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A TRIAL ON IMMUNIZATION OF RABBITS AGAINST HYALOMMA ANATOLIC ANATOLICUM TICKS INFESTATIONS

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Abstract

The present study was conducted in Sulaimaniya Iraq. Unfed larval stage of Hyalomma anatolicum anatolicum ticks were used for preparation of antigen for local breed rabbits. Each rabbit was inoculated with 0.1 ml (6.8 mg/ ml) in three successive doses at weekly interval. Following immunization rabbits developed significant level of protective immunity to infestation of ticks. There was significant increase in WBC, monocytes and neutrophils, while decrease in lymphocytes, eosinophils and basophils. There was increase in phagocytic index in immunized rabbits than controls. There was significant increase in skin fold thickness after 2 hours reaching maximum after 24 hours. The level of total serum protein, albumin and globulin were elevated in immunized rabbits. The immunoglobulin G level was higher in immunized rabbits than controls.In post immunized rabbits congestion, papules and oedema in the site of the infestation. In preimmunized rabbits there was high number of engorged larvae still sucking blood.

It is concluded that the larval extract of Hyalomma anatolicum anatolicum showed significant protection against tick challenge in immunized rabbits..

INTRODUCTION

A successful host-parasite relationship is a balance between limiting the host defense and the ability of the parasite to modulate, evade or restrict the host response and could enhance the ability of the arthropoda to obtain a blood meal and facilitate pathogen transmission (Wikel et al., 1994). The degree of host resistance to ixodide infestations can be measured by the following indicators, decrease of body weight after engorgement with consequent reduction of oviposition, increase of feeding period, and reduction of larval hatchability rate and molting rates (Wikel et al., 1996). Effective control measures against tick borne disease are best achieved through combination of tick control, prevention of disease through vaccination and treatment (Minjauw and de-Castro, 1999).

Brossard and Wikel (2004) revealed that both innate and specific acquired immune defenses are involved in the responses of vertebrate hosts to infestation, ticks have evolved countermeasure to circumvent host immune defenses.

Innate immunity mediates resistance of tick infestation in some animal species. These non-adaptive immune factors are capability of some breeds of animals that can consciously move their skin, self grooming activity, swelling, skin color and thickness, area of skin available for tick infestation or length of fur (Kashino et al., 2005).

Acquired or specific immunity is immunity induced by exposure to an antigen, naturally or using vaccination (Kuby, 1994), can be further divided into two subcategories: humoral and cell mediated immunity. The humoral branch of the immune system involves the interaction of B cells with extracellular antigen and their subsequent proliferation and differentiation into antibody-secreting cells that are specific for a certain antigen.

Antibodies secreted by B cells function as the effectors of the immune humoral response by binding to an extracellular antigen and neutralizing and/or facilitating its elimination. Cell-mediated immunity involves the interaction of T cells and their associated cytokines to eliminate intracellular pathogens (Galyean et al., 1999).

During the secondary infestation, mast cells and basophill degranulation and the release of mediators are much greater than during the primary infestation, and basophils rapidly invade the feeding lesion, degranulate and liberate vasoactive mediators leading to edema, erythema and rejection of ticks (Morrisan, 1989).

Brossard and Wikel (2008) reported that the tick-induced suppression of host immune defense is characterized by reduced ability of lymphocytes from infested animals to proliferation and diminished primary antibody responses to T-cell dependent antigen, and decreased elaboration of macrophage (IL-1 and TNF-α) and Th1 lymphocyte cytokines.

The aim of this study was to trial of immunization of rabbits against *Hyalomma anatolicum anatolicum* tick infestation against rabbits.

MATERIALS AND METHODS

Rearing and breeding of hard tick Hyalomma anatolicum anatolicum under laboratory condition.

In vitro engorged female of *Hyalomma anatolicum anatolicum* were collected in sterile glass with bijou bottle and covered by muslin cloth and incubated at 27±1.6°C, kept in desiccator jars with relative humidity (RH%) 85±1.4% (using saturated solution of potassium chloride for humidity control) both temperature and humidity measured by (Humidity-Temperature meter, France). Observation was made twice daily at the morning and evening hours until eggs were laid, in oviposition times of (27 ± 1.32) days after incubation, with hatching times (22.4± 2.26) days. The eggs were hatched to larvae, with duration of hatching 6.16±1.02 days. Larvae were left in the incubator (WTC-binder, Germany) at the same conditions of temperature and humidity which required for hatching within two weeks, the larvae (pre-feeding period) became sclerotized and Hardening . The larvae were placed into a deep freezer at –20°C for preparation of antigens for immunization.

