The mean engorged weights of A. variegatum larvae fed on resistant and naive rabbits did not differ significantly. They were 2.44 and 2.5 mg respectively (Table 33). Failure to moult was evident in larvae fed on resistant hosts. Some larvae initiated moults but failed to cast away larval cuticle.

Feeding performance of 10 female R. e. evertsi on rabbits sensitized with larvae-nymphae stages of the tick species

Rabbits repeatedly infested with 100 larvae allowed 97% of adult R. e. evertsi females to engorge to repletion (Table 34). Similar challenge with higher numbers of larvae (500 and 1500) allowed about 70% of applied female to engorge successfully. Although the majority of adult ticks fed to repletion, their engorgement weights were significantly ($P<0.05$) reduced compared to those of the control animals.

Immunological reaction among antigens from R. e. evertsi, R. appendiculatus and A. variegatum

Double diffusion analysis confirmed that anti-R. e. evertsi serum arising in rabbits after tick feeding was able to form a precipitin band with antigens from A. variegatum (Fig. 27) and R. appendiculatus (Fig. 28). Two precipitin lines were formed between the antisera and the homologous R. e. evertsi antigens, while one

TABLE 33

FEEDING AND MOULTING PERFORMANCE OF 100 A. VARIEGATUM LARVAE ON RABBITS RESISTANT TO R. E. EVERTSI ADULTS

Rabbit group (No. animals)	Immune status	% repletion	Mean feeding period (days)	Mean engorgement weight (mg)	% Moulting		
					Complete	Partial***	Failure
I (3)	Resistant	70.7	8.14 ± 1.41*	2.44 ± 0.44 (98%)**	68	32	10.38
II (3)	Naive	72	8.50 ± 1.12	2.50 ± 0.32 (100%)	90.21	9.79	10.19

* Mean ± standard deviation

** Engorged weights expressed as a percentage of the control

*** Moulting was initiated but nymphs failed to emerge from larval cuticle

TABLE 34

FEEDING PERFORMANCE OF ADULT R. E. EVERTSI ON HOSTS SENSITIZED TO THE LARVAE-NYMPHAE OF THE SAME TICK SPECIES

Rabbit group (No. animals)	Larval sensitizing dose	% repletion	Mean engorgement weight (mg)
I (3)	100	97 (29)* (30)	641.71 ± 184.87
II (3)	500	73 (22) (30)	600.23 ± 259.46
III (2)	1500	65 (13) (20)	559.28 ± 241.55
IV (3)	0	87 (26) (30)	936.17 ± 187.81

* Number of replete females/total female applied

FIGURE 27

Recognition of R. e. evertsi and A. variegatum antigens with R. e. evertsi infested rabbit serum in immunodiffusion. Centre well contains R. e. evertsi infested rabbit serum. Wells 1 and 2, A. variegatum fed larval homogenate; wells 3 and 6 respectively contain fractions 2 and 3 of R. e. evertsi SGA eluted by Sephacryl S-300 column chromatography (Chapter 8). Wells 4 and 5 contain PBS.

FIGURE 28

Recognition of antigenic fractions of R. e. evertsi and R. appendiculatus SGA eluted by Sephacryl S-300 column chromatography (peripheral wells) with R. e. evertsi infested rabbit serum (centre well). Well 1, R. e. evertsi SGA fraction 2; well 2, R. appendiculatus SGA fraction 2; wells 3, 6, R. e. evertsi SGA fraction 3; wells 4, 5 PBS. The immunogenic fractions were prepared as given in Chapter 8.

line was observed with each heterologous antigen from A. variegatum and R. appendiculatus. One precipitin line (Figs. 27 and 28) fully fused with another band composed of antibody reacting with antigens of the three tick species. The common antigens shared between R. e. evertsi and R. appendiculatus were contained in 2 of the 5 R. e. evertsi SGA fractions eluted by column chromatography.

DISCUSSION

Although some ixodid ticks seem to show common antigenicity (Trager 1939a,b; McTier et al., 1981; Brown and Askenase, 1983; Brown et al., 1984) protection is in general, species specific. This was evident from the present study where tick loss, deaths and reduced feeding were more pronounced for R. e. evertsi than either R. appendiculatus and A. variegatum instars. The number of R. e. evertsi instars engorging successfully on resistant hosts was 7.43% (Table 28) while it was 60.3% (Table 32) and 70.7% (Table 30) for the instars of R. appendiculatus and A. variegatum respectively. The larval engorged weights (as percentage of control) were 31.5, 68.5 and 98% respectively for R. e. evertsi, R. appendiculatus and A. variegatum (Tables 28, 31 and 33). The adverse effects of resistance were more severe for R. appendiculatus than A. variegatum instars, indicating that the heterologous resistance was stronger for ticks of the same than of different genus.

Published studies on ixodid cross resistance have reported on tick feeding ability, deaths and depressed moulting success

(Brown and Knapp, 1981; McTier et al., 1981; Brown et al., 1984). In this study, R. appendiculatus and A. variegatum larvae that fed on rabbits sensitized with adult R. e. evertsi initiated moults but the nymphs failed to emerge from larval cuticle. The absence of such moults, together with heavy loss of R. e. evertsi instars, compared to the other two tick species is a further indication that R. e. evertsi- induced resistance may be more effective in regulating physiological process of the species than other tick species.

Resistance stimulated by adult R. e. evertsi, assessed in terms of percent reduction in engorged weights was stronger for females of A. variegatum than R. appendiculatus (Tables 29 and 30). Reduction in engorged weights ranged from 50-68% for R. appendiculatus and 15.4 to 41% for A. variegatum. A similar trend was observed in the egg masses oviposited. This finding contrasts with that by Norval (1975, 1978) that, ticks which have long mouth parts, for example Amblyomma species can penetrate deeply into the host skin and appear to be less vulnerable to host resistance mechanisms than the "shallow" feeders such as Rhipicephalus ticks which have short mouth parts. In this study, the feeding period of A. variegatum was about 50% longer than that of adult R. appendiculatus, suggesting that the long mouth parts may have assisted in securing firm anchorage in the host skin to ensure adequate feeding. It has been reported that resistance to tick infestation may be acquired as early as the first week after initial exposure (Boese, 1974; Brossard, et al., 1982). Hence the 2 week feeding period of A. variegatum may have stimulated specific

resistance in the hosts, unlike the 7 day feeding period of R. appendiculatus. It is suggested that specific resistance possibly provoked by A. variegatum may have acted synergistically with that elicited by R. e. evertsi to suppress the feeding and reproductive performance of the species.

Rabbit sensitized with larvae-nymphs of R. e. evertsi developed resistance which failed to prevent adult ticks from attaching and feeding to repletion (Table 34). Although there was a reduction in engorgement weights, it was not significantly (P 0.05) different from that of ticks fed on rabbits made resistant by adult ticks (Chapter 1). Brown et al. (1984) observed the same feature with A. americanum and attributed it to a dual mechanism existing in the two life stages of the tick and responsible for priming host immune system. Therefore one mechanism operates to prevent tick attachment and the other interfers with assimilation of blood for ticks already attached. While accepting this finding, the observation that the adults of R. e. evertsi immunosuppress hosts while feeding (Chapter 4) cannot be ruled out since Wikel (1982, 1985) has shown the phenomenon to facilitate ectoparasite feeding on already resistant hosts.

Double diffusion assays performed to compare the heterologous antigen preparations showed that these antigens can be recognized by antibodies produced in rabbits infested with adult R. e. evertsi. The antibody preparation formed 2 precipitin lines with R. e. evertsi antigens and one line with each antigen from R. appendiculatus and A. variegatum. Each of the R. appendiculatus and A. variegatum bands

fused fully with one of the 3 bands formed between R. e. evertsi SGA and the immune serum. It is suggested that the resistance observed between R. e. evertsi on one hand and R. appendiculatus and A. variegatum on the other, is due to some shared antigenic determinants.

SUMMARY

A study was carried out to investigate cross resistance between R. e. evertsi, R. appendiculatus and A. variegatum. Rabbits sensitized with adult R. e. evertsi were challenged with the life stages of R. appendiculatus and A. variegatum. Similarly rabbits sensitized with R. e. evertsi instars were challenged with the adults. The results indicated that sensitization with adult R. e. evertsi conferred strong protection (homologous) against the instars of the same species and some cross protection (heterologous) against R. appendiculatus and A. variegatum,. Sensitization with R. e. evertsi instars conferred weak cross protection (homologous) against adult of same species, suggesting the resistance to be unidirectional in nature. The common antigenicity of the three tick species as a possible cause of cross protection was investigated.

CHAPTER 6

THE DEVELOPMENT OF DELEAYED TYPE HYPERSENSITIVITY TO HOMOLOGOUS AND HETEROLOGOUS ANTIGENS IN RABBITS INFESTED WITH RHIPICEPHALUS EVERTSI EVERTSI

INTRODUCTION

Many manifestations of cell-mediated immune responses (CMI), for example graft rejection, host versus parasite and delayed type hypersensitivity (DTH) are allergic inflammatory reactions, mediated primarily by T cells (Valdimarsson, 1978). Primed T cells, on recognition of specific antigens, produce lymphokines (Valdimarsson, 1978). These lymphokines are thought to amplify the reaction to the level of expression, by nonspecific recruitment of circulating lymphocytes and monocytes. Several investigators have demonstrated the significance of CMI which is stimulated by different ixodid tick species (Bagnall, 1975; Wikel et al., 1978; Brown, 1982; Wikel and Osburn, 1982; Wikel, 1985). Studies by these workers indicated that ticks feeding on resistant hosts may suffer injurious effects with subsequent rejection. On the other hand such ticks can adopt strategies to thwart, subvert or coexist with the host immune response. Wikel and Osburn (1982) reported that T cells from hosts infested with the paralysis tick, D. andersoni, responded poorly to mitogenic stimulation, suggesting a weakened CMI. The present study was designed to evaluate possible influence of tick feeding on the development of DTH in rabbits sensitized by R.e. evertsi.

MATERIALS AND METHODS

The design of the experiment

Twenty one adult New Zealand White rabbits, males and females, 6-8 months old, weighing approximately 2.5 kg were reared under the conditions of the experiment as described in Chapter 1. They were divided into seven groups of three rabbits each (Table 35). Three groups were derived from animals used as reported in Chapter 1 and comprised animals, previously sensitized four times with 20, 50 and 100 adult R. e. evertsi. These rabbits were inoculated intradermally with tick SGA, 30 days after completion of the last tick challenge to test the ability of the animals to respond immunologically to the antigens. Of the remaining four groups, one group contained animals previously infested once with 20 adult ticks while animals in the other three groups were naive. Rabbits in the latter four groups were inoculated intramuscularly (IM) with 2 ml of 1% SRBC. A week after inoculation with SRBC, three of the four rabbit groups were challenged with 20 or 60 adult R. e. evertsi as shown in Table 35 and the skin test was performed simultaneously using SRBC. The other group acted as an uninfested control.

Skin test procedure

The procedure for the skin test was as follows: The skin on the thoracic wall of the rabbits was shaved with a clipper. All 9 rabbits that had been sensitized 4 times in succession with different numbers of adult R. e. evertsi were inoculated intradermally at 4 different sites on the shaved skin with 0.1 ml SGA (about 0.38 mg protein) as shown in the experimental protocol in table 35. The

TABLE 35

THE DESIGN OF AN EXPERIMENT TO INVESTIGATE EFFECTS OF TICK INFESTATION ON DEVELOPMENT OF DELAYED TYPE HYPERSENSITIVITY IN RABBITS TO SRBC AND SGA

Skin test procedure (3 animals/group)	Host status	Number of ticks applied per rabbit
SGA	Resistant - four repeated exposures to 20 ticks	-
SGA	Resistant - four repeated exposures to 50 ticks	-
SGA	Resistant - four repeated exposure to 100 ticks	-
SRBC	Resistant - a single exposure to 20 ticks	20
SRBC	Naive	20
SRBC	Naive	60
SRBC	Naive	0

remaining 12 rabbits were similarly inoculated with 0.1 ml, 1% SRBC. Four other shaved sites on each rabbit were inoculated with 0.1 ml PBS and served as controls. The increase in skin thickness was measured with a pair of callipers before injection with the antigens and daily thereafter for 11 days. A mean value for each rabbit group was calculated and plotted against the period of observation.

