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Increased early local immune responses and altered worm development in high-dose infections of mice susceptible to the filaria *Litomosoides sigmodontis*

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Abstract The relationship between the number of larvae inoculated and filarial infection outcome is an important fundamental and epidemiological issue. Our study was carried out with BALB/c mice infected with the filaria *Litomosoides sigmodontis*. For the first time, an immunological analysis of infection with various doses was studied in parallel with parasitological data. Mice were inoculated with 200, 60 or 25 infective

larvae (third stage larvae, L3), and monitored over 80 days. At 60 h post-inoculation the immune response was stronger in the 200 L3 group than the 25 L3 group. Cells from lymph nodes draining the site of inoculation proliferated intensely and produced large amounts of IL-5 and IL-4. In the pleural cavity, leukocyte populations accumulated earlier and in larger quantities. IgG1, IL-4 and IL-10 serum concentrations were transiently higher. During the first 10 days the worm recovery rates were identical in all groups, but decreased thereafter in the 200 L3 group. In this group, the development of the worms was altered, with reduced lengths, diminished intra-uterine production of microfilariae and abnormalities of male copulatory organs. Whereas mice inoculated with 25 L3 became microfilaraemic, only one third reached patency in the 200 L3 group. However, detrimental effects of high numbers of worms are not seen in studies using different inoculation protocols. This suggests that the very early events determine subsequent immune response and infection outcome rather than competitive interactions between the worms.

Keywords Filariasis · Murine model · Immunity · Infection dose · *Litomosoides sigmodontis*

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Introduction

Understanding the relationship between the intensity of filarial infections and the number of filarial infective larvae delivered to the host has both fundamental and epidemiological relevance [8, 43]. In the past much work was done on the parasitological aspect of filarial infections with several models of filariases including rodents, primates, and cats. Assessing the filarial outcome with the microfilarial densities yielded a large range of results from an almost proportional increase [19, 35, 49, 50] to a drastic reduction [12] when the number of larvae

delivered increases. This diversity of results suggests a great complexity of mechanisms involved. The inoculation of high doses of infective larvae deserves to be investigated further with the more recent model of filariasis *Litomosoides sigmodontis* Chandler, 1931 to investigate the mechanisms involved in controlling the development of filariae.

The filaria *L. sigmodontis* in mice allows both immunological and parasitological studies throughout the infection, from the inoculation of larvae until the arrival of microfilariae in the blood. Its potential in laboratory mice has been demonstrated over the last decade [5, 37] with important results on the control of worm development in primary infection [2, 3, 6, 27, 30, 32, 38, 39, 46, 47] or following vaccination [25, 26, 31, 33], and on microfilarial survival in blood [20].

Preliminary parasitological studies had suggested that the infection of BALB/c mice with increasing doses of *L. sigmodontis* infective larvae (25, 60 or 200 third stage larvae, L3) resulted in alterations of microfilarial outcome because the three groups exhibited similar rather than increased microfilaraemiae [28]. In the present study, to assess the effect of infective larvae numbers on the immune response as well as on filarial outcome, doses of 25 or 200 L3 were administered in single subcutaneous inoculations to susceptible BALB/c mice. A kinetic analysis of the infection and immune response was performed over 80 days of infection, including investigation in the different host compartments visited by the filaria: the lymph nodes, pleural cavity and blood. In addition, an intermediate 60 L3 dose served to further detail the parasitological study.

The effect of large numbers of infective larvae delivered at once is not only theoretically interesting, but probably reflects natural conditions found in the human filariasis caused by *Loa loa*. The *Chrysops* vectors may harbor as many as 800 infective larvae [22], which have been described to “pass on to the skin-surface in a continuous stream” during the fly’s blood meal on humans [10], an observation that has been confirmed [16]. It thus seems that a considerable number of larvae could be deposited on the patient’s skin in loiasis. Two opposing manifestations are notorious in this filariasis; consistently high prevalence of occult filariasis (i.e., without blood microfilariae) and the highest microfilaraemiae found in humans [14, 48]. These are associated with distinct immune patterns [1, 7], and raise the question whether these contrasting outcomes might be, at least in part, a consequence of this mode of transmission.

Material and methods

Mice and inoculation protocol

Maintenance of the filaria *L. sigmodontis* Chandler, 1931 and recovery of infective larvae (L3) from the mite

vector, *Ornithonyssus bacoti*, were carried out as previously described [13, 37].

Female BALB/cAnNHsd mice, 6–7 weeks old, were purchased from Harlan Olac (Gannat, France). All mice were maintained in microisolators with filter-topped cages and received sterilized food and water to avoid any exposure to other microorganisms, and particularly gastrointestinal nematodes like pinworms, known to share antigenic molecules with filaria [21]. Mice were divided into three groups inoculated subcutaneously with a single dose of either 25, 60 or 200 larvae. Necropsies were performed at several time points: 60 h (H60) post inoculation (p.i.) (6 mice per group); day 10 (D10) p.i. (7 or 6 mice per group); day 30 (D30) p.i. (10 mice per group); day 60 (D60) p.i. (7 or 6 mice per group); day 80 (D80) p.i. (6 mice per group).

All experiments and procedures conformed to the French Ministry of Agriculture regulations for animal experimentation (1987). Additionally, natural infections of BALB/c mice by *L. sigmodontis*-infected mites were performed as previously published [47].

Recovery of worms at necropsy and protocol of morphological study

At D10, D30, D60 and D80 p.i., filariae were recovered with pleural exudate cells (PleC) and pleural microfilariae by flushing of the pleural cavity with 10 ml PBS according to the technique described previously [6]. The peritoneal cavity was also observed under a dissection microscope in case rare filariae were present. Location, motility and aspect of the filariae were noted. Filariae (F) were harvested, counted and fixed in hot 70% ethanol for morphological analysis; worms were cleared in lactophenol and observed under a microscope. The filarial development was evaluated by means of the following parameters: (1) percentage of mice with microfilariae; (2) percentage of mice with filarial worms; (3) recovery rate of filariae: number of worms recovered/number of larvae inoculated $\times 100$ (F/L3); (4) number of live worms partially surrounded by inflammatory cells: these worms were included in the calculation of the recovery rate; (5) number of dead worms or pieces of worms in granulomas (not included in the recovery rate); (6) size of worms; (7) stage of worms; and (8) sex ratio of recovered worms: number of female/total worm recovered. More detailed analysis was carried out on the male spicules, spermatozoa production, and females uteri contents, including presence/absence of spermatozoa. At D60 and D80 p.i., the whole body of each female filaria was observed and the density of uterine microfilariae was estimated according to a previously established scale [9]. These densities were scaled as 0 to 5, which corresponds to the maximum observed in female *L. sigmodontis* from the highly susceptible host *Meriones unguiculatus*. The same index, 0 to 5, was used for divided eggs and ovulae.

