

Pleural cellular reaction to the filarial infection *Litomosoides sigmodontis* is determined by the moulting process, the worm alteration, and the host strain

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Received 20 July 2007; received in revised form 8 January 2008; accepted 13 January 2008

Available online 4 February 2008

Abstract

The filarial nematode *Litomosoides sigmodontis* model was used to decipher the complex *in vivo* relationships between filariae, granulomas and leukocytes in the host's pleural cavity. The study was performed from D5 p.i. to D47 p.i. in resistant C57BL/6 mice, to D74 p.i. in susceptible BALB/c mice, and to D420 p.i. in permissive jirds. We showed that, during the first month, leukocytes only clustered as granulomas around shed cuticles (exuviae) and with eosinophils as the major constituents. In addition, carbohydrates residues became abundant on exuviae only, suggesting a glycan-dependent mechanism of eosinophil attachment. Neutrophils were absent from the pleural cavity of all rodents and from the murine granulomas, but they made up 25% of the granuloma cell population in jirds. After the first month of infection granulomas formed around developed adult worms and morphological evidence suggested that leukocytes preferentially clustered around altered, but still motile, worms. No carbohydrates were detected on these worms and neutrophils were abundant in those granulomas. Finally, a rare third type of granuloma was observed in the resistant mice only; they contained young newly moulted adult worms; typically these granulomas were attached to the lateral lines of the worm via eosinophils; this feature correlated with the persistence of carbohydrate residues on the worms' lateral lines. Neutrophils were always in low proportion in all granulomas from resistant mice, suggesting difference in their adhesive properties in these mice. *In vitro* neutrophil recruitment in resistant mice was similar to that observed in susceptible mice although they expressed less cell surface CD11b.

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Keywords: Filariae; Exuviation; Worm alteration; Granulomas; Carbohydrates; Wolbachia

1. Introduction

Litomosoides sigmodontis infections of rodents are a well-established model of filariasis [1,2]. The jirds *Meriones unguiculatus* and BALB/c mice are both susceptible to *L. sigmodontis* infections and allow complete parasite devel-

opment and reproduction, whereas resistant C57BL/6 mice are resistant to the full development as demonstrated by the absence of a patent phase [3].

Regardless of the host, *L. sigmodontis* infective larvae go through a phase of lymphatic migration and settle in the pleural cavity as early as day 4 p.i. [3]. The larvae moult a first time 8–10 days p.i. and moult again to the adult stage one month p.i.±1 week. Concomitantly, a cellular reaction is mounted against the worms and gradually eliminates them. Previous work on *L. sigmodontis* granulomas and their formation had revealed contrasting results: in albino rats or CBA/Ca mice overexpressing

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interleukin-5 the granulomas were shown to be composed of eosinophils and macrophages [4,5], whereas in late infection of BALB/c mice they were mainly composed of neutrophils [6,7].

In this study we describe three different types of granulomas, two of which hitherto undescribed, whose occurrence depends on the filarial stage or the host strain. The aim of the study was to investigate these three types of granulomas, to determine their cellular composition and their relation to the moulting process, the extent of associated worm alterations and the presence of cuticle surface carbohydrates. Examinations of filariae were performed from the third larval stage to the late adult stage in resistant mice, susceptible mice, and jirds.

Three main questions were addressed: (i) The identification of leukocytes in granulomas observed for the different larval stages and hosts. Special attention was given to granulocytes since *Wolbachia*, an endosymbiotic bacteria present in *L. sigmodontis* [2,8] has been shown to have a role in neutrophil recruitment [9–11], filarial biology [12,13] and pathology [14,15] through their LPS-like components [15,16]. (ii) The cell–worm interface. Specific patterns of cell–cell recognition and adhesion in granulomas were analysed focusing on glyco-conjugates which are important components expressed on the surface of nematodes [17,18], because they account for much of the anti-inflammatory and Th2-biasing observed [17,19–22]. (iii) The genesis of the granulomas. It is unclear why one can be observed in the same immunological environment free worms and worms associated to granulomas. We focused on the role of moulting and release of exuvium [23,24] and worm alteration on granuloma formation.