Tick feeding performance

The feeding performance of ticks on hosts was examined. For rabbits that were sensitized 4 times in succession with different numbers of ticks, the proportion of ticks which died *in situ* or were rejected by the host while still attached to the bite site was calculated in relation to all ticks applied that failed to feed. For the remaining four rabbit groups, mean engorged weights of the ticks were calculated. Similarly, their maximum skin response to SRBC was calculated as a percent of that observed on the uninfested control rabbits (taken as 100%).

Histopathology

Feeding sites with ticks *in situ* were excised from the ears of rabbits. Most of these sites were parasitized by ticks that were observed to feed poorly between day 3 and 6 of the infestation. Biopsies of skin nodules at 72 hours after intradermal inoculation of *R. e. evertsi* SGA were similarly taken, including skin from ears of naive rabbits, not infested with ticks and inoculated with 0.1 ml, PBS (control). Excised tissues were fixed in 10% formalin for 72 hours, dehydrated, cleared in xylene, embedded in paraffin wax and serially sectioned at 6 um thickness on a rotory microtome. Sections

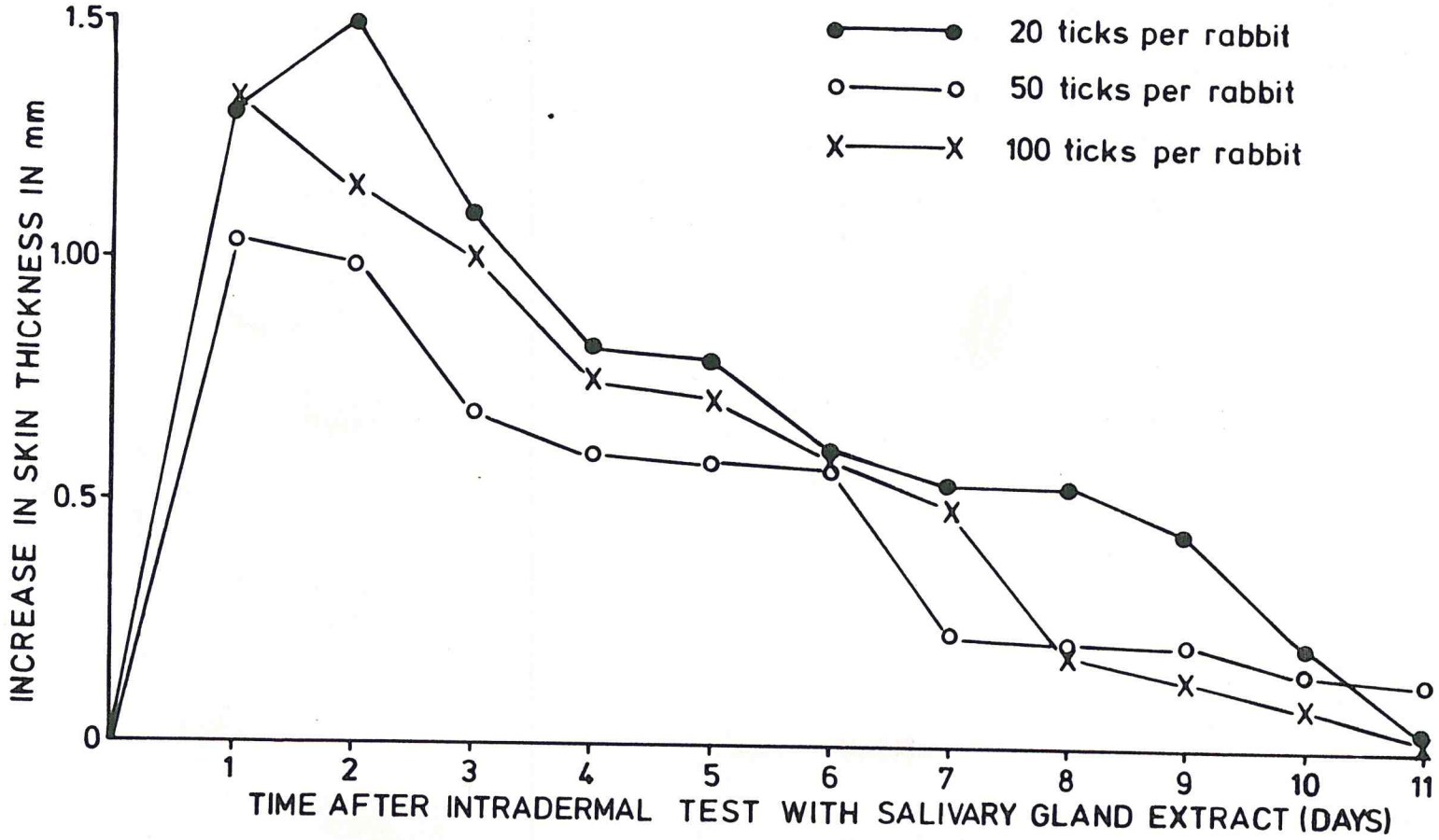
were stained with haematoxylin and eosin (H&E) according to the technique of Lillie (1954). Microscopic assessment of tick-induced pathology was performed on the lesions which were in the form of dermal cellular infiltrate.

RESULTS

Skin reactivity to antigens

As shown in Fig. 29, maximum DTH responses to SGA demonstrated in 50 and 100 tick challenged groups were smaller than observed in 20 tick challenged rabbits. These responses in terms of increased skin thickness were 1, 1.3 and 1.5 mm, for 50, 100 and 20 tick challenged rabbits respectively.

The pattern of development of DTH to SRBC in hosts simultaneously infested with R. e. evertsi was similar to that described for SGA (Fig. 30). The skin reactivity of tick-infested hosts to inoculation with SRBC were significantly reduced compared to uninfested control rabbits (Table 36, Fig. 30). Peak dermal responses, expressed as a percentage of the control, were 58, 33 and 17% respectively for tick resistant rabbits concurrently infested with 20 ticks, naive rabbits infested with 20 and 60 ticks. Naive rabbits infested with 60 ticks developed the poorest cutaneous response. This was abolished by day 6 of tick feeding which was the period of mating and active feeding. The DTH response curves for most of the rabbit groups had single peaks and were suppressed significantly by tick infestation.


20 ticks per rabbit
50 ticks per rabbit
100 ticks per rabbit
INCREASE IN SKIN THICKNESS IN mm
1.5
1.00
0.5
0
1
2
3
4
5
6
7
8
9
10
11
TIME AFTER INTRADERMAL TEST WITH SALIVARY GLAND EXTRACT (DAYS)

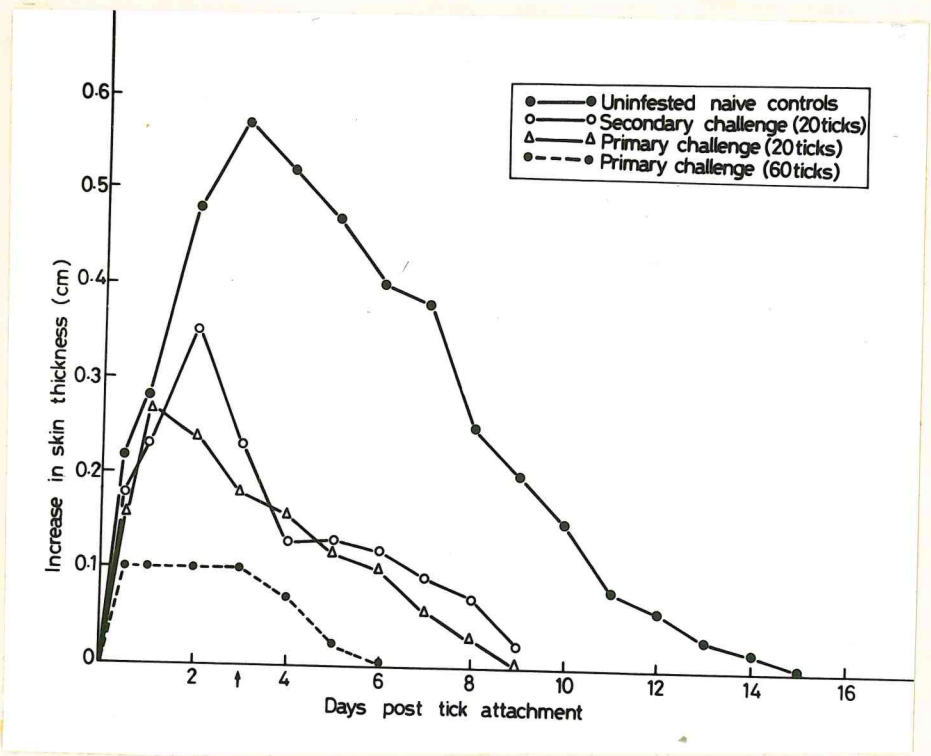

Uninfested naive controls
Secondary challenge (20 ticks)
Primary challenge (20 ticks)
Primary challenge (60 ticks)
Increase in skin thickness (cm)
0
0·1
0·2
0·3
0·4
0·5
0·6
2
4
6
8
10
12
14
16
Days post tick attachment

TABLE 36

RELATIONSHIP BETWEEN DERMAL RESPONSE OF RABBITS INFESTED WITH ADULT R. E. EVERTSI AND INOCULATED WITH SRBC AND TICK WEIGHTS AFTER FEEDING

Rabbit group (No. of rabbits)	Host status	Number of ticks applied	% repletion	Mean engorged weight (mg)	Range of feeding period (days)	Maximum dermal response* (%)
A(3)	resistant	20	80	348.48 ± 128.42	5 - 8	58
B(3)	naive	20	100	594.39 ± 132.3	5 - 8	33
C(3)	naive	60	97	777.94 ± 106.09	5 - 8	17
D(3)	naive control	0	0			100

* As % of the control

Rabbits in group A were previously sensitized with 20 ticks

Relationship between DTH response to SRBC and tick engorged weights

The maximum skin reactivity of tick resistant rabbits later exposed to 20 adult ticks was 58% of uninfested control (taken as 100%) (Table 36) and the ticks had mean engorged weight of 348.48 mg. A group of naive rabbits exposed to 60 ticks had poor cutaneous response to SRBC being 17% of the control, and ticks fed to a mean engorged weight of 777.94 mg.

Tick feeding period

The feeding period of ticks ranged from 5 ± 8 days (Table 36).

Factors affecting ticks feeding on resistant hosts

Factors controlling the number of ticks which fed to repletion during repeated tick challenges are analysed in Table 37. Forty percent of the ticks fed on hosts repeatedly sensitized by 20 ticks died *in situ*. A further 23.38% of the ticks were walled off and rejected with their feeding sites . There was no mortality *in situ* observed for the rabbit group challenged with 50 ticks although they rejected a total of 4.60% of all female ticks applied. Inspite of the similarity in the percent of ticks rejected by the 50 and 100 tick challenged rabbit groups, 25% died *in situ* in the latter group. More ticks failed to attach on rabbits challenged with 50 and 100 ticks compared to the 20 tick challenge rabbits.

Histopathological study

Histopathological examination of skin nodules taken 72 hours after tick attachment or following inoculation of SGA showed

TABLE 37

PERCENT SUCCESS OR FAILURE TO FEED OF FEMALE R. E. EVERTSI DURING SUCCESSIVE INFESTATION OF RABBITS WITH DIFFERENT NUMBERS OF THE TICK

Rabbit group (No. animals)	Sensitizing tick dose (No.)	Number of ticks observed*	Effects on ticks		
			dead in situ	rejected with host tissue	failed to attach
I(3)	20	30	40	23.40	36.60
II(3)	50	65	0	4.60	95.40
III(3)	100	96	25	4.20	70.80

* Cumulative observations in four repeated infestations

mononuclear cell infiltration of the dermis and subcutis.

