THE FATE OF THE FILARIA *LITOMOSOIDES SIGMODONTIS* IN SUSCEPTIBLE AND NATURALLY RESISTANT MICE

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Summary:

The fate of *Litomosoides sigmodontis* was compared in susceptible BALB/c and resistant B10D2 mice, presenting the same major histocompatibility complex ($H\text{-}2^d$), with an attempt to dissociate the different elements of the life cycle in order, later, to dissociate the different mechanisms involved. Each female mouse was inoculated once with a small dose of infective larvae (25 L3) or a large dose (100 or 200 L3). In total, 92 BALB/c and 49 B10D2 were studied.

Necropsies were performed up to D85 following infection with 25 larvae. The early fate was similar in B10D2 and BALB/c mice; particularly the recovery rate of worms was almost identical during the first month p.i. and represented a quarter of the inoculated larvae. Resistance in B10D2 mice appeared progressively, as judged by retardation of growth and of the fourth moulting, the presence of very small sterile female worms and male worms with abnormal left spicule, and a high frequency of live filariae coated with inflammatory cells and encapsulated dead worms. The *L. sigmodontis* life span in B10D2 was about half that in BALB/c.

Necropsies were carried out up to D20 following infection with 100-200 L3. The recovery rate was increased in BALB/c. Growth was retarded earlier in B10D2 mice, this crowding effect already apparent at D10; this may indicate a role for metabolic factors.

The pattern of the life cycle in both mouse strains confirms recent conclusions on Onchocercinae: the recovery rate is established as soon as the second day during "phase 1 of massive destruction", then it is stable during "phase 2 of insignificant mortality". During phase 1, the infective larvae are immediately destroyed in the subcutaeous tissue if they are not able to escape the inflammatory process by penetrating in local lymphatic vessels. By contrast, phase 2, which is longer than the duration of the third larval stage, indicates there is no mortality linked to the third moulting, at least following a single inoculation.

KEY WORDS : filariae, mice, quantitative analysis, lymphatic biology, resistance, competition, *Litomosoides*.

MOTS CLÉS : *filaire, souris, analyse quantitative, biologie lymphatique, résistance, compétition métabolique,* Litomosoides.

Résumé : DÉVELOPPEMENT DE LA FILAIRE *LITOMOSOIDES SIGMODONTIS* CHEZ LA SOURIS SENSIBLE ET NATURELLEMENT RÉSISTANTE

Le développement de Litomosoides sigmodontis *est comparé chez deux souches de souris de même complexe majeur d'histocompatibilité, la BALB/c sensible et la B10D2 résistante, en cherchant à dissocier les différents éléments du cycle pour pouvoir dissocier ultérieurement les différents mécanismes mis en jeu.*

Chaque souris femelle est inoculée en sous-cutané avec une faible dose de larves infectantes (25 L3) ou une forte dose (100 ou 200 L3). Au total 92 BALB/c et 49 B10D2 ont été étudiées. Les autopsies ont été effectuées jusqu'à J85 après un petit inoculum. Le début du développement est semblable chez la souris B10D2 et la BALB/c; particulièrement, le pourcentage de larves qui se développent est presque identique pendant le premier mois et représente le quart du nombre inoculé (respectivement 21 et 26 %). La résistance de la B10D2 apparaît progressivement. Elle se traduit, après le stade 3, par un retard de croissance et de la mue 4, la formation de femelles très petites et stériles et de mâles ayant un spicule gauche anormal, ainsi que par une fréquence plus élevée de filaires vivantes mais entourées d'un manchon de cellules inflammatoires, et de kystes. La durée moyenne de vie de L. sigmodontis *chez la B10D2 est presque deux fois plus courte que chez la BALB/c.*

Les autopsies ont été faites jusqu'à J20 après un fort inoculum. Celui-ci élève le pourcentage de larves qui se développent chez la BALB/c, et rend plus précoce le retard de croissance chez la B10D2. Dans ce cas, la compétition est déjà visible dix jours après l'inoculation, ce qui suggère l'intervention de facteurs métaboliques.