Hematological examination in rabbits

Total white blood cells counts (WBC X103/µl) and differential leukocyte counts were estimated as described by (Coles, 1986).

Preparation of tissue antigens extracts:

The unfed larvae weighed and placed onto pre-sterilized Petri dishes containing an ice-cold 0.15 M Phosphate buffered saline (PBS), pH 7.2. They were thoroughly washed thrice in PBS, air- dried and then wrapped with aluminium foil papers. Approximately 3.0 gm of unfed larvae were homogenized in 10 ml of 0.15 M PBS, pH 7.2 containing 1.0 µm/l disodium ethylene diamine tetra acetic acid (EDTA), employing sterile pestle and mortar placed on an ice-bath. The homogenate suspensions were filtered free of cuticle and debris with a double layered muslin cloth into a sterile beaker. Washing by PBS and centrifuged (Speed centrifuge-80-1, Italy) at 5000 rpm for 10 min three times, 5 ml of PBS was added to the precipitate protein and kept in sterile glass vial at -20°C until further use (Van den Broek et al., 2003).

Determination of total protein concentration of the antigen

Total protein concentration of larval antigen was determined by modified Lowry's method, which depends on reduction of phosphotangestate or phosphomolybdate salts by amino acids, tyrosine and tryptophan. Bovine serum albumin (BSA) 1.0mg/ml of distilled water was used as a standard solution with an extinction coefficient of 0.670 at a wavelength of 280nm (Holme and Peck, 1988). Employing UV/Visible spectrophotometer (Eppendorf, bioPhotometer, Germany). Standard solution as form (50, 100, 200, 400, 600, 800 µg/ml) versus the absorbance of (0.08, 0.2, 0.33, 0.55, 0.76, 0.90) at 280 nm. Then (5µl of antigen+995 µl of distilled water) were measured. The absorbance of antigens was plotted into the standard curve to determine protein concentration of each antigen.

Experimental design

Twenty local breed rabbits (rabbit more available and easy handling in vitro and suitable for rearing and molting of stages of the ticks), 3-6 month old rabbits were used (10 rabbit for control and 10 rabbits for immunization) each rabbit was shaved the back of neck region and wiped with 70% ethanol followed by tincture iodine, and was inoculated subcutaneously, using a sterile syringe and needle of 23 gauge with 0.1 ml (6.8 mg/ml) larval antigen mixed with vegetable oil in three successive doses at weekly interval. The control group rabbits were inoculated with phosphate buffer saline. At fourth weeks post inoculation, 5 ml of blood was drawn directly from heart by cardiac puncture of each rabbit, one ml deposited in disposable clean plastic tube with anticoagulant (DMD-Dispo, S.A.R.) used for estimation of hematological parameters and the remainder four milliliters of blood deposited without anticoagulant in free plastic tube, to obtain serum by ordinary centrifuge 5000 rpm (Speed centrifuge-80-1, Italy) spanned for five minutes and the separated serum was kept at - 20C° for the estimation of the following serological analyses of both control and immunized rabbits (Van den Broek et al., 2003).

Serological analyses

These analyses include the activity of total serum protein, total serum albumin and total serum globulin execute on the experimental rabbits (pre-immunization and post-immunization rabbits), using spectrophotometer (Apel-AD-303, Japan) used for determination of specific wavelength. Biuret method was used for estimation of total protein, in which Cu+2 reacts in alkaline solution with the peptide linkages of proteins to form a violet-colored complex. The intensity of the color produced is proportional to the protein concentration minutes and read at 540nm (Schalm et al., 1975). Albumin concentration in serum was measured by using Biolabo kits. The determination of globulin concentration in blood serum was done through a simplified mathematical method by subtraction of the albumin concentration that previously estimated from total protein concentration.

