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Studies on *Dipetalonema viteae* (Filarioidea)

I. Microfilaraemia in hamsters in relation to worm burden and humoral immune response[1]

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Summary

The course of a primary infection with *Dipetalonema viteae* was studied in one randomly bred and in one inbred strain of hamster. Worm recovery and the duration and intensity of the microfilaraemia were analyzed and related to the humoral immune response of the host by using the indirect immunofluorescent antibody test on frozen sections of female worms, on eggs and on intact microfilariae. The inbred strain showed a greater susceptibility to the parasite. This was evidenced by high worm recovery and prolonged microfilaraemia. The duration of microfilaraemia did not depend on the number of recovered female worms. Most of the randomly bred hamsters suppressed microfilaraemia by week 30 post infection whereas some hamsters of the inbred strain were still microfilaraemic. Splenectomy prior to infection did not affect the duration of microfilaraemia. Antibodies to the cuticle of microfilariae always appeared in the sera after immunity to circulating microfilariae had been built up.

Key words: Dipetalonema viteae, immunity to microfilariae, hamster, humoral immune response.

Introduction

The course of a primary infection with *Dipetalonema viteae*, a filarial parasite of the jird, has been studied in the golden hamster (Weiss, 1970). In this experimental host an acquired immunity to circulating microfilariae was established after a short transient microfilaraemia. The adult worms, however, were

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tolerated for a much longer time. Female worms from a latent hamster still had the capacity to produce microfilariae when transferred to a clean host or when maintained in vitro. Such an amicrofilaraemic state was also induced by repeated injections of microfilariae into clean hamsters (Weiss, 1970). Recently Neilson and Forrester (1975) confirmed these results by demonstrating that no recrudescence of microfilaraemia was observed after secondary and tertiary infections in *D. viteae* infected hamsters.

An acquired immunity to microfilariae has been observed in *Litomosoides carinii* infected albino rats (Ramakrishnan et al., 1962) and in experimental Dirofilariasis in dogs (Wong, 1974). Filariasis without microfilaraemia is known to occur in humans too (reviewed by Beaver, 1970). In eosinophilic lung – as well as in Meyers-Kouwenaar's – syndrome microfilariae can only be found in the affected tissue and not in the circulating blood. In patients presenting a clinical picture of occult filariasis antibodies to microfilariae have been demonstrated (Wong and Guest, 1969). The reasons for the absence of microfilaraemia in human filariasis are still unknown.

The rodent filaria model, *Dipetalonema viteae*/hamster, was chosen to analyze the antigenicity of the parasite and the immune responses against the different worm stages. The present study includes parasitological results and the course of the humoral immune response in relation to the ability of the hamster to control the microfilaraemia. Results obtained in two strains of hamster are compared.

In addition, the effect of neonatal thymectomy and splenectomy on the course of a primary infection is described.

Material and methods

Animals

Male golden hamsters from a randomly bred colony (strain LAKZ, Institut für Zuchthygiene der Universität Zürich, Zurich, Switzerland) and inbred hamsters (strain LSH, Charles River Lakeview Hamster Colony, Newfield, N.J., USA) were infected at 5 to 6 weeks of age.

Splenectomy was carried out one week before infection as previously described (Weiss, 1970). Neonatally thymectomized hamsters were obtained from Charles River Lakeview. Successful thymectomy was assessed by the abolition of reactivity of lymphnode cells to phytohemagglutinin in a blasttransformation assay, according to Weiss et al. (1976).

Dipetalonema viteae was maintained on jirds *(Meriones unguiculatus)* and on soft ticks *(Ornithodorus moubata)* as intermediate host.

Infection

Third stage larvae were isolated from infected ticks, counted and subcutaneously injected into hamsters (Weiss, 1970). The infection doses were 100, 150 and 300 larvae for LAKZ- and 150 larvae for LSH-hamster. In addition, one group of outbred hamsters were subcutaneously injected with 150 larvae which were frozen and thawed three times before use.

Parasitological examinations

Microfilaraemia was followed fortnightly after week 7 post infection (p.i.). Hamsters were anaesthetized and blood taken from the retro-orbital sinus using a Pasteur pipette. Microfilariae (mf) were counted on thick blood smears (20 mm^3). For the analysis of peak mf-counts in the different groups of animals the geometric mean and standard error (s.e.) was calculated.

Adult worms were recovered following the procedure described by Worms et al. (1961). The number of living male and female worms as well as the number of calcified worms were counted. The number of recovered worms expressed as the percentage of the injected larvae represents the recovery rate (arithmetic mean ± s.e.).

Serological methods

Antigen preparation: Frozen sections (7 microns) of adult female worms and eggs (obtained after dilaceration of uteri) were used after acetone fixation as antigen. In addition mf were harvested from in vitro maintained females. RPMI 1640 (Flow) supplemented with 200 μg resp. units of a streptomycin-penicillin mixture (Difco) was used as a culture fluid. Worms were incubated in a 5% CO_2-air atmosphere at 37° C. Mf were washed with phosphate buffered saline (PBS) pH 7.2 and fixed with formaline (1% in PBS). In addition, mf were partly digested by incubation (5 min at 37° C) in a 0.3% solution of papain and then fixed on slides, according to Gonzaga dos Santos et al. (1976).

