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# THE INDUCTION OF RESISTANCE TO LICE BY ARTHROPODS

Principal Investigator: Prof. Rachel Galun

Grantee Institution: Department of Parasitology, Hebrew University-Hadassah Medical School, Jerusalem, Israel.

Collaborator: Dr. J.O. Ochanda

Institution: Dept. of Biochemistry, University of Nairobi, Kenya

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**Executive Summary**

Resistance to human body lice, Pediculus humanus humanus, induced by feeding on rabbits immunized with an extract of the louse midgut was studied. Lice fed on immunized rabbits showed higher mortality, took smaller quantities of blood, laid less eggs, their eggs were less viable, and the nymphal stages took longer to develop to the next stage, as compared with lice fed on control animals. The proportion of dead nymphs and female lice with ruptured guts was significantly higher in lice fed on immunized rabbits. All these parameters together resulted in a steady decrease in the louse population. From an extract of the louse midgut 18 proteins ranging between 12 KDa and 117 KDa were detected by electrophoresis. Serum from rabbits immunized with a louse midgut extract recognized nine immunogenic proteins. Four of them were electoeluted from the gel and laboratory animals were immunized with the individual antigens. Although each protein induced significant protection, it seems that for an effective vaccine at least two proteins should be used. Four of the immunogenic proteins were also detected by immunoblot in the feces of the louse. Preliminary studies showed that host animals could successfully be immunized with fecal extracts of the parasite. Most of the immunogenic antigens were localized on the microvilli of the midgut epithelial cells. One proteolytic enzyme, a leucine aminopeptidase, was detected in the midgut of the louse. At least four proteins were identified which were common to the human body louse, the human head louse, Pediculus humanus capitis, the cattle louse, Hematopinus africanus and the goat louse, Linognatus stenopsis. In the framework of this project the Kenyan scientists were introduced to the techniques of lice rearing, artificial membrane feeding, removal and collection of louse guts, extraction of gut antigens, and bioassays for assessing resistance of immunized host animals to lice. They were also introduced to different aspects of louse biology and control which could enable them to deal with lice and louse-borne diseases in Kenya and in neighboring developing countries. They are continuing their studies on the kinetics of proteolytic enzymes of the louse midgut and have the background to clone and analyze the genes encoding for the most protective proteins. They are also able to overproduce these proteins in appropriate expression systems. Similar studies using hidden antigens of the parasite, were conducted previously with a few hematophagous arthropods.

**Research Objectives**

The purpose of this project was to develop a new mode of louse control in domestic animals. Resistance would be induced by immunizing rabbits with an extract of human body louse midgut. Animals with louse infestation are treated today with topically applied insecticides. Recent awareness of insecticide danger to our health and the environment, combined with the emergence of lice strains resistant to insecticides, indicate a need for developing alternative control methods.

Interest in vaccination of host animals with "hidden" or "concealed" antigens of the parasite has increased markedly in recent years. A good example is the vaccination of the cattle with the cattle tick, Boophilus microplus, by inducing an immunologic reaction against antigens in the tick gut. The uptake of antibody during feeding leads to rupture of the tick gut. One of the responsible tick gut antigens has been purified and characterized (Bm86) (Willadsen & McKenna, 1991; Willadsen et al. 1989). A recombinant form, rec-Bm86, was produced in E. coli and contained 640 of the 650 amino acids of the native tick protein (Rand et al. 1989). Today the vaccine is commercially available under the name TickGARD. Similar studies were carried out on blowfly (Lucilia cuprina) (Tellam et al. 1994) as well as with mosquitoes, stable flies and tsetse flies (Willadsen et al. 1993).

Preliminary studies showed that host animals can successfully be immunized using fecal extracts from body lice. We also showed that different lice species share common antigens, and therefore human body lice, which are very easy to colonize, could provide the antigen to be used for the vaccination of different domestic animals against their own lice. To our knowledge this was the first time where midgut antigens were isolated from fecal pellets of a blood-sucking arthropod.

