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Leishmania infections damage the feeding mechanism of the sandfly vector and implement parasite transmission by bite

(chitinase inhibition)

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ABSTRACT ***Leishmania* parasites are transmitted by the bites of infected female sandflies by a mechanism that has not been clarified. *Leishmania* infections in the vector develop only in the gut, and the parasites' exit is through the food channel in the proboscis. The problem is how during the bite, when blood flows in, parasites are emitted through the same channel in the opposite direction. It is well documented that infected sandflies maintained on sugar diets are potent vectors, whereas transmission fails after constant feeding on blood. Hence to study the mechanism of transmission, we fed these diets to *Phlebotomus papatasi* infected with *L. major*. Histological examination demonstrated that only in the sugar-fed flies did the cuticle lining of the cardiac valve detach and other valve tissues degenerate gradually. The injury of the main valve of the food pumps hindered gorging of most flies when force-fed from capillaries, and they regurgitated the gut contents with fluids from the capillaries. We suggest that infections are caused by parasites regurgitated from the stomach that are deposited in the host tissue. We found that secretion of chitinolytic enzymes by cultured *L. major* parasites is inhibited by blood or hemoglobin, and hence these enzymes are apparently absent from the blood-fed infected flies, where the cardiac valve appears undamaged. We therefore presume that lysis of the chitin in the cuticle lining of the valve leads to exposure and degeneration of the underlying tissues.**

Protozoa parasites of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) are causative agents of several diseases that are important public health problems in many countries. The vectors of these parasites are sandflies, and they infect humans and animal reservoirs of the diseases. The developmental cycle of *Leishmania* in the sandfly is exclusively in the gut, and parasites reach the host tissue via the food channel in the proboscis. During the bite of infected sandflies the ingested blood is pumped into the gut while at the same time and through the same channel, parasites are deposited in the opposite direction. Evidently, in this system the transmission of the parasites is not implemented by the normal process of ingestion, and additional factors have to be involved. The common opinion was that transmission depends on the presence of parasites in part of the feeding system of the sandfly. The debate was whether parasites facilitate transmission by disturbing the flow of ingested blood or whether they are directly deposited from the proboscis by the act of probing (reviewed in refs. 1–7). These theories were not correlated to transmission experiments, and both the manner and the circumstances of transmission were (8) and still are (9) a major problem in the study of leishmaniasis. We have previously reported that *Leishmania major* infections in the vector *Phlebotomus papatasi* cause degeneration of the cardiac valve, the main valve of the feeding system, presumably by the chitinolytic activity of the parasites' enzymes (10). Here we bring evidence that this damage can be prevented by conditions that inhibit the secretion of these enzymes. Furthermore, we show that dysfunction of the degenerate valve impairs the process of feeding in a manner that can enable the transmission of parasites by bite. According to these observations we propose a mechanism of *Leishmania* transmission.

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MATERIALS AND METHODS

Cell Cultures. The *L. major* was MHOM/IL/90/LRC-L585, an isolate from the Jordan Valley, Israel. Parasites were grown in Dulbecco's modified Eagle's medium with high glucose content (Biological Industries, Beth Haemek, Israel) supplemented with 10% (vol/vol) fetal calf serum, 4 mM L-glutamine, streptomycin at 200 μg/ml, and penicillin at 200 international units/ml (Teva, Jerusalem). Experimental cultures were grown in the same medium with additions of 10% (vol/vol) rabbit blood or bovine hemoglobin (Sigma) at 30, 50, 100, or 150 mg/ml.

Enzyme Assays. The enzyme used was fraction II preparation, which had the activities of both chitinase and *N*-acetylglucosaminidase (NAGase). This preparation was an ammonium sulfate precipitate of proteins from the supernatant of cultures at the stationary phase of growth, as described by Schlein *et al.* (10). Protein concentration was measured by the method of Bradford (11), using a standard of bovine serum albumin. The undigested hemoglobin in the overlay of cultures precipitated with the proteins, and its quantity in enzyme preparations was measured by Drabkin's cyanmethemoglobin method (12). Hemoglobin in the highest proportion found, 20 mg/mg of protein, was added to enzyme preparations from cultures grown without this substance in control assays of enzyme activity.