Quantitative evaluation of immunoglobulin concentration in rabbits

Glutaraldehyde coagulation and Sodium sulfite precipitation tests was performed according to Mary et al. (2004).

Evaluation of delayed-type hypersensitivity cutaneous reaction

The test was carried out according to the method of Kaura and Sharma (1982). The right side of flank skin of each immunized (post-immunization) rabbits was carefully freed of hair, using hair clipper. A circular area was marked and wiped with 70% ethanol. 0.05 ml (3.4 mg) of whole crude larvae extract were inoculated I/D, using 1.0 ml tuberculin syringes, while 0.05 ml of normal saline was done into the left side in all rabbits. Observation was made within 0 hr, 2 hr, 4 hr, 6 hr, 12 hr, 24 hr, 36 hr, 48 hr, 72 hr, 96 hr and 7days post-inoculation to exclude an immediate-type cutaneous hypersensitivity reaction and monitor degree of swelling and redness of skin. The mean diameter and skin fold thickness were measured by a caliper (Vernier/ Electronic digital caliper/ 0-150mm, Lezaco, China.

Evaluation of phagocytosis

The ingestion of Staphylococcus aureus test: S. aureus colonies from Children General Hospital, Sulaimani were taken with sterile platinum loop, into diluted (1:10) peptone broth (1gm of peptone diluted to 9 ml distilled water) in two test tubes and incubated at 37°C for 18 hrs, 0.05ml of the culture medium was mixed with 1.0 ml of freshly prepared heparinized blood obtained from the immunized and control rabbits. The mixture was incubated at 37°C for 1hr. Smears were prepared on clean glass slides, air-dried and stained with 10% Giemsa stain for 30 min. Examined under oil immersion lens (× 100) for estimation of cells engulfing bacteria (S. aureus) as well as the number of phagocytic cells (Burrell, 1979).

Determination of the lgG protein

Determination of the lgG protein (Shaheed Hadi Consultation Clinic), using radial immunodiffusion plate (Radial immunodiffusion plates, IgG RID, Italy) which contained specific antiserum in agarose gel, 0.1M phosphate buffer pH 7.0, 0.1% sodium azide as a bacteriostatic agent, 1 mcg/ml amphotericin B as an antifungal agent. The plates contained 0.002 M ethylene-diaminetetraacetic acid. The kit included plates (Radial immunodiffusion plates, IgG RID, Italy), the plates contained agraose gel and a specific antiserum IgG. Radial immunodiffusion was based on the diffusion of antibody from a circular well radial into a homogeneous gel containing specific antiserum IgG. A circle of precipitated antigen and antibody formed, and continued to grow until equilibrium is reached. After 48 hr of incubation, the zone diameter of control and samples were measured

Challenge larval infestation

After two weeks of pre-feeding, the sclerotized uncalculated numbers of larvae in vitro were obtained for attachment and feeding process as indicated by accumulation of flat larvae onto muslin covers and their attempt to suck blood from nearby finger. The content of each bijou bottle (unfed larvae of Hyalomma a. anatolicum) was placed on and clipped and shaved ear-pinnae of immunized (post-immunized) and control (pre-immunized) rabbits put inside tubular ear-bags (muslin cloth) (5 X 10) cm and fixed at the base of each ear by thread (cotton) which present in the edge of ear-bags using for prevent ticks escaping from ears. Ear movement was limited by tightening tips of the ear-bags with a nylon thread around the neck, after 3 days changes in each ear of rabbits detected for attachment and feeding.

Statistical analysis

Statistical analysis was conducted using SPSS for windows (version 7). The analysis of variance (ANOVA), as general test and the comparison between the means conducted by least significant difference at the significant level (0.01) (JMP7, 2007).

RESULTS

Leukocyte and differential count:

Evaluation of responses of immunized rabbits with whole larval crude extracts by estimation of cellular defense activity, mainly by estimation of white blood cells (total leukocyte number) and differential leukocyte count (LDC). The results indicated a significant increasment in the values of total Leukocyte 13.5×103/µl ± 0.58 in immunized rabbits in comparison with control group 7.4 ×103/µl ± 0.22. There was statistically increase in numbers of monocyte 18.1 ± 1.1 and neutrophils 50.1 ± 1.17 in immunized rabbit in comparison to control group 14.5 ± 1.1 and 42.1 ± 1.63 respectively ((Table 1). .

