

Studies on "Dipetalonema viteae" (Filarioidea). Teil II, Antibody dependent adhesion of peritoneal exudate cells to microfilariae in vitro

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Objektyp: **Article**

Zeitschrift: **Acta Tropica**

Band (Jahr): **35 (1978)**

Heft 2

PDF erstellt am: **22.05.2024**

Persistenter Link: <https://doi.org/10.5169/seals-312378>

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Studies on *Dipetalonema viteae* (Filarioidea)

II. Antibody dependent adhesion of peritoneal exudate cells to microfilariae in vitro¹

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Summary

Peritoneal exudate cells from normal uninfected hamsters adhered in vitro to microfilariae in the presence of 19S antibody fractions from hamsters which had suppressed or were going to suppress their microfilaraemia. The adhering cells were predominantly mononuclear, although eosinophils were occasionally found. Experiments with sensitized microfilariae and peritoneal exudate cells indicated that the macrophage probably recognizes the microfilariae/antibody complex. Macrophage cytophilic antibodies did not seem to be involved. This adhesion reaction may initiate the trapping of microfilariae in vivo, thus contributing to the observed acquired immunity to circulating microfilariae in the hamster.

Key words: *Dipetalonema viteae*, hamster, microfilariae, adherence, peritoneal exudate cells, antibody.

Introduction

Antibody dependent cell-mediated effector mechanisms against parasitic helminths have recently been demonstrated. The interaction of antibodies with various cell types such as eosinophils (Butterworth et al., 1977), neutrophils (Dean et al., 1974) and macrophages (Capron et al., 1977; Perez and Smithers, 1977) has been reported to play specific effector roles in the phenomenon of acquired resistance to schistosome infections.

The in vitro study of possible cellular effector mechanisms against microfilariae in human filariasis is also of interest, particularly as histopathological

¹ Supported by the Swiss National Science Foundation (grant no. 3.689.76)

findings from patients suffering from eosinophilic lung as well as from Meyers-Kouwenaar syndrome have indicated the contribution of cellular effectors to the host's response against microfilariae (Webb et al., 1960; Bras et al., 1951).

The end of microfilaraemia in *Litomosoides carinii* infected albino rats is associated with adhesion of macrophages, lymphocytes and polymorphonuclear cells to microfilariae. This was observed in vivo and subsequently analyzed in vitro (Bagai and Subrahmanyam, 1970; Subrahmanyam et al., 1976). Experimental *Dipetalonema viteae* infections in hamsters lead after a short microfilaraemia to an amicrofilaraemic state which was shown to represent an acquired immunity to circulating microfilariae (Weiss, 1970). The appearance of antibodies to microfilariae cuticles was shown to be closely correlated with the onset of latency (Weiss, 1978).

The present in vitro study was planned to investigate a possible involvement of antibody dependent cellular effector mechanisms in the acquired immunity to circulating microfilariae in vivo.

Material and methods

Animals

Male golden hamsters strain LAKZ (80–100 g) came from a randomly bred colony (Institut für Zuchtthygiene, Zürich, Switzerland).

The life-cycle of the filarial parasite, *Dipetalonema viteae*, was maintained as described by Worms et al. (1961). Worm donor hamsters were infected with third stage larvae according to Weiss (1970).

Microfilariae (Mf)

Mf were obtained from adult female worms maintained in vitro (Weiss, 1978). Mf were recovered from the culture medium by centrifugation (250 g, 15 min), washed twice and subsequently adjusted to 5000/ml with serum free RPMI 1640 (Flow, laboratories). Mf for the indirect immunofluorescent test were prepared according to Weiss (1978).

Sera

Sera were obtained by bleeding infected hamsters (three groups with 15–20 hamsters each) from the retro-orbital sinus during the prepatent period (week 4 post infection [p.i.]), during the patent period (week 12 p.i.) and during the latent period (week 20 and 30 p.i.). After clotting, the sera were pooled, inactivated (56° C, 30 min) and stored at –70° C. Fresh absorbed guinea pig serum (kindly provided by the Institute for Microbiology, Basel, Switzerland) served as complement source.