**Methods and Results**

A. Materials and Methods

1. Lice. A colony of *P. h. humanus* has been maintained in our laboratory since 1983 and was originally obtained from Dr. Maunder (London School of Tropical Medicine and Hygiene). The lice were fed on rabbits and maintained at 30°C and 70-80% relative humidity.

2. Rabbits. Outbread albino New Zealand rabbits weighing 2-4 kg, with no previous ectoparasitic infestation, were used for the feeding of lice and for immunization with lice extracts.

3. Lice midgut. Female and male lice starved for 24 hrs were dissected in bleeding buffer (0.01 M phosphate buffered saline (PBS), pH 7.2) containing 5 mM EDTA and the anti-protease P.M.S.F.) under a stereo microscope. The lice were pinned on sticky tape in a petri dish. Using a fine scalpel, the head and abdomen were gently separated until the whole gut was visible. The midgut was then removed and the salivary glands and symbionts removed. The guts were rinsed and stored in bleeding buffer.

4. Antigen preparation. The midgut extract of adult body lice were used to immunize rabbits and for the biochemical and immunological studies. The number of midguts used varied according to the experiment from 300 to 11,000. The guts were rinsed in 0.01 M PBS and homogenized in 1 ml of extraction buffer consisting of the non-ionic detergent Triton X-100 (0.1%). All extractions were carried out at 4°C. The homogenate was centrifuged at 13,000 g for 30 min. The supernatant was stored at -20°C until used. Protein concentration was determined by the Lowry et al. (1951) method.

Fecal pellets of the human body louse which had been deposited during the previous 15 hrs, were collected from containers in which the lice were kept when not feeding. The faecal material was first separated from other louse particles by sieving (0.2 mm mesh); 0.8 g of fecal material was dissolved in 3.3 ml of 0.01 M PBS, centrifuged

at 2,000 g for 5 min, and the supernatant was collected. Rabbit blood, diluted 1:2,000 in PBS was used for comparison with fecal extracts using electrophoresis and immunoblotting techniques.

One hundred each of body lice, head lice and cattle lice were homogenized in 1 ml PBS, and 30 goat lice were separately homogenized in 0.3 ml PBS using Polytron (Kinematica, Switzerland) and stirred overnight at 4°C. The homogenates were centrifuged at 6,000 g for 10 min and the supernatant was collected.

5. Immunization. Rabbits were injected with 500 μg of louse midgut extract (LME) proteins at 3-week intervals. Freund's complete adjuvant (Difco) was used for the first injection given intramuscularly in both hind legs. Freund's incomplete adjuvant was used for the two subsequent injections given subcutaneously in the neck area. Serum samples were collected before immunization, 2 weeks after the last injection and 1 week after lice feeding had terminated. Control rabbits were injected with chicken ovalbumin (Sigma) as described by Ben-Yakir et al. (1986).

6. Enzyme-linked immunosorbent assay (ELISA). This technique was used to determine the rabbit anti-louse IgG titer. An extract of unfed first nymphal lice was used as the antigen for this assay in order to avoid high background due to ingested rabbit IgG. The antigen was plated at a protein concentration of 1 μg/ml directly on the plate. Other conditions were similar to those described by Ben-Yakir et al. (1987).

7. Evaluation of immunity. The initial population allocated for each rabbit totalled between 300 and 600 lice from all developmental stages. Lice were fed on a rabbit for 1 hr every 48 hrs; for a total of five times. A different area of the rabbit was used for each feeding in order to reduce possible adverse effects to the lice resulting from localized skin reactions (Bell et al. 1982). The mean weight of blood taken by a female louse, the number of eggs produced per female, and percentage hatching were calculated. The lice mortality, the percentage of lice that died with a ruptured gut and the developmental rate of the

different nymphal stages were also recorded. Rabbits were checked for 5 months after the initial experiment, in order to determine the duration of protection provided by the lice immunization.