Chitinase activity was assayed by using *p*-nitrophenyl β-D-*N,N'*-diacetylchitobiose as the substrate and the activity of the NAGase was assayed by using *p*-nitrophenyl *N*-acetyl-β-glucosaminide (Sigma), following the modified (10) procedure of Polacheck and Rosenberger (13). One unit of chitinase or NAGase is defined as the amount of enzyme that generates 1.0 pmol of *p*-nitrophenol in 1 hr under the standard assay conditions.

Experiments with Sandflies. Experimental *P. papatasi*, 1 to 7 days old, were from a colony originated with flies from the Jordan Valley. Artificial infection was with 10^6 *L. major* per ml in rabbit blood according to Schlein *et al.* (14). Uninfected controls were fed on blood without parasites. Subsequently, one series of the infected flies and the uninfected controls were maintained on sugar meals containing: 5.5 g of sucrose, 0.6 g of glucose, 0.35 g of fructose, 0.3 g of mannose, 0.3 g of bovine serum albumin, 0.3 g of lactose, 0.3 g of raffinose, and 30 mg of *N*-acetylgalactosamine (Sigma) ground together

Abbreviation: NAGase, *N*-acetylglucosaminidase.

and dissolved 1:9 (wt/vol) in water. This food was intended to imitate the complexity of natural sandfly diet from plant sources.

A second series of infected flies was fed on a rabbit at 1-day intervals. All flies were offered water and were maintained at 24°C and 80–100% relative humidity. Flies of the three series were used for histology and for feeding experiments.

For light microscopy flies were fixed in Carnoy's fluid at 1-day intervals, between the 4th and 18th days, and embedded in paraffin, cut in sagittal serial sections, and stained in Mayer's haemalum stain according to Culling *et al.* (15).

Flies with 12- to 14-day-old infections and contemporary uninfected flies were used for transmission electron microscopy. They were fixed in 2.5% glutaraldehyde in 0.1 M cacodylic acid, pH 7.2, for at least 24 hr, post fixed with buffered 1% osmium tetroxide (Polaron, Watford, U.K.), dehydrated, embedded in either SPI-Pon 812 (SPI Supplies, West Chester, PA) or Polarbed 812 (Bio-Rad), sectioned sagittally, and stained according to El-On and Messer (16).

Similar flies of the same series were force-fed from glass capillaries (NSRI, Christian Bardram, Denmark) by using the modified technique (17) of Hertig and McConnell (18). The feeding solution contained 5% bovine serum albumin, 0.45% NaCl, and 0.5% (wt/vol) food dye marker (indigotine 73015, Stern, Natania, Israel) in water. The feeding performance was observed under a stereoscopic microscope and flies with inactive food pumps were discarded. For the others, the degree of gorging and the extent of entrance of feeding solution into the gut (indicated by the marker) was recorded. Afterwards, the gut was dissected and examined by a phase-contrast microscope to confirm the presence of parasites.

RESULTS AND DISCUSSION

Design of the Study. In this study we used sandfly diets that determine the vector competence to transmit *Leishmania*. The early investigators of leishmaniasis thought that blood is the only food of adult sandflies. Consequently they used many thousands of blood-fed infected sandflies, including *P. papatasi*, in unsuccessful attempts to cause infection by bite and demonstrate that sandflies are the vectors of *Leishmania* (19–21). Feeding sandflies on sugars is now a routine that is also practiced in successful transmission experiments (22–28).

Since the chitinolytic enzymes of *L. major* infections were associated with the degeneration of the cardiac valve of *P. papatasi* (10), we examined the effect of blood on these enzymes and compared the condition of the cardiac valve and feeding performance in three series of flies: "nontransmitters" (blood-fed infected flies), sugar-fed flies that are capable of transmission, and uninfected flies.