DISCUSSION

In the present study, rabbits that completed four successive infestations with 20, 50 and 100 adult R.e. evertsi and skin tested with homologous tick SGA displayed DTH reactions (Fig. 29). Similarly, natural and laboratory hosts, previously infested with ticks develop DTH when subsequently challenged with homologous antigens (Wikel et al., 1978). The inflammatory cutaneous responses are responsible for lethal effects and rejection of ticks parasitizing resistant hosts (Wikel 1979, Brown et al., 1982). Therefore, they are important in regulating ixodid tick populations on hosts.

Rabbits, challenged with 50 ticks displayed the poorest cutaneous response reaching a peak smaller ($P < 0.05$) than that observed for the 20 tick challenge group which was highest of all the three rabbit groups tested. The peak DTH response of the 100 tick challenge group was not significantly higher than that in the 50 tick infested group. The different levels of DTH response observed among the three rabbit groups indicated a tick-load dependent immunosuppression. The cause of the low DTH reactions in rabbits challenged with 50 and 100 ticks was not determined in this experiment. However, impaired T-lymphocyte function in tick-infested hosts has been reported (Wikel, 1982c, 1985). Ribeiro et al. (1985) demonstrated a prostaglandin in the saliva of I. dammini which inhibits the activity of T cells and therefore provide some evidence

that ticks suppress DTH responses.

Development of DTH to a heterologous antigen, SRBC, was impaired during the course of tick feeding (Fig. 30). The degree of impaired development was influenced by the resistance status and numbers of ticks feeding on the host. Thus, the response was severely reduced or abolished in naive hosts infested with 60 ticks.

Suppression of DTH to SGA in 50 and 100 tick-challenged hosts suggest involvement of mechanisms other than stress. This hypothesis was based on the fact that, skin tests performed one month after infestations were completed on hosts repeatedly infested with ticks, showed yet a reduced response to the antigens.

Acquired resistance to ticks is associated with high mortalities for the immatures (Brown and Knapp, 1981) and significant rejection of adults (Brown and Askenase, 1983,). Brown et al. (1984) reported that these effects exerted on the tick by the host are T cell dependent. The results in Table 37 show that resistance induced by the 20 tick challenge regimen caused more deaths of ticks in situ compared to that stimulated by 50 and 100 tick challenges. However, of the ticks that were applied on the 50 and 100 tick-challenged hosts, most failed to attach and feed. Histopathological studies of the epidermal layer of the skin where a few ticks that attached were feeding demonstrated accumulations of inflammatory cells, predominantly mononuclear cells. From these results, it was postulated that the skin trauma caused by high tick challenges was one of the factors deterring ticks from feeding.

The fate of ticks deterred from feeding in the field has not been intensively examined. In this study such ticks fed satisfactorily on naive hosts within 48 hours of being rejected (Chapter 1). Resistance initially stimulated by low repeated tick challenges apparently contributed significantly towards killing of the tick population (Table 37). However, the observation made in Chapter 1 and by Wikel and Osburn (1982) that low level repeated infestations immunosuppressed hosts indicates that such resistance does not kill ticks. Ribeiro et al. (1985) provided evidence that tick-infested hosts that manifest weak DTH reactions fail to reject ticks. Therefore immunosuppressed and naive hosts in a herd may perpetuate the rejected ticks.

SUMMARY

Salivary gland antigens from adult R. e. evertsi elicited antigen-specific delayed type hypersensitivity (DTH) in groups of rabbits previously infested repeatedly with 20, 50 and 100 adult ticks. The highest cutaneous responsiveness to the antigen was observed in the 20 tick challenge group and the lowest in the 50 tick challenge group. Development of DTH to sheep red blood cells in groups of rabbits challenged with different numbers of adult R. e. evertsi was retarded and reduced as a result of tick infestation. Reduction was more severe for tick-naive than previously tick-exposed rabbits. Sixty adult ticks, applied on naive rabbits, suppressed and abolished the development of DTH to SRBC. These results suggest that infestations with adult R. e. evertsi affect development of DTH responses to homologous and heterologous antigens.

CHAPTER 7

THE ROLE OF T CELLS IN THE ACQUISITION OF RESISTANCE TO ADULT RHIPICEPHALUS EVERTSI EVERTSI INFESTATIONS IN RABBITS

INTRODUCTION

Thymus derived (T) lymphocytes have been shown to mediate immunologic functions including helper cell activity for antibody production (Gershon and Kondo, 1971) and cell-mediated immune (CMI) responses such as graft rejection and delayed type hypersensitivity (DTH) (Louis et al., 1982). Ticks feeding on hosts that have had prior exposure to these ectoparasites, have been shown to elicit dermal infiltrates rich in different cell types. Basophils have been reported to be abundant in cutaneous reactions to ticks in resistant rabbits (Krinsky et al., 1982), cattle (Allen et al., 1977; Brown et al., 1984) and guinea pigs (Allen, 1973; Brown and Knapp, 1981; Brown and Askenase, 1981). Wikel et al. (1978) reported dominance of lymphocytes in such host responses at the skin lesions. Tick rejection was observed to occur when these cells arrived at the feeding site (Allen, 1973; Wikel and Allen, 1976a; Krinsky et al., 1982). Eosinophils were reported to dominate such responses for guinea pigs infested by fleas (Johnston and Brown, 1985). Brown et al. (1982) suggested that these basophil-eosinophil-lymphocyte rich cutaneous responses are part of T-lymphocyte immune response to ectoparasites. They also showed that basophils and eosinophils were important for effective expression of resistance to ticks.

This study investigated the role of T cells in acquisition of resistance to infestations with R. e. evertsi.

MATERIALS AND METHODS

Rabbits

Six New Zealand White rabbits weighing approximately 2 kg each were used in this study.

Preparation of thymocytes and lymphocytes

Three rabbits three-months old were euthanized and their thymuses, popliteal lymph nodes and spleens were harvested separately. Thymocytes were separated from the thymuses and lymphocytes from the popliteal lymph node and spleen following the method described by Sabolovic et al. (1977) and Kisielowi et al. (1984). Briefly the organs were placed between two strips of fine nylon mesh, in twice their volume in Hanks Balanced Salt Solution (HBSS), containing 100 mg of streptomycin and 100 units of penicillin. The organs were then squeezed by scrapping the upper surface with a syringe plunger in order to release the cells. The thymocytes were separated on Ficoll-paque gradient (Pharmacia Fine Chemicals, Sweden) as interphase cells. The cells were washed three times in HBSS, counted in a haemocytometer chamber and the cell viability was tested with trypan blue (Garvey et al., 1979). Finally,a suspension containing of 2×10^7 cells/ml in HBSS was made. Lymphocytes were similarly separated from lymph nodes and spleen.

Identification of lymphocytes from thymus and popliteal lymph node of normal rabbit

Lymphocytes derived from thymus were detected by immunofluorescent antibody technique and rosette assay as described by Sabolovic et al., (1977). The rosette assay was performed as follows: Two hundred microlitres of the thymocyte cell suspension adjusted to 2 x 10^7 cells per ml in Eagle's minimum essential medium (MEM) was placed in two test tubes. Into one tube 200 µl of 1% SRBC was added while into the second tube a similar amount of rabbit red blood cells was introduced and the contents mixed gently. Fetal calf serum (50 µl) (Flow Labs, UK), heat-inactivated at 56°C, 30 mins and absorbed with rabbit red blood cells was added. The mixture was left to stand at room temperature for ten minutes and then centrifuged at 200 g for another ten minutes. The tubes were then placed into an ice bucket for five minutes before the pellet was resuspended with a pasteur pipette. A sample of the cell suspension was filled into the two chambers of a hemocytometer and 200 thymocytes were counted. A nucleated cell which bound three or more red blood cells was scored as a rosette forming cell (RFC). The procedure was repeated with lymphocytes from the popliteal lymph node.

In the immunological study, live thymocytes and lymphocytes suspended in one ml MEM at the same concentration as shown above were mixed with 2 ml of fluorescein-conjugated mouse anti-rabbit IgG (Cappel Labs. Inc. USA) diluted 1:8. The mixture was incubated overnight at 4°C. The cells were then washed four times in MEM and

200 cells were examined by fluoresence microscopy for the ability to pick up the dye.

Preparation of goat anti-rabbit thymocyte serum (ATS)

Three adult goats were used to raise antiserum to the rabbit thymocytes. These goats were immunized subcutaneously with 2×10^7 thymocytes at four different sites. Two booster immunizations were performed at 2 weekly intervals. The goats were bled two weeks after the last injection and serum was prepared. The serum was absorbed three times with washed, pelleted rabbit red blood and bone marrow cells. The serum was then heat-inactivated ($56^{o}C$, 30 mins.) and stored at $-20^{o}C$ in 20 ml aliquots.

Cytotoxicity of ATS on thymocytes and lymphocytes

The specificity of the ATS was determined by a series of experiments as follows. Thymocytes and lymphocyte cell suspensions were adjusted to 10^7 cells/ml in MEM. Lymphocytes and thymocytes contained in 0.5 ml of the suspension were incubated $37^{o}C$ for 30 mins. with an equal volume of ATS, diluted 1:8. An equal volume of complement (guinea pig serum)(Flow Labs. Rockville, MD, USA) (diluted 1:4) was then added and incubated for another 60 min. Four sets of tubes were used for each lymphocyte and the thymocyte sample in the following manner: lymphocyte + ATS + complement; lymphocyte + ATS + medium; lymphocyte + complement + medium and lymphocytes + medium. The cells were centrifuged at 100 g for 5 min. and resuspended to the original volume in MEM. Cell viability was assessed using trypan blue as described above.

Treatment of rabbits with ATS followed by tick infestation

Three rabbits six months old were each inoculated intravenously (i.v) with ATS at a dose of 2 ml/kg body weight daily for 4 days before and after they were infested with 20 adult ticks (50:50 sex ratio) as described in Chapter one. These rabbits were designated as ATS^{+}. A total of three tick infestations and similar ATS treatments were performed on the ATS^{+} rabbit group after a tick-free period of seven days between infestations. A second set of three rabbits was introduced, inoculated with phosphate buffered saline pH 7.2 (PBS) and infested similarly with the same number of ticks. This rabbit group was designated as ATS^{-} control. During the second and third infestations two such similar controls were introduced and infested once with 20 ticks. Rabbits were bled weekly, serum collected and stored at $-20^{o}C$ until needed.

Antibody response of the rabbits to tick antigens in the course of ATS treatment

The passive haemagglutination test as described in Chapter 1 was used to assess host immune response to tick antigens presented during feeding. The tick SGA used was prepared as outlined in Chapter 1.

Evaluation of DTH

The skin test was performed during the third tick challenge by injecting intradermally 0.1 ml SGA (approx. 0.38 mg protein) into clipped skin sites on the thoracic wall of both tick infested ATS^{+} and ATS^{-} rabbits. Four other sites were inoculated with PBS to serve as controls. The skin thickness was measured with a pair of callipers before and after injection with test antigen. Subsequent

measurements were performed initially after 6 hours and thereafter every 24 hours post injection (pi) for 17 days. The difference in skin thickness between the sites inoculated with SGA and PBS was regarded as the net increase in skin thickness.

Tick feeding and reproductive performance

Three main parameters were used to assess effects of resistance on the adult tick: tick engorged weights, ability to lay eggs (fecundity), ability of the eggs to hatch (fertility) and tick survival 20 days after detachment. These parameters were observed in all ticks placed individually in glass tubes and maintained at 25^{o}C, 85% RH according to Bailey (1960).

RESULTS

Identification of lymphocytes from thymus and popliteal lymph nodes of normal rabbits.