2. Materials and methods

2.1. Hosts and infection protocols

The *L. sigmodontis* filariae were maintained in our laboratory, and infective third-stage larvae (L3) were recovered by dissection of the mite vector *Ornithonyssus bacoti* as previously described [1,3,25].

Six- to seven-week-old female C57BL/6 and BALB/c mice were purchased from Charles River (France). Female and male *M. unguiculatus* were bred in the MNHN animal facilities. Forty or 60 infective L3 in 200 µl of RPMI 1640 were inoculated subcutaneously into the right lumbar area of mice or jirds. All experiments and procedures conformed to the French Ministry of Agriculture regulations for animal experimentation (1987).

2.2. Necropsy and worm, granuloma and pleural leukocyte recovery

Times of necropsies were chosen according to the third and fourth moult periods and adult worm survival. They differed depending on hosts.

The rodents were anesthetized, bled and sacrificed. For necropsies at D8–11 post infection, the pleural cavity was washed with 10 ml of cold phosphate buffered saline (PBS), as previously described [3,26,27]. The infiltrating cells as well as the

granulomas were collected from the pleural wash for further analysis.

2.3. Study of the worms and granulomas

The following parasite features were analysed by light microscopy on materials maintained in cold PBS: i) stage — L3; moult 3; L4; moult 4; adults; ii) gender; iii) presence/absence of attached leukocytes; iv) normal or altered state of the cuticle, hypodermis and muscles, ovaries and genital tracts, the intra-uterine embryogenesis, presence of spermatozoa and the presence or absence of the spicules and their shape [3,28]. Then, for further morphological and histological analysis, worms were fixed *in toto* with heated 70% ethanol or cold 4% paraformaldehyde in PBS (PFA), to avoid any body shrinkage. Granulomas (G) were measured and dissected; they were classified according to their contents, worm or exuviae. In early granulomas, exuviae or worms were easily separated from the host cell coat with a brush. Granulomas were fixed in 4% PFA or 4% glutaraldehyde in 0.1 M Millonig's buffer [29].

2.4. Characterization of leukocytes from pleural exudates and granulomas

Pleural exudates cells (PleCs) were centrifuged at 600 g for 6 min at 4 °C, resuspended in 200 µl of foetal calf serum (FCS) and then, two smears were prepared per rodent. Granulomas were spread on SuperFrost Plus slides by moving them slowly with thin needles, allowing leukocytes to adhere as well as the exuviae and worms. Slides were dried in an incubator at 60 °C for 12 h and then, stained with May-Gründwald-Giemsa. For PleCs, proportions of the different leukocyte populations were determined by light microscopy by counting 200 leukocytes in consecutive fields. For granulomas, 200 leukocytes were counted by taking in account the heterogeneity of each preparation. It should be noted that 2% to 10% of cells were not identifiable because they were either too dense or apoptotic/dead.

2.5. Immunohistochemistry

Samples fixed in 4% PFA were embedded in paraffin and 5-µm-thick sections were prepared. Some sections were stained with haematoxylin–eosin for histological analysis. For immunohistochemistry, sections were placed on SuperFrost Plus slides, then pre-treated in a microwave oven in 10 mM, pH 6 citrate buffer for three 5 min cycles at 350 W and stained according to the following methods.

To detect *Wolbachia*, sections were incubated with a rabbit polyclonal antibody against *Wolbachia* surface protein (WSP) following the previously described biotin–streptavidin–HRP method [30,31]. Briefly, sections were incubated for 1 h with anti-mouse CD16/CD32 (5 µg/ml, Fc block, BD Pharmingen) diluted in PBS — 0.1% BSA — 0.1% Saponin. Endogenous peroxidases were blocked with 3% H₂O₂ followed by blockade with 10% BSA. Sections were incubated for 1 h with anti-WSP (1:2000) diluted in PBS — 0.1% BSA — 0.1% Saponin. Slides were treated with a biotinylated goat anti-rabbit IgG (10 min),

washed and incubated with a streptavidin–HRP complex (10 min). Reactivity was revealed with the red chromogen 3–3-aminoethylcarbazine (AEC, LSAB2 kit, DAKO, France) and sections were counterstained with Mayer's haematoxylin (Reactifs RAL, France). As a positive control for *Wolbachia*, sections were stained with a polyclonal antibody against bacterial hsp60 (*Escherichia coli* GroEL, Sigma, France) diluted at 1:50. Negative controls were carried out by omitting the primary antibody.

