PN-ABG-711
CS-136

FIELD AND LABORATORY STUDIES ON NEW ASPECTS OF EPIDEMIOLOGY OF LEISHMANIASIS

Final Report on Program Project No. DPE-5544-G-SS-6008-00(CS-136)
June 1986- - June 1990

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Addendum

It was noticed in the last moment that the last study was erroneously not included in the report. A reprint was attached to each copy and the number of publications resulting from this project is 10.

INTRODUCTION

The report summarizes the 9 published papers that resulted from the cooperation between the Hebrew University of Jerusalem and the International Center of Insect Physiology and Ecology, Nairobi, Kenya, in a project supported by the United States Agency for International Development (see list at the end). The responsible investigators were Dr. M.J. Mutinga in Kenya and Dr. Y. Schlein in Israel. The summary was divided into studies carried out by the Israeli team (chapters 1 to 5), cooperative work (chapter 6), and studies from the Kenyan team (chapter 7). The approach and method in the work described in chapter 7 are based on earlier work of the Israeli team (Schlein and Warburg, 1986. J. Med. Ent. 23:1-15). The two resulting manuscripts were summarized for the Sixteenth Annual Report, 1988, of ICIPE, and this summary was included in this report. It should be noted that the order of authors in the joint Israeli-Kenyan publication was decided by Dr. Mutinga.

We would like to take this opportunity to express the gratitude of both the Kenyan and Israeli participants in the project, for the generous support of the AID CDR.

SCIENTIFIC BACKGROUND

The subjects of this study are specific and scientists with a broad knowledge of parasitology will not necessarily be acquainted with the problematics. Therefore, the summary was extended to include some of the background and previous results which are needed for a rational presentation.

The adaptation of the Leishmania parasites to the sandfly is the basic requirement for vector competence. This adaptation is rather strict and in many cases the vectors exhibit various degrees of resistance to infections with Leishmania strains of species other that the one they transmit in nature. This was demonstrated in several experiments with various sandflies and Leishmania species. In the field however, the vector and the adapted pathogen occur together, the vector susceptibility is probably a stable factor which does not cause fluctuations in the incidence of leishmaniasis.

Another factor in vector competence which apparently influences the epidemiology of leishmaniasis is the diet of the sandfly vector. The entire development of the Leishmania in the vector takes place in the gut and the parasites are therefore permanently in a medium consisting of sandfly food and digestive enzymes. Sandfly diet is determined by its behaviour in the search for food and the local availability of sources. Thus, the only medium of reliable quality for the Leishmania is the infective meal of blood taken with the parasites from the reservoir animals. Subsequent blood meals or sugar meals can vary in their composition and have different effects on the parasites.

Accordingly, the main subject of our studies was the effect of sandfly food, either blood or sugar meals, on the Leishmania parasites and the potential of sandfly diet as a factor in the transmission of leishmaniasis.

1. Effects of blood meal on the vector potential of Phlebotomus papatasi.

The resistance of vectors unable to support the growth and transmission of Leishmania other than the one they transmit naturally has been described several times. However, there has only been one early study (Adler, 1938. Harefuah, 14:1-6) which was aimed at investigating the phenomenon.

In the sandfly gut, the levels of proteases rise after the ingestion of blood and they could be effectors of Leishmania selection. We compared the effects of the naturally transmitted L. major and of L. donovani, on the gut enzymes of Phlebotomus papatasi, using gut homogenates of artificially infected flies and uninfected controls as enzyme preparations. The digestion of C^{14} labelled globin substrate by preparations from flies fed 24 hrs earlier on serum containing L. major was about a third less that that of controls, while that from flies infected with L. donovani was about one third greater. When added to control homogenate, in vitro, promastigotes of other species promoted proteolysis (Schlein and Romano, 1986. Exp. Parasitol. 62:376-380). Similar preparations of flies fed on rabbit blood were used in further experiments, in which the relative quantity if trypsin-like enzymes was measured at different times after the infective meal (Borovsky and Schlein, 1987).

The overall amount of the enzymes in L. major infections was reduced to 50% and 34% of the controls, 20 and 30 hrs post-feeding, and increased to

184% at 52 hrs. The general levels measured for flies infected with L. donovani did not vary considerably from the controls, however, the quantities of different enzymes that were measured from gel-electrophoresis by densitometry were different. The greater effect of L. major leads to the suggestion that the massive manipulation of gut enzymes by this species represents its defence mechanism against enzymes of its vector. To substantiate this assumption, it was necessary to demonstrate that the trypsin-like enzymes of P. papatasi were harmful to L. donovani. This was confirmed by adding 1% soybean trypsin-inhibitor to infective meals, which resulted, 3 days later, in 63% of the flies harbouring L. donovani compared to 10% in the control.