Of the thymocytes examined by immunofluorescence method only 5.5% picked up the dye (positive) as compared to 29% of lymphocytes from the popliteal lymph node (Table 38). Both thymocytes and lymphocytes formed rosettes only with homologous but not heterologous red blood cells. Eighty four percent rosettes were formed with thymocytes and 63.8% with the lymphocytes derived from the popliteal lymph nodes.

Cytotoxicity of ATS on thymocytes and lymphocytes

The results in Table 39 show that ATS killed 97.75% of thymocytes derived from rabbits. Treatment of lymphocytes from

TABLE 38

ROSETTE FORMATION AND ANTIBODY BINDING ABILITY OF LYMPHOCYTES DERIVED FROM RABBIT THYMUS AND LYMPH NODES

Lymphocyte origin	% rosette formation with red blood cells: Rabbit	Sheep	Immunofluorescing Cells %
Thymus	84	0	5.5
Popliteal lymph node	63.8	0	29

TABLE 39

CYTOTOXICITY OF GOAT-ANTI RABBIT THYMOCYCTE SERUM ON LYMPHOCYTE AND THYMOCYCTE VIABILITY (MEAN* % DEAD CELLS).

TREATMENT	SOURCE OF CELLS		
	THYMUS	POPLITEAL LYMPH NODE	SPLEEN
ATS + C'	97.75	72.25	80
ATS + media	8.2	5.25	6.4
C' + media	1.25	6.25	9.7
Media	2.75	8.3	12.5

* Observed on four sample replicas

C' Complement

popliteal lymph node and spleen with ATS killed 72.25% and 80% respectively of the cells from these organs. In the absence of complement, ATS killed less than 10% of the cells derived from the three types of organs.

Antibody response to tick antigens by rabbits treated with ATS

Agglutinating antibodies to SGA were demonstrable in all rabbits during the course of tick infestation (Fig. 31) whether ATS^+ or ATS^-. Antibody activity was not detected during the primary infestation in ATS^+ rabbits but developed after the second exposure compared with the primary exposure for the ATS^- controls. The antibody response of the ATS^+ rabbits was significantly lower than the ATS^- controls. The ATS^- rabbits showed a progressive increase in antibody production with further tick challenge.

Cutaneous response to SGA of rabbits treated with ATS

Circumscribed skin swellings appeared at the test sites inoculated with SGA in both the ATS^+ and ATS^-rabbits, 12 hours after inoculation. Maximum dermal response was observed within 72 hours and differed significantly between the two groups of rabbits (Fig. 32). The peak dermal response was lower ($P<0.05$) for ATS^+ than the ATS^- control rabbits. Detachment of ticks from the hosts started on day 5 and 10 after the period of maximum dermal response in the ATS^- and ATS^+ rabbits respectively. Ticks applied to ATS^+ rabbits completed detachment by day 17 of feeding compared to the 8 on ATS^- rabbits.

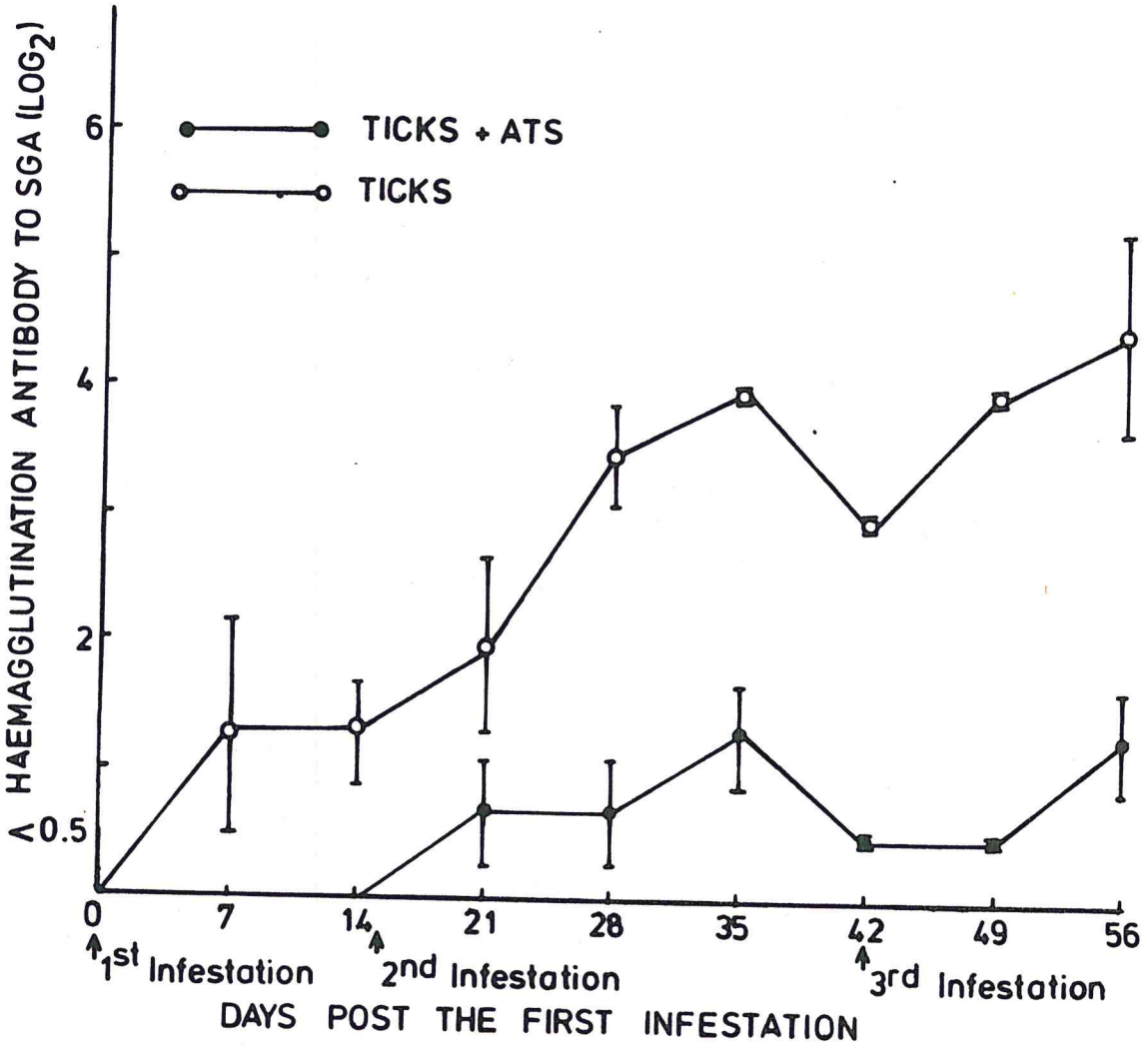

TICKS + ATS
TICKS
HAEMAGGLUTINATION ANTIBODY TO SGA (LOG2)
6
4
2
0.5
0
7
14
21
28
35
42
49
56
1st Infestation
2nd Infestation
3rd Infestation
DAYS POST THE FIRST INFESTATION

FIGURE 32

Development of delayed type hypersensitivity to R. e. evertsi SGA in rabbits inoculated with ATS (Ticks+ATS) including ATS-untreated controls (Ticks) during third repeated challenge with 20 adult ticks of the same species. Each point is a mean increase in thickness(cm) of 12 skin test sites inoculated with SGA above that of control sites inoculated with PBS for each rabbit group (4 test sites per rabbit). Arrows indicate the start and completion of tick detachment from hosts.

Engorgement weights of ticks fed on ATS-treated rabbits

The mean engorgement weights of adult ticks fed on the ATS^+ and ATS^- during the primary infestation were 953.82 and 951.62 mg respectively (Table 40). There was no significant difference between these engorged weights. Reductions in engorged tick weights were observed in both the ATS^+ and ATS^- rabbit groups during the second infestation. During the second infestation, ticks detached replete with mean engorged weights of 223.3 and 435.7 mg respectively for the ATS^- and ATS^+ rabbit groups. Mean engorged weights of ticks fed on ATS^+ hosts during third infestation was 340.3 mg compared to 65.97 mg observed on ATS^- hosts. These weights compared with those of the previous infestations indicate that they were more reduced for ticks fed on ATS^- than ATS^+ hosts. However ticks fed adequately on naive hosts as indicated by mean engorged weights of 921.84 mg and 897.16 mg respectively on the second and third infestation controls.

Tick mortality

During primary tick infestation, 3.33% and 10% of the females fed on ATS^+ and ATS^- rabbits respectively were dead by the fourth week of repletion (Table 41). The mortalities increased with the advance in the frequency of tick challenge in the two groups of rabbits. In general higher mortalities were observed in the ticks fed on ATS^- rabbits.

TABLE 40

MEAN ENGORGEMENT WEIGHTS (MG ± STANDARD DEVIATION) OF ADULT R.E. EVERTSI FED REPEATEDLY ON ATS$^+$ AND ATS$^-$ RABBITS

Rabbit groups[a] Treatments	A ATS$^+$	B ATS$^-$	C Control	D Control
Infestation 1	953.82 ± 137.55* (30)/(30)	951.62 ± 261.60 (30)/(30)	-	-
Infestation 2	435.7 ± 214.03 (20)/(20)	223.3 ± 186.04* (16)/(20)	921.84 ± 104.05** (28)/(30)	-
Infestation 3	340.3 ± 212.22 (18)/(20)	65.97 ± 44.37 (12)/(20)		897.16 ± 201.71* (25)/(30)

Each asterisk represents a dead rabbit after developing paralysis

[a] Three rabbits per group

Numbers in parentheses indicate replete females over total female ticks applied

TABLE 41

PERCENT MORTALITY IN FEMALE R. E. EVERTSI FED REPEATEDLY ON IMMUNOSUPPRESSED (ATS^+) AND CONTROL (ATS^-) RABBITS*

Rabbit group[a]	A	B	C	D
Treatments	ATS^+	ATS^-	Controls	
Infestation 1	3.33	10	ND	ND
Infestation 2	10	25	0	ND
Infestation 3	25	92	ND	3.33

* Cumulative deaths observed for 20 days after tick repletion

[a]Three rabbits per group

ND Not done

Fecundity and fertility of female R. e. evertsi fed on ATS^+ or ATS^- rabbits

During primary tick feeding on the ATS^+ and ATS^- rabbits, comparable egg masses were oviposited by the ticks (Table 42). There was inconsistency in tick egg production between these rabbit groups with repeated infestations. For example, during the second infestation ticks fed on ATS^+ rabbits laid an average of 214.39 mg eggs compared to 102.54 mg for those fed on ATS^- rabbits. Egg hatch failures, were more pronounced for ticks fed on ATS^- rabbits (Table 41). The percent mean egg hatch rate was slightly higher for eggs laid by ticks fed on ATS^+ rabbits.

DISCUSSION

The study showed that, treatments of rabbits with ATS, during each repeated infestation with R. e. evertsi suppressed the immune status of the rabbits to the ticks as reflected by higher tick engorgement weights in the treated compared to the control (Table 40). The mean tick engorged weights was comparable for the two rabbit groups after primary exposure. After the second challenge, the mean engorgement weights for the two host groups were significantly different ($P<0.005$, $F1, 34 = 9.18$), being 435.7 and 223.3 mg respectively for ATS^+ and ATS^- groups. During the third exposure, the mean engorged weights for the ticks was 340.3 and 65.97 mg in the same order as reported above for the treatment groups. The declining trend in tick engorgement weight observed on ATS^+ as opposed to ATS^- hosts, was inconsistent with that reported for other tick species in repeatedly infested hosts (Bowessidjaou et al., 1977; Brossard et al., 1982 and Mongi, 1982). The reduction in

TABLE 42

MEAN WEIGHTS AND PERCENT HATCHING OF EGGS LAID BY R.E. EVERTSI FEMALES FED REPEATEDLY ON IMMUNOSUPPRESSED (ATS^+) AND CONTROL (ATS^-) RABBITS

Infestation number	Treatment (No.animals)	Mean egg weight (mg ± s.d)	Failure to hatch of entire egg mass* %	Egg hatching rate %
1	ATS^+ (3)	501.27 ± 114.23 (28)**	0	72.28
	ATS^- (3)	518.68 ± 140.60 (25)	0	57.46
2	ATS^+ (2)	214.39 ± 135.58 (18)	5.56	81.5
	ATS^- (2)	102.54 ± 110.40 (13)	23.08	60.80
3	ATS^+ (2)	147.15 ± 106.20 (18)	0	74
	ATS^- (2)	30.65 ± 17.12 (7)	0	55.7

* As % of fecund female ticks

** Number of fecund female ticks

engorged weights was more severe for ticks fed on ATS^- than ATS^+ hosts, suggesting that T cells are important in the acquisition of resistance to ticks by hosts.