Microfilaraemia and pleural microfilariae

Percentage of mice with blood or pleural microfilariae was noted. Peripheral and cardiac blood microfilarial density (number of microfilariae/10 μ l) was determined at D60 and D80 p.i. on a 10- μ l-thick blood smear stained with Giemsa. Pleural microfilariae were enumerated on a Malassez counting chamber.

Recovery of leukocytes from lymph nodes and the pleural cavity fluid

Lymph nodes that are close to the inoculation site (right inguinal lymph node and iliac + lumbar lymph nodes) from each mouse were collected and each lymph node population studied separately. The lymph nodes were crushed with a rubber plunger and allowed to pass through a 100- μ m nylon cell strainer (Becton Dickinson, Le Pont de Claix, France).

Lymph node cells (LNC) and PleC were studied as follows. After centrifugation at 1,900 *g* for 6 min at 4°C, the cells were resuspended in 1 ml RPMI 1640 and counted in PBS-0.04% Trypan Blue (Sigma-Aldrich, St Quentin Fallavier, France) using a Malassez hemocytometer containing 1-mm³ sample.

Proportions of polynuclear eosinophils, polynuclear neutrophils and mast cells in the pleural exudate were determined on cytospin cell preparations: a suspension of 100,000 PleC in 150 μ l PBS-1%FCS was centrifuged (500 rpm, 3 min, Shandon Cytospin 3) against slides, and stained with May-Grünwald-Giemsa. LNC and PleC were further phenotyped by flow cytometry. Data were collected using a FACSCalibur flow cytometer and analyzed using the CellQuest software (Becton Dickinson, Le Pont de Claix, France). The following rat anti-mouse antibodies were used: anti-CD19 (ID3 clone) conjugated to FITC, as a marker of B cells; anti-CD5 (53-7.3 clone) conjugated to PE, a marker found on B1 cells and not B2 cells; anti-CD3 (17A2 clone) conjugated to Cy-Chrome anti-CD4 (RM 4-5 clone) conjugated to FITC; anti-CD8 (58-6.7 clone) conjugated to PE. All antibodies were from BD Pharmingen except anti-F4/80 (C1:A3-1 clone) conjugated to PE (Tebu-Caltag, Le Perray-en-Yvelines, France), a marker of macrophages.

LNC and PleC stimulation in vitro

LNC and PleC were cultured in duplicate in 96-well plates at 3×10^5 cells/well in RPMI 1640 supplemented with 10% newborn calf serum, 2 mM L-glutamine (Sigma Aldrich), 100 U/ml penicillin and 100 μ g/ml streptomycin (Life Technologies-Gibco BRL, Cergy-Pontoise, France) and stimulated for 72 h with L3 and mixed sex adult filarial extract (female worms contained microfilariae) at 10 μ g/ml or Con A at 5 μ g/ml (Sigma) at

37°C in 5% CO₂-enriched atmosphere. DNA synthesis was assessed by incorporation of [³H]thymidine (Amersham, Orsay, France) at 1 μ Ci/well for another 16 h.

Cytokine ELISA of culture supernatants, pleural wash fluids and sera

Culture supernatants from LNC and PleC that had been stimulated for 72 h (diluted 1:3 for IL-4, IL-5 and IL-6 and 1:6 for IL-10 and IFN- γ), pleural wash fluid (not diluted) and sera (1:5 for IL-4 and IL-5, and 1:10 for IL-10 and IFN- γ) collected at 60 h (LNC only), or 10, 30, 40, 60 or 80 days p.i. from individual mice were assayed for cytokine content by ELISA in triplicate, as detailed previously [6]. Serum was separated from blood by centrifugation at 13,000 *g* for 4 min in Serasieve (Hughes and Hughes, UK), which rapidly separates serum based on a density gradient without the need for clotting. Results are expressed in pg/ml. Detection limits, determined as twice the mean value of blank wells (as recommended in the ICH Harmonized Tripartite Guideline, Validation of Analytical Procedures: Methodology, 1996), were set at 2 pg/ml for IL-4, 20 pg/ml for IL-5, 90 pg/ml for IL-6, 10 pg/ml for IL-10 and IFN- γ .

IgG1 and IgG2a ELISA

The amounts of IgG1 or IgG2a reactive to filarial adult extract were assessed in the sera of mice on D10, D30 and D40 p.i. The plates were coated overnight at 4°C with adult worm extract at a final concentration of 5 μ g/ml obtained as described previously [30]. After incubation with mouse sera (1:400), the plates were processed as described [6]. Results are expressed in optical densities. The detection limits were 0.007 and 0.016 ELISA units (OD) for IgG1 and IgG2a, respectively.