All the experiments to investigate the fit between vector and pathogen were carried out using the blood of one host, the rabbit. However, there is evidence that the choice of host can also alter vector competence, as shown by the death of L. major in P. papatasi that fed on turkey before or after the infective meal (Schlein et al., 1983. Acta Tropica, 40:65-70). Chicken blood given 24 hrs following an infective meal of parasites in saline had a similar effect and no parasites were observed in flies dissected 3 days later. As with L. donovani, it appears that trypsin-like enzymes were responsible for the vector's resistance, since the infection succeeded in 63% of the flies, when 1% soybean trypsin-inhibitor was added to the chicken blood-meal (unpublished data). According to these results, it appears that even the adaptation of L. major to P. papatasi is limited and it cannot cope with the composition of trypsin-like enzymes elicited in this sandfly by avian blood.

2. The development of sandfly forms of Leishmania in sucrose solutions.

We have previously shown that Phlebotomus papatasi that had been fed on sucrose solution was capable to transmitting L. major. Since with this diet the parasites were able to complete their cycle it was of interest to observe the effect of sucrose based media on cultured parasites.

The dominant form of Leishmania in proteinaceous cultures is the promastigote. In the sandfly the ingested amastigotes develop into elongate promastigotes and in P. papatasi these give rise to a great number of infective promastigotes, paramastigotes, and aflagellates. Paramastigotes

and aflagellates appear in small numbers in cultures and were considered degenerate. In the sandfly the parasites develop first in medium of the infective blood meal and then in the subsequent sugar meals. This study was undertaken to see whether development of the specific sandfly-forms of Leishmania can be imitated by exposure of parasites reared in NNN medium to sucrose solution. The medium used was sucrose solution alone or in mixture with Ringer's salt solution. The parasites were initially grown in NNN medium and starters of sucrose cultures were taken at different stages of the development of the NNN mother culture. In sucrose solutions L. major continued to divide and multiply by 38-46%. Paramastigotes and aflagellates constituted 79% in sucrose medium compared to maximum 15% in NNN started cultures. Motility was lost in sucrose solutions, but it was retained in a mixture of sucrose and Ringer's solutions. In the latter, the parasites exhibited transformation as well as attachment to the substrate followed by thickening of the flagellum. The thickening appeared similar to the hemidesmosomes formed by attachment of the flagellum to the cuticle in the gut of the vector. The transformation of L. major in sucrose solution resembles, and is apparently similar to the development in P. papatasi.

It is difficult to study the Leishmania forms in the sandfly as the infection of flies and harvesting of the parasites are complicated and the number of parasites obtained finally would be limited. By growing in sucrose medium it is possible to obtain greater numbers of parasites and to study the particular traits of the stages occurring in the sandfly vector.

3. Marking of Phlebotomus papatasi by feeding on sprayed coloured sugar baits.

There is no information on the actual sources of natural sugar meals of sandflies, except for the observation that in the laboratory P. papatasi feeds selectively from plants and on aphid secretion of honeydew (Schlein and Warburg, 1986. J. Med. Ent. 23:1-15). This sandfly-plant interaction suggested the possibility of marking the flies by feeding them on dyed-sugar baits in the field, assuming that the sprayed baits would compete with naturally available sources of food.

P. papatasi occurs in great numbers in the burrows of the sand-rat Psammomys obesus in the endemic focus of leishmaniasis in the Jordan Valley.

We sprayed sugar solution mixed with 1% food dye on plants in a limited area around the rodent colonies and the flies were afterwards caught with CDC light traps. The dye marked an average of 25.9%, 50% and 32.9% of the P. papatasi caught respectively after the 1st, 3rd and 6st night following spraying.

4. Dispersal of the Phlebotomus papatasi in the Jordan Valley.

The sandfly Phlebotomus papatasi is the main vector of cutaneous leishmaniasis in the Jordan Valley, where it enjoys a close ecological relationship with the main reservoir host of the disease, the burrow dwelling sand rat Psammomys obesus. In recent years intensive agriculture has changed the physical features of the Jordan Valley environment - the natural habitat is receding as more land is cultivated. Most agricultural fields lie fallow during the summer months. Nevertheless, many sandflies are to be found crossing them.

The dispersal of sandflies has been studied in the past, mainly with the mark-release-recapture technique. The overall picture resulting from these studies is that sandflies are generally sedentary, but they may travel from 200 to 2,000 meters during a night.

Assuming that samples trapped exiting borrows consist of both dispersing and non-dispersing flies, whereas samples trapped over the fallow fields would contain mainly dispersing flies, we compared them in order to characterize the dispersing population. The parameters used for comparison were proportions of males and females in the two populations, nutritional status, and in the females - parity, ovarian development, insemination and infection with Leishmania promastigotes.

In all the comparisons between flies caught over the bare field and those exiting burrows, a significant difference was found. The ratio of males to females in our laboratory colony and in numerous samples from burrows does not deviate significantly from 1:1. In the bare field the females constituted 68.5-76% of the various samples, a consistently significant departure from the expected 50%, which allows the conclusion that dispersal is mainly a female activity in P. papatasi

From the data presented in Table 1 it is apparent that gravid, blood fed females do not disperse: 26% of those exiting burrows were in stages

III-V of the gonotrophic cycle, as opposed to 11.6% of the dispersing ones. The sugar feeding status of the blood fed females exiting and entering burrows (Table 2) indicates that the main reason for their activity outside burrows is sugar feeding, which explains the short duration and localization of this activity. Blood fed females who test negative to anthrone are young females who take a blood meal in the burrows before taking a sugar meal, and are compelled to exit the burrow during the digestion of the blood meal in order to find a sugar meal.