Le développement de L. sigmodontis *chez les deux souches de souris suit les règles énoncées récemment pour les Onchocercinae: le rendement est établi dès le deuxième jour pendant la «phase 1 de destruction massive des larves » : seule échappe à la destruction par la réaction inflammatoire la proportion des larves infectantes qui entrent dans les vaisseaux lymphatiques. Ensuite le rendement est stable pendant une période relativement longue, la « phase 2 de mortalité insignifiante ». Sa durée est plus grande que celle du troisième stade et montre que la mortalité n'est pas liée particulièrement à la mue 3.*

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INTRODUCTION

The capacity of *Litomosoides sigmodontis* to reach maturity and produce patent infections in immunocompetent laboratory mice provides an excellent opportunity to study the immunology of filarial infections. Petit *et al.* (1992) have established the pattern of infection in various inbred strains of mice. BALB/c are the most susceptible and all mice inoculated with infective larvae harbour adult worms at necropsy and 51 % of these animals also have a patent microfilaraemia. Mice of the B10 background are more resistant to infection and between no more 1 % of animals inoculated with L3 larvae were found to harbour adult worms, and none of these animals developed a patent microfilaraemia.

Petit's observations were made 2 months post-infection and in order to develop the *L. sigmodontis*/mouse model further, a chronological study of the fate of invading and migrating larvae was undertaken to establish the precise time and site at which resistance is first demonstrable. In anticipation of detailed immunological investigations, the histocompatible (H-2d) BALB/c and B10D2 mice were chosen for this work. An infective dose of 25 larvae was used as the basis of the study but comparisons were also made using larger inocula of 100 and 200 L3. For each experiment, the recovery rate, filarial growth, moulting and stages of the parasites were recorded.

MATERIAL AND METHODS

Maintenance of the strain of *L. sigmodontis* Chandler, 1931 and harvesting of infective larvae from the vector *Bdellonyssus bacoti* were described by Diagne *et al.*, 1989 and Petit *et al.*, 1992.

Inoculation and necropsy

One month old female BALB/c (Charles River) and B10D2 (Harlan Olac) were inoculated. Each mouse received a single dose of infective larvae in 0.2 ml of RPMI 1640 supplemented with 20 % calf serum by subcutaneous inoculation in the right lumbar area. Several experiments were performed; generally they each contained a few BALB/c and a similar number of B10D2 mice inoculated at the same time with larvae harvested from the same batch of mites; some experiments were performed with BALB/c alone. Either a large dose of larvae (100 or 200 L3 in BALB/c, and 100 L3 in B10D2) or a small dose (25 L3) was inoculated. Necropsies were performed early after inoculation (D2-3 p.i.), close to the third moulting (D10-12), during the fourth stage fourth moulting period (D20-21 and D28), during the adult phase, subdivided into prepatent phase (D40-57) and patent phase (D70-85). The dissection procedure of mice was that described by Bain *et al.*, 1994 for other rodents. In addition, sera and spleen were removed at autopsy from most of the mice inoculated with 25 L3 for the host immune response analysis (Maréchal *et al.*, in preparation).

Worms were localised and identified as live (motile) or dead (unmotile) worms (shown respectively as F and K). Only the live worms were used to calculate the percentage of mice with filariae (% F) and the recovery rate (number of filariae/number of larvae inoculated × 100: F/L3). A proportion of live worms might be partially embedded in a coat of inflammatory cells (coated filariae: cF). The dead worms were generally encapsulated and more or less broken into debris (here called cysts); a few were not surrounded by host cell reaction (dead free filariae); their internal structure was apparently normal or lysed.

Microfilaraemia (mf) was measured from D70 in blood taken from the retro-orbital sinus, and expressed as the number of microfilariae/10 mm^3. Two thick blood smears of 10 mm^3 were performed per mouse and stained with Giemsa.