Blood leukocyte counts

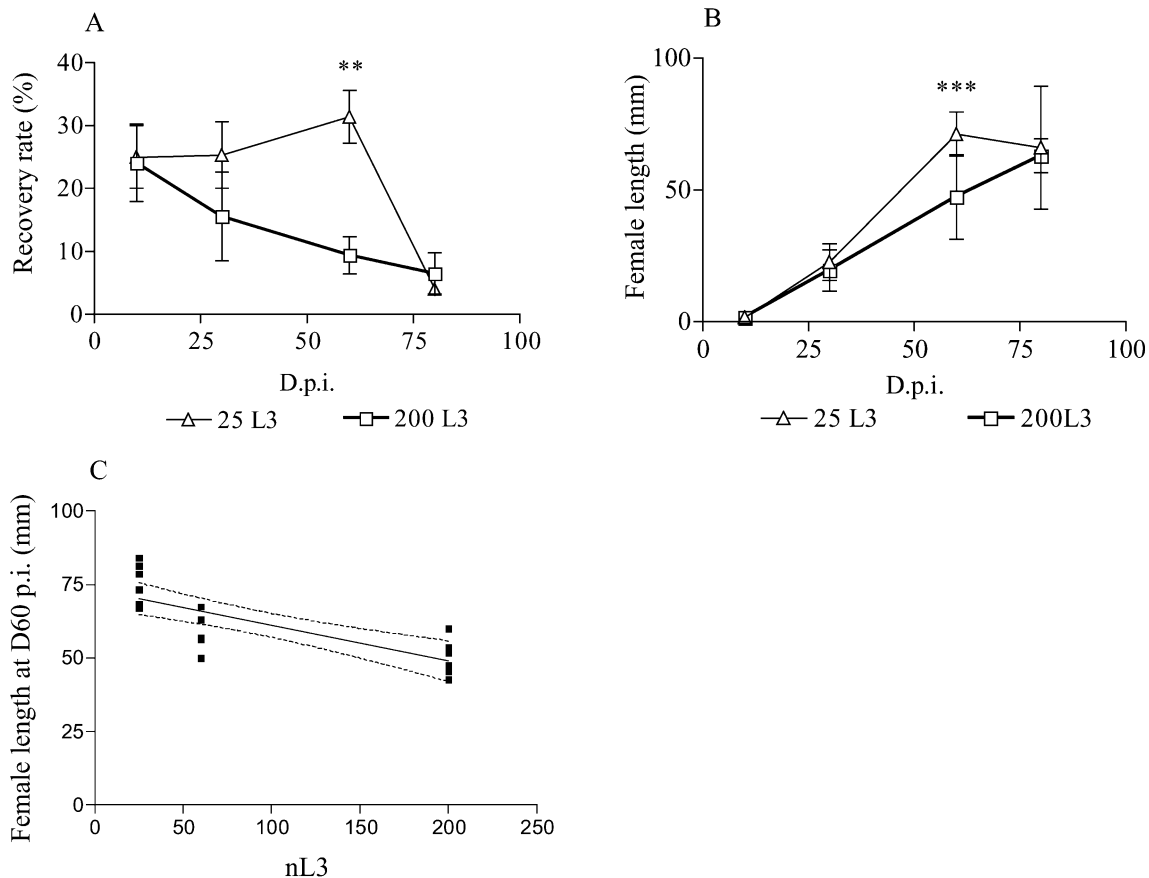
Blood leukocytes were counted before challenge inoculation (D0), on D10 and D30 p.i. for the three groups and on D60 p.i. for the groups inoculated, respectively, with 25 or 200 L3. Smears of tail blood were stained with May-Grünwald-Giemsa and the percentages of different leukocyte populations were determined for 300 cells. Total leukocytes were enumerated with a Malassez counting chamber (volume 1 μ l), in a 5% (v/v) peripheral blood in 1% acetic acid solution; they are expressed in number per 1 ml blood.

Statistical analysis

Non-parametric Kruskal-Wallis H-test was used to assess non-normally distributed parameters to compare

filial worm recovery rates, percentages of fourth stage larvae, worm sizes, cytokine and immunoglobulin levels between groups. Two-way ANOVA with Bonferroni adjustment were performed to assess the influence of dose and time p.i. on worm recovery rates, PleC accumulation, and cytokine and immunoglobulin levels. Additionally, when only two groups were compared, the non-parametric Mann-Whitney U-test was used in all cases except for comparing filarial worm lengths, for which Student's *t*-test for unpaired groups was used. *P* of less than 0.05 was considered to be a significant difference.

Fig. 1 **A** Kinetics of *Litomosoides sigmodontis* recovery rates from BALB/c mice injected with 25 (open triangles) or 200 (open squares) infective larvae. Error bars represent SEM, $**P=0.005$ (Mann-Whitney's U test). At D10 p.i. $n=4$, D30 p.i. $n=7$, D60 p.i. $n=6$ and D80 p.i. $n=5$. **B** Growth of *L. sigmodontis* over 80 days p.i. Data shown are mean female filarial worm lengths \pm SD in mm of filaria recovered at D10, D30, D60 and D80 p.i. in BALB/c mice. $***P<0.001$ (Student's *t*-test). There were too few worms left at D80 p.i. in 25 L3 group to compare the sizes at that time point. **C** Female *L. sigmodontis* lengths at D60 p.i. in mice inoculated with doses of 25, 60 or 200 L3. Black squares represent the mean of all female filarial lengths per mouse ($n=6$); the line shows the linear regression between nL3 and Lf, $y = -0.12 \pm 0.025 (x) + 73.24 \pm 3.006$ ($R^2=0.60$), with 95% confidence intervals as dotted lines (L3 third stage larvae, D.p.i. days post inoculation)



Results

Worm recovery rates are similar at early time points but decrease progressively in the 200 L3 group compared to the 25 L3 group

Doses of 200 or 25 *L. sigmodontis* infective larvae were inoculated into BALB/c mice to study the effect of inoculation dose size on filarial establishment. Another group of mice was also inoculated with 60 L3. All data from that group were intermediate between the two others and thus unless specified, only the 200 and 25 L3 group data are shown. When we examined worm recovery over the course of infection, we found that at each time point, the recovered filariae were far more numerous in the 200 L3 group than in the 25 L3 group. When assessed in terms of percentages, the recovery rate at D10 p.i. was similar in both groups, about 30% (Fig. 1A). However, the worm recovery in the 200 L3 group decreased progressively, as assessed at D30 p.i. ($15.6 \pm 7.1\%$), and reached $9.4 \pm 2.9\%$ at D60 p.i., whereas it remained around 30% in the 25 L3 group ($P=0.006$ at D60 p.i., Mann-Whitney U-test). Consistent with the rate at which the recovery rate decreased over the infection, granulomas were frequent and abundant only in the 200 L3 group: 8.7 and 5.5, on D30 and D60 p.i. respectively, instead of 1.7 and 0.9 in the 25

L3 group. Live coated filariae were rare in both groups (≤ 1).

By D80 p.i., the recovery rates had fallen in the 25 L3 group and was as low as in the 200 L3 group (3% and 5%, respectively).

Large inoculation doses induce late filarial growth retardation

We analyzed the filarial development stage and worm length of almost a thousand worms recovered from D10 to D80 p.i. During the first month, sizes and stages did not differ between the 200 L3 and 25 L3 groups (Fig. 1B), although a tendency toward retardation of growth was seen at D30 (statistically significant in male worms because of the lower dispersion of the data). At D60 p.i., the retardation of growth became more evident in the 200 L3 group: females were 47.3 ± 2.2 mm as compared to 71.1 ± 1.6 mm in the 25 L3 group ($P < 0.0001$, unpaired Student's *t*-test). At D80 p.i., female worms from 200 L3 group had continued to grow to 62.9 ± 0.9 mm (Fig. 1B) and seemed to have caught up with the 25 L3 group, although too few had survived to perform an accurate statistical test. However, we compared the mean length of worms in the 200 L3 group at D80 p.i. with that of worms at D60 p.i. from the 25 L3 group: in the 200 L3 group, the worms had not compensated for their growth retardation at D80 p.i. (respectively 62.95 ± 0.9 mm and 71.15 ± 1.6 mm, $P < 0.0001$).