On average 55.4% of the dispersing females were parous, as opposed to 39% of those exiting burrows, which indicates that the age of the dispersing flies is higher, and that they have already successfully completed one gonotrophic cycle. The insemination rates of burrow vs. field caught females points to burrows as the mating centers. Leishmania infections were found in 9% of exiting females, and 2.2% had no vestiges of blood in their gut. In the bare field 2.6% of the females were infected and had no blood in the gut, and none were found who had not completely digested the infective blood meal. Therefore, the proportion of host seeking infective flies in the dispersing population is similar to that of flies exiting burrows. As P. obesus is practically the only source of Leishmania in this region, and these rodents (or their tracks) were never seen in the fallow fields, it is certain that Leishmania infected females dispersed away from their burrows. It was found that in Turkemenistan the dispersal of P. caucasicus and P. papatasi was limited by the availability of burrows of the great gerbil Rhombomys opimus and in the new world several species of Lutzomyia do not cross bare, arid clearings in the rain forest. It is possible that in the Jordan Valley the abundance of humid, fertilized fallow fields enhances the tendency of P. papatasi females to disperse, therefore increasing the risk of infection. Several workers have suggested the creation of a cordon sanitaire devoid of rodent burrows around human settlements in endemic areas. This may be inadequate even if they are wider than the noctural flight range of the vector, if suitable shelter (other than rodent burrows) may be found in them. The blood fed females exiting burrows in need of a sugar meal are a more suitable target for control, in as much as the target population (Leishmania infected females) presents itself in a well defined area (near burrows) with a well defined appetitive behaviour - sugar feeding.

5. Oviposition diapause and other factors affecting the egg-laying of Phlebotomus papatasi in the laboratory

The maintenance of a sandfly colony is tedious and precarious as is evident from the variety of methods described since the first successful breeding of Phlebotomus papatasi by Wittingham & Rook (1922. Brit. Med. J. 3285:1144-1151). One of the main problems is the death of females before or during oviposition and the ensuing loss of eggs and adults. Our initial intention was to study the oviposition response of P. papatasi to stimuli in the closed environment of the cages, however, during the work we encountered the appearance of oviposition diapause, i.e., the retention of a very large proportion of eggs by gravid females towards the winter. The phenomenon was relevant, and the study was extended to include the observation on the influence of light on the diapause.

In this study we have observed that the rate of oviposition by P. papatasi females is initially a seasonal phenomenon since egg retention occurred at the onset of winter under standard insectary conditions. Exposure of gravid females to various environmental stimuli demonstrated the influence of such factors on the degree of oviposition.

Oviposition promoters.

Oviposition of P. papatasi was increased to a highly significant extent above control levels using two kinds of stimuli, availability of furrows or serrations of "egg niches" in the plaster substrate of cages, and the presence of aged cow manure (larval medium), or a combination of both these factors. The greatest number of eggs, around 40/female/week, were obtained in the presence of manure regardless of the structure of the plaster (Table 3). Preference for furrowed surface of the oviposition substrate was apparently a reaction of the roughened texture of the plaster of Paris since, smooth depressions made while the plaster was soft, had no such effect (unpublished data). A similar physical distinction between surfaces occurs with mosquitoes; for example Aedes vaxans prefers laying among a certain size of sand particles.

Increased oviposition did not cause higher fly mortality. This indicates that the series that laid significantly more eggs than controls, following exposure to manure or furrowed plaster (Table 2), had a higher survival rate at oviposition.

Oviposition inhibitors.

Reduction of oviposition occurred when larvae or pupae were present in the cage and when the flies were confined in a smaller volume. With larvae present, oviposition dropped to about a half of the control rate of 15.0 eggs/fly/week. The effect of larvae apparently diminished to some extent during the experiments, as many died, pupated or were cannibalized.

The effect of presence of pupae on oviposition was smaller and limited in time, being significantly different from the control 6.2 vs. 11.0 eggs only on the 4th day of the experiments (Table 3).

The reaction of flies to confinement in a volume smaller that that of controls was similar whether they had a standard surface area or half of it available for oviposition. In each case most of the oviposition occurred until the third day (Fig. 1) and the weekly total was only about 5 eggs/female whereas controls in larger cages produced 12.5 eggs/female/week. As we have found that reduction of fecundity is an effect of the crowding of gravid females, it is necessary to further investigate the optimum living space required for P. papatasi adults in insectary cages.

In these experiments the reaction of flies to the various test conditions was expressed in the degree of oviposition. Egg laying in nature is preceded by a search for a suitable site. Larval survival and maturation of adults will be achieved in a breeding site that includes larval food, humidity, physical protection and low larval density. Such factors would be used as cues in the search behaviour guiding the selection. Hence, experimental factors which stimulated or inhibited oviposition would under natural conditions function primarily as attractants or repellents in the search of a oviposition site.

Seasonal rate of oviposition.