Slides with frozen sections or eggs as well as serum samples were stored at –20° C, fixed mf in suspension were kept at 4° C.

Indirect immunofluorescent antibody test (IFAT): IFAT on slides with egg or adult worm antigen were carried out as described by Coudert et al. (1968). IFAT on mf was carried out in suspension using approximatively 2500 mf per tube. Mf were washed twice before test and after each incubation step (30 min at 37° C) with PBS.

Hamster sera were tested in twofold dilutions starting at a reciprocal titre of 40 for adult worm and egg antigens and fourfold dilutions starting at 80 for mf antigen. The results were evaluated by calculating geometric mean titres and standard deviations (s.d.).

Anti-hamster IgG fluorescein-isiothiocyanate (FITC) conjugated antisera (Miles) was used at a dilution of 1 in 40. Readings were made using a Zeiss-microscope equipped with a Ploem illumination system and special interference filters for FITC.

Results

Parasitology

Recovery of adult worms

All animals studied developed a patent infection. Table 1 shows the recovery rates at different times p.i. In LAKZ-hamsters infected with 100 or 150 larvae the mean recovery varied between 40 and 50%. The individual values showed marked variations. Calcified worms were rarely seen at week 20 p.i., but became more numerous at week 30 p.i. or later. This phenomenon was related to the decrease in worm burden. There was no difference in the number of worms recovered from splenectomized compared to control animals infected with the same number of larvae. In heavily infected hamsters (300 larvae) the mean recovery rate was 63% at week 16 p.i., but significantly decreased to 23% at week 30 p.i. (t-test: $2P < 0.005$). In LSH-hamsters infected with 150 larvae a higher worm burden was always observed at week 8 and 12 p.i. compared to the

Table 1. Recovery of adult worms (male, female and calcified) and recovery rate (in % of injected larvae) in relation to time of autopsy, infection dose and strain of hamsters

Strain	Infection	Number of animals	Autopsy (weeks p.i.)	Adult worms (mean ± S.E.)			Recovery rate in % (±S.E.)
				female	male	calcified	
LAKZ	100 L	14	30	21 ± 3	21 ± 4	2 ± 1	44 ± 7 a
	150 L	10	8/12	34 ± 6	36 ± 8	0	46 ± 9 b
		10	20	34 ± 6	41 ± 7	< 1	50 ± 8 c
		18	30/36	27 ± 4	27 ± 5	3 ± 0.5	39 ± 7 d
	150 L spx	7	30	31 ± 6	31 ± 8	< 1	42 ± 8 e
	300 L	5	16	90 ± 7	98 ± 12	0	63 ± 6 f
		6	30	34 ± 10	33 ± 10	2 ± 1	23 ± 8 g
LSH	150 L	12	8/12	56 ± 8	63 ± 7	0	79 ± 10 h
		6	20	39 ± 4	37 ± 5	6 ± 1	55 ± 6 i
		5	30	13 ± 4	13 ± 2	6 ± 2	17 ± 4 k

L = larvae
spx = splenectomized animals
t-test: a–b, b–c, b–d not significant; b–h $2P < 0.05$; d–e not significant; h–i not significant; f–g $2P < 0.005$; i–k $2P < 0.005$

corresponding group of LAKZ-hamsters (79% vs. 46%; t-test: $2P < 0.005$). Unlike in outbred hamsters, calcified worms were detected in the inbred strain in considerable numbers as soon as week 20 p.i. The recovery rate at week 30 p.i. was very low (17%) and close to the value of heavily infected LAKZ-hamsters. The mean number of calcified worms at week 20 and 30 p.i. was significantly higher among worms found from the inbred strain (6.2 vs. 1.0; t-test: $2P < 0.001$).

Microfilaraemia

Circulating mf could be detected in the peripheral blood in all hamsters studied. Table 2 summarizes data on the length of the patent period and the density of microfilaraemia for LAKZ-hamsters infected with different numbers of third stage larvae. The onset and the mean duration of microfilaraemia was independent of the infection dose.

Splenectomy had no effect on the persistence of bloodstream mf although peak mf counts were significantly higher compared to the control group (t-test: $2P < 0.01$). A similar effect occurred in heavily infected animals.

Fig. 1 shows the relation between maximal mf-counts and female worms in LAKZ-hamsters. With an increasing number of recovered females a higher peak mf-count could be observed. In most animals peak mf-count occurred between week 10 and 13 p.i., except in some of those animals which did not suppress their microfilaraemia (see below).