At D60 p.i., the 60 L3 group measurements were intermediate, 58.4 ± 2.1 mm in females. The relation between female worm length (*y*) and infective dose (*x*) at that time point, assessed as a linear regression was $y = -0.12 \pm 0.025 (x) + 73.24 \pm 3.006 (R^2 = 0.60)$, with 95% confidence interval (CI) slopes ranging from -0.17 to -0.069 and intercepting the Y axis between 67 and 80 (Fig. 1C).

Development of reproductive organs is impaired in female and male worms from mice inoculated with high larval doses

The sex ratio was stable but the genital development of adult worms and female fecundity were altered with higher larval doses. At D60 p.i. two thirds of the female worms from the 25 L3 group contained microfilariae but less than 20% in the 200 L3 group. The density of microfilariae in the uteri of the females was also lower in this group (Table 1). The 60 L3 group was intermediate with 37% of the females with microfilariae and a uterine microfilarial index of 0.9.

A striking finding was that, while males of the 25 L3 group had normal right and left spicules (Fig. 2A), among the males of the 200 L3 group, 84% had an abnormal (Fig. 2B), or even absent, left spicule (6 of the 7 randomly chosen males at D30 p.i., and 10 of the 12 males recovered at D80 p.i.).

By D80 p.i., no female filariae had any microfilariae left in the 25 L3 and 200 L3 groups; ovulae, eggs, and male gametes were not found in either group. No spermatozoa were identified in males, except in one specimen from the 200 L3 group.

Consistent with development alteration induced by high larval doses, patency is delayed and reduced

At D60 p.i., the mice inoculated with 25 L3 had microfilariae in the peripheral blood, with the exception of one mouse which had worms of a single sex (Table 1). The patent mice had microfilariae in the cardiac blood, in higher densities, and in the pleural cavity. In contrast, in the 200 L3 group only one mouse had peripheral and cardiac blood microfilariae and none had pleural microfilariae (Table 1).

At D80 p.i., 2 out of 6 mice in each group were microfilaraemic, but with lower densities of microfilariae in the peripheral and cardiac blood than at D60 p.i. in

Table 1 Microfilarial density in relation to adult worm numbers and morphology in BALB/c mice inoculated with 25 L3 or 200 L3 on D60 p.i. and D80 p.i. Results are expressed in means, with extreme values in brackets. Uterine microfilariae density is given on a scale from: 0, no uterine microfilariae; to 5, uterine densely filled with microfilariae (*D* days, *p.i.* post inoculation, *L3* third stage larvae, % *mice Mf* percentage of microfilaraemic mice,

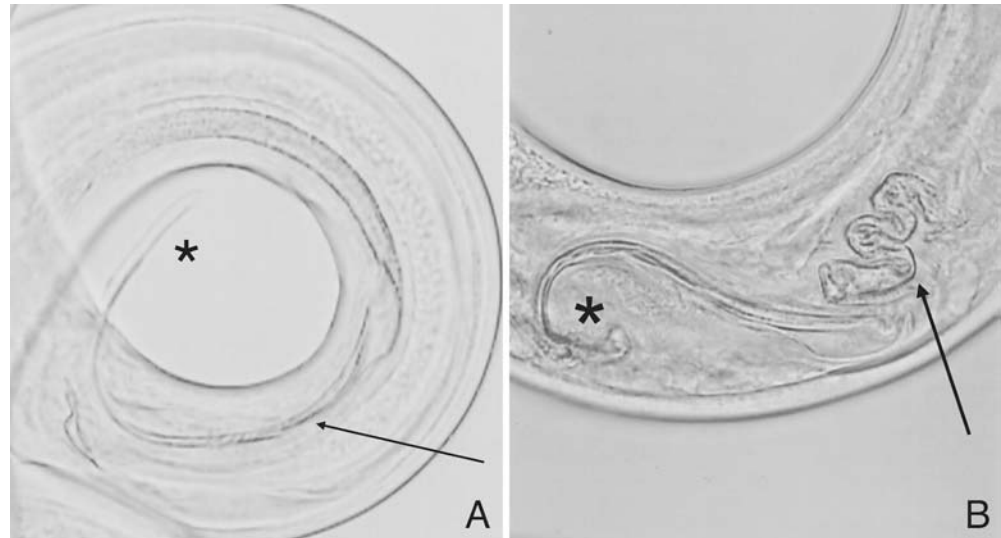
Periph. Mf number of microfilariae/10 μ l peripheral blood, *Cardiac Mf* number of microfilariae/10 μ l cardiac blood, *Pleural Mf* number of microfilariae in pleural cavity fluid, *nF* number of filariae recovered, *Lf* mean length of female filariae, %*f. Mf* percentage of female filariae with uterine microfilariae, *Uterine Mf* mean density of uterine microfilariae, % *abn spicule* percentage of males with an abnormal left spicule)

Dose/D p.i.	% mice Mf	Periph. Mf	Cardiac Mf	Pleural Mf	nF	Lf	%f. Mf	Uterine Mf	% abn spicule
25 L3/D60	86 (6/7 mice)	6.3 (0–26.5)	42.8 (0–105)	1790 (0–7,600)	8 (5–12)	71.1	64	2.5 (0–4)	0
25 L3/D80	33 (2/6 mice)	4.3 (0–14)	27.3 (0–58)	0	1 (0–2)	66	0	0	0
200 L3/D60	17 (1/6 mice)	0.25* (0–1.5)	24.3 (0–146)	0	19.7 (4–46)	47.3***	16	0.5 (0–2)	85.7
200 L3/D80	33 (2/6 mice)	1.3 (0–6)	30 (0–86)	0	10.7 (0–37)	62.9	0	0	83

*Statistical difference between 25 L3/D60 and 200 L3/D60, $P < 0.05$, Mann-Whitney's U test

***Statistical difference between 25 L3/D60 and 200 L3/D60, $P < 0.0001$, Student's *t*-test

Fig. 2 Posterior region of adult male *L. sigmodontis*. **A** Normal left spicule (arrow), with protruded blade (asterisk). **B** Abnormal left spicule with crumpled handle (arrow) and coiled blade (asterisk). Scale: 1 cm = 23 μ m (\times 40)



the 25 L3 group. Mice of neither groups had pleural microfilariae at this time point.