Comparison of the control series during the months from April to December demonstrated that oviposition of P. papatasi was subject to seasonal variation. the average number of eggs per female between April and mid-October ranged from 11.5 to 18.6 dropping to only 1.4 eggs/female/week after mid-October. For this extreme reduction on the oviposition we suggest the term oviposition-diapause.

These changes took place in a stock of P. papatasi maintained in constant conditions of 28°C temperature, high humidity and a summer

photoperiod of 17:7 L.D. The rate of oviposition of P. papatasi in the laboratory is thus evidently controlled by a circannual rhythm. It is interesting to compare the abundance of P. papatasi in nature with the rate of oviposition in the laboratory. It seems, that the increase of oviposition in August (Fig. 2) gives rise to the build up of peak populations of adults in September-November (unpublished observations), whereas the decline of oviposition in November precedes the winter disappearance of adult flies in the Jordan Valley at about the end of December. The seasonal rhythm of oviposition therefore seems to be an important factor in determining the seasonal density of P. papatasi adult populations in the field.

The results demonstrate that oviposition diapause can be broken by ultra violet light. UV in the sunlight that reaches the earth is of waves longer than [illegible]0 nm. Comparison of the UV averages of oviposition of P. papatasi [illegible]onstrate a good correlation (Fig. 2). Furthermore, between October and April, there is no UV in the sunlight until 8.^{00} hrs and after 16.^{00} hrs. Thus, very little UV irradiation can reach P. papatasi adults when they are active at dawn or dusk at the end of the sandfly season. It is therefore tempting to suggest particular wave-lengths of UV light set the cycle of oviposition-diapause of P. papatasi. This differs from the way that photoperiod, in the form of day-length, governs winter diapause of many organisms, for example the mosquito Culex pipiens in Israel.

Since this is the first observation of oviposition diapause in sandflies, it is not yet known whether it occurs in species other than P. papatasi and whether this phenomenon is correlated to larval diapause. Possibly UV light is the primary cue for both diapauses and this possibility could be investigated in a species such as P. ariasi in which larval diapause could not previously be interrupted.

6. Evaluation of food-dye marking in the study of sandfly behaviour in Baringo district, Kenya

Evaluation of the food-dye marking method for the study of sandfly behaviour was carried out in Baringo district, Kenya. This method was developed by us and tried successfully in the Jordan Valley (see previous chapter in the report).

Bait solution of sucrose and food-dye was sprayed on the vegetation around an animal burrow which was the source of sandflies. The extent of

marking and the distribution of coloured flies within a radius of 200 m was monitored for 4 nights. The species caught were Sergentomyia clydei, S. schwetzi, S. antennatus, S. squamipleuris, S. bedfordi, S. africanus, S. ingrami and S. affinis. Feeding on the bait marked 68.9% of the flies caught at burrow's entrance. The total number of flies caught at a distance of 50, 100, 150 and 200 m exceeded the theoretical expectation. The varied marking of species in the different locations was discussed in the paper.

The usual method for studying sandfly dispersal is based on the application of dyes to captured flies, such marking interrupts the normal behaviour of the sandflies and the recapture is usually low.

The present method can be used for several purposes in the study of sandfly behaviour feeding and dispersal. It could also be used for the assessment of control agents that will be added to the sprayed solution. The influence on caught flies can be observed back in the laboratory, using unmarked flies as controls. It is also possible to study the behaviour of populations since the marking can continue for a long period. Two major problems in the epidemiology of visceral leishmaniasis in Kenya are the identification of the reservoir animals and the location of the sources of infection. Monitoring the behavior of sandflies with the method may help to solve these problems and elucidate other aspects in the behaviour of vector sandflies.

7. Ability of the sandfly S. ingrami to feed on plant juices.

The diet of sandflies is likely to affect the development of Leishmania, the causative agent of leishmaniasis which they carry, and thus influence the transmission and distribution of the disease. Plant sugars are part of the diet of sandflies, and it is suspected that many species obtain sugars directly from plants. We therefore investigated the ability of S. ingrami to feed on various species of plants. A total of 4,838 female and 3,509 male, teneral, laboratory-reared sandflies 1-2 days old were caged in a number of replicates and offered shoots of a total of 46 plant species, both indigenous and exotic, belonging to 21 families of Kenyan plants. The tests lasted for 16 hours at 25±1°C and 90% r.h. Control sandflies were kept under the same conditions, but were not offered plants. The sandflies were then dissected and tested for the presence of sugars using the Anthrone

test. The feeding performance of the sandflies was measured by the percentage feeding rate (PFR):

$$PFR = 100\ (b+c+d)\ /\ a+b+c+d$$

where a = number of sandflies which failed to feed, b = number of fed sandflies with faint blue staining intensity, c = number of fed sandflies with intermediate blue staining intensity, and d = number of fed sandflies with deep blue staining intensity in the Anthrone test. A statistical analysis was carried out and significance was determined at the 5% level.