8. Statistical evaluation. The difference in survival time between lice fed on the immunized and those fed on control rabbits, was tested using the log rank test (Lawless, 1982). The Mantel-Hanzel statistic was used to test odds-ratio (Fleiss, 1981). Changes over time in both bloodmeals and number of eggs were evaluated using Spearman's D-test for trend and Friedman's Q-test (Lehmann, 1975). The differences, at each post-treatment time point, between the immunized and control rabbits in the percentage of hatched eggs was tested using the Mantel-Hanzel test and the Bonferroni's procedure for simultaneous interference (Miller, 1981).

9. Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was carried out in the presence of SDS with a discontinuous buffer system (Laemmli, 1970).

10. Staining for lipids and carbohydrates. Protein samples separated by gradient SDS-PAGE were stained for both carbohydrates and lipids using the Periodic Acid Schiff reagent (PAS) (Kapitany & Zebrowski, 1973) and Sudan black (Narayan, 1975), respectively.

11. Western blotting. Electrophoretic transfer of the protein polypeptides from the lice midgut extract from SDS-PAGE to nitrocellulose membrane (NCM) filters was performed according to the method of Burnette (1981).

12. Electroelution of immunogenic proteins. The proteins were run on a 6-15% SDS gradient PAGE. After running the gel, the bands were cut and loaded into the electroeluter glass tubes (Bio-RAD). Electroelution was carried out at 10 mA per tube for 8 hrs. At the end of elution the current was reserved for 1-2 min. The run was stopped and proteins were collected immediately for the silicon adaptors according to the manufacturers instructions.

13. Precipitation of serum antibodies. Antibodies in immune serum were concentrated using the ammonium sulphate precipitation method according to Harlow & Lane (1988).

14. Immunoaffinity chromatography. Immunogenic proteins were isolated by coupling antibodies to CNBr-activated Sepharose 5MB beads (Pharmacia), and eluting antigens from an immunoaffinity column according to the method described by Harlow & Lane (1988).

15. Limited trypsin digestion. Louse midgut proteins of 35 KDa and 63 KDa were subjected to limited trypsin digestion by incubation of 20 µg of each protein with a constant amount of trypsin at a trypsin:protein weight ratio of 1:5 for a period of 90 min at 37°C, in a water bath. The reaction was initiated by addition of trypsin in PBS to each protein sample and stopped by addition of SDS-PAGE sample buffer and boiling for 5 min (Robbs et al. 1988). Both samples were subsequently resolved on a 18% SDS-PAGE. Protein fragments on half the gel were blotted onto a nitrocellulose membrane and their immunogenicity analyzed.

16. Aminopeptidase assay. For soluble enzyme assays 1,000 midguts were homogenized in cold 0.15 M NaCl in a glass-glass homogenizer and then centrifuged at 10,000 g for 10 min at 4°C. For membrane associated enzymatic activity, midguts were homogenized in 0.15 M NaCL containing 1% Triton X-100. The supernatants were then assayed for aminopeptidase activity. The reaction mixture (950 µl of 50 mM phosphate buffer, pH 8.0 at 30°C, and 25 µl of 0.16M leucine-paranitroanilide (Sigma) in DMSO) was equilibrated at 30°C for 15 min. Midgut homogenate (25 µl) was added and the reaction was allowed to run for 30 min, and then stopped by the addition of 0.3 µl of 30% acetic acid. Absorbance was determined at 405 nM using a Beckman Du spectrophotometer. The enzyme activity was then calculated using an extinction coefficient of 8,800 mm $cm^{-1}$. For inhibition studies, the substrate at different concentrations was incubated with the given concentration of inhibitor

before the midgut homogenate was added.

17. Indirect immunofluorescence technique. Louse midguts were embedded in Tissue Tek (Miles) and stored at -20°C overnight. Sections of 4 µm were cut in a cryostat at -18°C and transferred to a slide coated with chromium gelatine. The gut sections were incubated with 70 µl of the antisera (1:10) for 30 min at room temperature, rinsed 3 times with PBS and incubated again with 70 µl of FITC-conjugated anti-rabbit IgG (1:20) (Sigma) for 30 min. They were then rinsed with PBS and covered with a drop of glycerine:PBS (1:1) mixture and a cover glass. Examination of the sections was carried out using a Bausch & Lomb balplan fluorescence microscope at a magnification of x 500.