Inhibition of the Chitinolytic Enzymes by Hemoglobin. Activities of both the chitinase and the NAGase of *L. major* were inhibited by the presence of either blood or hemoglobin in culture media. The addition of 10% blood resulted in a decrease of 55.0% of chitinase activity and a decrease of 48.0% of NAGase activity, in comparison with the respective control values of 6.7 and 36.0 units/mg of protein. This inhibition by hemoglobin was dose dependent (Fig. 1), producing a reduction of 69.0% in chitinase activity and 65.0% in NAGase activity at a hemoglobin concentration of 150 mg/ml. Most of the hemoglobin in cultures (73.0–85.0%) was digested by the parasites. However, the remnant (12–20 mg of hemoglobin per mg of fraction II enzyme preparation) in the precipitated preparations could interfere with the enzyme assays, and its effect was therefore measured. Addition of hemoglobin in a proportion of 20 mg of hemoglobin per mg of fraction II of control cultures had no effect on the enzymatic activities. Chitinase activity of 5.28 units/mg of protein in the presence of hemoglobin was similar to the 5.4 units/mg of protein of the control. NAGase activity was 53.8 units/mg of protein with the hemoglobin and 64.4 units/mg of protein in the control. This lack of effect on the activity implies that in cultures the hemoglobin inhibits the secretion of enzymes.

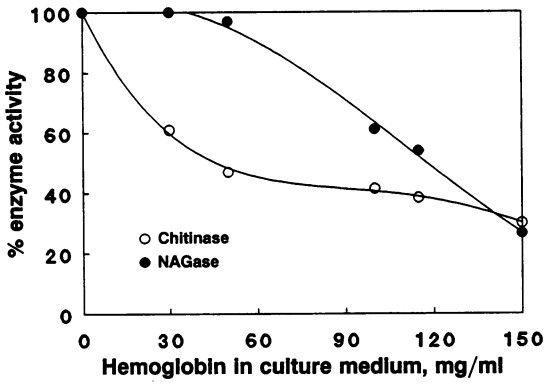

FIG. 1. Effect of hemoglobin in the culture medium on the secretion of chitinase and NAGase by *L. major* parasites. Enzyme activity is in percent of average control activity of 6.7 units/mg for chitinase and 35.0 units/mg for NAGase.

The presence of hemoglobin at 150 mg/ml in our cultures, similar to its level in human blood, arrested most of the secretion of chitinase (69.0%) and NAGase (65.0%) into the medium. In the sandfly blood meal this process is probably more pronounced initially when the excretion of fluids raises the hemoglobin concentration, while enzyme secretion would increase with the digestion of the blood.

Infections Result in Damage to the Cardiac Valve of Sandflies. The cardiac valve (Fig. 2*A*) is the gate between the food pumps in the head of the sandfly and the midgut cardia. It consists of a ring of cuticle-covered high epithelial cells that bulges as an inverted collar into the cardia. The closure of this valve is by a circular sphincter muscle located at the base of the epithelial ring. When this muscle contracts the circumference of the ring is diminished by folding of the epithelial ring, and these folds shut the entrance to the midgut. It was observed (10) that in infected *P. papatasi* (Fig. 2*B*), the cuticle cover of the valve appears to be separated and the epithelial cells are drastically diminished.

We compared the condition of the cardiac valves in three groups of *P. papatasi*, including infected flies maintained on diets of either sugars or blood and uninfected sugar-fed controls. Damage to the valve tissues was observed by electron microscopy in sugar-fed flies with mature infections. The following summary includes only the characteristics that

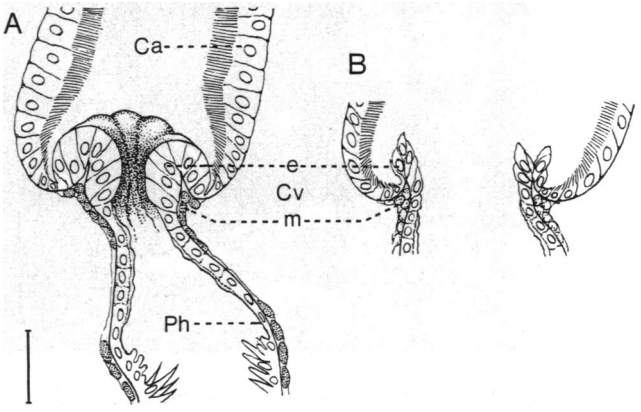

FIG. 2. Cardiac valve region of *P. papatasi*, drawn from sections prepared and stained as described in *Materials and Methods*. (Bar = 20 μm.) (*A*) Uninfected fly after 14 days. (*B*) Infected sugar-fed fly after 14 days, with atrophied cardiac valve. Ca, cardia; Cv, cardiac valve, Ph, pharyngeal pump; e, epithelial ring; m, sphincter muscle.