In the intermediate 60 L3 group, no peripheral nor cardiac microfilariae were detected, but one mouse had 1,400 pleural microfilariae at D60 p.i. This is at odds with observations in a preliminary study where this group harbored blood microfilariae (2.2 ± 1.2 microfilariae/10 μ l, [28]). No observation was made at D80 p.i. for this group.

Early after inoculation, LNC from mice infected with high larval dose exhibit dramatically higher responses to Con A and a striking Th2 bias

Since the infective larvae migrate through the lymphatic system the early local immune response was measured in the lymph nodes that drain the site of inoculation. In the presence of the mitogen Con A, T cell proliferation was

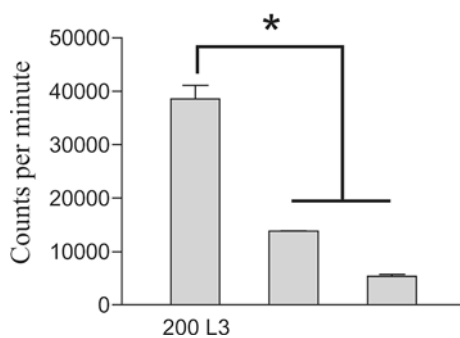


Fig. 3 LNC proliferation after Con A stimulation at 60 h p.i. LNC from mice infected with 200 or 25 infective larvae, and from naive mice were stimulated with 5 μ g/ml Con A for 72 h. DNA synthesis was assessed by [3 H]thymidine incorporation over 16 h, and is shown as counts per minutes. Error bars indicate SEM, * $P < 0.05$ (Mann-Whitney's U test) (LNC lymph node cell)

strongly enhanced in mice that received 200 L3 as compared to 25 L3 ($P < 0.05$, Fig. 3) and Th2 cytokine production showed a strong tendency to be increased: 14-fold more IL-4, 7-fold more IL-5, twice as much IL-10. In contrast, half the amount of IFN- γ was produced, whereas IL-6 was produced in similar quantities (Table 2). The response was grossly similar in the inguinal and iliac + lumbar lymph nodes (data not shown).

Only IL-5 production was detectable in LNC in response to L3 extracts in the 25 L3 and 200 L3 groups. Lymphocytes from inguinal or iliac + lumbar lymph nodes at 60 h p.i. did not produce detectable amounts of IL-4, IL-6 or IFN- γ in response to L3 extracts, whereas IL-5 was detected in the iliac + lumbar lymphocytes (Table 2). In response to adult worm extracts, IL-5 was produced in the 200 L3 group at a 5-fold higher level than in the 25 L3 group, whereas the other cytokines were produced sporadically and similarly in both groups (data not shown).

Table 2 In vitro cytokine production from LNC collected 60 h p.i. from BALB/c mice inoculated with 25 or 200 L3. LNC were stimulated with L3 extracts, or Con A at 3×10^5 cells/well for 72 h in 96-well culture plates. Results are in pg/ml, and represent the cytokine concentrations minus values from 'medium-only'-stimulated cells (hence, some values at 0 pg/ml). Results are from the inguinal LNC, except for IL-5, Iliac + lumbar LNC (LNC lymph node cells)

Cytokine	Dose	L3	Con A
IL-4	200 L3	0	4,578
	25 L3	0	334
IL-5	200 L3	65.4	1,832
	25 L3	40.6	784
IL-6	200 L3	0	1,652
	25 L3	0	1,597
IL-10	200L3	0	10,978
	25L3	0	5,466
IFN- γ	200L3	0	4,936
	25L3	0	8,241

Blood leukocyte numbers evolve similarly in the mouse groups, whereas the pleural leukocyte populations increased earlier and steeper in mice inoculated with 200 L3

To assess how the inoculation dose and immune responses seen in the lymph nodes would be reflected in leukocyte population dynamics, we studied the dynamics of different leukocyte subsets (T, B, polynuclear cells and macrophages) in the blood and pleural compartments. Blood leukocyte numbers evolved similarly in the mouse groups, except for a slightly earlier increase of neutrophils with the highest larval dose. In the 200 and 25 L3 groups the total leukocyte numbers had increased twofold by D30 p.i. then remained at the same level the second month of infection (D80 p.i. not studied). Eosinophil numbers peaked at D10 p.i., and neutrophils increased regularly during infection but twice as fast in the 200 L3 group.

The local inflammatory response was studied in the pleural cavity, in which the filariae settle. The number of PleC was threefold higher in mice that received 200 L3 ($P < 0.05$) at D10 p.i., tended to be higher at D30 p.i., and was still significantly higher at D80 p.i. ($P < 0.05$, Fig. 4A). We then further characterized the cell populations present in the pleural cavity. At D10 p.i., a sharp increase of the numbers of macrophages, T cells, B cells and eosinophils was observed in the mice that received 200 L3, whereas this increase occurred 20 days later and was weaker in the 25 L3 group (Fig. 4B–G). At D80 p.i., the number of macrophages, CD8⁺ T cells, B cells and eosinophils decreased in all groups of mice, whereas the CD4⁺ T cell population remained high in the 200 L3 group. Interestingly, at D80 p.i., a dramatic increase in neutrophils occurred only in the 200 L3 group (Fig. 4H).

IgG1, IL-4 and IL-10 but not IL-5 concentrations in the serum increase with the number of larvae inoculated

We studied the *L. sigmodontis*-specific antibody production over 40 days p.i. and found similar concentrations of specific IgG1 at D10 p.i. between 25, 60 and 200 L3 groups. At 1 month p.i. the production of specific IgG1 had increased vastly in the 200 L3 group as compared to the 25 L3 group ($P < 0.0001$), and was intermediate in the 60 L3 group ($P < 0.001$), but then its concentration in all groups reached similar levels by D40 p.i. (Fig. 5A). In contrast, the production of specific IgG2a, whose switch is driven by IFN- γ , showed no clear difference of production between the groups at any time point, although it had a tendency to be produced more in the 200 L3 group (Fig. 5B).