Microscopical morphological study of worms

Stages, moulting and sex were identified. The third stage presents two caudal lappets and a regularly thin buccal capsule; no sclerotized segment is present between the capsule and the mouth. The fourth stage has no caudal lappets; the buccal capsule is thickened at the end of the first third and a transparent sclerotized segment is present between the capsule and the mouth (Fig. 1); the male larva is swollen in the precloacal region by the spicular primordia; the female larva is

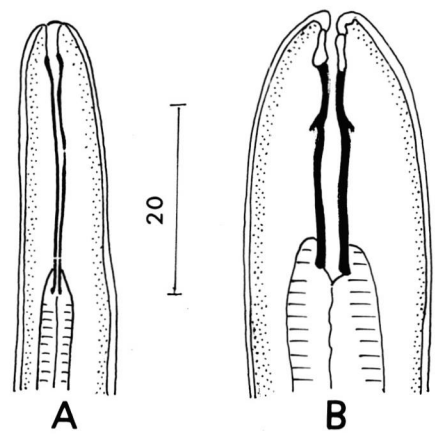

Fig. 1. — Buccal capsule of *L. sigmodontis* third (A) and fourth (B) stage larvae.

swollen in the oesopheal region by the vaginal primordium, the vulva is closed by the cuticle. The adult worm has a thick buccal capsule, the vulva is open in the female and the caudal papillae of the male are present. The uterine condition and spicular morphology of adult worms were observed.

Measurements were made on worms from D10-28 and D57-70 p.i. after fixation in hot 70 % alcohol. Worms were randomly measured in mice infected with a large dose of larvae. All worms recovered from mice inoculated with a small dose were measured.

STATISTICAL ANALYSIS

The non parametric U-test of Mann-Whitney was used to compare the adult worm numbers, microfilaraemiae, recovery rates and sizes of worms. The χ^2-test was used to compare the percentages of mice with worms (% F). For each test, the confidence interval was established at 95 %.

RESULTS

Results were based upon 24 BALB/c *versus* 10 B10D2 mice inoculated with a large dose of larvae and necropsied from D2 to D20, and 68 BALB/c *vs* 39 B10D2 inoculated with a small dose of larvae and necropsied respectively from D2 to D85 and D11 to D85.

MIGRATION AND LOCALISATION (Table I)

No differences were noted between the two strains, following a small or a large dose of larvae inoculated. At D2 the intra-lymphatic localisation was the most frequent (40 to 60 % of the larvae). The remaining larvae were recovered from the medium used to rinse the abdominal and pleural cavities, or were associated with the subcutaneous tissue, heart and lungs. From D10 p.i., most worms were recovered from the coelomic cavities. The majority were found in the pleural cavity but peritoneal localisations could be important; in about 1 in 10 mice, 50 to 100 % of the filariae were intraperitoneal.

STAGE AND SIZE OF FILARIAE (Table II)

Large dose of larvae

At D10 (200 L3 in BALB/c mice *vs* 100 L3 in B10D2 mice). Larvae from BALB/c were fourth stages; those from B10D2 were third stages, at third moulting or fourth stages. Their average size was significantly greater in the BALB/c than B10D2 mice: respectively 1.4 and 1.1 mm long and 30.8 and 23.2 µm wide (test U, $p < 0{,}0001$).

At D20 (100 L3 in the two mouse strains). Larvae were still fourth stages. Male and female worms from BALB/c were approximately twice as large in average as those from the B10D2 mice (test U, $p < 0{,}0003$).

Strain	nL3	*D*	Sc	ExtLy	IntLy	AC	He	Lu	TC	nR
BALB/c	100-200	*2*	29	16	35.1	3	7.1	7.6	2.2 ± 5.4	6
		10	0.9	0	8.9	28.8	0.5	1	60.9 ± 35.2	9
		20	0	0	0	19.7	0	0	80.3 ± 32.2	9
	25	*2*	9.6	10.8	27.5	20.6	0	5.4	26.1 ± 25.4	9
		10	0	0	0	31	0	1.9	67.1 ± 28	13
		20	0	0	0	21.37	0	2.6	76 ± 32.4	10
		28-57	0	0	0	14.4	0	0	85.9 ± 15.2	18
B10D2	100	*2*	5.9	21	45	5.8	7	13	2.3 ± 2.7	4
		10	0	0	0	15	0	0	85 ± 7.5	4
		20	0	0	0	4	0	0	96 ± 5.2	2
	25	*10*	0	0	4	52	0	0	44 ± 42	3
		20	0	0	0	27	0	0	73 ± 33	4
		28-57	0	0	0	15	0	0	85 ± 31	18