There was no overall significant difference between the sexes when comparisons were made on the combined plant families. Among the families most preferred (with mean PFR±s.e.) were: Musaceae (21.3±2.8), Araceae (5.3±0.4), Compositae (5.1±0.1), Umbelliferae (4.5±0.5), Myrtaceae (4.6±0.1), while the least preferred were Camelineae (0.7±0.1) and Alliaceae (0.1±0.04). The control sandflies were all negative in the Anthrone test.

The data were also examined for species preferences within families. For example in the Compositae, Bidens pilosa (86.8±4.3) and Galinsoga parviflora (78.0±2.1) were the most preferred, compared to Chrysanthemum coccineum (2.0±0.2) which was the least preferred. Similarity in feeding preference by male and female sandflies was confirmed, for instance, for the following species (with mean PFR values for female and male, respectively, in parenthesis): Asystasia schimperi (31.2 and 30.2), Bidens pilosa (67.1 and 70.3), Conyza floribunda (20.0 and 22.1), Veronia lasiopus (36.2 and 38.2), Ficus natalensis (19.2 and 19.9), Neonotonia wightii (33.1 and 33.6), Lycopersicon esculentum (5.0 and 5.0) and Solanum incanum (2.0 and 2.0).

Detailed chemical analysis of the host plants will be needed to elucidate the feeding performance of S. ingrami and other sandflies. This is essential if we are to understand the influence of sandfly diet on the transmission of Leishmania.

PUBLISHED STUDIES SUPPORTED BY CDR GRANT

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TABLE 1. Ovarian development, parity and insemination in female *P.papatasi* trapped while exiting from burrows or dispersing.

Trap site	Percentage of total in each physiological category*						*n*
	I–II; +; P	I–II; –; P	I–II; +; N	I–II; –; N	III–V; +	III–V; –	
Fallow field	10.8	54.7	19.5	4.3	2.1	8.6	92
	6	57.7	18.3	6	6	6	66
	12	32	28	8	12	8	50
	44.5	18.5	22.2	7.4	0	7.4	64
	8	32	48	4	4	4	62
$\bar{x}$±SD	16.2±2.9	39.2±3.8	27.1±3.5	5.9±1.8	4.8±1.7	6.8±2	334
Burrows	34.2	8.5	31.4	2.8	17.4	5.7	70
	18.1	18.1	31.8	4.5	27.5	0	44
	27.2	5.5	38.8	0	19.4	8.3	71
	40.7	3.7	29.6	0	18.5	7.4	54
$\bar{x}$±SD	30.1±4.1	8.9±2.5	33.2±4.2	1.8±1.2	20.7±3.6	5.3±1.9	239

*I–V indicate stages of ovarian development; + or – indicate insemination; P=parous; N=nulliparous; *n*, number collected per trap site.

TABLE 2. Fructose in guts of bloodfed and non-bloodfed female *Phlebotomus papatasi* exiting or entering sand rat burrows.

Behaviour	Bloodfed	No. examined	Positive (%) (±SD)
Exiting burrows	+	52	38.4±4.2
	–	46	78.2±5.7
Entering burrows	+	49	93.8±3.9
	–	48	81.2±6.8

TABLE 3. Average 1-week oviposition of *P.papatasi* females on substrates with different treatments (April–October).

Treatment	Day	Experiment (plaster with treatment)		Control (plain plaster)		*P*
		Mean	SE	Mean	SE	
Furrows present, cow manure absent	1	1.6	0.87	0.9	0.45	NS
	2	2.5	0.93	1.6	0.72	NS
	3	5.2	1.84	2.9	0.57	NS
	4	8.4	3.39	3.5	0.64	NS
	5	15.1	4.77	5.3	0.38	<0.05
	6	19.8	3.66	6.7	1.08	<0.005
	7	29.6	1.38	10.2	1.96	<0.001
Cow manure present, furrows absent	1	16.9	4.4	7.1	2.58	NS
	2	20.3	3.3	7.7	2.62	<0.02
	3	25.8	4.0	9.5	2.04	<0.01
	4	27.9	3.47	10.2	1.81	<0.002
	5	33.9	4.9	11.4	1.9	<0.005
	6	36.8	5.39	14.2	3.54	<0.01
	7	39.7	3.63	28.6	5.31	<0.02
Cow manure and furrows both present	1	12.0	3.47	7.2	3.00	NS
	2	19.0	4.77	8.0	2.98	NS
	3	25.7	5.1	8.9	2.85	<0.05
	4	29.8	4.31	9.0	2.81	<0.01
	5	34.4	4.37	9.4	2.82	<0.005
	6	39.1	3.04	13.5	4.6	<0.002
	7	40.9	2.44	20.9	6.33	<0.01

SE=standard error; NS=not significant.

TABLE 4. Average 1-week oviposition of *P.papatasi* females in presence of third–fourth stage larvae or pupae compared to control females in the absence of immature stages (April–October).