Fractionation of sera

Four ml of pooled hamster sera were applied to a 26 × 100 cm column of Sephadex G 200 (Pharmacia Fine Chemicals). The running buffer was 0.1 M tris-HCl, pH 8.0, containing 0.5 M NaCl and 0.1% NaN₃. Fractions (2.5 ml each) were eluted by descending chromatography at 4° C. The protein content was recorded at 280 nm with an Uvicord analyzer. Particular fractions were pooled and diafiltrated against RPMI 1640 and concentrated (10 times) on a Diaflo PM 10 ultrafiltration membrane (Amicon Inc.).

Medium

Microfilariae and peritoneal exudate cells were stored and tested in RPMI 1640 containing 200 units/ml penicillin and 200 μ g/ml streptomycin (Difco).

Peritoneal exudate cells (PEC)

Hamsters were anaesthetized with ether and washed with alcohol (70%). After stripping back the fur 12 ml of Hanks balanced salt solution (Flow, laboratories) containing 5 IU/ml heparin, 200 units/ml penicillin and 200 μ g/ml streptomycin were injected into the peritoneal cavity. The abdomen was gently massaged for about 3 min and subsequently opened (incision of 2–3 cm) to collect the peritoneal fluid by holding the hamster above a funnel. PEC were washed twice with RPMI 1640 by centrifugation (250 g, 12 min, 4° C). PEC-suspensions containing many erythrocytes were treated with NH_4Cl -tris according to Boyle (1968) to eliminate the erythrocytes.

The nucleated and living (Trypan Blue exclusion test) cells were counted in a Neubauer haemocytometer and after adjusting the cell suspension to 10^7 /ml the PEC were kept on ice until use. The phagocytic activity of PEC was tested by their ability to ingest Latex beads (\varnothing 1.101 μ , Dow Inc.).

For the experiments with activated macrophages, hamsters were injected intraperitoneally with 3 ml of a 2% starch broth three days before cell harvesting.

Adherence assay

Experiments were carried out in sterile glass tubes with a screw cap and a capacity of 1.5 ml (Anwander Inc., Basel, Switzerland). Approximately 500 living mf and 10^6 PEC were added to each tube containing medium supplemented by serum or serum fractions. The final volume was 1 ml (pH 7.2).

The tubes attached to a disk were kept rotating (16 rpm) in a 37° C waterbath for 3 h. This system prevented PEC from adhering to the tube and enabled interactions of PEC with mf.

After incubation the tube content was transferred to flat-bottomed (\varnothing 16 mm) tissue culture plates (Costar) and the adherence (% mf with adhered PEC) as well as the number of adhered PEC/mf was estimated by microscopical examination of at least 100 mf. Adherence was classified as negative (no PEC adhered), + (≤ 5 adhered PEC), ++ (6 to 12 adhered PEC) and +++ (> 12 adhered PEC).

Sensitization of mf and PEC

Mf or PEC were incubated in medium containing 10% sera for 30 min at 37° C (5% CO_2 -atmosphere), subsequently spun down (250 g, 15 min) and added (directly or washed twice) to the PEC or mf respectively for adherence tests.

In addition, mf previously treated with sera (see above) were washed and subsequently incubated with 1/30 dilution of guinea pig serum in 0.15 M barbital (Veronal)-buffered glucose pH 7.4 with Ca^{++} , Mg^{++} and 0.1% gelatin for 30 min at 37° C. Mf were washed once and resuspended in medium. The subsequent interaction with PEC occurred as described above.

Indirect immunofluorescent antibody test (IFAT)

IFAT on mf and on frozen sections of adult female worms as antigens were carried out according to Weiss (1978).