18. Immunogold labeling. Midguts of the body louse were fixed in 3% glutaraldehyde buffered in PBS (pH 7.3) for 3 hrs at 4°C. Post fixation was carried out in 1% glutaraldehyde. Dehydration was performed in a graded ethanol series followed by 20 min in propylene oxide. Specimens were embedded in Araldite, and ultrathin sections (600-700 A°) were cut with an LKB ultramicrotome. The grid-mounted tissue sections were initially treated with saturated sodium metaperiodate for 1 hr at room temperature. They were washed in PBS and then with 1% ovalbumin for 5 min. The samples were then incubated with the antiserum (1:10) for 2 hrs. After rinsing with PBS they were incubated with 1% ovalbumin for 5 min and then with a 0.02% protein A-gold (Biocell, gold particle size 10 nm) solution for 30 min. The sections were stained with 2% uranyl acetate and lead citrate. Control sections were incubated with sera from control rabbits.

19. Electron microscopy. The specimens were viewed with a Philips CM 12 electron microscope operating at 100 KV.

B. Results

1. Induced resistance. The mortality of lice fed on immunized rabbits was 73%, significantly higher than that of lice fed on control rabbits (52%) ($p < 0.01$). The proportion of dead nymphs and female lice with ruptured guts was significantly higher in lice fed on the immunized rabbits ($p < 0.01$). The size of the bloodmeal was 35% greater in female lice fed on control rabbits than on immunized rabbits. Lice fed on immunized rabbits laid 40% less eggs than those fed on the controls, they also demonstrated a significant decrease in the number of eggs per female over time ($p < 0.01$). 86% of the eggs laid by lice fed on immunized animals hatched, compared with 92% hatching of eggs laid by the lice fed on control animals ($p < 0.01$). With the exception of the first bloodmeal the percentage of hatched eggs which were laid between any two bloodmeals was significantly smaller (p ,0.01) in the lice fed on immunized rabbits than in the control group. The first nymphal stage of lice fed on immunized rabbits took an average of 5.2 days to moult to the second stage, compared with 4 days for those fed on control rabbits. Immunization of rabbits with LGE induced a high titer (1:10,000 using ELISA) of specific IgG in all animals. The antibody titer remained high without significant changes during six months post immunization. The resistance acquired by the immunized rabbits lasted for at least 6 months (Ben-Yakir et al. 1994).

2. Characterization and properties of the immunogenic proteins. Eighteen proteins ranging between 12 KDa and 117 KDa were found in the midgut extract. Antibodies raised against the midgut extract reacted strongly with nine midgut proteins of relative molecular weights of 17 KDa, 29 KDa, 35 KDa, 40 KDa, 50 KDa, 55 KDa, 63 KDa, 97 KDa and 117 KDa when tested by the Western blot technique. The analysis of the immunogenic proteins revealed that the 17 KDa, 29 KDa, 35 KDa and 97 KDa proteins were glycosylated and none of them contained a lipid moiety. Using the electroelution technique four major proteins with

a MW of 17 KDa, 29 KDa, 35 KDa and 63 KDa were purified. On trypsinization, the proteins of 35 KDa and 63 KDa produced four major fragments when resolved on a 18% SDS-PAGE. However, only fragment F1 of the 35 KDa protein appeared to be a potential immunogen (Ochanda et al. 1994). Although amino acid composition and sequence analysis was done the data did not provide much information. The analysis is to be redone once more for both 35 KDa and 63 KDa proteins.

3. Protective immunogens. The immunization of rabbits with each of the four electroeluted proteins produced significant protection, but not one of these proteins provided a protection as high as the whole midgut extract, meaning that for an effective vaccine at least two proteins should be used (Ochanda et al., in preparation).