Treatment	Day	Experiment Mean	Experiment SE	Control Mean	Control SE	*P*
Larvae	1	0.4	0.3	0.2	0.14	NS
	2	0.5	0.3	1.0	0.46	NS
	3	0.6	0.31	3.4	0.74	<0.01
	4	0.9	0.31	6.5	1.8	<0.01
	5	1.4	0.52	9.1	2.11	<0.01
	6	4.3	2.5	12.3	3.37	NS
	7	7.7	4.05	15.0	4.73	NS
Pupae	1	2.4	1.26	5.6	2.55	NS
	2	3.4	1.21	6.0	2.61	NS
	3	5.0	1.19	8.7	1.87	NS
	4	6.1	1.04	11.0	1.56	<0.05
	5	9.2	1.09	12.8	1.72	NS
	6	14.2	1.89	17.0	3.33	NS
	7	16.7	2.29	21.8	5.39	NS

SE = standard error; NS = not significant.

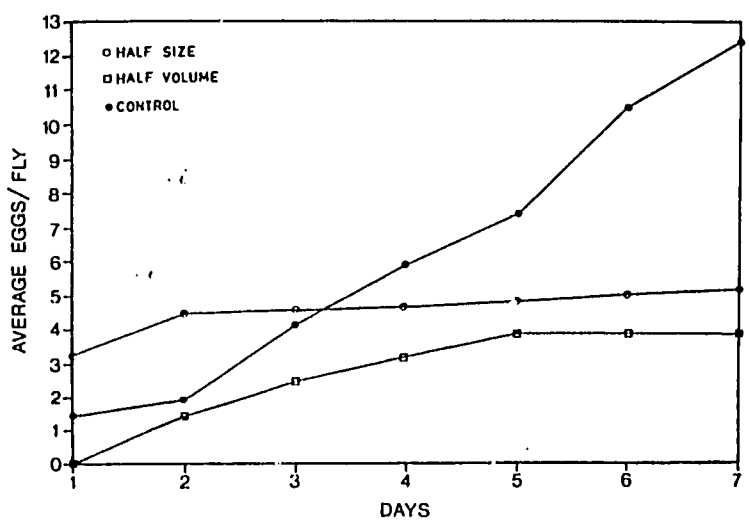

FIG.1. Oviposition by *P.papatasi* females in standard control cages, cages with half standard area of plaster substrate, or cages of half-volume with half-sized area of plaster substrate; 15 females/cage; average eggs/fly/day for 7 days.

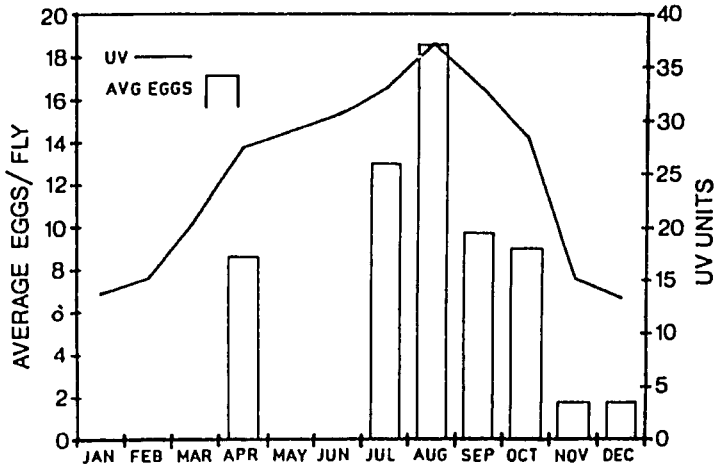

FIG. 2. Monthly levels of fecundity of laboratory-bred *P.papatasi* (female weekly averages) compared to concurrent maximal sun ultraviolet radiation in Jerusalem; values unavailable for January–March and May–June; UV in relative units (Ashbel, 1937).

Released glycoconjugate of indigenous *Leishmania major* enhances survival of a foreign *L. major* in *Phlebotomus papatasi*

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Abstract

The effect of *Leishmania* glycoconjugate in the vector was investigated using *Phlebotomus papatasi* artificially infected with a *Leishmania major* strain that this vector does not transmit in nature. Glycoconjugate of the vector-specific strain of *L. major* was added to the infective meals of some fly groups and the success of infections with or without this substance was compared 4 d later. In the absence of glycoconjugate the parasites survived in 15·6% of the flies, while the addition of 0·5 mg/ml and 2 mg/ml raised the rate of infection to 34·3% and 63·0% respectively. Undigested blood was found in the stomach of 7·8% of the infected flies, whereas following meals with parasites and 2 mg/ml glycoconjugate it was present in 37% of the flies. The results demonstrated that this glycoconjugate increased the viability of the parasites in the unsuitable vector and delayed digestion of the infective meals. In a parallel experiment the glycoconjugate of *L. donovani* did not affect the survival of the parasites.

Introduction

Recent research on the role of *Leishmania* glycoconjugates has concentrated on the interactions of the parasites with the vertebrate host. The main suggested functions were the facilitation of attachment and entry of the parasites into host cells (HANDMAN & GODING, 1985; HANDMAN *et al.*, 1986; PUENTES *et al.*, 1988) and protection of amastigotes against enzymatic activity in the phagolysosome (EL-ON *et al.*, 1980; EILAM *et al.*, 1985; MCNEELY & TURCO, 1987; BUCHMULLER-ROUILLER & MAUEL, 1987). However, glycoconjugates are produced and released in abundance by promastigotes (SCHNUR *et al.*, 1972), the form occurring in the sandfly. This suggests that the glycoconjugates have a role in the development of the parasites in the vector. This study investigated the possibility of the glycoconjugate of a vector-specific strain of *Leishmania* affecting the survival of promastigotes in the arthropod host.