Table 1. Adherence activity of pooled sera and their Sephadex G 200 fractions at different time post infection (p.i.). The reciprocal geometric mean IFAT-titres of the pooled sera on intact mf are added

Serum	Stage of infection	% mf with PEC	Number PEC/mf	IFAT on mf
None	—	0	—	neg
NHS total	uninfected	0	—	neg
NHS fraction I	uninfected	0	—	neg
NHS fraction II	uninfected	0	—	neg
HS week 4 p.i. total	prepatent	0	—	neg
HS week 7 p.i. total	patent	0	—	neg
HS week 12 p.i. total	patent	65 ± 22*	++	neg
HS week 20 p.i. total	latent	>95	+++	101
HS week 20 p.i. fraction I ..	latent	>95	+++	80
HS week 20 p.i. fraction II ..	latent	5 ± 4*	+	neg
HS week 30 p.i. total	latent	>95	+++	320
HS week 30 p.i. fraction I ..	latent	>95	+++	40
HS week 30 p.i. fraction II ..	latent	2.5 ± 3*	+	neg

NHS = normal hamster serum, HS = hamster serum

* = arithmetic mean ± standard deviation

fraction I: first peak from Sephadex G 200 containing 19S antibodies

fraction II: second peak from Sephadex G 200 containing 7S antibodies

+++ : >12 adhered PEC/mf, ++ : 6 to 12 adhered PEC/mf, + : ≤5 adhered PEC/mf, —: no adhered PEC/mf

Results

Table 1 shows the adherence activity of pooled sera and of their Sephadex fractions. Adherence of PEC to mf was detected with sera from week 12 p.i. onwards. The different pools of sera from week 12 p.i., from microfilaraemic hamsters, showed marked variations in the percentage of mf with adhered PEC (Table 1), and there were always less than 12 PEC/mf adhered. However, pooled sera from week 20 and 30 p.i., from latent animals, always caused strong (>95%) adherence with more than 12 PEC/mf adhered. The same results were observed using sera from twelve individual hamsters. This demonstrated that pooled sera did not represent the properties of a single serum in the pool.

PEC were attached along the whole mf with no predilection for a particular site. The motility of mf decreased when the number of adhered PEC/mf increased. Heavily covered mf (Table 1, +++ and ++) moved slowly and no longer exhibited the rolling and unwinding typical of mf. Weakly covered or uncovered mf (Table 1, + and —) did not show any decrease in their motility. Sera alone did not affect the motility of mf within the four-hour observation period.