A delay in the humoral response to tick antigens presented during feeding was observed in the ATS^+ groups (Fig. 31), suggesting the delay to be associated with reduced level of response. Similarly the DTH response was lower in the ATS^+ than ATS^- groups (Fig. 32). Interruption of tick feeding on ATS^- hosts coincided with maximum DTH response while ticks continued to feed during the peak DTH response on ATS^+ rabbits. The data obtained in this study support that by Larsh et al., (1964 a, b); Brossard and Girardin, (1979) and Brown et al., (1982) that specific antibodies and cutaneous reactions to tick SGA are required for effective establishment and expression of resistance to ticks by hosts.

Tick mortalities were more from ATS^- than ATS^+ rabbits within 20 days of detachment (Table 41). R. e. evertsi fed on naive hosts have a preoviposition period of about 5 days (Chapter 1), followed by 20 days of active oviposition (Londt et al., 1977). Brown and Knapp (1981) reported similar mortalities for ticks fed on resistant hosts. Brown (1982) and Willadsen et al., (1979) attributed such mortalities to mediators released by cells of inflammation at the tick feeding sites on hosts. Presence of appropriately sensitized T cells at the tick feeding sites of the hosts is a prerequisite for the mediator release (Valdimarsson, 1978;

Brown and Askenase, 1985a). It is suggested that ectoparasite mortality associated with host resistance was reduced by suppressing T cell activity.

It has been reported that T cells enhance hydrolytic capacity of macrophage enzymes over a variety of substrates:proteins, lipids, polysaccharides and nucleic acids (Valdimarsson, 1978). The broad range of substrates for these host cellular enzymes may suggest also inactivation of tick proteins which are crucial for survival and also nucleic acids.

Rhipicephalus e. evertsi fed initially on both rabbit groups laid approximately similar egg masses, average 500 mg (Table 42). Rechav et al. (1977) reported the same results and associated such a high rate of egg production to the plurality of livestock pathogens transovarially transmitted by the tick. The rate of egg production decreased relative to the observed engorged weights.

Failure to hatch of whole egg mass occurred during the second infestation only in 5.56% and 23.08% of all egg masses laid by ticks fed on ATS^+ and ATS^- host groups respectively. However, most egg batches laid, hatched the percent hatch rate being higher for eggs oviposited by ticks fed on ATS^+ than ATS^- rabbits. Evidence that ticks parasitizing resistant hosts feed poorly and ingest factors detrimental to the formation of eggs and embryogenesis was provided by Bowessidjaou et al. (1977) and Allen and Humphrey (1979). In the present study tick feeding and biotic potential was better on ATS^+

than ATS^- rabbits suggesting that usage of ATS leads to reduction in host factors inimical to feeding and embryogenesis. However, it was not clear from this study why the failure to hatch of some egg batches was observed only during the second challenge both rabbit groups.

Humoral and DTH responses to tick SGA expressed in ATS^+ rabbit group, suggest together with others (Redelman et al., 1976; Ratajczak et al., 1979) that the antithymocyte sera presently used did not deplete all T lymphocytes from blood circulation. Larsh and Weatherly (1975) and McConnell (1978) reported that T cells which escape the effects of immunosuppressive sera and proliferated in response to an antigen, are resistant to further actions from such sera and may express the partial resistance observed in the ATS^+ group. Furthermore, repeated use of immunosuppressive sera for example antilyphocytic serum (ALS) was shown to elicit an immunologic response to heterologous ALS antibodies and a concomitant rise in blood lymphocyte population (Taub and Deutsch, 1977). Such an immunological response to heterologous ATS antibody may weaken its immunosuppressive potential and result in failure to abrogate host resistance to ticks.

SUMMARY

Rabbits treated with goat-anti rabbit thymocyte serum (ATS), during each repeated challenge with adult R. e. evertsi, acquired inferior resistance to the ectoparasite, compared to that of untreated controls. The delayed type hypersensitivity (DTH) and antibody mediated responses to tick antigens presented during feeding were significantly depressed in the ATS-treated hosts. In the subsequent exposures, feeding and reproductive fecundity were less affected for ticks fed on ATS-treated than those on ATS-untreated controls. The ability to reject ticks in ATS-treated rabbits was abolished and a prolonged tick feeding period was observed. This finding highlights the importance of host T cells in mediating immune resistance to the tick species. The failure to detect significant humoral antibodies to the tick antigens in ATS-treated hosts suggests such antigens to be mainly T cell dependent.

CHAPTER 8

IDENTIFICATION OF ANTIGENS IN SALIVARY GLAND EXTRACTS OF ADULT R. E. EVERTSI USING IMMUNE SERUM OF RABBITS RESISTANT TO THE TICK SPECIES

Introduction

Ticks and tick-borne diseases cause heavy economic losses annually in the livestock industries of various countries (Steelman, 1976). Dipping animals in acaricide is the most widely used method of tick control (Wharton, 1976). The success of this control method has been diminished with the advent of acaricide resistance by ticks (Drummond, 1970; Wharton and Roulston, 1970; Wharton, 1976), necessitating the need to develop alternative methods of control.

One approach to tick control suggested by Galun (1978) is direct chemical action on tick antigens, hormones and/or organs using biological insecticides. This approach has not been followed for lack of basic data. Biological control of ticks using microbes and wasps (Cole, 1965; Lipa, 1971) has been attempted but without success. The observation that animals acquired resistance to tick infestations (Trager, 1939a) accelerated studies about use of resistance induced either by artificial infestation (Riek, 1962; Wagland, 1975; Brossard, 1976; Willadsen et al., 1978) or by injecting crude tick extracts (Riek, 1958; Bagnall, 1975;

Allen and Humphrey, 1979; McGowan et al., 1981) to control tick infestations. The results indicated that it is possible to induce resistance by repeatedly infesting animals, but the nature of this resistance has remained obscure.

Attempts have been made to identify tick antigens responsible for inducing host resistance. Brown et al. (1984) demonstrated in the salivary gland extracts of Amblyomma americanum a protein of molecular weight 20,000 daltons that was responsible for the elicitation of host resistance to the tick infestation. Two proteins of molecular weights 30,000 and 60,000 daltons each were shown to be associated with host resistance induced by Boophilus microplus (Geczy et al., 1971; Willadsen and Williams, 1976). Mongi (1982) detected about 10 possible target antigens ranging in molecular weight between 82,000 to 160,000 daltons in various tissues of Rhipicephalus appendiculatus.

In this study, an attempt was made to identify R. e. evertsi salivary gland antigens which react with immune serum from rabbits and which might be responsible for the induction of protection.

MATERIALS AND METHODS

Tick salivary gland antigens (SGA)

Salivary gland antigens were prepared from adult R. e. evertsi as described in Chapter 1.

Analysis of R. e. evertsi SGA by Immunoelectrophoresis

Immunoelectrophoresis (IE) of the SGA was carried out as described by Grabar and Williams (1953) and as modified by Scheidegger (1955). Three microscope slides (90 x 80 mm) pre-rinsed in chromic acid and distilled water were layered with 1.2% molten agarose solution (containing 0.05% sodium azide) in barbital acetate buffer (pH 8.6), on a horizontal surface. The gel was left to set and allowed to harden at 4°C in a moist chamber. The gels were then placed on a template and five wells (about 1.2 mm diameter) were cut. The wells were evacuated by suction and filled with the SGA. The gels were placed in an electrophoresis tank containing the buffer described above and a constant current of 3.0 mA/cm gel (30 mA 90 mm plate) was applied for one hour. The gel was removed from the tank, placed on a template and troughs for antiserum were cut with a double bladed knife. The agar (Difco Special Agar-Noble) was lifted gently out, the troughs were then filled with antisera of R. e. evertsi infested rabbits and the slide incubated at room temperature in a moist chamber. When precipitin lines had formed, the gels were washed in 9% NaCl, dried and stained with Coomassie brilliant blue.

Immunodiffusion analysis of R. e. evertsi SGA

Immunodiffusion was carried out according to the method of Ouchterlony (1949). The antigen and anti-sera of tick-infested rabbits were placed in the centre and peripheral wells respectively. Diffusion was allowed to proceed at room temperature for 24 hr in a humid chamber.

Analysis of R. e. evertsi SGA by Polyacrylamide gel electrophoresis (PAGE)

Identification of the tick SGA was carried out by polyacrylamide gel electrophoresis (PAGE) on slab gel using the method described by Smith (1976). The separating gel was in the form of 15% acrylamide in 1.5 M Tris-HCl, pH 8.3. The acrylamide solution used contained acrylamide and methylene bisacrylamide in a ratio of 30:1.0 and ammonium persulphate (10%). The acrylamide polymerization solutions were introduced into a gradient mixer and fed into the gel moulds. The gel dimensions were 1.5 mm thick x 160 mm long x 170 mm wide. After gel polymerization was complete, spacer gel containing 3% acrylamide was layered on top of the separating gel. Sample wells were obtained in the spacer gel by a plexiglass comb pushed firmly into the surface of the weak upper end of the gel. Fifty µl of SGA mixed with 5 µl of 0.1% tracking dye (Bromophenol blue) was introduced into the gel. Similarly 50 µl of high molecular weight markers, (Pharmacia Fine Chemicals, Sweden) were loaded. Electrophoretic buffer was Tris-HCl, pH 8.3, containing Trizma base and glycine. Electrophoresis was carried out in the cold ($4^{o}C$) at 30 mA and was terminated when the dye in the samples had started to move out of the gel into the buffer tank. The gels were removed from the glass plates and stained in a gel solution containing 225 ml methyl alcohol, 50 ml acetic acid, 1.25 gm Coomasie brilliant blue in 500 ml distilled water for about 6 hours. After this period, the gels were destained in several changes of a solution consisting of 90 ml acetic acid, 50 ml methyl alcohol and 880 distilled water.

Analysis of R. e. evertsi SGA by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis of the SGA, in presence of SDS (SDS-PAGE) was carried out according to the method of Hames and Riikwood (1981). The separating gel was 15% acrylamide in 1.5 M Tris-HCl, pH 8.8 with 10% SDS. This solution contained acrylamide and methylene-bis-acrylamide in a ratio of 30:0.8 and also 10% ammonium persulphate. The polymerization solution was introduced into the gel moulds as described for PAGE above. A spacer gel containing 3% acrylamide and 10% SDS was layered on top of the separating gel. Sample wells were obtained as described for PAGE. Test sample and low molecular weight markers were mixed with the running buffer in presence of 10% SDS and Beta mercaptoethanol at a final concentration of 3% SDS, 1% Beta mercaptoethanol and 0.1% Bromophenol blue. This mixture was heated for 5 minutes at 100^{o}C. Fifty ul of each sample was loaded into the gels. The buffer for running the test contained 40 mM Tris-HCl, 350 mM glycine and 0.1% SDS. Electrophoresis was performed by running a constant current (30 mA) in the separating gel and stopped when the dye in the test sample had run into the tank containing the running buffer. The gels were removed, stained and destained as described above.