The major cell surface glycoconjugate of *L. donovani* (KING *et al.*, 1987) and *L. major* (MCCONVILLE *et al.*, 1987) was defined as lipophosphoglycan. We used the released carbohydrate-rich glycoconjugate purified from spent culture medium (excreted factor: SLUTZKY *et al.*, 1979), assuming that the substance detached from the parasites affects the ambient conditions in the sandfly gut. This molecule probably comprises only the distal components of the membrane-anchored glycoconjugate (TURCO, 1988).

Materials and Methods

Sandfly stock. The *Phlebotomus papatasi* were from a colony founded and restocked annually with flies from the Jordan Valley. The colony is maintained at 29±1°C and relative humidity of 80%.

Leishmania *strains and glycoconjugates.* The parasites used were *L. major* LRC-L137 (MHOM/IL/67/Jericho II), a human isolate from the Jordan Valley, and an isolate of *L. major* LRC-L119 (MTAT/KE/00/T4) from *Tatera nigricauda* in Kenya (HEISCH *et al.*, 1959). Glycoconjugates of *L. major* LRC-L137 and Indian *L. donovani* (MHOM/IN/54/LRC-L52) were purified from spent culture media according to a modification by SCHNUR (1982) of the method of SLUTZKY *et al.* (1979). Before being dissolved in the blood meal, the glycoconjugates were sterilized dry in glass vials, immersed in boiling water for 1 h.

Experimental procedure. Experimental fly groups consisted of 1–5 days old females that were membrane-fed on sterile infective meals as described by SCHLEIN *et al.* (1983). The meals contained 10^6/ml late logarithmic-phase promastigotes in rabbit blood inactivated at 56°C for 30 min. Purified glycoconjugate was added to some infective meals in quantities of 0·5 or 2·0 mg/ml. The calculated number of parasites in a fly's meal was between 100 and 200 promastigotes and the quantity of glycoconjugate was 0·5–1 μg per fly in one series and 2–4 μg in the second. After feeding, the engorged females were separated, maintained for 4 d on 20% sucrose solution and water, and then dissected. The gut of each female was examined, using a phase contrast microscope. The location of parasites in the gut was observed and their number was estimated. The results were statistically analysed using Fisher's exact test (ZAR, 1984).

Results

The initial observation was that the susceptibility of *P. papatasi* to strain LRC-L119 was low. Only 15·6% of the flies that ingested parasites retained the infection for 4 d (Table). In addition the infections did not develop as in a normal vector, but remained limited to the abdominal midgut. Parallel experiments with the strain LRC-L137, which occurs naturally in *P. papatasi*, produced infections in 86% (142/165) of the flies.

The presence of 2 mg/ml *L. donovani* glycoconjugate in the infective meals did not improve the viability of strain LRC-L119, the parasites being found, mostly in low numbers, in 17·7% of the flies (Table).

The survival of strain LRC-L119 in the sandflies was enhanced when the glycoconjugate of strain LRC-L137 was added to the infective meals. Addition of 0·5 mg/ml raised the proportion of infected females to 34·3% and 2 mg/ml raised it to 63·0%. The difference between controls and each of these series was statistically significant ($P<0·001$). The improvement of survival was mainly expressed by an increase in the number of flies with heavier infections (level III, Table).

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Table. Survival rate of *Leishmania major* LRC-L119 promastigotes in groups of *Phleobotomus papatasi* following infective meals with or without glycoconjugate of the vector-specific *L. major* LRC-L137

Glycoconjugate: source & quantity	No. of replicates	Number of sandflies: Total	With blood in gut	With infection level[a]: I	II	III	Total no. infected
None	6	192 (100%)	15 (7·8%)	13 (6·8%)	10 (5·2%)	7 (3·6%)	30 (15·6%)
L. donovani LRC-L52 2 mg/ml	2	96 (100%)	9 (9·4%)	10 (10·4%)	4 (4·2%)	3 (3·1%)	17 (17·7%)
L. major LRC-L137 0·5 mg/ml	2	70 (100%)	7 (10·0%)	8 (11·4%)	6 (8·6%)	10 (14·3%)[b]	24 (34·3)[b]
L. major LRC-L137 2 mg/ml	3	54 (100%)	20 (37·0%)[b]	8 (14·8%)	9 (16·7%)[b]	17 (31·5%)[b]	34 (63·0%)[b]

[a]Estimated numbers of parasites: I, <10; II, 10–100; III, >100.
[b]Result significantly greater than that for LRC-L119 without glycoconjugate (Fisher's exact test): *$P<0·01$; **$P<0·001$.

The ingestion of glycoconjugate also caused a delay in the digestion of blood meals, as indicated by the number of flies in which this process was not completed. Undigested blood was observed in only 7·8% of the flies that had infective meals with parasites of strain LRC-L119 with no addition, compared to 37·0% of the flies fed an infective meal of LRC-L119 mixed with 2 mg/ml LRC-L137 glycoconjugate. This difference was statistically highly significant ($P<0·001$).