Table 2. Mean duration of microfilaraemia, mean mf peak count (per 20 mm^3 of peripheral blood) in different groups of hamsters

Strain	Infection	Number of animals	Microfilaraemia	
			length (weeks) mean ± S.E.	peak count (mf/20 mm^3) geom. mean (log. mean ± S.E.)
LAKZ	100 L	26	11 ± 1	100 (2.00 ± 0.16) a
	150 L	36	9 ± 1 b	119 (2.08 ± 0.11) b
	150 L spx	10	9 ± 1	582 (2.76 ± 0.19) c
	300 L	11	11 ± 2	414 (2.62 ± 0.18) d
LSH	150 L	13	14 ± 2 e	484 (2.69 ± 0.14) e
L = larvae spx = splenectomized animals		t-test:	b–e $2P < 0.02$	a–b not significant b–c $2P < 0.01$ a–d $2P < 0.05$ b–e $2P < 0.01$

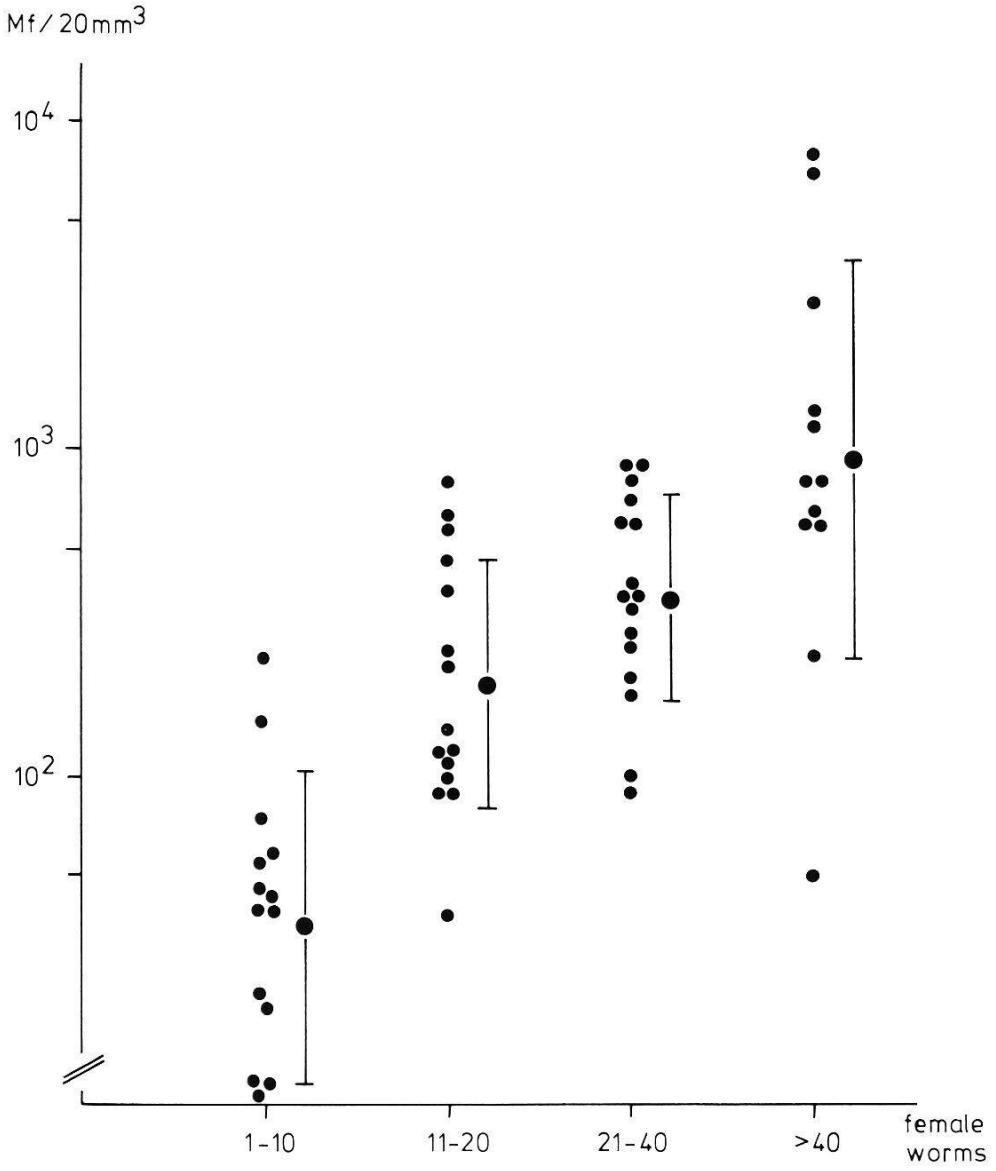

Fig. 1. Maximum microfilarial counts (per 20 mm^3 of peripheral blood) plotted against the number of female worms. Each point represents an individual value of a LAKZ-hamster (bars indicate geometric mean ± standard deviation).