pertain to the present study, and a detailed description will be published elsewhere. The cuticle layer of the valve was covered by numerous attached parasites and in large areas it was separated from the epithelial cells (Fig. 3*B*). Similar detachment of the valve cuticle can be seen also in micrographs of other *Leishmania*-infected sandflies of the species *Lutzomyia flaviscutellata* (29) and *Lutzomyia abonneci* (30). The underlying exposed epithelial cells showed various degrees of disruption, including loss of cell membrane (Fig. 3*C*), decrease in the volume, and separation of individual cells from the tissue. Cells of the sphincter muscle were sometimes hypertrophied and appeared degenerated with empty-looking nuclei, loss of cytoplasmic structures, and various degrees of loss of muscle fibrils (Fig. 3*F*). In infected blood-fed sandflies fewer parasites were attached to the cardiac valve, and this valve and the sphincter muscle generally resembled their normal counterparts in uninfected flies (Fig. 3 *A*, *D*, and *E*).

It appears that the passage to the stomach can close only partially when the opening is widened and distorted by the atrophy of the valve epithelium. This is probably aggravated and the valve remains completely open when the sphincter muscle degenerates. The prevalence of damaged cardiac valves was estimated in light microscopy histology. The shape of the cardiac valve was normal in all the 32 uninfected flies after more than 10 days (Fig. 2*A*), and at the same time the infections had no apparent effect on the valves of 16 blood-fed flies. In contrast, damage to the cardiac valve was manifested in all of the 23 infected flies that were maintained on sugar meals. Of these, in 3 flies on the 4th day of infection, when only small numbers of parasites reach the cardiac valve, the damage was localized in groups of cells in the epithelial ring. Infections of more than 6 days caused massive epithelial degeneration in 15 flies (Fig. 2*B*) and partial atrophy in 5 flies.

Normally the epithelial cells of the valve have a protective layer of cuticle which, like other insect cuticles, consists