The capacity of sera to evoke strong adherence of PEC to mf correlated

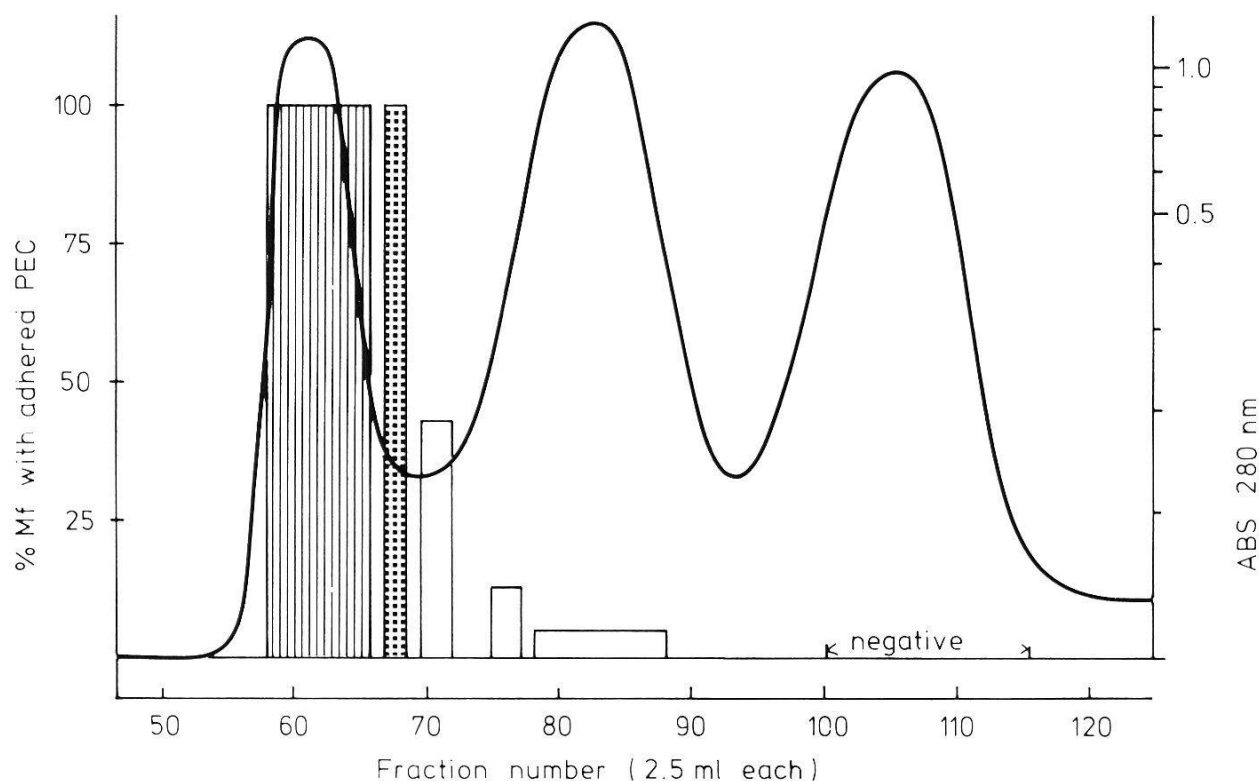


Fig. 1. Adherence activity of Sephadex G 200 fractions of pooled sera from amicrofilaraemic hamsters. Microfilariae (mf) were incubated for 3 h with medium containing fraction and 10^6 hamsters peritoneal exudate cells (PEC).

striped area: >12 adhered PEC/mf

dotted area: 6 to 12 adhered PEC/mf

empty area: ≤ 5 adhered PEC/mf

with the appearance of humoral antibodies against the mf surface in these sera, as was shown by IFAT on intact mf (Table 1). With pooled sera from week 12 p.i., weak adherence was demonstrated, although the IFAT did not reveal antibodies to the mf surface. Sera from hamsters which did not suppress their microfilaraemia at week 27 p.i., but showed antibodies to mf surface by IFAT, also caused adherence.

Fractionation of the sera on G 200 Sephadex demonstrated that 19S antibodies were active in fluorescence and in promoting adherence to mf (Fig. 1). In contrast immunofluorescent activity to adult female antigens could predominantly be detected in the 7S antibody fractions (Weiss, 1978).

Normal hamster serum, its fractions and PEC alone did not cause adherence (Table 1). It was also noted that there is no difference between normal and induced (2% starch) PEC. Moreover, PEC harvested from infected hamsters at different time after infection (week 6, 8, 12, 20 and 30 p.i.) behaved in the adherence assay like normal PEC from uninfected hamsters. Fig. 2 shows the titration of the adherence activity of two different pools of serum from two different groups of hamsters. The capacity of these sera to cause adherence was weak during microfilaraemia, but became very strong at the time when the hamsters suppressed their microfilaraemia. At the same time the IFAT-titre on mf in-