To detect neutrophils or eosinophils, sections were incubated with a rat anti-neutrophil mAb NIMP-R14 (Hbt) or a rat polyclonal antibody anti-murine Major Basic Protein (MBP, from S.S., Bonn, Germany). Briefly, sections were incubated for 1 h with anti-mouse CD16/CD32 (5 µg/ml, Fc block, BD Pharmingen) diluted in buffer (0.05 M tris buffered saline (TBS) — 1% BSA — 0.1% saponin). Slides were incubated for 2 h with the rat anti-NIMP-R14 mAb (1:10) or with the rat anti-MBP polyAb (1:1000) diluted in buffer then for 1 h with a biotinylated goat anti-rat Ig (BD Pharmingen; 1:50) and finally for 45 min with a streptavidin-AKP (BD Pharmingen; 1:100). The colour reaction was developed using Fast Red Substrate System containing naphthol phosphate and levamisole (kit DAKO, France). Slides were counterstained with Mayer's haematoxylin for 10 min. Negative controls were carried out by omitting the primary antibody.

Slides were mounted with Aquatex and analysed by light microscopy.

2.6. In vitro worm exuviations

After live observation of filariae under optical microscope, moulting 3 and moulting 4 filariae were isolated, washed 3 times in PBS then cultured overnight in RPMI 1640 Glutamax

(Gibco) supplemented with 10% FCS, 100 U penicillin/ml, 100 µg de streptomycin/ml (Life Technologies-Gibco BRL, Cergy-Pontoise, France), 37 °C, 5% CO₂.

2.7. Fluorescent lectin binding

Lectin binding was performed as previously described [19] to reveal surface carbohydrates on the following stages: L3 from mites, pleural L3, L4 and adults or exuviae. Briefly, filariae or exuviae were placed in round 12-well plate and were incubated for 30 min at 4 °C in 50 µg/ml Fluorescein isothiocyanate (FITC)-labelled Wheat Germ Agglutinin (WGA) or *Helix Pomatia* Agglutinin (HPA) diluted in PBS. After 3 washes in ice cold PBS, filariae or exuviae were mounted on slides with 10 µl of anti-fadant mounting media; slides were examined under a fluorescent microscope (Nikon Optiphot, Japan). WGA selectively recognises *N*-acetyl-glucosamine residues (GlcNAc) and sialic acid (NeuNAc) while HPA binds *N*-acetyl-galactosamine residues (GalNAc). As a control, larvae or exuvium were incubated simultaneously with lectins and 0.2 M of the appropriate inhibitory saccharides (WGA: GlcNAc; HPA: GalNAc). All reagents were purchased from Sigma-Aldrich, France.

2.8. CD11b expression on migrated neutrophils

Young adult worms (D27–D30 p.i.) from BALB/c or C57BL/6 mice were isolated, washed 3 times in sterile PBS then put in culture in RPMI supplemented as above for 4 h or overnight at 37 °C, 5% CO₂ as follows: groups of 2–3 females plus 1–2 males (for a total of 4 filariae) were placed in individual wells of a 12-well cell culture plate containing 1 ml of media. Then the filariae were removed and the culture medium used in chemotaxis assay as previously described [32].