Table (1): Total and differential leukocyte count in immunized and control rabbits.

Experime-ntal. Rabbits	WBCs × $10^3/\mu l$	Differential Leukocyte count Mean ± SE				
	Mean ± SE	Monocyte	Lymphocyte	Neutrophil	Eosinophil	Basophil
Control	7.4 ± 0.22	14.5 ± 1.1	38 ± 1.46	42.1 ± 1.63	6.1 ± 0.5	2.6 ± 0.3
Immunized rabbit	13.5 ±0.5**	18.1 ± 1.1*	30.9± 0.93**	50.1± 1.17**	3.10.34**	0.0± 0.0**
t (18) = 2.10 * 0.05	t (18) = 2.87 ** 0.01					

Phagocytic ingestion activity

The phagocytic ingestion activity was observed in immunized rabbits with larval crude antigen extract by estimation of phagocytic Index. It was revealed that the significantly increasing in number of phagocytes that ingested the Staphylococcus aureus 7.2 ± 0.61 in immunized rabbits was higher than control group of pre-immunized rabbits 1.8±0.24 as indicated in Table (2) .

Table (2): Phagocytic index in control (pre-immunized) and immunized in

rabbits with whole crude larval extract. (Mean ean ±SE)

Experimental rabbits	Phagocytic cells
Control (pre-immunized)	1.8 ± 0.24
Immunized rabbit	7.2 ± 0.61**
T (18) = 2.87	**0.01

Delayed-type hypersensitivity by cutaneous reaction (Intradermal skin test):

Skin intradermal test reaction was used with whole crud larval extract for typical type of hypersensitivity when introduced intradermally, and significantly ($p \leq 0.01$) increased in the skin thickness and inflammatory response occurred after two hours and reached maximum after 12 to 24 hr (10.20 ± 0.38 mm, 11.10 ± 0.26 mm) respectively, and reaction was subsided and reached to thickness as zero time (2.60 ± 0.19 mm) at 7 days post-inoculation (immunized) rabbits (Table 3).

Table (3): Skin fold thickness (millimeter) of rabbit's inoculated (l/d) with whole crude larval extract (Hyalomma anatolicum anatolicum).

Mean ±SE

Experimntal Rabbits	Skin fold thickness (mm) at	Post- inoculation(l/d) of whole crude larvae/ skin fold thickness(mm)									
		2h	4h	6h	12h	24h	36h	48h	72h	96h	7 days
Post-immunized rabbit	2.10± 0.15	3.80 ±0.34**	4.80 ±0.47**	7.90 ±0.52**	10.20 ±0.38**	11.10 ±0.26**	8.70 ±0.64**	6.70 ±0.49**	4.90 ±0.24**	3.20 ±0.30**	2.60ns ±0.19**

T (18) = 2.87 t (18) = 2.10 ns= not significantly

** 0.01

Total protein (Albumin/ Globulin:.

The content of serum total protein, albumin and glubulin were determined in immunized rabbit with whole crude larval extract. These values showed highly significant elevation ($P \leq 0.01$) 9.677 ± 0.16 gm/dl, 4.349 ± 0.08 gm/dl and 5.328 ± 0.13 gm/dl respectively in immunized rabbits compared to control group 6.466 ± 0.21, 3.858 ± 0.10 and 2.608 ± 0.14 respectively (Table 4).

Table (4): Level of total protein, albumin, and globulin in immunized and

control rabbits.

Mean values±SE

Experimental Rabbits	Total serum protein gm/dl	Total serum albumin gm/dl	Total serum globulin gm/dl
Control	6.466 ± 0.21	3.858 ± 0.10	2.608 ± 0.14
Immunized	9.677 ± 0.16 **	4.349 ± 0.08**	5.328 ± 0.13**

T (18)=2.1	0.01**		

Biochemical reaction for determination of immunoglobulin (Ig)concentration

The biochemical analysis was used for determination of the quantitative values of concentration of nonspecific immunoglobulin (lg) in serum of immunized rabbits with whole crude larval extract. The result of glutaraldehyde coagulation test used to estimate the concentration of lg in serum of immunized rabbits. It was higher than 600 mg/dl (0.6gm/dl). The results of sodium sulfite precipitation reaction, the concentration of lg in immunized group was more than 1500 mg/dl (1.5 gm/dl) in comparison with control group which was between 500-1500 mg/dl (0.5-1.5 gm/dl).