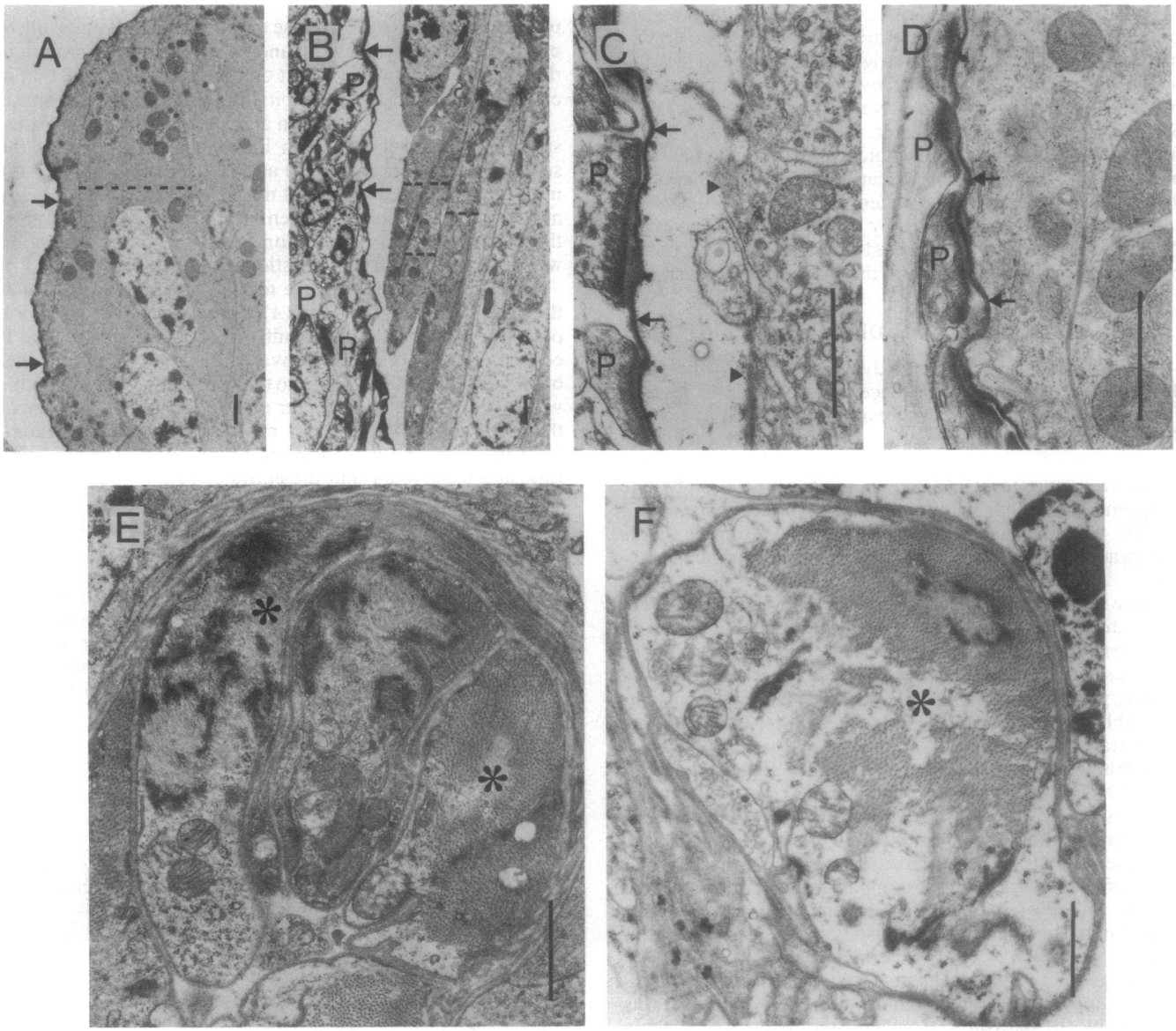

FIG. 3. Electron micrographs of sections of the cardiac valve of *P. papatasi* 13 days after treatment. (Bars = 1 μm.) (*A*) Uninfected fly, epithelium (cell width indicated by broken line) covered with cuticle (arrows). (*B*) Infected sugar-fed fly, narrow atrophied cells (broken lines). The cuticle cover (arrows) is detached with a mass of adhering parasites (P). (*C*) Similar to *B*, shows loss of membrane and irregular border of exposed cell (arrowheads), separated cuticle (arrows) with attached individual parasite flagella (P). (*D*) Blood-fed infected fly. The cuticle (arrows) with the attached parasite flagella (P) is closely apposed on the epithelial cells as in uninfected flies. (*E*) Valve-sphincter muscle cells of an uninfected fly with densely packed myofibrils (*). (*F*) Degenerate hypertrophied muscle cell of an infected sugar-fed fly, containing loose disorganized myofibrils (*).

mostly of chitin fibers embedded in a proteinaceous framework. We presume that the enzymes of the parasites function like the insect chitinases that are involved in the process of molting (31), and they degrade only the inner softer layer of the cuticle. This degradation may cause detachment of the remainder of the cuticle and expose the underlying tissues to harmful effects of the sandfly digestive enzymes and to secretions of the *Leishmania* parasites. In this study we have seen that the cardiac valve, including the cuticle cover, remains intact in infected flies that have been constantly fed on blood. We have also shown that blood or hemoglobin inhibits the production of chitinolytic enzymes by cultured parasites. Thus the activities of these enzymes or other parasite-secreted substances are probably instrumental in the cardiac valve degeneration in the absence of blood meals.