Discussion

There is a close association between *Leishmania* species and their specific vectors, and the sandflies that transmit similar leishmanial strains are closely related (KILLICK-KENDRICK, 1985).

LRC-L119 is a strain of *L. major* (CHANCE *et al.*, 1978; SCHNUR, 1984), and its isoenzyme profile is identical to that of strain LRC-L137 (LE BLANCQ *et al.*, 1986), which is transmitted by *P. papatasi* in the Jordan Valley, the region of origin of the experimental flies. Moreover, promastigotes of strain LRC-L119 do not release serologically evident glycoconjugate (SCHNUR & ZUCKERMAN, 1977; SCHNUR, 1982). Hence, the vector-parasite combination studied was presumably suitable to show the effect of artificially added glycoconjugate in the vector.

The initial experiments showed that *P. papatasi* was refractory to infection by strain LRC-L119, as these parasites survived in only 15·6% of the flies. However, the addition of the glycoconjugate of LRC-L137 to the artificial meals caused a dose-dependent increase in the proportion of viable LRC-L119 infections in the sandflies. Evidently the glycoconjugate of the *Leishmania* naturally transmitted by *P. papatasi* conferred protection on a parasite strain that does not normally develop in this vector.

Leishmania strains produce various glycoconjugates, as indicated by their interaction with immune rabbit sera, which is the basis for the serotype classification of the genus (SCHNUR *et al.*, 1972). Further differences within the serotype groups have been demonstrated with monoclonal antibodies (JACOBSON *et al.*, 1988). This heterogeneity of the glycoconjugates may reflect functional adaptations of *Leishmania* to the conditions in their vector sandflies.

To test the assumption that glycoconjugates are vector-specific we used the substance released by *L. donovani*. *P. papatasi* is unsuitable for the development of *L. donovani*, as is evident from the low rate of infection in flies fed on hamsters infected with a Sudanese strain (2/51, 3·9%: ADLER, 1947) and the similar infection rate (4/82, 4·8%) observed in flies artificially fed 2×10^6/ml parasites (Schlein, unpublished data). In accord with these observations, in our experiments the presence of *L. donovani* glycoconjugate in the infective meals did not promote the survival of LRC-L119 parasites.

By comparing the activity of the 2 glycoconjugates we demonstrated the vector-specific function of the substance shed by LRC-L137 promastigotes. Furthermore, the lack of activity of the *L. donovani* glycoconjugate in *P. papatasi* is apparently the reason for the failure of this species of *Leishmania* to infect this vector. Therefore glycoconjugates apparently facilitate the establishment of *Leishmania* infections in the specific vectors.

The resistance of sandfly vectors to various species of *Leishmania* can be overcome by increasing the number of parasites ingested. This was observed following feeding *P. papatasi* with artificial meals containing different concentrations of amastigotes (ADLER & THEODOR, 1930, 1931) or promastigotes of *L. infantum* (see ADLER & THEODOR, 1927), *L. chagasi* (see ADLER & THEODOR, 1939) and *L. donovani* (see ADLER, 1947). It may be that the glycoconjugates are only partially effective in an inappropriate vector, and in these experiments their function was cumulatively enhanced by the increase in the number of parasites introduced into the flies.

In addition to the promotion of infections, the glycoconjugate of LRC-L137 caused a delay in the digestion of the blood meal. The death of *Leishmania* parasites in an inappropriate vector is apparently caused by the level of proteolytic enzymes secreted for the digestion of the infective meal, as indicated by the increased survival of *L. donovani* promastigotes in *P. papatasi* that had been fed soybean trypsin inhibitor (BOROVSKY & SCHLEIN, 1987). Live parasites of *L. major* (LRC-L137), or crude extract of glycoconjugate from used culture medium, reduced proteolytic activity in the gut of *P. papatasi*, whereas this type of preparation from *L. donovani* or its live promastigotes had an opposite effect (SCHLEIN & ROMANO, 1986). This evidence and our present observations suggest that the function of the *Leishmania* glycoconjugate in the vector involves modulation of proteolytic enzymes. All the phenomena described in these studies were induced by either a high number of parasites or a large quantity of experimental material. Vector sand-

flies ingest only a small number of amastigotes (KILLICK-KENDRICK, 1979), which transform to promastigotes and multiply. The normal secretion of proteolytic enzymes in *P. papatasi*, as in other haematophagous Diptera, is induced by the ingestion of a blood meal, and their quantity increases gradually to a maximum at 30 h (BOROVSKY & SCHLEIN, 1987). Hence the parasites taken in with the blood meal are initially not exposed to these enzymes. It is possible that the rapidly growing numbers of promastigotes are cumulatively sufficient to influence the concurrent digestive processes in the *Leishmania*-specific vector.

Acknowledgement

This work was supported by the leishmaniasis component of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases and AID grant CDR C5-136.

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Received 14 June 1989; revised 7 March 1990 (unavoidably delayed); accepted for publication 7 March 1990