We also examined the serum cytokine concentrations and found that IL-4 was detectable at D30 p.i. in the 200 L3 group, and then increased, but was still very low at D40 p.i. in the 25 L3 group. The IL-10 serum concentration was first detectable in the 200 L3 group at D40

p.i.; by D60, IL-10 was found in both groups, with a concentration proportional to the number of inoculated larvae (Fig. 6). The IL-5 serum concentration increased regularly and similarly during the first 2 months in both groups. The concentration of IFN- γ , a cytokine characteristic of Th1 profiles, was low in the serum during the first month, although slightly higher in the 200 L3 group, and it increased strongly and similarly the second month in all mouse groups (Fig. 6).

Discussion

To determine the effect of the number of infective larvae on the course of filarial infections, we inoculated BALB/c mice with rising quantities of *L. sigmodontis* L3. Microfilaraemia and its prevalence were not increased when increasing doses of infective larvae were inoculated (Table 1), confirming a preliminary study [28]. In fact, at D60 p.i., these parameters were negatively associated with the number of inoculated larvae. If we except the rare case of a single-sex infection, all mice became microfilaraemic with 25 L3, as previously stated [24, 29, 37], but only 17% did in the 200 L3 group. These differences in microfilaraemia were abolished later as the infection diminished in the 25 L3 group and slightly progressed in the 200 L3 group (Table 1). However, microfilaraemia in the latter group remained low and the poor condition of their worms indicates that a late production of microfilariae was unlikely. Indeed, although the mean length of the surviving female worms increased between D60 and D80 p.i. (Fig. 1B), their tissues were altered, their uteri were empty and their ejaculatory duct rarely contained spermatozoa. Moreover, 85% of the males had an abnormal left spicule, and no microfilariae were present in the pleural cavity.

Amicrofilaraemia in a susceptible host can have several causes, among which the destruction of circulating microfilariae has been mostly documented [51, 52]; specific antibodies targeted against microfilariae play an important role in this suppression [17, 18, 23, 51]. More rarely, amicrofilaraemia may be due to the absence of microfilarial output by fecund female worms, as after ivermectin treatment, which is thought to momentarily block the laying of microfilariae [42, 44]. Here, as it has been observed in CBA/Ca mice inoculated with 25 *L. sigmodontis* L3 [37], the absence of blood microfilariae is associated with the very low production of microfilariae by the adult female worms. At D60 p.i., no microfilariae were found in the pleural cavity of the 200 L3 group, in contrast to the 25 L3 group (Table 1). At this time point the adult filariae are twice as abundant in the 200 L3 group, but each female produces fewer microfilariae (microfilaria uterine index 0.5 instead of 2.5 in the 25 L3 group). This reduced fecundity may be linked to two parasitological factors: (1) the small size of the females: they do not exceed a mean 50 mm at D60 p.i., and we have not previously observed any female with microfilariae below 55 mm (results from wild-type and transgenic

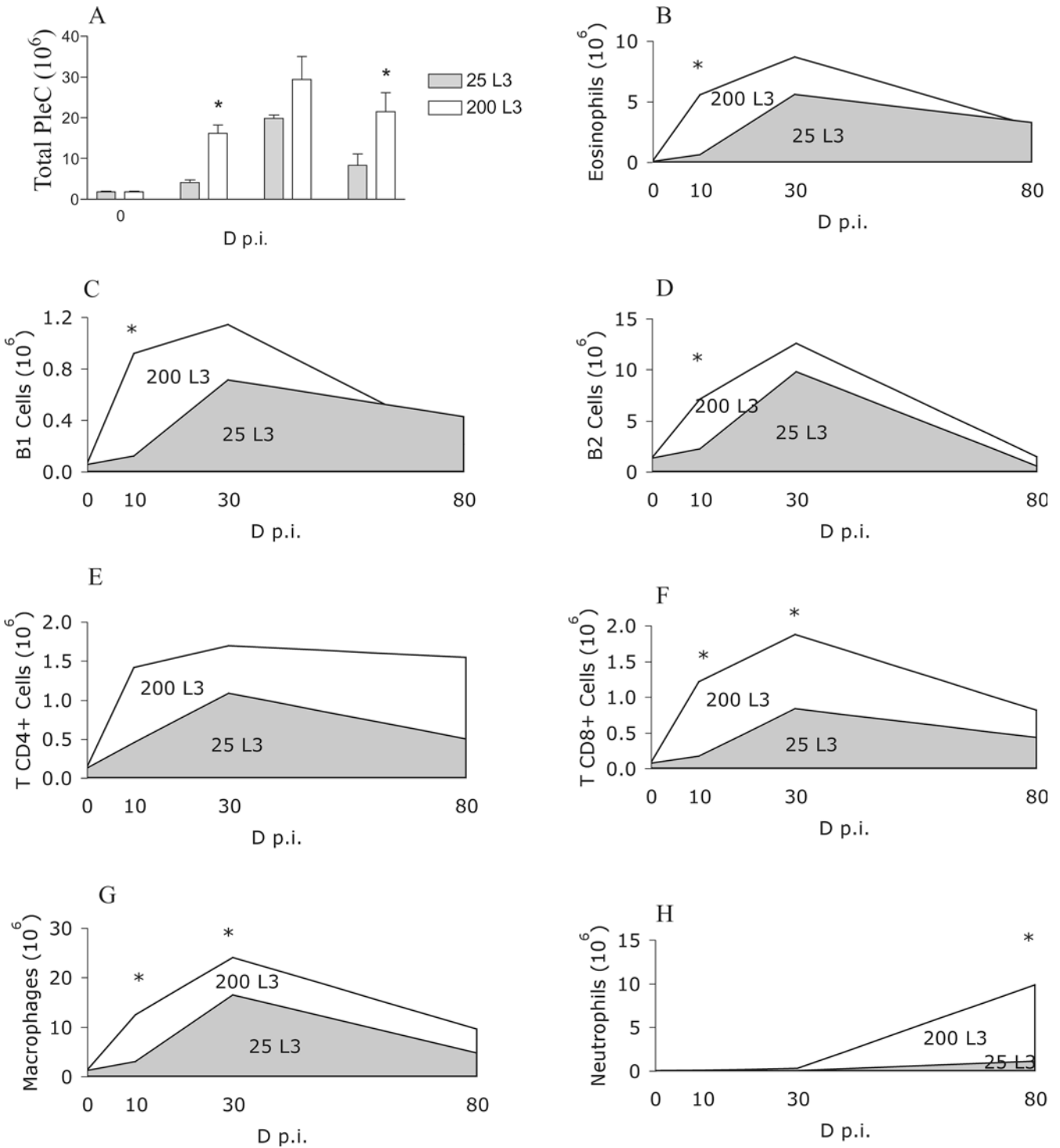


Fig. 4 Kinetics of pleural leukocyte accumulation during the course of infection. Cell numbers were assessed before infection (D0), at D10, D30 and D80 p.i. in BALB/c mice inoculated with 25 (gray area) or 200 L3 (white area). **A** Total PleC numbers; **B** polynuclear eosinophils, determined on cytopins; **C** B1 (CD19⁺ CD5⁺) cells; **D** B2 (CD19⁺ CD5⁻) cells. **E** CD4⁺ T cells; **F** CD8⁺ T cells; **G** macrophages (F4/80⁺); and **H** polynuclear neutrophils, determined on cytopins. At D0, $n=6$; at D10 p.i. $n=4$; at D30 p.i. $n=4$ and at D80 p.i. $n=6$. * $P < 0.05$ (Mann-Whitney's U test) (PleC pleural exudate cell)