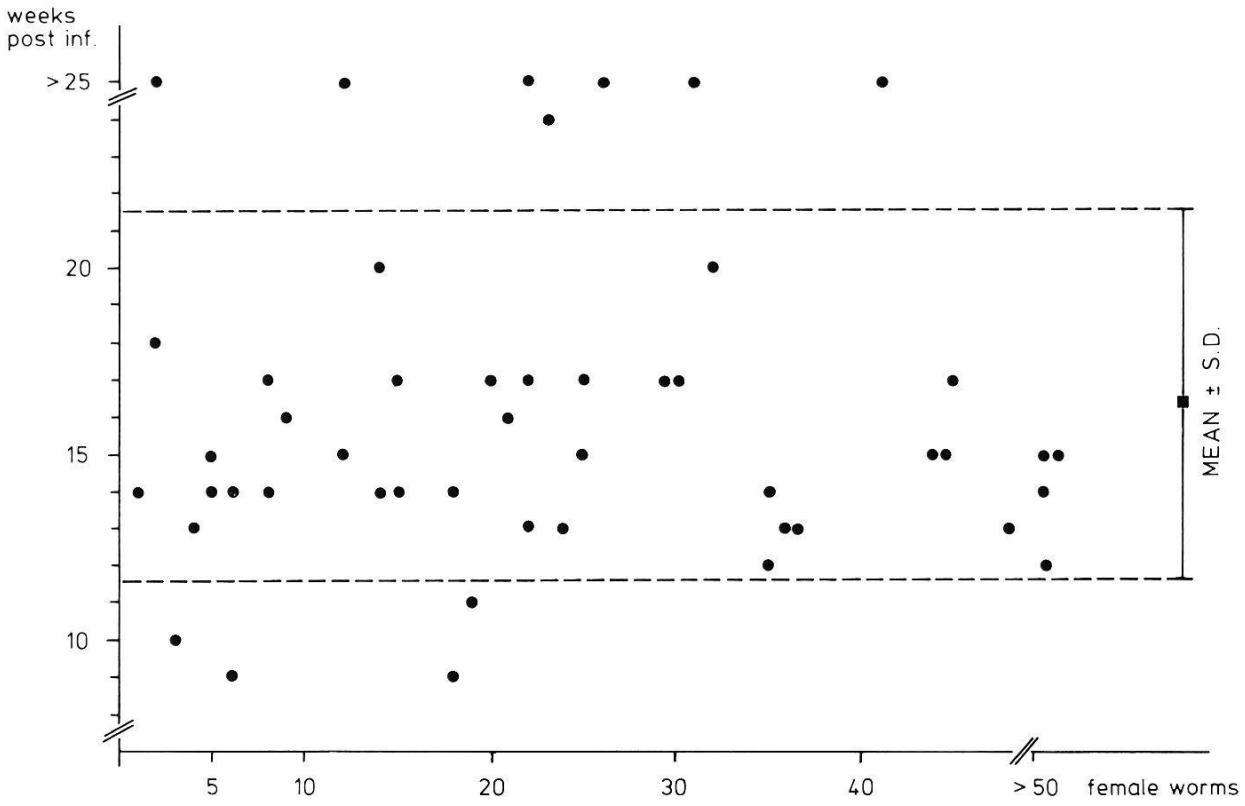

Fig. 2. End of microfilaraemia (weeks post infection) plotted against the number of female worms. Each point represents an individual value of a LAKZ-hamster. The bar indicates the mean endpoint of microfilaraemia (± standard deviation) of 48 hamsters.

The individual length of microfilaraemia in LAKZ-hamsters was independent of the number of female worms recovered at autopsy as demonstrated in Fig. 2. 41 out of 48 animals (= 81%) suppressed mf in the blood at week 20 p.i. The average number of female worms of these hamsters did not differ from hamsters with a long persisting microfilaraemia (24 ± 3 vs. 22 ± 5 as mean ± s.e.). Long persistant microfilaraemia were decreasing to low mf-counts (<100 mf/20 mm^3) or exceptionally increasing parallel to the age of infection to very high densities (>5000 mf/20 mm^3).

The limited number of LSH-hamsters studied so far did not allow an extensive analysis. Preliminary results indicate that the duration of microfilaraemia is also independent of the number of adult worms. The LSH-hamsters are not as efficient in suppressing circulating mf as the LAKZ-strain. The mean duration of patency was significantly longer than in equally infected randomly bred animals (Table 2; t-test: $2P < 0.01$). Only 14 out of 30 animals (= 47%) had reached latency at week 20 p.i. The percentages of latent animals of the two hamster strains in the course of a primary infection are compared in Fig. 3. Statistical analysis revealed significant differences at week 15 and 20 p.i. (χ^2-test, $p < 0.05$). One third of 18 animals followed up to week 30 p.i. had not controlled microfilaraemia. Three neonataly thymectomized LSH-hamsters suppressed microfilaraemia at week 20, 23 and 25 p.i.