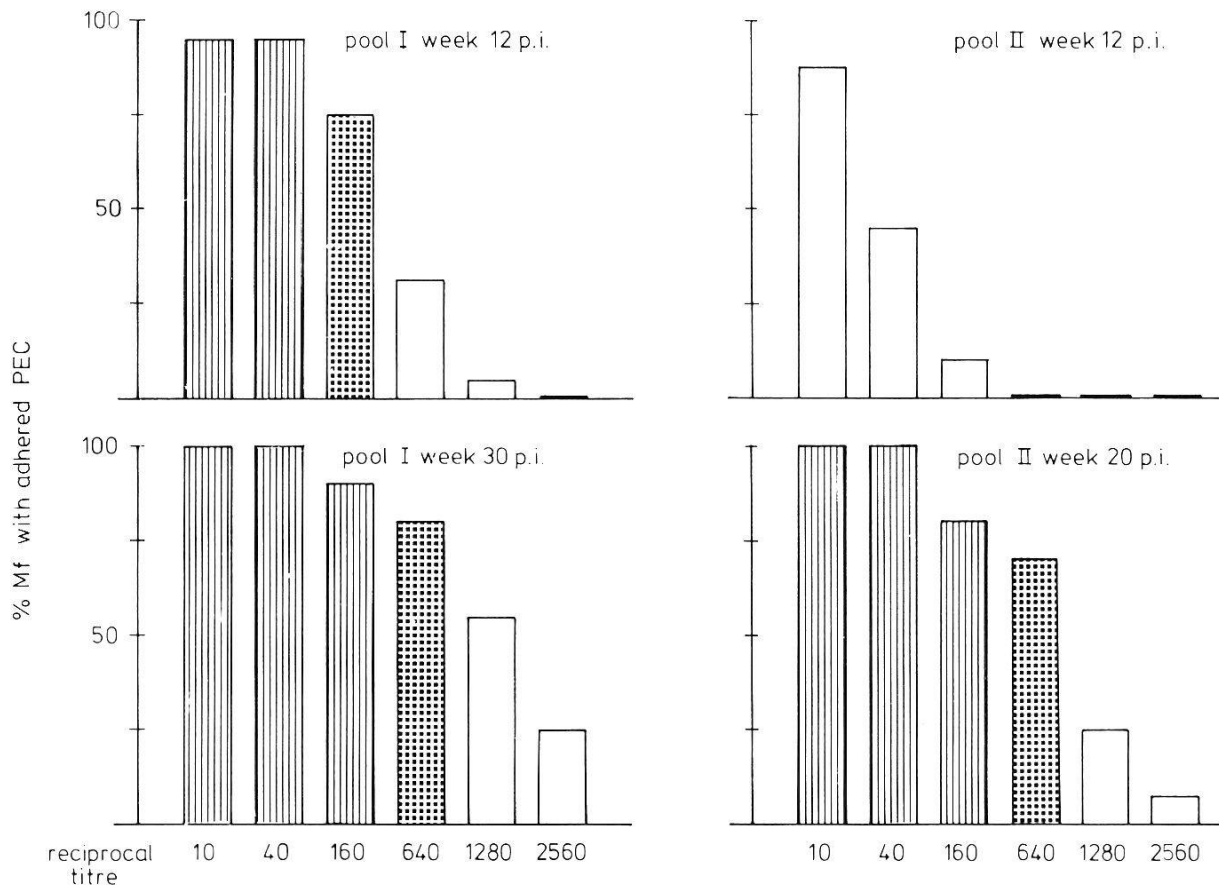


Fig. 2. Titration of adherence activity of pooled sera from two groups of hamsters harvested in the patent (week 12 p.i.) and in the latent (week 20 p.i. and 30 p.i. respectively) period of infection.
 striped area: >12 adhered PEC/mf
 dotted area: 6 to 12 adhered PEC/mf
 empty area: ≤5 adhered PEC/mf

creased from negative (week 12 p.i.) up to 1/160 (week 20 p.i.) and 1/320 (week 30 p.i.) respectively.