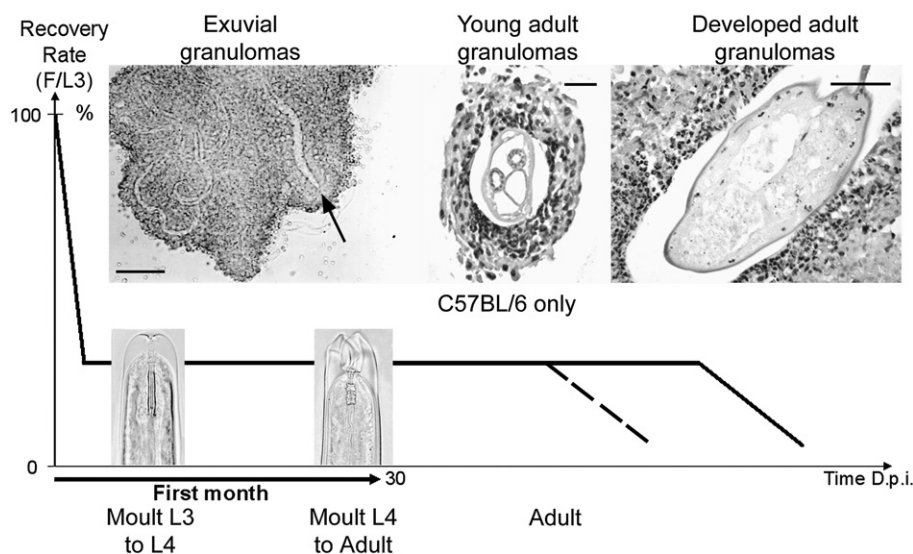


Fig. 1. Three types of granulomas were recovered during *L. sigmodontis* infection, depending on the time p.i. and the host. (i) Exuvial granulomas ($n=92$): around exuviae of third ($n=41$) and fourth ($n=51$) moults, during the first month in jirds and in susceptible or resistant mice; arrow: caudal extremity of exuvium (Scale bar: 100 µm). (ii) Young adult granulomas ($n=20$): around newly moulted adult filariae in resistant C57BL/6 mice only from D27 to D39 p.i. (Scale bar: 25 µm). (iii) Developed adult granulomas ($n=16$): around developed adult worms during the second month and later on, in jirds and mice (Scale bar: 50 µm). The time post-infection is represented by the kinetical curve of the recovery rate. F/L3=percentage of inoculated larvae which were recovered in the pleural cavity (broken line for the resistant mice). Anterior part of worms at beginning of exuviation at moults 3 and 4 are figured in the lower quarter (buccal capsule length=20 µm).

Murine bone marrow leukocytes from naïve BALB/c or C57BL/6 mice (2×10^6 cells in 0.2 ml assay buffer) were placed in the upper chamber of Transwell filters, Millipore (3 μ m pore diameter) that were in turn placed in individual wells of a 24-well cell culture plate containing 0.3 ml of assay buffer. Chambers were incubated for 60 min at 37 °C, 5% CO₂. The number of neutrophils that migrated into the bottom chamber was determined by a FACSCalibur flow cytometer (BD Biosciences, Oxford, U.K.), with relative cell counts obtained by acquiring events for a set time period of 30 s. The neutrophil population was gated according to its characteristic forward/side scatter profile. Neutrophils collected from the bottom chamber of the above system were blocked with CD16/CD32 mAb for 30 min on ice then incubated with FITC-conjugated rat anti-mouse CD11b mAb or the appropriate isotype control mAb for another 30 min on ice. After washing, cells were resuspended in Cellfix™ and analysed using a FACSCalibur flow cytometer. The expression of CD11b was determined by measuring the fluorescence intensity using CellQuest® software.

2.9. Environmental scanning electron microscope (ESEM)

Samples to be examined were placed, without preparation, on the stage of a Peltier cooler (2 °C) of an FEI XL30-ESEM-FEG environmental electron microscope, operated at 15 kV. Partial vapour pressure is maintained at 3.6 Torr to avoid any dehydration and subsequent damage to samples.

2.10. Electron microscope (EM)

The samples were fixed for 2–3 h, at ice-water temperature, in 4% glutaraldehyde in 0.1 M Millonig's buffer [29], pH 7.4, washed in three changes of cold Millonig's buffer containing 1 drop of 10% sucrose/ml of buffer, 20 min/wash, then post fixed for 2–3 h in 1% osmium tetroxide in Millonig's buffer. The samples were subsequently washed three times in plain, cold Millonig's buffer, 20 min/wash, allowed to reach room temperature after the last wash, then rinsed twice in distilled water, dehydrated in graded series of acetone and embedded in Spurr's low-viscosity embedding medium [33], (Electron Microscopy Sciences, Fort Washington, PA, USA). After polymerization, the blocks were trimmed and then sectioned with a diamond knife mounted in an LKB Microtome III. Ultra thin sections, collected on 100 or 150 mesh copper grids coated with Formvar-carbon membrane (Electron Microscopy Sciences), were stained first with uranyl acetate, then with lead citrate, and examined in a Philips 200 transmission electron microscope operated at 80 kV acceleration voltages. Thick sections were stained with a solution of Azure II 1%–Methylene blue 1% (Electron Microscopy Sciences) and observed under optical microscope.