4. Collection of antigens from other sources. Three midgut proteins with a MW of 17 KDa, 29 KDa and 35 KDa were isolated using immunoaffinity chromatography and SDS-PAGE electrophoresis. Electrophoresis of the fecal pellets of the louse and subsequent immunoblotting using antisera to louse midgut extract revealed 8 proteins ranging between 17 and 86 KDa. The 17 KDa, 29 KDa, 35 KDa and 63 KDa proteins were common to both the fecal material and the midgut. Antisera to a midgut protein extract also recognized a 63 KDa protein in rabbit blood on which the lice were fed.

Comparison of the proteins of the human body louse with those of the head louse, the goat louse and the cattle louse, using electrophoresis and Western blotting with sera raised against a whole human body louse extract, revealed the presence of at least 4 common proteins ranging between 38 and 44 KDa (Mumcuoglu et al. 1994).

5. Aminopeptidase activity. The midgut of body lice has both non-detergent and detergent-soluble leucine aminopeptidase active in alkaline pH 8.0. The enzyme release is stimulated by a blood-meal. Using a constant enzyme concentration and increasing substrate concentration, a typical Michaelis Menten curve (sigmoid/hyperbolic)

was obtained. Thus an increase in substrate concentration resulted in a very rapid rise in the velocity of reaction rate of both soluble- and detergent-soluble enzymes. However, the activity of aminopeptidase in the latter was higher than in the former. It was also shown that the water-soluble and detergent-soluble enzyme is the same one but it has two different catalytic rates for substrates. One might be required when the substrate concentration is low while the second is required when the substrate concentration is higher, according to their affinities. The Lineweaver-Burk plot for the inhibition studies indicates that aminopeptidase activity is competitively inhibited by the substrate analogue (Ochanda et al. in preparation).

8. Localization of the immunogenic antigens. Localization of immunogenic midgut antigens was carried out by reacting the midgut tissue with antisera to a louse-midgut extract and subsequently probing with fluorescent and gold-labelled antibodies. A strong fluorescence was observed on the outer membrane of the epithelial cells of the midgut. The immunogold technique revealed that most of the antigens were localized on the microvilli of the midgut cells (Mumcuoglu et al. 1994).

**Impact, Relevance and Technology Transfer**

An anti-lice vaccine for animals would reduce monetary losses to farmers and help to increase the source of food supply by improving livestock production in developing countries. Development of a vaccine will help to eliminate problems of lice resistance to insecticides and the toxic effects on animals, animal products and environment caused by use of such compounds.

Dr. Ochanda and his team are now able to conduct research on lice and louse-borne diseases in Kenya and in neighboring developing countries. In the future they should be able to use recombinant DNA technologies for the mass production of protective antigens. They are

also able to develop vaccines against other hematophagous arthropods Dr. Ochanda is currently involved in the development of a vaccine against tsetse flies. To our knowledge the Department of Biochemistry in the University of Nairobi is the only center in Kenya working on the research of lice and lice control.

The findings show that there is a good potential for the development of a louse-vaccine which could be used on a large scale on domestic animals and sold commercially.

**Project Activities/Outputs**

Dr. J. Ochanda visited Israel twice (17.6.-1.7.1991 and 27.4.-3.5.1993) and he sent one of his students, Mr. Jacob Okuru, for a two-week stay (21.1.-3.2.1992) to our department in Jerusalem. Dr. Mumcuoglu visited Kenya during the period 6. - 21.6.1994.

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Two further manuscripts on the enzymatic activity of the gut

extracts and a manuscript on the bioassay with individual antigens and the fecal material are being prepared.

*Abstracts/Lectures*

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**Project Productivity**

We believe that the project accomplished all its proposed goals.

**Future Work**

More work remains to be done concerning the characterization of the protective antigens and their ability to induce resistance separately, or in combination with other immunogenic proteins. The genes encoding for the most protective proteins should be cloned and analyzed and later overproduced in appropriate expression systems. The efficacy of the individual recombinant antigens -each separately and in

combination- should be bioassayed on laboratory animals. Similar vaccines could be prepared for the immunization of domestic animals such as cattle and pigs against their specific lice or with antigens shared with other lice species.

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