nL3: number of infective larvae inoculated; *D*: number of days between inoculation and necropsy; Sc: sub-cutaneous tissue; ExtLy: external lymphatic system (inguinal, sub-iliac, popliteal, axillary and neck lymph nodes, with associated lymphatic vessels); IntLy: internal lymphatic system (lumbar, iliac, mesenteric, sacral, thoracic lymph nodes, with associated lymphatic vessels); AC: peritoneal and vagino-peritoneal cavities; He: right heart; Lu: lungs; TC: pleural and pericardial cavities; nR: number of mice necropsied. Standard deviation is given for the pleural localisations.

Table I. — Distribution of *L. sigmodontis* larvae in BALB/c and B10D2 mice, expressed in percentages of the total recovery.

nL3	*D*	mm/µm	BALB/c	B10D2	n/n'
100-200	*10*	L	1.4 ± 0.2	1.1 ± 0.1	20/13
		W	30.8 ± 2.5	23.2 ± 2.2	
	20	Lm	7.2 ± 1.1	3.6 ± 0.6	9/10
		Wm	70 ± 8.3	47.3 ± 8.6	
		Lf	10 ± 1.9	4.4 ± 2.5	11/7
		Wf	75 ± 9.5	59.4 ± 19	
25	*10*	L	1.5 ± 0.2	—	19/0
		W	29.4 ± 2.5	—	
	11-12	L	1.7 ± 0.3	1.7 ± 0.3	8/9
		W	38.7 ± 5.2	36 ± 6.5	
	20-21	Lm	8.5 ± 1.2	6.7 ± 1.8	5/4
		Wm	86.4 ± 3.5	77.5 ± 15	
		Lf	10.2 ± 3.7	9.5 ± 1.6	4/5
		Wf	84.2 ± 21.2	80 ± 11.7	
	28	Lm	13.3 ± 1.2	6.7 ± 2	8/4
		Wm	110.5 ± 8.6	76.3 ± 25.3	
		Lf	11.6 ± 7.2	6 ± 3.6	2/3
		Wf	100 ± 28.3	87.5 ± 26.3	
	57	Lm	21.4 ± 1.3	14.7 ± 1.1	8/3
		Wm	123.1 ± 9.2	100 ± 0	
		Lf	65.8 ± 19.2	41.5 ± 16.2	5/2
		Wf	170 ± 15.4	130 ± 0	
	70	Lm	22.3 ± 1.1	—	7/0
		Wm	122.9 ± 11.1	—	
		Lf	76 ± 1.4	49	2/1
		Wf	217.5 ± 45.9	175	

nL3: number of infective larvae inoculated; *D*: number of days between inoculation and necropsy; mm/µm: length L in mm, width W in µm; f: female worm; m: male worm; n/n': number of worms measured respectively in BALB/c and B10D2 mice; ± standard deviation.

Table II. — Dimensions of *L. sigmodontis* in BALB/c and B10D2 mice.

Small dose of larvae

Until D20 all worms were fourth stages. At D28, male worms from BALB/c mice were adult stages; one of the two female worms was a fourth stage and the other one moulting to adult. Male filariae from B10D2 were fourth stages or at fourth moulting, female filariae were fourth stages.

Concerning the size, worms recovered from BALB/c mice at D10 were not significantly larger than those at the same time following the inoculation of a large dose. At D11-12, larvae from both mouse strains were 1.7 mm long. Later, a great variation of size of the female worms was noted in both strains. Nevertheless a retardation of growth was apparent in the period D20-28. About two months p.i., male worms were approximately 2 cm long in BALB/c *vs* 1.5 cm in B10D2; female worms were almost 8 cm long in BALB/c mice and 5 cm in B10D2.