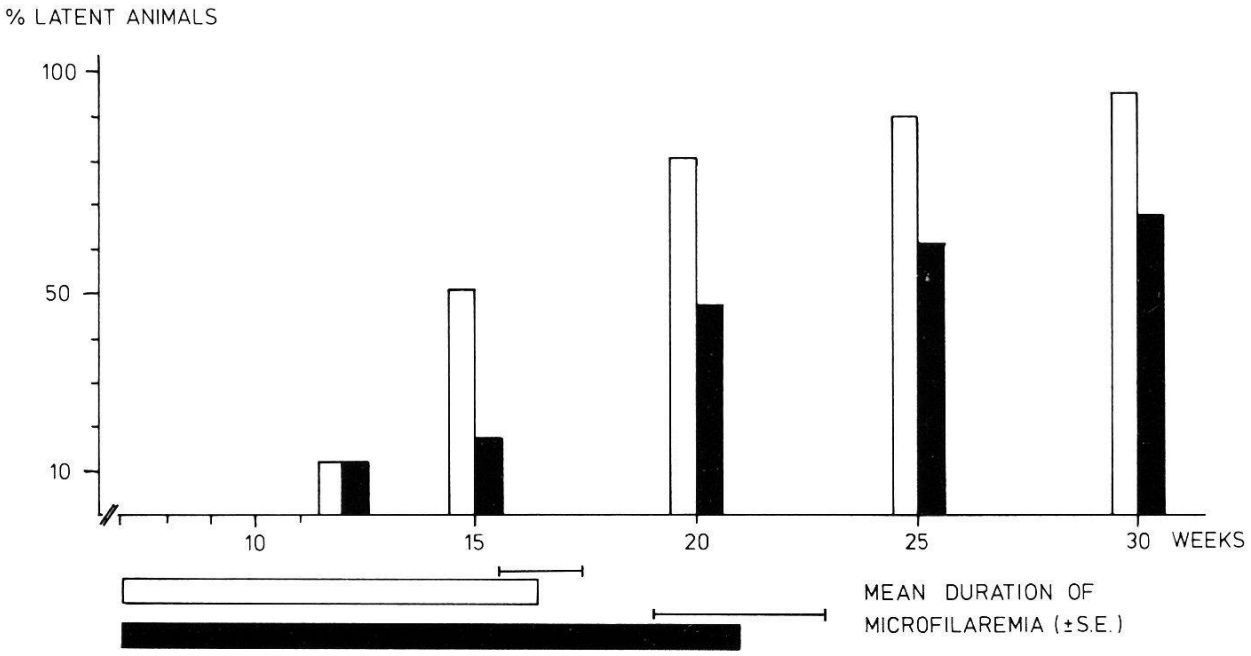

Fig. 3. Cumulative percentage of latent animals during the course of a primary infection. White columns represent LAKZ-hamsters (59 animals), black columns LSH-hamsters (30 animals for week 12, 15 and 20; 18 for week 25 and 30 post infection).

Serology

Antibodies against antigens of adult worms

The kinetic of the humoral immune response was followed with the immunofluorescent antibody test (IFAT) on frozen sections of adult worms (Fig. 4). From week 2 p.i. onwards, antibodies to various structures of the worm (as noncontractile part of somatic muscle cells, interstitial spaces between muscle cells, amorphous content of uterus and egg-membranes) were detectable in the sera of hamsters of the two strains infected with 150 larvae. It is relevant to note that even the injection of dead larvae elicited antibodies to tissues of adult female worms at week 4 p.i. However, the titres were lower than in infected animals (Fig. 4). After week 4 p.i. the rate of the titre increase is delayed. After week 12 p.i. the antibody titres remained stable during the whole observation period. Antibody titres in splenectomized and thymectomized hamsters (only measured once at week 30 resp. 23 p.i.) did not differ from that of normal hamsters (Fig. 4). Pooled hamster sera from week 4, 12, 20 and 30 p.i. were fractionated by gelfiltration on Sephadex G 200 (Tanner and Weiss, 1978). Antibodies to adult worm antigens could be demonstrated predominantly in the 7 S and a minor portion in the 19 S fraction.

Antibodies against antigens of microfilariae

Antibodies to somatic antigens of mf unmasked by papain digestion were detectable from week 2 p.i. In contrast antibodies to the cuticle of intact mf were only present in the sera after week 12 p.i. Table 3 shows the correlation between