Impaired Ingestion by Sugar-Fed Infected Flies. The process of ingestion was observed in infected *P. papatasi* that had been maintained for 12–14 days on a diet of sugars or blood and in sugar-fed uninfected controls. The flies were fed from capillaries (17, 18) with dye-marked fluids that could be traced in the various parts of the gut. All of the 17 uninfected control flies as well as the 11 infected blood-fed flies were fully fed. In contrast, no visible gut distention occurred in 13 of the sugar-fed infected flies. Another 5 flies of this series were half gorged, and 5 flies fed normally. In the flies that showed no apparent increase in the gut volume, the dye marker indicated that fluids were taken into the midgut and in two cases they reached the hindgut. Thus, evidently, these flies exchanged fluids between their stomach and the capillary. It is reasonable that this inability to feed results from the degeneration of the cardiac valve, and both phenomena occurred only in the flies that were potential transmitters.

Furthermore, flies that probe without feeding have been repeatedly seen in successful transmission experiments (summarized in ref. 9), and their efficiency as vectors was confirmed in specific observations (17, 28, 32, 33). These observations apparently indicate that transmission is facilitated by an impairment of the feeding system similar to that described by us. Accordingly, we suggest a mechanism of transmission which is based on the dysfunction of the cardiac valve of infected flies.

Mechanism of Transmission by Flies with a Damaged Cardiac Valve. *P. papatasi* ingests food by the action of two pumps, pharyngeal and cibarial, that are located in the head. The cibarial valve separates the two pumps and the main sphincter, the cardiac valve, is behind the esophagus (34–36). In normal flies (Fig. 4*A*), when the cibarial and pharyngeal pumps are extended in unison (according to our observations), the cibarial valve has to be open and the cardiac valve must be closed to permit only unilateral flow from the food channel into both pumps. In infected flies (Fig. 4*B*) the damaged cardiac valve remains open; therefore the suction of the pumps is in both directions and the midgut contents, including parasites, are drawn into the pumps and mixed with the ingested blood. Afterwards the pumps empty in a similar manner regardless of the condition of the cardiac valve (Fig. 4*C*). This valve is opened or already open and the cibarial valve is closed while the contents of the pharynx are released into the midgut, whereas the cibarial pump (Ci) delivers its contents back into the food channel. Thus when infected flies bite, with a permanently and fully open cardiac valve, the pulsation of the pumps only whirls their contents and each beat draws and delivers fluids in both directions. Partial function of the cardiac valve causes less regurgitation and allows for partial engorgement of the fly. During the bite this regurgitation engulfs parasites from the midgut and deposits them in the host tissue.

Suggested Model of Transmission and Previous Theories. In mature *Leishmania* infections most of the parasites, including infective promastigotes (37), are in the midgut, and in many infections they reach the pharynx and sometimes the food channel in the proboscis. Opinions are divided about the initial location and manner of transfer of parasites that finally infect the host. One approach is that concentration of parasites in different regions of the pharyngeal or cibarial food pumps can disturb or block the flow of ingested blood, thus causing a backflow that carries the parasites (6). This means that only the small population of parasites in the pumps is