BALB/c) [46, 47]; and (2) the left spicule malformation in the males (Fig. 2), which is likely to impede subsequent mating.

Our study shows that the immune response in mice of the 200 L3 group differed from that of the 25 L3 group over the course of infection. As early as 60 h p.i., nonspecific T cell proliferation increased in the lymph nodes of the first group (Fig. 3) with a marked

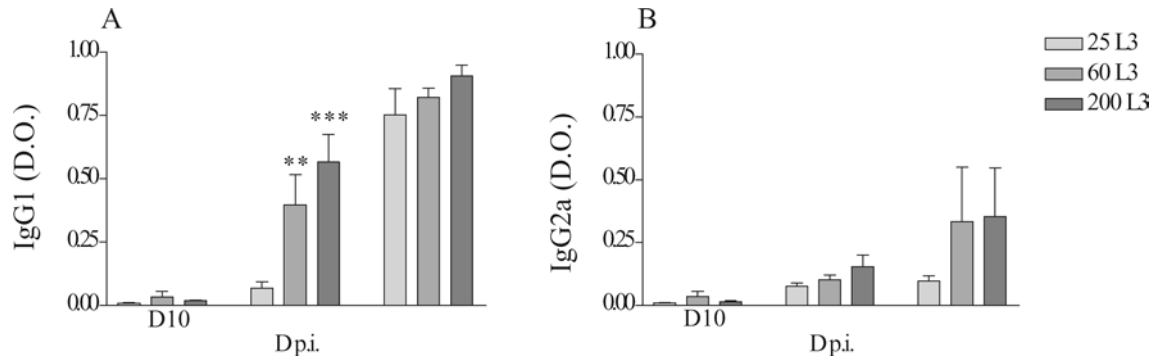


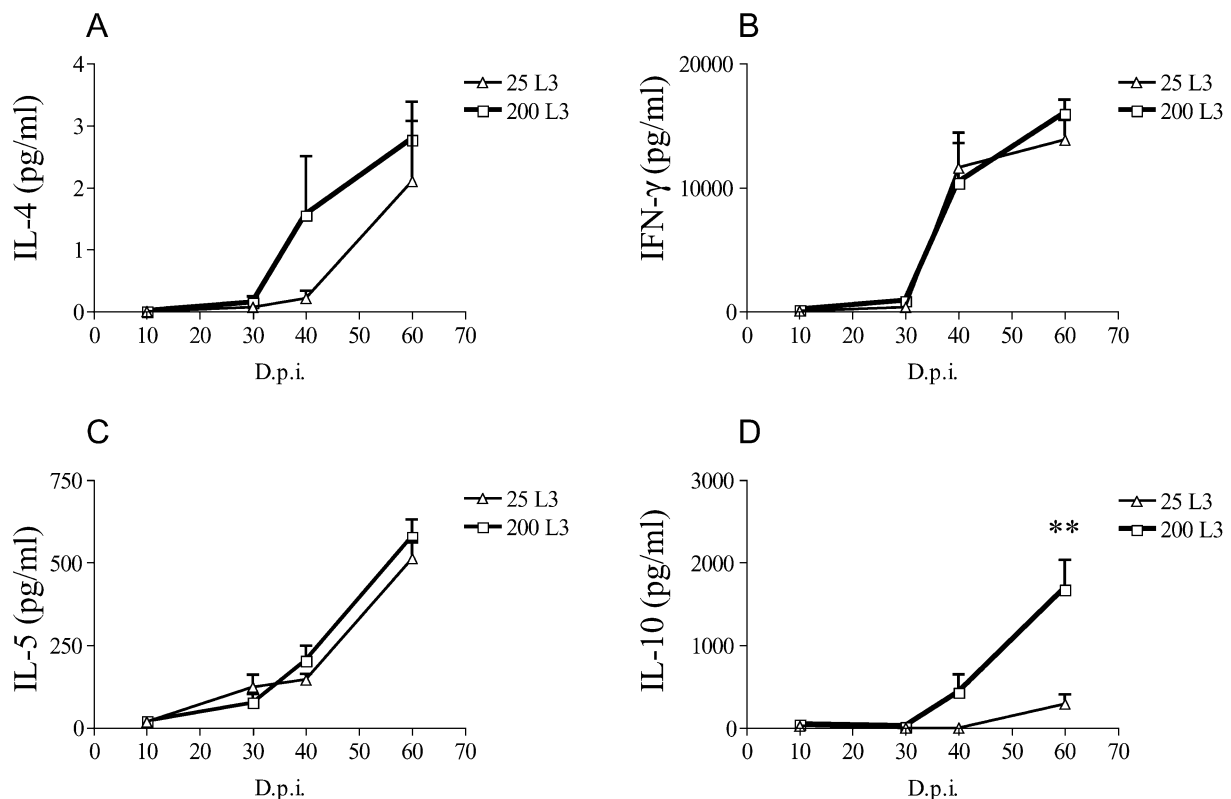
Fig. 5 Specific antibody isotype levels. Levels of IgG1 (A), and IgG2a (B) at D10, D30 and D40 p.i. in mice inoculated with 25, 60 or 200 L3 are expressed in arbitrary units of optical density \pm SD. ** $P < 0.001$, *** $P < 0.0001$ (Bonferroni post-test on two-way ANOVA), as compared to the 25 L3 group of the same time point

tendency to produce more Th2-type cytokines, mainly IL-4 and IL-5 (Table 2). IL-5 is suspected to favor the larval growth of *L. sigmodontis* [6, 31, 32] and indeed, growth was not impaired during the first month in heavily infected mice compared to 25 L3 group (Fig. 1B). However, IL-4 and IL-5 were also shown to

have protective roles in *L. sigmodontis* infections [25, 26, 31, 32, 46, 47]. These increased cytokine levels are likely to be responsible for the more rapid expansion of T cell, B cell, macrophage and eosinophil populations in the pleural cavity (Fig. 4). Interestingly, neutrophils increased much later and predominantly in the 200 L3 group (Fig. 4H). Neutrophils, previously implicated in worm destruction [4, 39], have repeatedly been seen to arrive late in the pleural cavity of mice infected by *L. sigmodontis* [4, 6, 33].