Sephacryl S-300 column chromatography of R. e. evertsi and R. appendiculatus SGA

Sephacryl S-300 (Pharmacia Fine Chemicals, Sweden) was loaded on a 1.6 x 90 cm column according to the procedure described in the gel filtration technical manual (Pharmacia Fine Chemicals, Sweden) .

The column was equilibrated with 0.1 M Tris-HCl Buffer pH 7.8 with 0.5 M NaCl and 0.02% sodium azide. Eight and four millilitres of concentrated R. e. evertsi and R. appendiculatus SGA respectively were loaded separately into two such columns, and eluted with the same buffer at 4°C. The flow rate was 25 ml per hour and 3 ml portions of the eluate were collected. Protein concentration was determined in these fractions by the Folin - Lowry method (Lowry et al., 1951).

Analysis by immunodiffusion of the Sephacryl S-300 SGA elutes

Immunodiffusion was carried out on the different SGA, S-300 eluted peaks as described above after they were concentrated by ultrafiltration (Smith, 1976). Rabbit serum obtained 6 weeks after primary challenge with adult R.e. evertsi, in a series of repeated infestations was used. Diffusion was allowed to proceed at room temperature in a humid chamber for 24 hr.

Determination of carbohydrate content in the Sephacryl S-300 SGA eluates

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis on slab gel was carried out as described above. Periodic Acid Schiff's reagent (PAS) was used to stain for glycoproteins as described in the gel electrophoresis and staining techniques manual (Pharmacia Fine Chemicals, Sweden).

RESULTS

Immunoelectrophoresis of R. e. evertsi SGA

Serum from rabbits infested with adult R. e. evertsi gave a positive reaction showing at least four distinct precipitin lines when diffused against electrophoresed SGA (Fig. 33).

Immunodiffusion

One precipitin line was formed in a reaction between the tick SGA and sera from most rabbits repeatedly infested with 20, 50 and 100 ticks (Fig. 34). Some paralysed rabbits, challenged with 50 ticks showed 2 lines.

Analysis of R. e. evertsi SGA by PAGE

Two protein bands (stained with Coomassie blue) were seen after PAGE of SGA (Fig. 35). Their molecular weights were 67,000 and 232,000 daltons.

Analysis of R. e. evertsi SGA by SDS-PAGE

Three distinct protein bands were demonstrated (Fig. 36). Two of the bands had identical molecular weights of about 67,000 daltons. The third band had molecular weight above 94,000 daltons.

Sephacryl S-300 column chromatography of R. e. evertsi and R. appendiculatus SGA

Four fractions, designated 1, 2, 3, 4, were eluted for R. appendiculatus SGA (Fig. 37). However, five fractions similarly

(33

FIGURE 33

Immunoelectrophoresis of R. e. evertsi SGA. The wells (W) contain the tick SGA and the troughs (T) contain rabbit serum obtained one week after secondary challenge with 100 adults of this tick species (Chapter 1). Four distinct precipitin arcs are demonstrated.

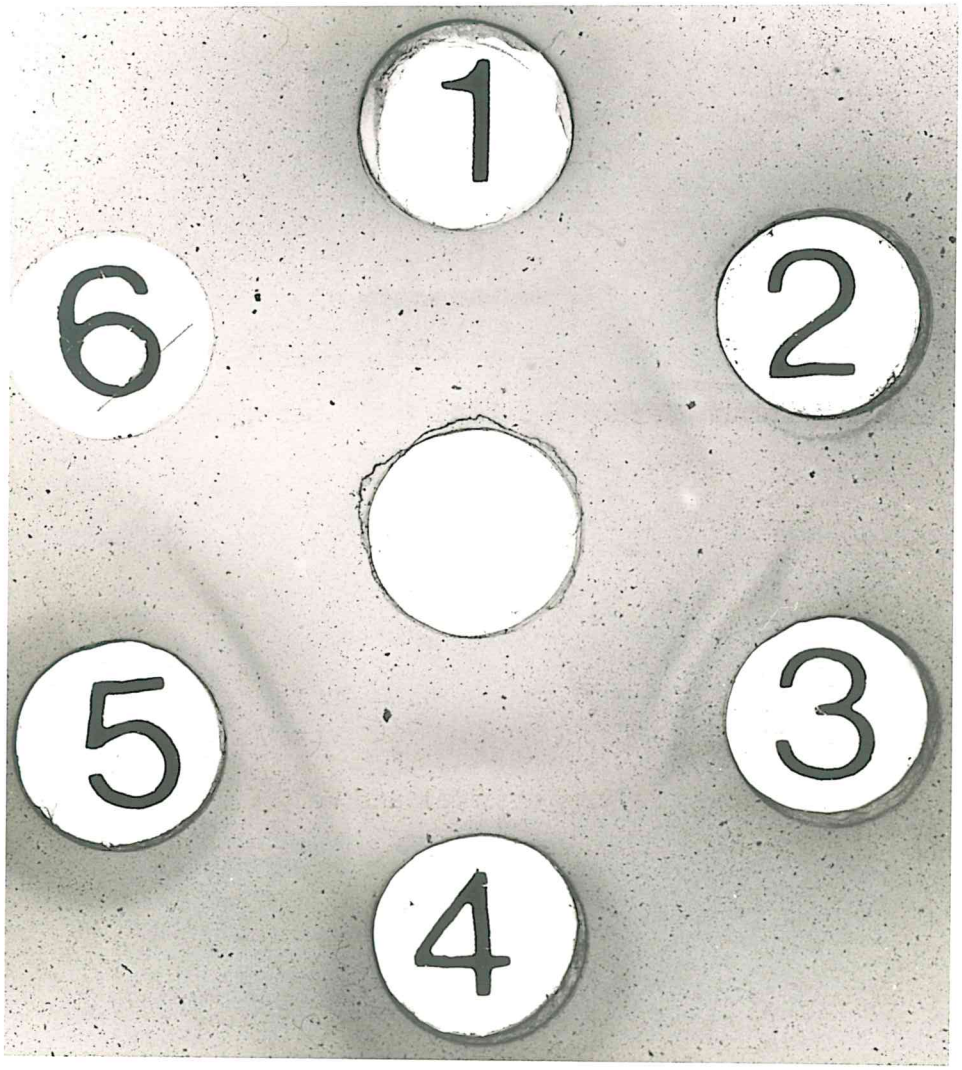
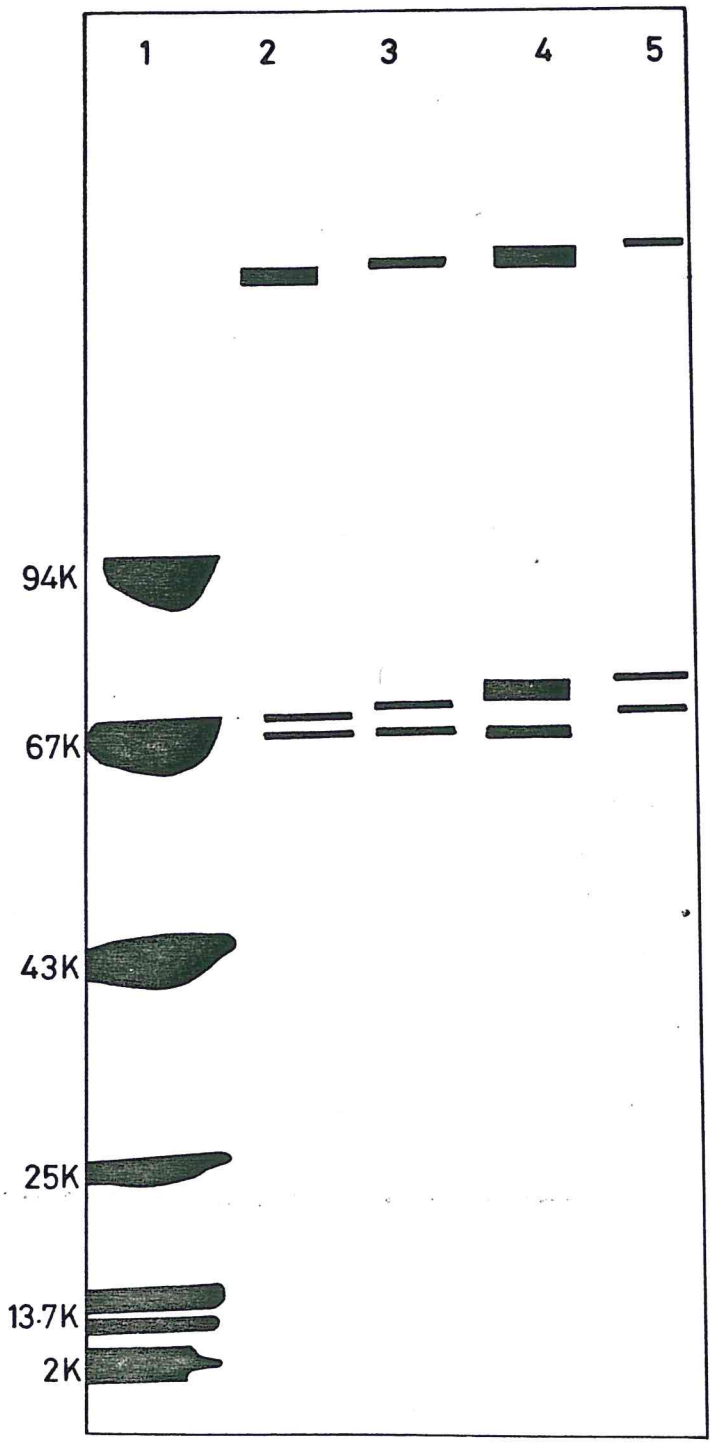
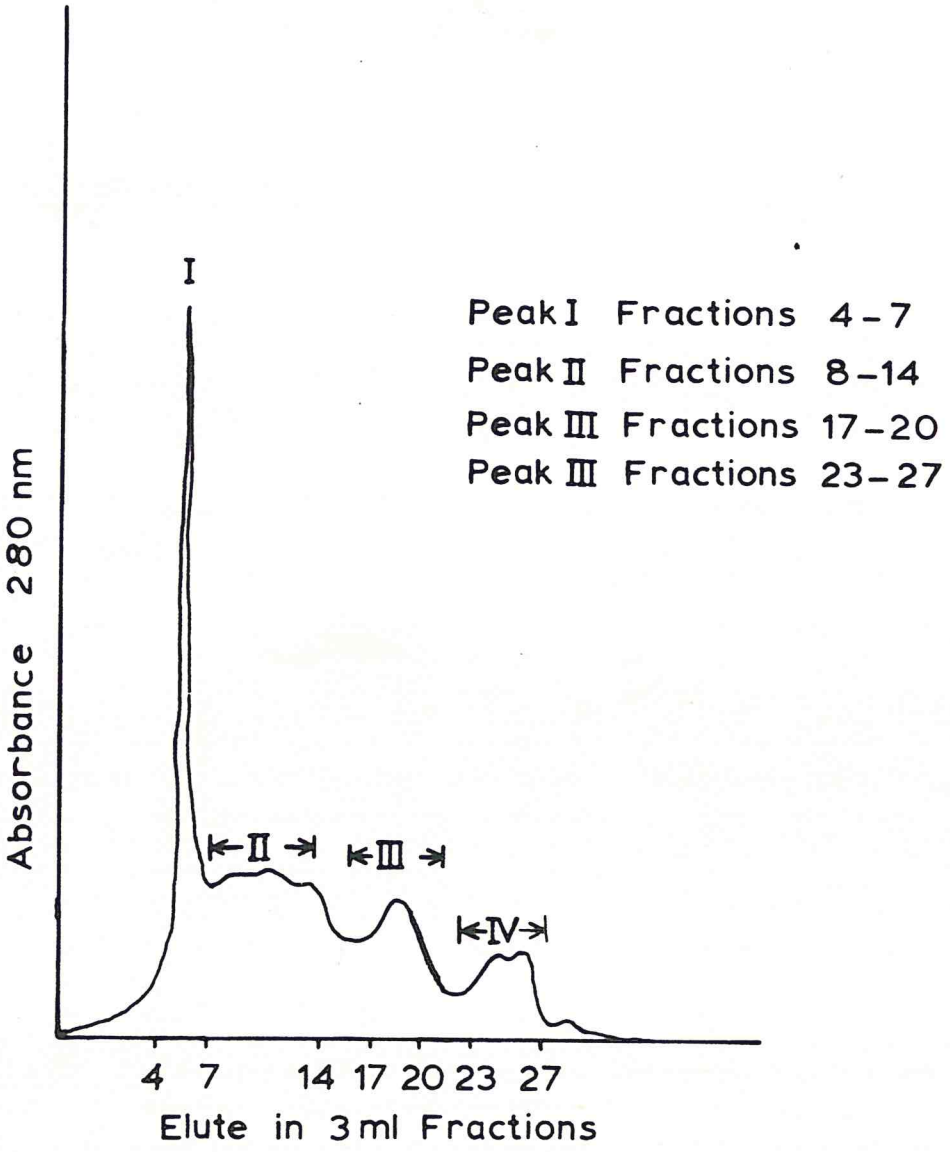

1
2
3
4
5
6

FIGURE 35

Polyacrylamide gel electrophoretic separation of R. e. evertsi SGA showing presence of 67 K and 232 K proteins. Lanes numbered 1 - 5 have the antigen preparation and lane 6 high molecular weight markers.