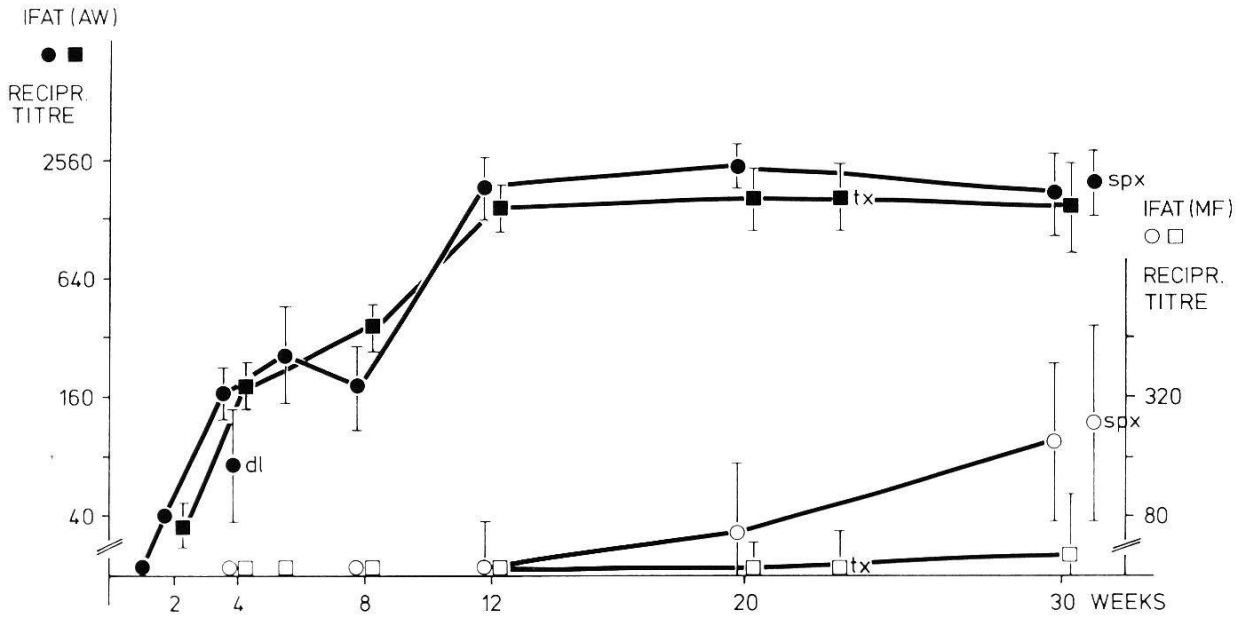

Fig. 4. Humoral immune response during the course of a primary infection with 150 larvae. Symbols and bars represent geometric mean titre (± standard deviation) of at least 6 sera tested by the indirect immunofluorescent antibody test (IFAT) on adult worm (AW) and on intact microfilariae (MF) as antigen. ● ○ results for LAKZ- ■ □ for LSH-hamsters. spx = splenectomized hamsters (5), tx = thymectomized hamsters (3), dl = hamsters (6) injected with 150 dead larvae.

Table 3. Results of individual sera in the indirect immunofluorescent antibody test on microfilariae as antigen in relation to the presence or absence of circulating microfilariae for LAKZ- and LSH-hamsters

Hamster strain	Number of positive results (in %) at	
	patency	latency
LAKZ	5/37 (14) a	34/38 (90) b
LSH	5/35 (14) c	11/13 (85) d

χ^2-test: a–b $P < 0.01$
c–d $P < 0.01$
a–c not significant
b–d not significant

microfilaraemia and anti-cuticular antibodies. Cuticular fluorescence was demonstrated in 85 and 90% of sera tested from latent LAKZ- and LSH-hamsters respectively. During microfilaraemia these antibodies could only sporadically be detected in the serum. The correlation between the course of the microfilaraemia and the appearance of anti-cuticular antibodies to mf was similar in both strains of hamsters. Fig. 5 demonstrates this correlation in eight animals (four of each strain) which were selected according to the course of microfilaraemia. LAKZ-hamsters predominantly showed pattern A and B whereas with LSH-hamsters no favoured pattern could be observed.