Male and female worms recovered from BALB/c from D57 to D70 presented respectively normal spicules and developing eggs and microfilariae. The 13 males recovered from B10D2 during the period D40-D70 were studied; 12 had a small and slightly sclerotised left spicule; the three female worms recovered from D57-70 had no developing eggs and microfilariae.

Percentage of mice with filariae (Table III)

Following a large or a small dose, all mice of the two strains presented worms until D20. All later observations were done on mice inoculated with a small dose. The percentage of BALB/c mice with worms was still 100 % until D70 then decreased to 75 % at D85. The percentage of B10D2 mice with worms was reduced to 60 % at D40-49 and was nil at D85.

Recovery rate (Table III, Fig. 2)

Large dose of larvae

From D2 to D20, no reduction of the recovery rate was observed in the two mouse strains.

In BALB/c mice, the recovery rate was statistically higher at D20 (test U, $p < 0{,}0112$). We ascribe this difference to the individual variation, which is great, and to the difficulty in recovering all the larvae when they are younger and smaller. The series D2-D20, taken as a whole, showed a mean recovery rate of 40.2 ± 14.7 %.

		F/L3		% F		
nL3	*D*	BALB/c	B10D2	BALB/c	B10D2	n/n'
100-200	*2-3*	32.2 ± 5.8	29.5 ± 12.1	100	100	6/4
	10	36.4 ± 17.8	20.5 ± 10.5	100	100	9/4
	20	49.2 ± 11.3	26 ± 1.4	100	100	9/2
25	*2*	18.7 ± 11.1	—	100	—	9/0
	10	24.9 ± 13.8	21.3 ± 12.2	100	100	17/3
	20	26.4 ± 16.8	18.2 ± 10	100	100	10/4
	28	24 ± 33.9	26 ± 2.8	50	100	2/2
	40-49	27.3 ± 13.2	5.6 ± 6	100	60	6/5
	50-57	34 ± 12	5.9 ± 6.7	100	63.6	10/11
	70	18.9 ± 13.4	0.6 ± 1.6	100	14.3	7/6
	85	2.8 ± 3	0	75	0	7/8

nL3: number of infective larvae inoculated; *D*: number of days between inoculation and necropsy; F/L3: recovery rate (number of worms recovered/number of larvae inoculated × 100); % F: percentage of mice with worms; n/n': respective numbers of BALB/c and B10D2 mice necropsied (the mouse found without parasites at D28 is an exception since about 200 BALB/c mice were examined at present and all harboured worms at two months p. i.).

Table III. — Evolution of *L. sigmodontis* infection in BALB/c and B10D2 mice.