1
2
3
4
5
94K
67K
43K
25K
13·7K
2K


I
II
III
IV
Absorbance 280 nm
4 7 14 17 20 23 27
Elute in 3 ml Fractions
Peak I Fractions 4-7
Peak II Fractions 8-14
Peak III Fractions 17-20
Peak III Fractions 23-27

designated were eluted for R. e. evertsi SGA (Fig. 38). Factions 2 and 3 of R. e. evertsi and 2 of R. appendiculatus precipitated with serum from rabbits infested with R. e. evertsi (Fig. 39).

SDS-PAGE of R. e. evertsi SGA eluates

Only elute fractions 2 and 3 had demonstrable polypeptides after SDS-PAGE analysis. Six distinct protein bands, two with approximate molecular weights of about 67,000 daltons each and the rest below this range were seen in fraction 2 (Fig. 40). Fraction 3 had a single protein of m.w. 67000 daltons.

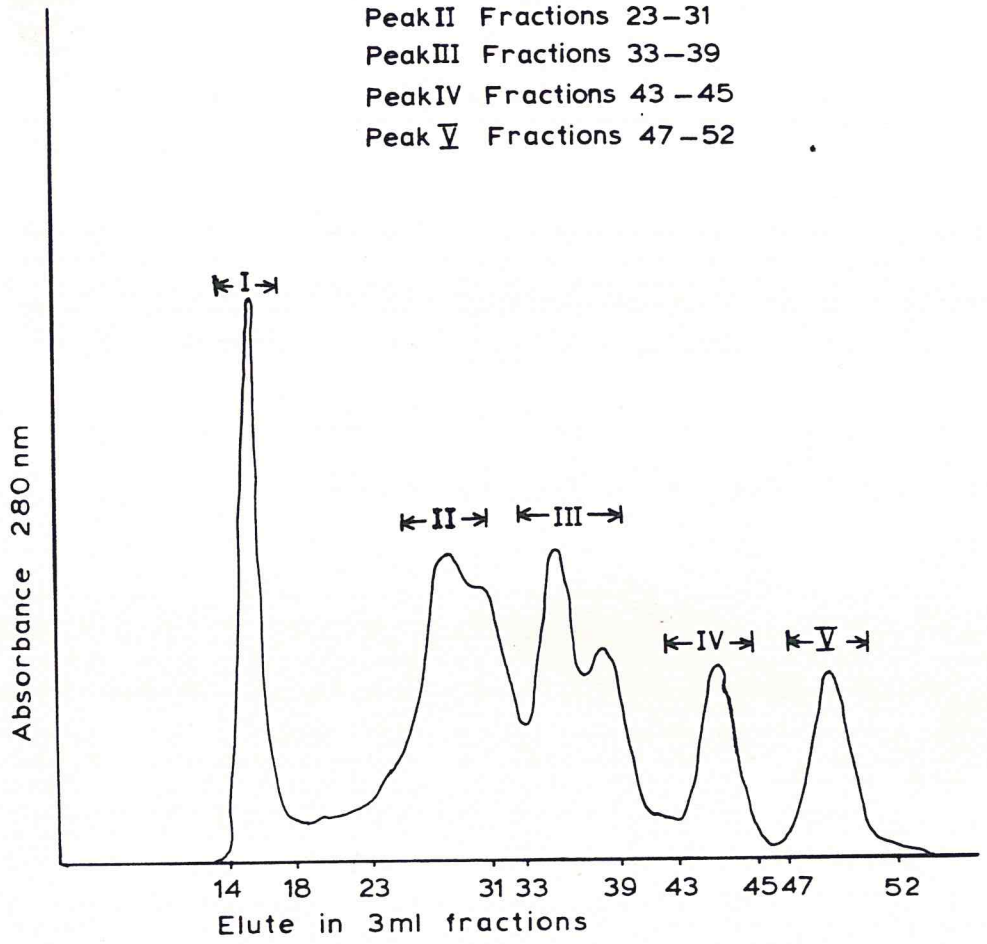
Staining for carbohydrates

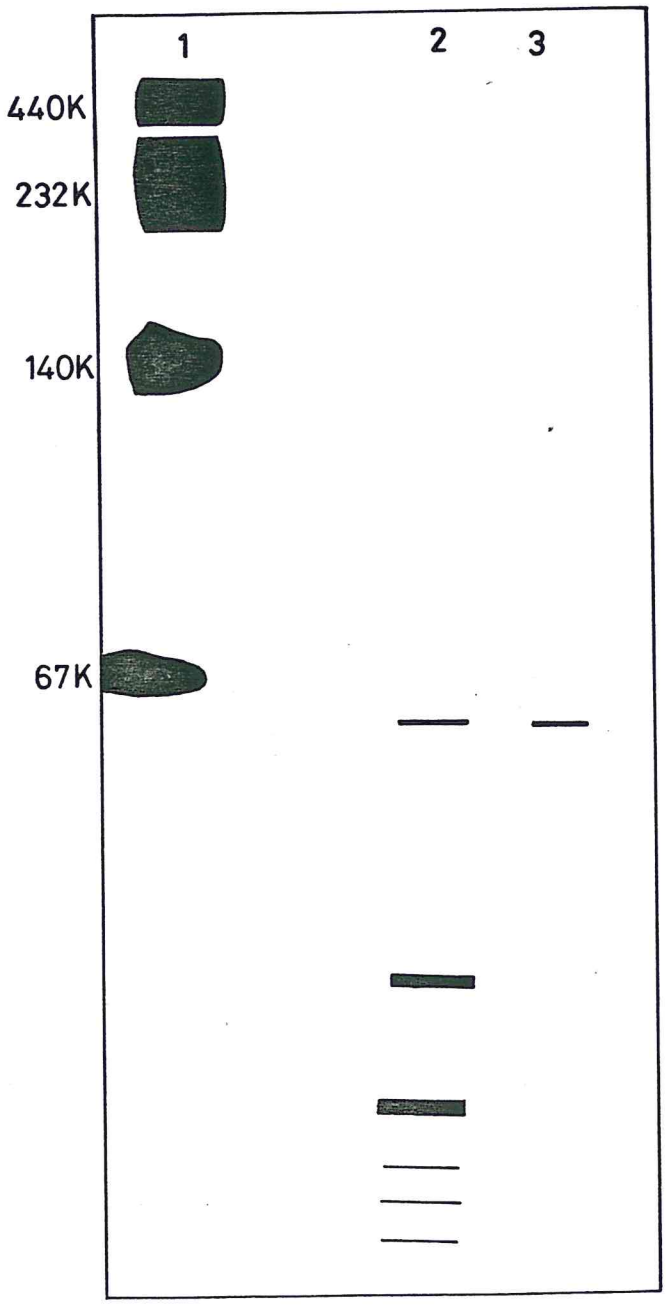
None of these reduced SGA fractions stained with PAS for carbohydrates.

DISCUSSION

Antigen prepared from salivary glands of partially fed adult R. e. evertsi, formed at least one precipitin band against most rabbit sera obtained 9 weeks after primary infestation with different loads of 20, 50 and 100 adult ticks of the same species, on agar gel double diffusion (Fig. 34). However, two precipitin bands were observed with some sera of paralysed rabbits in 50 tick challenge regimen (Fig. 34). Brossard (1976) reported that 7-9 precipitin lines were demonstrable in the sera of rabbits immunized with tick extracts while 2-3 bands could be demonstrated in the sera of animals naturally infested with ticks. Fujisaki (1978) observed 1-4

Peak I Fractions 14 – 18
Peak II Fractions 23 – 31
Peak III Fractions 33 – 39
Peak IV Fractions 43 – 45
Peak V Fractions 47 – 52




1
2
3
440K
232K
140K
67K

precipitin bands in sera of rabbits repeatedly infested with H. longicornis. Four precipitin lines were similarly demonstrated on immuno- electrophoresis (Fig. 33) in this study.

Polyacrylamide gel electrophoresis (PAGE) of R. e. evertsi SGA carried out in the absence of reducing conditions, identified two polypeptides with molecular weights of approximately 67 and 232 kilodaltons (Fig. 35). Tatchell (1971) and Willadsen and McKenna (1983) obtained 4 polypeptides with saliva of B. microplus.The same test repeated in presence of SDS, under reducing conditions demonstrated three polypeptides, two of molecular weight 67 kilodaltons each and the remaining subunit above this range (Fig. 36). In a similar analysis of R. appendiculatus SGA, as many as 52 polypeptides were recorded (Mongi, 1982). Brown et al., (1984) using SDS-PAGE obtained 4 immunoreactive polypeptides in A. americanum SGA. At present it is difficult to account for the difference between the SDS-PAGE results of this study and that by Mongi (1982).

The antigen was fractionated by Sephacryl S-300 column chromatography into five fractions (Fig. 38), of which two (fractions 2, and 3) were immunogenic and formed a precipitin band against homologous serum from rabbits infested with adult R. e. evertsi. When these two SGA fractions were subjected into further separation by SDS-PAGE, they yielded more polypeptides than observed with whole SGA (Fig. 40). Sephacryl S-300, fraction 2 was resolved into six protein subunits, 2 of identical molecular weights (67 kilodaltons each) while the remaining 4 polypeptides had molecular weights below

this range. However, Sephacryl S-300 fraction 3 had a single, faint staining, protein subunit of molecular weight 67 kilodaltons. None of the five Sephacryl S-300 fractions stained distinctly for glycoproteins. It is apparent that R. e. evertsi SGA can be cleaved into more protein subunits by alternating column chromatography and SDS-PAGE separation methods than use of SDS-PAGE alone, suggesting existence of subunits relatively resistant to proteolytic attack. These results support those of Willadsen and McKenna (1983) that tick proteins polymerize using strong bonds. As a result of this they are difficult to denature. Proteins, resistant to normal degradation have been observed to paralyse host immune systems (Felton, 1949). These data suggest that impaired host immunological vigour arising from tick induced immunosuppression (Wikel, 1985), may be complemented with immune tolerance resulting from persistence of poorly metabolizable tick antigens. Therefore the host immune responsiveness is significantly affected resulting in enhanced tick feeding.

Mongi (1982) observed that R. appendiculatus SGA fractionated by Sephadex G-75 revealed 2 protein peaks and that homologous serum recognized the first peak. In a comparative study using Sephacryl S-300, four and five fractions were eluted from SGA of R. appendiculatus and R. e. evertsi respectively. Only fraction 2 of R. appendiculatus SGA was recognized by serum from rabbits infested with adult R. e. evertsi. The difference in the number of peaks in R. appendiculatus SGA observed in this study and the previous worker may be attributed to superior resolution power of Sephacryl S-300 over

Sephadex G-75. It was concluded that R. e. evertsi SGA is more complex than that of R. appendiculatus but why such differences do not occur in SDS-PAGE analysis is still unexplained.