Efforts were made to identify the cell types involved in the adherence phenomenon by using Giemsa stain, eosinophil specific Phloxin stain and mast cell-specific Neutral Red stain. The PEC of 14 normal uninfected hamsters were composed of $95\% \pm 3.5$ (arithmetic mean \pm standard deviation) mononuclear cells, $3\% \pm 2$ eosinophils, neutrophils and lymphocytes (1% each). The adhering cells appeared to be of mononuclear nature displaying varying amounts of cytoplasm. These cells firmly adhered to plastic and were able to ingest Latex beads. Interestingly, one or two eosinophils were sometimes detected in cases when the mf was heavily packed with adhering macrophages. Other polymorphonuclear leucocytes and lymphocytes were never observed adhering to mf surfaces.

Mf and/or PEC previously incubated (sensitized) with immune sera resulted in inconsistent and low adherence with always less than 12 adhered PEC/mf (Table 2). PEC sensitized with sera from latent hamsters evoked only 20% adherence to mf. In contrast mf sensitized with the same immune sera

Table 2. Percentage of mf with adhered PEC (arithmetic mean \pm standard deviation) after sensitization of microfilariae (mf) and peritoneal exudate cells (PEC) with normal hamster serum (NHS) and immune serum of latent hamsters (LIS). In all combinations shown less than 12 PEC/mf were observed. Number of experiments in brackets

	mf alone	mf + NHS	mf + LIS
PEC alone	neg (30)	neg (8)	55 \pm 36 (12) a
PEC + NHS	neg (5)	neg (5)	10 \pm 17 (5) b
PEC + LIS	20 \pm 16 (5) c	5 \pm 3 (8) d	15 \pm 14 (5) c

t-test: a-d: 2P <0.001, d-e: 2P <0.02, a-c: 2P <0.05, a-b, a-e, d-c: 2P <0.1

caused a significantly stronger adherence (55%) together with unsensitized PEC. This phenomenon was observed to a significantly lower degree when PEC were sensitized with normal hamster serum or immune serum and were subsequently added to mf sensitized with immune serum. If sensitized PEC or mf were washed twice, adherence was no longer observed.

Sensitization of mf with antibodies plus complement did not promote any adherence. However, mf sensitized by the same way caused immune-adherence (Nelson, 1953) to human red blood cells (in preparation). Antibody plus complement did not impair the motility of mf within a four-hour observation time.

Discussion

The adherence of PEC to mf seems to be a specific cell contact reaction since it only occurs in the presence of immune sera and since predominantly one cell type, a phagocytic cell, was involved. The origin of PEC in respect to the stage of infection of the PEC donor did not play an important role for the adhesion. Therefore the requirement of macrophages specifically armed by immune lymphocytes (reviewed by Evans and Alexander, 1976) is not likely. In this connexion, there was no correlation between the parasite-specific and unspecific T-cell responses during the course of a *D. viteae* infection with the microfilaraemia (Weiss, in preparation).

The source of serum plays the predominant role in this adherence reaction. When the IFAT reveals antibodies to intact mf surfaces, these sera also strongly evoked adhesion of PEC to mf. Antibodies to cuticular structures are detected in the latent infection and only exceptionally during microfilaraemia (Weiss, 1978). This was also observed with individual and pooled sera. As the adherence phenomenon already occurred with sera from the patent infection (week 12 p.i.), this could indicate together with the results from the titration of the adherence activity (Fig. 2) that the adhesion reaction is more sensitive in detecting antibodies to mf surfaces than the IFAT. One may also assume that the IFAT reveals only high affinity antibodies (which cannot be washed off during the test procedure) while the adhesion reaction might be based on a dynamic

equilibrium between the amount of low and high affinity antibodies binding to mf and the concentration of immune complexes in the serum. Therefore, serum from week 12 p.i. could have shown great variations due to the low amount of anti-microfilarial antibodies compared to the probably higher, relative immune complex concentration. The sensitization of mf with sera could have resulted in weak adhesion for similar reasons.