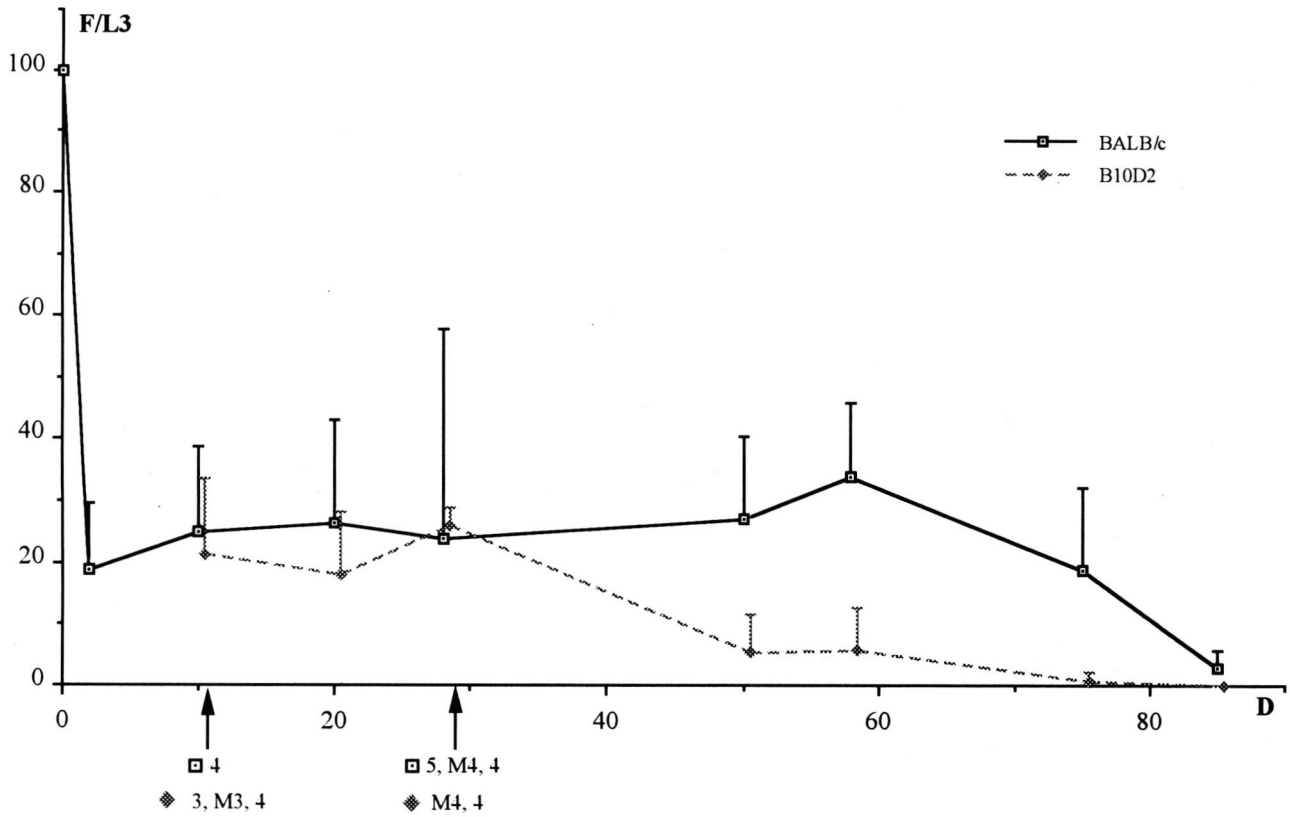

Fig. 2. — Evolution of the recovery rate of *L. sigmodontis* in BALB/c and B10D2 mice inoculated with 25 infective larvae.
D: number of days between inoculation and necropsy; F/L3: recovery rate (bars: standard deviation); 4, M4 and 5: fourth stage, fourth moulting and adult stage respectively.

In B10D2 mice, the chronological series D2 to D20 was homogeneous; the mean recovery rate was 25.4 ± 10 %.

Small dose of larvae

In BALB/c mice, the series was homogeneous from D2 to D50-57 with a mean recovery rate 26.0 ± 14.4 %. It was slightly reduced at D70 (not significant) and fell to 3 % at D85 (test U, $p < 0{,}0032$).
In B10D2 mice, the recovery rate was stable from D10 to D28 with a mean of 21 ± 9.3 %; it had already decreased greatly at D40-49 (test U, $p < 0{,}0053$) and was almost equal to zero at D70 (test U, $p < 0{,}0192$).

COATED FILARIAE, DEAD FREE FILARIAE AND CYSTS

In BALB/c mice, one dead free larva was found up to D28 (in a mouse inoculated with 25 L3) and no coated filariae nor cysts were found. Coated filariae were found from D40 to D70 in one fourth of the mice and represented respectively 4 % then 12 % of the worm burden at these times. Only one mouse in seven contained coated filariae at D85. Cysts were found from D70 and about 60 % of mice harboured cysts from this time to D85.

In B10D2 mice, a few dead free filariae were noted during the larval period (five, one and one larvae from the lungs, respectively at D3, 12 and 20) and the adult period from D40 (one in six female worms and one in 13 male worms were lysed). Coated filariae were found from D40 to D57, in two thirds of the mice and representing a third of the worm burden. No coated filariae were found later. Cysts were present from D40 in half of the mice until D70, and in all mice at D85.

MICROFILARAEMIA

In BALB/c mice, 31 % had blood microfilariae (2 to 27 mf/10 mm^3) and among B10D2 mice none displayed blood microfilariae.