SUMMARY

In agar gel double diffusion test, antigens extracted from salivary glands (SGA) of adult R. e. evertsi prefed for 5 days on naive rabbits, reacted against immune sera of rabbit groups infested with different numbers of the adult tick, forming 1 to 4 precipitin lines. Polyacrylamide gel electrophoresis (PAGE) of the native SGA carried out in the absence of sodium dodecyl sulphate (SDS), revealed presence of two polypeptides of molecular weights 67 and 232 kilodaltons. However, three polypeptides two of identical molecular weights (67 kilodaltons) and the third above this range were observed by SDS-PAGE. When the antigen extract was subjected to Sephacryl S-300 chromatography, 5 fractions were eluted of which only two (fraction 2 and 3) were recognized by the homologous immune serum. Fractions 2 and 3 were further shown by SDS-PAGE to contain 6 and 1 polypeptides respectively. A single protein subunit of molecular weight 67 kilodaltons was observed in Sephacryl S-300 fraction 3 and also 2 of the six subunits in fraction 2. The remaining 4 protein subunits in the latter fraction had molecular weights below 67 kilodaltons. None of these SGA factions stained for glycoproteins. Antigens in Sephacryl S-300 fractions 2 and 3, reacting with the sera of rabbits infested with the tick are potential candidates for studying the mechanisms of host-ectoparasite relationship.

GENERAL DISCUSSION AND CONCLUSIONS

Rabbits repeatedly infested with different numbers of R.e. evertsi life stages acquired resistance to the ticks after primary infestation. This resistance interfered with feeding, ability to lay eggs and limited the number of ticks, especially the instars, that matured on the hosts. The nymphs which matured on the hosts could be alloted to five weight categories, 0-4.9, 5-9.9, 10-14.9, 15-19.9 and 20-24.9 mg. A higher percent of females than males moulted from nymphs of weight 15-19.9 mg while the converse was true of nymphs of weight 5-9.9 mg. However, the moulting performance of the remaining 3 nymphal weight categories, was not undirectional but alternated between the sexes. Although the rate of egg production was significantly reduced, hatchability was not affected. It is suggested that host resistance does not block the life cycle of the tick species and that both high rates of egg hatch and nymphal moults are partially responsible for maintaining the tick populations in nature.

The histopathological picture of skin biopsies taken at tick feeding sites on the rabbits after primary infestation was comparable to that of other sites (on the same hosts) deliberately inoculated with tick salivary gland extract. These sites were intensely infiltrated with mononuclear cells.

Specific antibodies against tick salivary gland antigens were detected during the course of tick infestations

in the hosts sera by various serologic methods. These antibodies are ingested by the ticks in the course of feeding and evidence from other workers indicates that such antibodies may cross the mid-gut epithelium and exert deleterious effects on the ticks (Ackerman et al., 1981; Brossard and Rais, 1984). Indeed in tsetse flies, specific antibodies ingested with a blood meal are detrimental to this insect (Nogge, 1978, 1982; Nogge and Giannette, 1980). It is suggested from the studies here that the antibodies and cells of the inflammatory process at the tick feeding sites were mediators of the host resistance to the ectoparasites, although the mechanisms could not be determined in the course of the experiments.

Each of the 20,50 and 100 adult and 100, 500 and 1500 larvae of R.e. evertsi used to sensitize the rabbits stimulated high resistance noticeable to limit the numbers of ectoparasites feeding successfully on the hosts. Maximum antibody titres were attained after secondary tick infestation. Subsequent tick challenges did not increase these antibody titres; they were either maintained constant or in the case of hosts challenged with 20 adult ticks and instars decreased significantly. A small proportion of the applied ectoparasites in this study, especially the instars, was observed to feed well and this was also reported by Allen and Nelson (1982). Despite adequate feeding by the ticks some hosts did not acquire strong resistance. The constancy of the antibody titres despite an increased frequency of tick challenge is suggestive of a diminished state of immune

response in the hosts and has also been reported in other host-parasite relationships (Sprent, 1969; Hudson, 1973). The few ticks that feed on hosts under these circumstances do not provoke strong resistance (Randolph, 1979) possibly as a result of the host exerting selective pressure thus modifying their antigenic properties and reducing antigenic disparity (Dineen, 1963). Nelson et al. (1977) suggested that this phenomenon resulted in a state of balance favouring survival of both partners. These data indicate that two infestations of the host with ticks stimulates a threshold antibody titre which is not altered by subsequent infestations.

Rabbits infested repeatedly with adult R.e. evertsi, particularly those paralysed, responded poorly to a subsequent challenge with SRBC, indicating possible tick-induced immunosuppression, a pattern reported also in hosts infested with D. andersoni (Wikel and Osburn, 1982; Whelen et al., 1984; Wikel, 1985). The enhanced feeding of ticks in successive tick challenges reported in the present study supports similar results by Brossard et al. (1982) and Den-Hollander and Allen (1985). The hypothesis by Ribeiro et al. (1985) that tick-induced immunosuppression facilitates feeding and consequently the transmission of disease pathogens is supported by these results. Furthermore, the study complements the hypothesis by Wikel (1985) that such ixodid-induced immunosuppression could facilitate ticks to feed and maintain an adequate life cycle on resistant hosts.

Delayed type hypersensitivity reactions (skin tests) to tick SGA, manifested by rabbits repeatedly infested with 50 and

100 adult R.e. evertsi were in general weaker than those observed for hosts challenged with 20 ticks. However, responses were weakest in those rabbits that were paralysed and recovered, for example the paralysed animals in the 50 tick challenge group. This skin test demonstrates cell-mediated immunity in these experiments and exemplifies the ability of T cells to recruit effector leucocytes to tissue sites as a prerequisite to the protective response against etiological agents (Valdimarsson, 1978). The reduced skin reactivity to homologous tick SGA observed in the study supports the results of Wikel (1982, 1985). This observation suggests that T cell function is impaired in hosts repeatedly infested with R.e. evertsi. Brown and Askenase (1985) suggested that the immune response of hosts to ticks, for example ixodid rejection, is mainly T cell dependent. Thus, the reduced dermal reactions to tick antigens observed in the present study suggests that chronicity is characteristic of R.e. evertsi infestation.

It was shown in the study that the development of DTH to SRBC in groups of rabbits infested with adult R.e. evertsi was suppressed in the course of tick feeding and the cutaneous responses displayed were inversely related to the tick engorged weights. Sixty ticks fed on naive rabbits abolished the development of the cellular inflammatory skin reactions in response to inoculated SRBC while 20 ticks did not. Furthermore, two of the three rabbits challenged with 60 ticks became paralysed and died. On the other hand, 20 ticks fed on resistant hosts caused a minimal suppression of the responses

compared to those fed on naive hosts. The abolition of DTH and the death of hosts in some cases, is however, never so complete as to result in total severance of the tick life cycle. These results provide evidence that tick infestations impair host immune response to other antigens and the degree of impairment is influenced by the number and feeding vigour of the ticks. It is speculated that infestations may enhance the pathogenicity of concurrent infestations and render vaccinations ineffective.

Hosts parasitized with R.e. evertsi adults showed an initial antibody response within four days which became depressed during the later stages of feeding but the response recovered following completion of feeding. That this was a generalized immune suppression was shown by simultaneously injecting BSA and SRBC antigens and commencing tick feeding on naive hosts. The primary immune response to the BSA and SRBC antigens were both depressed.

When this experiment was repeated for the secondary response neither the responses to the feeding ticks or BSA/SRBC antigens were severely depressed and this was evidenced by the failure of the ticks to feed properly. In this experiment, precipitating antibodies to tick SGA were demonstrated in hosts sera within four days of tick feeding, indicating rapid acquisition of resistance. Taussig (1978) reported that competition occurs in vaccination programs where antigens are given as mixtures with the result that immune response to one antigen can be severely depressed while the response to the

other is unaffected. However, competition can be abolished when antibodies initially formed neutralize specific antigens on a subsequent encounter with the result that the immune response to less immunogenic or suboptimal amounts of antigens in a mixture is enhanced (Taussig, 1978). One possible explanation of the results reported here is that host antibodies formed in response to the primary antigenic stimulation by the two heterologous antigens (BSA, SRBC), neutralized the same antigens during secondary inoculation, abolished competition and thus allowed an enhanced immune response to tick antigens presented while feeding. These findings indicate further that tick infestations suppress host antibody response to a variety of antigens.

Resistance induced by adult R.e. evertsi protected the rabbits against infestations with immature stages of the same tick species, R. appendiculatus (all stages) and A. variegatum (larvae and adults). Cross resistance assessed by a decline in engorgement weights and number of ticks successfully fed was more significant with R. appendiculatus than A. variegatum. Cross resistance was not demonstrated between R. appendiculatus and A. variegatum (Heller-Haupt et al., 1981; de Castro et al., 1985). Trager (1939a) showed that cross immunity existed in laboratory animals between ticks grossly varying in sizes. Boese (1974) hypothesized increased host grooming activity as being responsible for the resistance and McGowan et al. (1979) concluded that resistance was more effective against large rather than small ticks. However the results of this study

indicate that cross resistance exists between R.e. evertsi, R. appendiculatus and A. variegatum and support the results of McTier et al. (1981) that resistance was more effective against smaller ticks. Such resistance was attributed to cross reacting antigens present in R.e. evertsi, R. appendiculatus and A. variegatum.

Martinod et al. (1985) hypothesized that the immune response to ticks is not specific and suggested that it is possible to protect animals against multiple tick species by immunization using antigens from a single species. Investigations on the biochemical and immunological characteristics of isolated antigens are required to test this possibility. In addition such studies are needed to clarify the findings that A. hebraeum larvae and nymphs do not provoke host resistance (Norval, 1978).

Rhipicephalus e. evertsi SGA was separated by PAGE to show two protein subunits of molecular weights 67 and 232 kilodaltons, while immunoelectrophoresis indicated the presence of five subunits. However, SDS-PAGE only demonstrated three subunits, two with a molecular weight of 67 Kdal and one with a weight above this range. These results of SDS-PAGE analysis are similar to those of Brown et al. (1984) where four subunits were demonstrated in A. americanum SGA. However, a total of nine polypeptides, seven staining distinctly for proteins, were demonstrated in R.e. evertsi SGA if SDS-PAGE was repeated with immunogenic fractions eluted by Sephacryl S-300 column chromatography. These polypeptides had molecular weights below

the 67 Kdal reported for A. americanum (Brown et al., 1984). Wikel (1981) showed that D. andersoni SGA constitutes approximately eight polypeptides. The SGA of the tick species studied presents complex molecules reducible to three to nine subunits and this differs significantly from the 52 polypeptides reported from R. appendiculatus SGA (Mongi, 1982). It is possible that a combination of gel filtration chromatography with SDS-PAGE would reveal further subunits of tick SGA which could be of value in the prophylactic control of these arthropods.

Analysis by SDS-PAGE revealed more protein bands in the immunogenic fractions after Sephacryl S-300 column chromatographic separation than in the whole SGA, indicating possible in vitro resistance of the antigens to denaturation. The polymerization of tick antigens, thought to prolong their retention at the feeding site to the advantage of the tick (Willadsen and McKenna, 1983) may also suggest reduced host ability to denature or neutralize them in vivo. Prolonged retention of parasite antigens in infected hosts may arise if a depressed primary immune response leads to a defective secondary response to the depressed antigens (Nantulya et al., 1982). Felton (1949) reported that the prolonged retention of antigens in the host suppresses the immune response. It is suggested by these findings that tick induced immunosuppression and poor antigen metabolism may be the reason for the limited ixodid resistance achieved either by artificial immunization or natural tick infestations.

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