It is still possible that sera induced adhesion partly because of antigen/antibody complexes. The inability of sera to sensitize PEC effectively (Table 2) and the fact that the adhesion causing antibodies were restricted to the fractions of the first Sephadex G 200 peak (containing 19S antibodies, immune complexes and aggregated IgG), are inconsistent with the characteristics of a macrophage cytophilic antibody reaction (Nelson and Boyden, 1967).

Sensitization of mf with antibodies plus complement did not result in adhesion, thus calling in question a mechanism via C₃ receptors on hamster phagocytes, particularly as immune-adherence (Nelson, 1953) of mf to human red blood cells could be demonstrated (in preparation). Thus, one tends to think of other macrophage receptors that are less well characterized and include a class of nonspecific receptors binding protein aggregates and other particles (Rabinovitch, 1967; Steinman and Cohn, 1972).

Similar adhesion mechanisms in vitro have recently been reported with schistosomulae (Perez and Smithers, 1977) and with *Trichinella*-larvae (Perrudet-Badoux and Binaghi, 1977).

As only sera from hamsters which suppressed or were going to suppress their microfilaraemia could cause adhesion, the relevance of the observed antibody dependent adhesion in vitro for the acquired immunity to circulating mf in vivo (Weiss, 1970) has to be considered. How the anti-microfilarial antibodies might be evoked has already been discussed in the preceding paper (Weiss, 1978). Although our results clearly show that cell contacts can occur, they do not indicate the fate of the adhesion complex: whether for example other cell types (perhaps eosinophils or neutrophils attracted by complement activation) are actually the first to damage mf or if the adhered cells also contribute to the phagocytosis of mf. The source of cells used in this study, PEC, may be unsuitable to demonstrate other cell types involved as effectors against mf, for 95% of PEC were formed by mononuclear cells.

The adherence of PEC to mf in vivo was occasionally observed in patent *D. viteae* infected hamsters which had exhibited haemorrhage into the peritoneal cavity (recent observations by the authors). This also leads to questions about the process of mf-trapping in hamsters, particularly as immune serum alone – also in combination with complement – does not seem to affect mf directly.

In spite of a possible influence of immune effectors on the mf-production by female worms (Weiss, 1978), mf are still produced during latency and must therefore be prevented from circulating in the blood. Female worms harvested from latent hamsters and kept in culture release mf (Weiss, 1970), and in histo-

logical studies on latent hamsters mf have been found in the connective tissue surrounding female worms (in preparation). The observation that splenectomy had no effect on the length of the microfilaraemia (Weiss, 1970; 1978) does not rule out a possible trapping of mf within the blood circulation. The liver could be considered as a possible site for the removal of mf. Wong et al. (1973) have shown that mf of *Dirofilaria immitis* are trapped in the lungs by eosinophils (freshly trapped mf) or by mononuclear cells (disintegrated mf).

These findings, the eosinophilic lung in human occult filariasis (reviewed by Beaver, 1970) and our observation that eosinophils were occasionally adhered to mf, raise questions on the possible contribution of eosinophils to the sequence of events that are responsible for the clearance of mf from the circulation and from tissue sites.

Mf might also be prevented from penetrating capillaries due to cellular adhesion reactions occurring in the tissues that surround female worms. Bagai and Subrahmanyam (1970) have already postulated an analogous mechanism based on an adhesion reaction observed in the peritoneal cavity of *Litomosoides carinii* infected albino rats during latency. As it is not yet clear how mf of *D. viteae* reach the circulation, mf could also be trapped in the regional lymph nodes. Such a trapping has been histologically demonstrated in experimental *Brugia pahangi* infections in hamsters (Malone et al., 1976) as well as in human filariasis (Bras et al., 1951; Webb et al., 1960).

Acknowledgments. The authors were most fortunate to receive the skillful technical assistance of Miss I. Unger and Mrs. G. Branscheidt. We gratefully acknowledge the helpful criticism and suggestions of Dr. L. Du Pasquier in preparing the manuscript.

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