Qualitative values of immunoglobulin:

A radial immunodiffusion plate was used for determination of the concentration of spe cific immunoglobulin type-G (lgG). The result was indicated by measuring the diameter of diffusion ring in which represented the concentration of IgG in serum. It was 2.758± 0.220 gm/dl which was significantly higher in immunized rabbits than control ones 1.068± 0.050 gm/dl at $P \leq 0.01$ (Table 5).

Table (5): The level of immunoglobulin type-G (IgG) in control and immunized

rabbits (gm/dl).

Experimental rabbits	Immunoglobulin type-G concentration gm/dl Mean ± SE
Control	1.068 ± 0.050
Immunized rabbit	2.758 ± 0.220**
T (18)=2.8	**<0.01

Challenge of larval infestations:

During the challenge of larval infestation, all rabbits terribly attempted to groom the infested ear-pinnae, particularly in the early stages of feeding and when the ear-bags removed after 3 days, the ear showed congestion, papules, edema and small abscesses have been developed on the infested region in post-immunized rabbits. In addition, totally fed-rejected and abnormality dropped larvae onto ear-bags, individual engorged larvae were noted and number of larvae were dead in post-immunized rabbits, while in non-immunized rabbits, high numbers of engorged larvae were still sucking blood, low number of fed-rejected and abnormality dropped larvae on to ear-bags were present

DISCUSSION

The immunity status in rabbits inoculated with whole crude larval extract were studied, which showed that in immunized rabbits, the total number of white blood cells, monocytes and neutrophils were higher while the lymphocytes, eosinophil and basophil were lower in immunized rabbits than in control groups. This may be due to migration from blood stream to biting site of tick lesion causes degranulation and produced histamine. It has been

shown that the skin reaction at the attachment site on resistance response appears as cutaneous basophil hypersensitivity reactions, and decreased lymphocytes with an increase in neutrophils can be an indication of an inflammatory or an immune response due to pathogen infection (Thomas, 2007).

Phagocytosis is a vital biological process in elimination of a foreign agent from the body, revealing a non-specific cell-mediated immune reaction; accordingly, the mean phagocytic index (PI) in immunized rabbit (7.2 ± 0.61) was significantly higher than control rabbits (1.8 ± 0.24). The phagocytic activity among the immunized animals may be attributed to increase of their neutrophill percentage.

In the study of delayed hypersensitivity reactions on the skin was done after inoculated (I/d) with 0.05 mg/ml of whole crude larvae antigen of *Hyalomma anatolicum anatolicum* significantly increased the thickness of the skin in immunized rabbits after 2 hrs and reached to peak at 24 hrs than decreased at 36 hrs to 96 hrs a, and recovered to normal in 7 days. While these changes does not occur on the skin and skin fold thickness in same immunized rabbits when inoculated with normal saline into the left side.. This may be due to the antibodies remained in immunized rabbits serum more than one month, this phenomena was shown by Allen (1973) who demonstrated that guinea-pigs infested with larval tick of *Dermacenter andersoni* had exhibited three month duration of antibodies in their sera. Ellenberger et al. (1984) found that the thickening and the reddening of the skin in immunized rabbits are attributed to vasodilation which increases capillary permeability lead to local influx of mononuclear cells to the site of inoculation.

Cutaneous hypersensitivity test was used to evalute host resistance to ticks and type of reaction (cellular immunity) to unfed larval extract. The tick injects numerous physiologically active agents into the feeding lesion inducing strong inflammatory, vasodilatory and immunological responses by the host (Krober and Guerin, 2007).

Qualitative and semi-quantitative estimation of immunoglobulin concentration by several screening tests are available. These tests can be performed in clinical practice and they can provide qualitative or semi-quantitative estimates of immunoglobulin concentration by using total protein, sodium sulfite precipitation test and glutaraldehyde coagulation test (Mary et al., 2004).