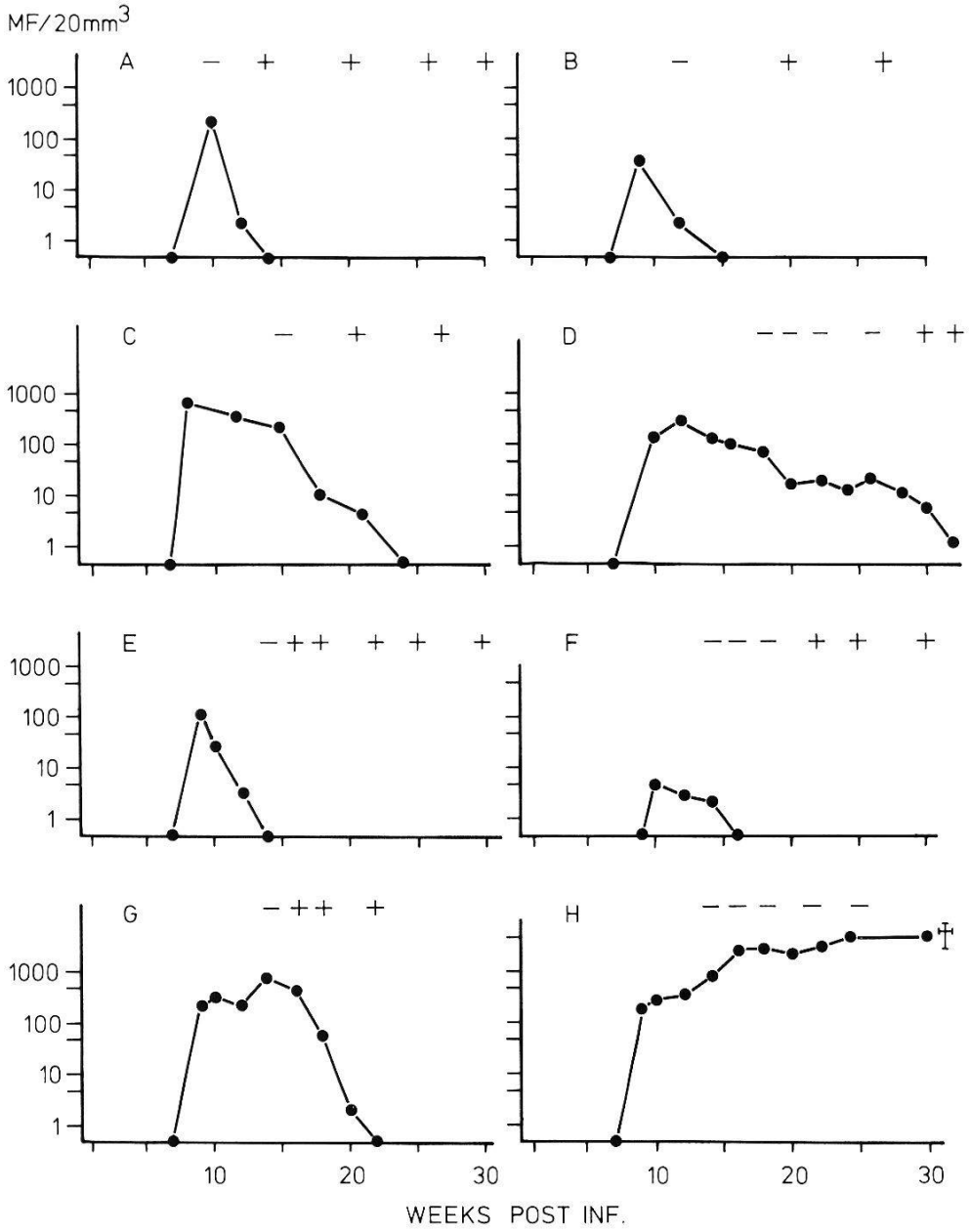

Fig. 5. Individual patterns of microfilaraemia and antibodies to the cuticle of microfilariae (results expressed as + or –). A–D: LAKZ-hamsters, E–H: LSH-hamsters.

LSH-hamsters were more tolerant to circulating mf as shown by higher peak counts as well as the higher mean duration of microfilaraemia (Table 2). This phenomenon was paralleled by the delayed appearance of anti-cuticular antibodies to mf as opposed to LAKZ-hamsters (Fig. 4).

Splenectomized hamsters controlled microfilaraemia as efficiently as normal infected outbreds and gave rise to a similar geometric mean titre at week 30 p.i. (Fig. 4). Anti-cuticular antibodies to mf were found in one out of the three thymectomized hamsters at week 23 p.i.

Antibodies against the cuticle of mf were only found in the first peak after separation of sera on Sephadex G 200 indicating that they were probably of IgM class.

Discussion

Golden hamsters of both strains (LAKZ and LSH) showed a high susceptibility to a primary infection with *D. viteae*. All animals developed a patent infection whereas in the natural host, *Meriones libycus*, an infection rate of about 80% has been reported (Worms et al., 1961; Weiss, 1970). The percentage of administered third stage larvae which reached maturity was significantly higher in the LSH-compared to the LAKZ-strain (see Table 1). The same result was obtained by Neilson (personal communication) who observed a higher susceptibility of LSH-hamsters compared to a randomly bred strain. Factors which determine host-parasite compatibility for helminths are largely unknown (Soulsby, 1976). Individual variations of the recovery rates were as great in the inbred as in the randomly bred strain of hamster. This might be due to individual differences in resistance or to differences in the infectivity of third stage larvae.

Adult worms were more vigorously and prematurely attacked in LSH- as compared to LAKZ-hamsters (see Table 1). One possible factor which might influence this destruction process could be the higher worm burden at the start of patency of the LSH-strain. With an infection of 300 larvae in LAKZ-hamsters a similar drastic decrease of the recovery rate was observed (Table 1).

In both strains of hamster microfilaraemia began between week 7 and 8 p.i. Thus, the length of the prepatent period was identical to that of the natural host (Weiss, 1970). 3 to 5 weeks later, peak microfilaraemia was reached in most LAKZ-hamsters (see Fig. 5A and B). The density of microfilaraemia was related to the female worm load (Fig. 1). The variations observed might be due to the method used as ether anaesthesia is known to influence the distribution of mf in the circulation (Hawking and Clark, 1967). Statistical analysis revealed higher mean mf peak counts in LSH-hamsters and in the group of heavily infected LAKZ-hamsters (Table 2). These findings were in agreement with the higher number of female worms recovered (see Fig. 1 and Table 1).

The persistence of mf in the circulation was highly strain dependent (see Table 2). The individual ability to control microfilaraemia was independent of the number of female worms recovered (as shown for LAKZ-hamsters in Fig. 2) and therefore also independent of the infection dose (see Table 2). The course of microfilaraemia was highly predictible in LAKZ- in contrast to LSH-hamsters which showed great individual variations. Whereas 81% of LAKZ-hamsters suppressed microfilaraemia by week 20 p.i. one half of the LSH-hamsters studied were still microfilaraemic (see Fig. 3). The same result was obtained in LSH-hamsters with a low infection dose (50 larvae); only 5 out of 12 animals controlled microfilaraemia at week 20 p.i. (data not presented).

Splenectomy one week prior to infection had no effect on the peristence of mf in the circulation of LAKZ-hamsters, although maximum mf-counts were significantly higher than in control animals (see Table 2). The comparative

analysis of the worm recovery in both groups of animals at week 30 p.i. revealed no difference. It cannot be excluded that a high worm recovery at early patency might explain the higher mf-density in the peripheral blood. It has already been shown that the spleen plays only a minor role in the removal of circulating microfilariae (Hawking, 1962). Splenectomy did not prolong the persistence of microfilaraemia after mf-injections into clean hamsters (Weiss, 1970). Also no effect of splenectomy could be demonstrated in worm transfer experiments with *Litomosoides carinii* into uninfected albino rats (Subrahmanyam and Chaudhuri, 1975).