DISCUSSION

Early necropsies are rarely performed for the study of filarial cycle, as underlined by Eisenbeiss *et al.* (1991). Although only about 80 % of larvae can be recovered during the first days p.i., these early data are essential to understand the mechanisms of natural or acquired resistance.

Resistance in B10D2 inoculated with 25 L3

Early development of *L. sigmodontis* was almost identical in the resistant B10D2 and the susceptible BALB/c strains: all mice had filariae and the recovery rate represented a quarter of the inoculated larvae (21 *vs* 26 %, not significant) and the pattern of lymphatic migrations (first described by Wenk, 1967) was similar.

The differences of the genetic background had little influence on the early stages of vertebrate life cycle, but they were critical later. In the B10D2 mice, a retardation of growth from D28 was observed by comparison with the development in BALB/c mice, and no male filariae passed the fourth moult whereas all filariae reached adulthood in BALB/c. Poor growth in B10D2 mice was reflected in development of an abnormal left spicule and an absence of embryogenesis.

Moreover, in the B10D2 mice, dead larval or adult filariae not associated with an inflammatory host reaction were more numerous; the frequency and density of coated filariae were higher, the cysts appeared earlier (from D40 instead of D70) and the filarial lifetime was shorter: the recovery rate decreased between D28 and D40 (test U, $p < 0{,}0084$), almost half the time recorded in BALB/c mice (Fig. 2).

Early effects of a large dose of larvae

A large dose of inoculated larvae had two effects, different in each strain.

Retardation of growth

Retardation of growth associated with increasing number of filariae has been described with *L. sigmodontis* in rat (Dahr & Singha, 1971) and is a common feature. In B10D2 mice, growth was retarded much earlier following 100 L3 than 25 L3: respectively at D10 and D20-28 (Table II). A similar crowding effect appeared also in BALB/c mice heavily infected but 10 days later.

Change of the recovery rate

The recovery rate increased in BALB/c mice to 40 % with a large dose, instead of 27 % following 25 L3 (Table III). On the other hand, in the B10D2 mice, no significant increase was observed. This well pronounced phenomenon of "facilitation" observed in BALB/c mice was unique. Data obtained with other pairs of Onchocercinae-vertebrate hosts indicated a fundamental constancy of the recovery rate (Bain *et al.*, 1994) though a very slight facilitation of the development of *Monanema martini* was demonstrated using Factorial Analysis of Correspondance (Wanji *et al.*, 1990).

CONCLUSION

Resistance against *L. sigmodontis* infection in B10D2 develops progressively. The early recovery rate is almost identical in this strain and in the susceptible BALB/c strain.

Competition is much more intense in B10D2 mice; the fact that it is already apparent at D10 suggests a role for metabolic factors.

From a general point of view, the development of *L. sigmodontis* in mice follows the same pattern as that observed in the other pairs of filaria-hosts (Bain *et al.*, 1994). In particular, the evolution of the recovery rate (Fig. 2) presents the two phases recently defined:

1) The "phase 1 of massive destruction of larvae". It lasts less than two days and occurs in the subcutaneous tissue. The infective stage is submitted on its arrival in the host to an inflammatory reaction and it escapes destruction by penetrating into the lymphatic vessels, where the medium is lacking important components (Greep & Weiss, 1973) and is consequently poorly agressive. The recovery rate, which corresponds to the capacity of a larva to penetrate into a lymphatic vessel, is characteristic of a filaria-host pair. In BALB/c mice, in which exceptionally an increase of the recovery rate is induced by a large dose inoculated (facilitation), the inflammatory reaction at the site of inoculation could be "overflowed" by an excess of larvae, or an increased dilation of lymphatic vessels could facilitate the penetration of the larvae in these vessels.

2) The "phase 2 of insignificant mortality". Like other filariae, it is noted here that the period of stability of the recovery rate (70 days in BALB/c and 28 days in B10D2 mice) exceeds the duration of the third larval stage (10-11 days). In case of mono-inoculation at least, the third moulting is not a particularly fragile stage of the life cycle.

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