The sodium sulfite precipitation test is based on the fact that immunoglobulins can be selectively precipitated from serum using concentration of a hydrous sodium sulfite ranging from 14%, 16% and 18%. A hydrous sodium sulfite concentration is required to cause precipitated in serum containing lower immunoglobulin concentration undergoing precipitation when mixed with a sodium sulfite with low concentration e.g. 14%, whereas sera with low Ig concentration do not undergo precipitation when mixed with the same solution of sodium sulfite. The latter sera may undergo precipitation when mixed with sodium sulfite solution of higher concentration e.g. 16%-18% depending on the Ig concentration of the serum (Mary et al., 2004).

The glutaraldehyde coagulation test is based on the fact that at low concentration, glutaraldehyde forms soluble complex with immunoglobulin, there by resulting in coagulation of the test mixture. The glutaraldehyde solution do not form coagulation in sera with Ig concentrate of less than 400mg/dl and complete or partial coagulation in sera with Ig concentration of greater than 600mg/dl.

The values of total serum protein, albumin and globulin in post-immunized rabbits were significantly higher than pre-immunized ones, after third inoculation with whole crude larvae of *Hyalomma anatolicum anatolicum*, these finding are similar have been observed by other researcher, such as Rechav et al. (1994) also concluded that the concentration of serum beta globulins increased only in Guinea pigs infested with immature ticks for the entire larval and nymphal feeding period and further added that the concentration of globulins, was highest in the Guinea-pigs exposed to medium and high numbers of ticks with long infestation intervals. Kumar and Kumar (1995) showed that the immunized rabbits had a significant reduction in tick yield weight and reduced feeding and reproductive performance of the ticks, when inoculated subcutaneously on days 0, 14 day 21 (three times) with a dose of 1 mg antigen (gut supernatant) per rabbit.

Determination of the lgG by using radial immunodiffusion plate executed in control and immunized rabbits in this study, showed that immunoglobulin concentration in immunized rabbits was significantly higher 2.758 ± 0.22 than control rabbits 1.068 ± 0.05 when inoculated intradermally with 6.8 mg protein (whole crude larval extract).

1987).

Kebede (2004) observed the highest reduction in the number of the ticks engorging and in the ability of the female ticks to lay eggs, when the female were feeding on the vaccinated cattle which leads to uptake of antibodies and other components of the host's humoral immune system, resulting in damage of the gut. Ochi (2004) showed significant elevation in the antibody titers in the sera of the immunized rabbits compared with control.

In this study, the challenge of larvae infestation with *Hyalomma anatolicum. anatolicum* in both experimental rabbits (control and immunized rabbits) using whole crude extract of larvae, showed that all rabbits attempted to groom the infested ear-pinnae, particularly in the early stages of feeding. When the ear-bags were removed after three days congestion, papules and edema observed in the site of the infestation, this may be due to cellular infiltration at the attachment site and the individual engorged, showed abnormal fed-rejected and high number of larvae was dead on to ear-bags in post-immunized rabbits. In pre-immunized (control) rabbit's high number of engorged larvae still sucking blood, low number of fed-rejected and abnormal dropped larvae to ear-bags were present. The abnormal rejection of larvae may be due to their inability to gain entrance to blood vessels as a result of the host immunological reactions , some what, similar observations were reported by Brown (1982) who found that the transfer of peritoneal exudates cell from immunized or immune serum to naive Guinea-pigs resulted in significant rejection of larval and adult (35.1%) of *Amblyomma americanum* tick associated with cutaneous basophill responses indicating humoral and cell-mediated immune response.

Das et al. (2005) found protective effect against tick infestation using purified 37 kDa larval antigens and demonstrated that the larvae of Hyalomma a. anatolicum are an important source of biological material for isolation of protective antigens. Only the larval extract of *Hyalomma anatolicum anatolicum* showed significant protection against ticks challenge in immunized rabbits. This may be due to the presence of higher immunoprotective antigen concentrations in larval extract (Moshaveri-nia et al., 2008). Three successive infestations of rabbits with adult of *Hyalomma dromedarii* induced significant immunity expressed as an inhibition of fertility of the ticks and laying fewer eggs than ticks fed on non immune animals (control) (Habeeb et al., 2009).

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