The course of the humoral immune response measured by IFAT on frozen worm sections was identical in both strains of hamsters (see Fig. 5). The first steep rise in antibody titre up to week 4 p.i. occurred during development and moulting of the third and forth larval stages as described by Chabaud (1954). As not all larvae reached maturity, dying larvae were an additional antigenic stimulus, as was demonstrated by the injection of dead larvae (see Fig. 5). From week 4 to 8 p.i. almost no increase in antibody titre was observed. Whether there was no important antigenic stimulus by the growing, young adult worms or whether humoral antibodies were absorbed or complexed is not known. A second increase in antibody titres to a maximal level went parallel with the early patent period. Besides mf shedding, egg-membrane material [possibly lysed in utero by membrane bound enzymes as suggested by McLaren (1972)] will be released in substantial amounts. This has been shown to be highly immunogenic (see below). From week 12 p.i. antibody titre remained at a high level independent on the number of worms recovered at autopsy. No impairment of the humoral immune response was detectable in splenectomized LAKZ- nor in thymectomized LSH-hamsters (Fig. 5).

Using intact mf as antigen in IFAT, antibodies to cuticular antigens appeared only in the sera of amicrofilaraemic animals or of hamsters just before all mf had been removed from circulation (see Table 3 and Fig. 5). The same results have been demonstrated in latent animals by the direct agglutination test in Dirofilariasis in dogs (Wong, 1964) as well as by IFAT in *Brugia pahangi* infected, amicrofilaraemic cats (Ponnudurai et al., 1974). Antibodies to microfilariae were also detectable in sera of amicrofilaraemic patients with clinical pictures of tropical eosinophilia and elephantiasis (Wong and Guest, 1969).

The first anti-filarial antibodies in the sera of infected hamsters were detectable at week 2 p.i., i.e. one week after the first moult to forth stage larvae (Chabaud, 1954). These antibodies reacted with adult worm antigens, egg-membranes and papain digested mf but never with intact mf. The same result was obtained with sera from hamsters immunized with dead third stage larvae. Thus, antibodies to common or closely related antigens of the different worm stages have been detected. Our results on the antigenic structures of the adult worm were essentially the same as described by Diesfeld and Kirsten (1975) studying sera of human filariasis patients on methacrylate embedded *D. viteae*

sections of adult worms. The antigenic similarity of filariae of different species has been extensively used for serodiagnosis of human filariasis (reviewed by Kagan, 1974; Ambroise-Thomas, 1974).

The antigenicity of the mf is of special interest, as the acquired immunity to circulating mf was shown to be stage specific and could also be elicited by repeated injections of mf into clean hamsters (Weiss, 1970). Antibodies to cytoplasmic mf-antigens were already present at the start of parasitaemia and were not effective in suppressing microfilaraemia. Only when mf have been removed and destroyed, antibodies to the cuticle were demonstrable. Their detection could have been delayed by absorption when mf-antigens are present in excess. Interestingly we could not detect antibodies fixed to the cuticular surface of circulating mf. One can assume that mf covered by antibodies have been removed from the general circulation. Whether living or only mf which were dying – due to the suboptimal physiological environment in the experimental host – were turning on a specific immune response is questionable. In this respect it is noteworthy that mf injected into clean animals circulated in the peripheral blood of hamsters for only 6 to 8 weeks, whereas mf circulate in the natural host for over 20 weeks (Weiss, 1970).

Why individual and important strain differences in the ability to control microfilaraemia did occur is unknown. Different levels of acquired immunity to *Schistosoma mansoni* have been observed in two strains of hamster (Smith and Clegg, 1976). There is probably a delicate balance of the immune response and the fresh supply of mf by the female worm. This balance could be abolished by immunosuppressives which have lead to a recrudescence of microfilaraemia in latent hamsters infected with *D. viteae* (Neilson, 1978). The excellent correlation between anti-microfilarial antibodies and latency is no proof for an exclusive humoral immune mechanism. Results on antibody dependent cellular adherence to microfilariae in vitro indicate that such a mechanism could probably also be effective in vivo (Tanner and Weiss, 1978). In this paper the questions of where and how mf are removed or trapped will be discussed.

From our results it is not possible to say how heavily the surviving adult worm population was affected by the host's immune responses. Embryogenesis has been shown to be functional in female worms from latent hamsters (Weiss, 1970). However, the possibility cannot be excluded that immune factors could slow down embryogenesis. Immune sera showed an inhibitory effect on microfilarial output in vitro (Weiss, 1970). Taliaferro (1948) pointed out the possibility that immune factors might depress metabolic activities influencing embryogenesis.

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Note added in proof

Unresponsiveness to phytohemagglutinin of lymphocytes from thymectomized LSH-hamsters cannot be regarded as proof for successful neonatal thymectomy since a depressed mitogenic response has recently been found in normal hamsters of this strain which were infected for more than 20 weeks (Weiss, manuscript in preparation).