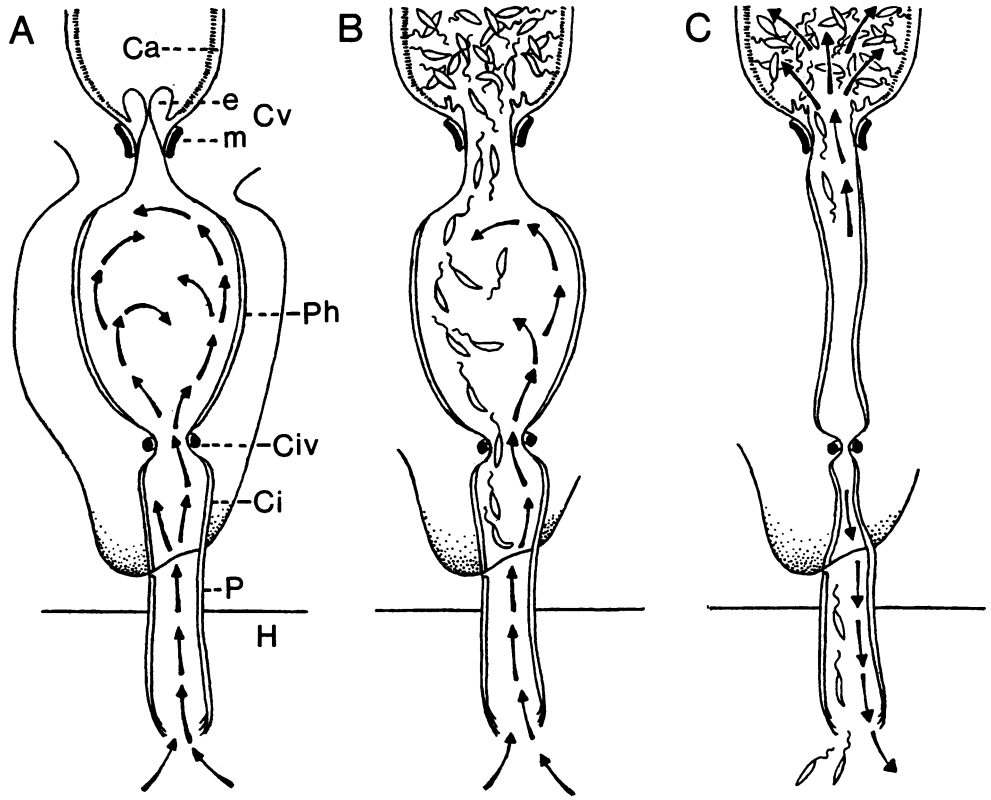

FIG. 4. Scheme of the feeding mechanisms of *P. papatasi*. (*A*) Normal blood suction (arrows) into the extended food pumps. Cardiac valve is closed and cibarial valve is open. (*B*) Bilateral suction of blood and parasites (spindle-shaped flagellates) when the cardiac valve is damaged and permanently open in an infected sugar-fed fly. (*C*) Contraction of the food pumps in the infected fly, similar to uninfected fly. Cardiac valve is open and cibarial valve is closed, pharyngeal pump empties into the stomach and cibarial pump releases blood and parasites into host skin. Ca, cardia; Cv, cardiac valve; e, epithelial cells; m, sphincter muscle; Ph, pharyngeal pump; Civ, cibarial valve; Ci, cibarial pump; P, proboscis (food channel); H, host skin.

available for transmission. Alternatively, it has been assumed that parasites from the proboscis are directly deposited by the probe of the sandfly (38). However there is no explanation for the scarcity, mortality, or absence of parasites in the proboscis of infective flies (4, 6, 9). Furthermore, there is no evidence on differences in the intensity of infections and the location of parasites between noninfective blood-fed flies and infective flies.

According to the mechanism of transmission suggested here the inability of flies to feed results from the permanently open cardiac valve and not from a block of parasites. The resulting regurgitation sweeps parasites from the mass in the midgut, where they multiply in the medium of ingested food, and circulates them in the food pumps and the proboscis. Thus during pump activity there is a constant supply of parasites for the repeated infective probes (summarized in ref. 38), while they are rarely seen in dissected sandflies.

Variations in Sandfly Diet and Transmission. The impairment of the feeding performance that apparently facilitates transmission was determined in our experiments by two diets of the infected flies. It is reasonable that it can be modulated also by other diets or feeding regimes. This is the suggested interpretation for the as-yet-unexplained success of Adler and Ber (39) in causing infections by the bite of blood-fed *P. papatasi*. In their experiment the initial infective meal consisted of diluted rabbit blood and presumably, the lower hemoglobin concentration permitted the degeneration of the cardiac valve and enabled transmission. Changes in the transmission capability of *P. papatasi* due to feeding infected flies on different sugars or sugar and protein (17) also imply that these meals influence the capability of *L. major* infections to induce degeneration of the cardiac valve.

It was previously postulated that in nature the gut environment of sandfly vectors can vary as a function of diet and influence the viability of *Leishmania* infections (40). In this study we show that the sandfly diets can apparently alter the vector capability to transmit disease by their effect on the *Leishmania* parasites.

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