Both parasitological and immunological features differed following low and high doses of infective larvae. However, the negative effects on parasite development were seen later than the increase of the immune response. Non-immune mechanisms of crowding effect might also explain the impaired development, such as competition for food or space, or, as shown with the cestode *Hymenolepis diminuta* [11], toxic effects of worm excretory products. A direct answer to this question

Fig. 6 Kinetics of cytokine concentrations in the serum of BALB/c mice inoculated with 25 (triangles) or 200 (squares) L3 at D10, D30, D40 and D60 p.i. A IL-4 (only the dynamics of IL-4 concentration should be taken into account as all values are below our detection limit), B IFN- γ , C IL-5, D IL-10 concentrations are expressed in pg/ml; $n=7$ in the 25 L3 group, $n=6$ in the 200 L3 groups; error bars represent SEM; ** $P < 0.01$ (Mann-Whitney's U test)



would be given by comparing the development in non-immunocompetent hosts. Paterson et al. [36] have shown that density-dependant effects that affect the intestine-dwelling nematode *Strongyloides ratti* are likely to be due to the host's immune response. By comparing the infection of immunocompromised nude rats with that of wild-type rats, they showed that the proportion of parasites that survived and developed and the worm fecundity were only identical, whatever the infective dose, in nude rats [36]. Although similar work needs to be done with *L. sigmodontis*, our results together with several published and unpublished data (detailed below) drive us to favor the hypothesis of an immunological control in this system as well.

Non-murine *L. sigmodontis* systems show either a negative relation between microfilaraemia and worm burden, as in the white rat [12], or increased microfilaraemia, as in *Sigmodon hispidus* [19] or *Meriones unguiculatus* (our unpublished analyses) in which female *L. sigmodontis* fecundity is not suppressed by high larval doses. However, we noted an early and transient negative dose effect evidenced by delayed worm maturation, as in mice.

In BALB/c mice, Taubert and Zahner [45] have recently used high infective doses of *L. sigmodontis* and their results contrast with ours. Their protocol also differs from ours: 5-day-old third stage larvae recovered from the murid *Mastomys coucha* were injected subcutaneously in the neck of 4- to 5-week-old BALB/c mice. When inoculated with 160 larvae, they detected microfilariae in 100% of the mice with a mean of 30 microfilariae/10 μ l and 90 adult worms (Table 3). Therefore, the high number of inoculated larvae per se is not the cause of worm development alteration and lower fecundity. The dose-dependent regulation of infection may not have occurred in this reported experiment because the protocol did not allow the early establishment of an immune response capable of inhibiting the filarial development. Two factors may be responsible: (1) the larvae inoculated were already developing third stage larvae, or (2) the mice used were young and, therefore, may not have had a fully mature immune system at the onset of infection, allowing the filarial worms to develop more successfully.

L. sigmodontis infection in BALB/c mice following the natural inoculation of infective larvae by blood feeding mites [40, 47] also seems to differ from our data using direct inoculation of L3 at a single site. We thus performed an experiment with the mite feeding protocol and analyzed the mean female length, which was found to be 55.3 mm, with a mean number of worms per mouse of 77 (Table 3). The linear regression between worm length and number of worms inoculated (see Results), although of limited power, provided a preliminary assessment for comparing natural infections with syringe infections. The calculated number of infective larvae with the mean female length observed in the natural infection would be 150 L3 (95% CI is between 85 and 215 L3, Table 3). The corresponding recovery rate would be of 51.4% (95% CI between 35.9% and 90.7%, Table 3). These discrepancies with the recovery rates observed after syringe inoculations are likely to be related to early events associated with mite feeding, for example mite saliva, which like other pathogen-transmitting arthropod saliva [15, 34] has known immunomodulatory properties, such as reduced T cell proliferation under Con A [41]. More successful infections by natural infections may also result from a modified response caused by mouse stress, or from the many small inoculations at multiple sites as opposed to a single site dose. However, more intermediate doses would be needed if we were to refine the dose predictions through filarial size alone.

The different results obtained in the *L. sigmodontis*-BALB/c model when different protocols of inoculation were used suggest that the early immune response impacts on the subsequent filarial development. The destruction of worms is a progressive event and the delay between the immune response and its effects on the parasite is to be expected. When parasitological data reach significant differences between mouse groups, they reveal that deleterious mechanisms had long before been at work. For example, the malformations of the left spicule in adult males are the result of alteration of the spicular pouch development during the L4 stage. Unraveling the relationship between dose, inoculation protocol and filarial infection therefore has important

Table 3 Infection of BALB/c mice according to the protocol of inoculation at 60 D p.i. (*nL3* number of larvae inoculated, *nF* number of filariae recovered, *F/L3* filarial recovery rate, *Lf* mean length of female worms \pm SD, % mice *Mf* percentage of microfilaremic mice, *Mf/10* μ l, number of microfilariae per 10 μ l of peripheral blood)

Inoculation protocol	nL3	nF	F/L3	Lf	% mice <i>Mf</i>	<i>Mf/10</i> μ l
L3 ^a	200	17	8.5	47.3 \pm 16.1	16.7	0
100 h - old L3 ^b	80	53	62	?	100	20
	160	90	53	?	100	30
Infected mites ^c	150 ^d	77	51.4 ^d	55.3 \pm 6.9	50	4

^aInfective larvae inoculated with a needle

^bThird stage larvae recovered 100 h p.i. from *Mastomys coucha* and inoculated subcutaneously in mice (data from [45], see Discussion)

^cInfective larvae naturally delivered to mice during the blood meal of infected mites

^dTheoretical values inferred from the data obtained with needle injections (see formulae in Results)

95% confidence intervals are 85–215 L3 and 35.9–90.7% for nL3 and F/L3, respectively

implications for our understanding of transmission and host specificity.

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