2.11. Statistical analysis

Data are expressed as the mean \pm SEM. The nonparametric Kruskal Wallis *H*-test coupled with posthoc Dunns test for multiple pair wise comparisons was used to assess non-normally

distributed parameters. Probability values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Granulomas were ascribed to three different types based upon the host and the progression of the filarial infection

Pleural worms were studied between (i) D5 and D48 p.i. in C57BL/6 mice (number of Rodents, nR=61; 318 worms), (ii) D5 and D74 p.i. in BALB/c mice (nR=35; 296 worms), and (iii) D5 and D420 p.i. in jirds (nR=31; 447 worms), with a majority of the specimens studied at early time points. Granulomas were found as early as D9 p.i. in the three hosts. We distinguished three different types of granulomas around filarial material: exuvial granulomas, young adult granulomas and developed adult granulomas, the first two being described for the first time.

During the first month p.i. granulomas only contained exuviae (Fig. 1) of the third moult between D8 and D10, and of

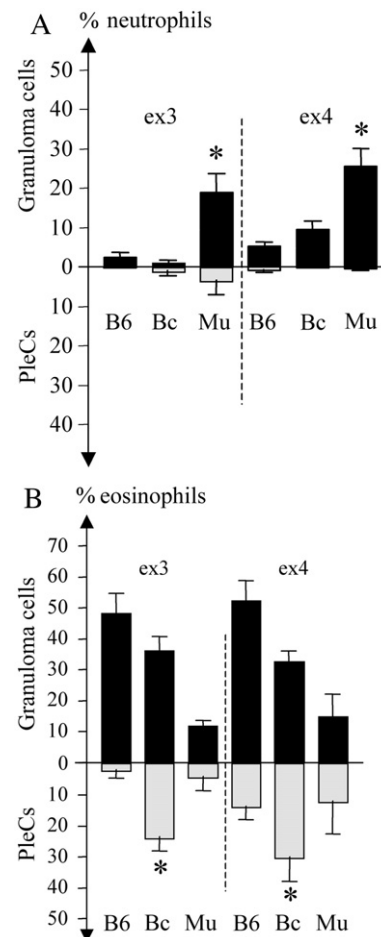


Fig. 2. Granulocyte composition compared in exuvial granulomas and pleural exudate cells following 3rd and 4th moults. A: Percentage of neutrophils. B: Percentage of eosinophils. ex3, ex4: exuvial granuloma of the third and fourth moults respectively. B6, Bc, Mu: C57BL/6 mice, BALB/c mice and jirds. 15, 12, 7 exuvial granulomas of the third moult from 3 B6, 3 Bc and 3 Mu (3 individual experiments) and 20, 15, 14 exuvial granulomas of the fourth moult from 9 B6, 9 Bc and 5 Mu (7 individual experiments) were analysed. Results are expressed as mean \pm SEM; *, $P < 0.05$ (Kruskall Wallis *H*-test).

the fourth moult between D25 and D39 p.i. These exuvial granulomas never contained larval stages. Granulomas of the third moult were 300–1000 μm long; those of the fourth moult were 1–3 mm long. One to 6 exuviae were enumerated per granuloma. For each exuvium, the long posterior part was well-preserved whereas the anterior part was never identifiable. Transverse striae 2.5 μm apart as well as basophilic lateral lines

were identified on exuviae of the fourth moult. No leukocytes were observed on filarial worms during the long process of moulting.

In order to understand how larvae moult, we performed *in vitro* cultures of third or fourth stage larvae. At the beginning of ecdysis, the cuticle was cleaved in the cephalic-cervical part and split lengthwise, withdrawing the buccal capsule and

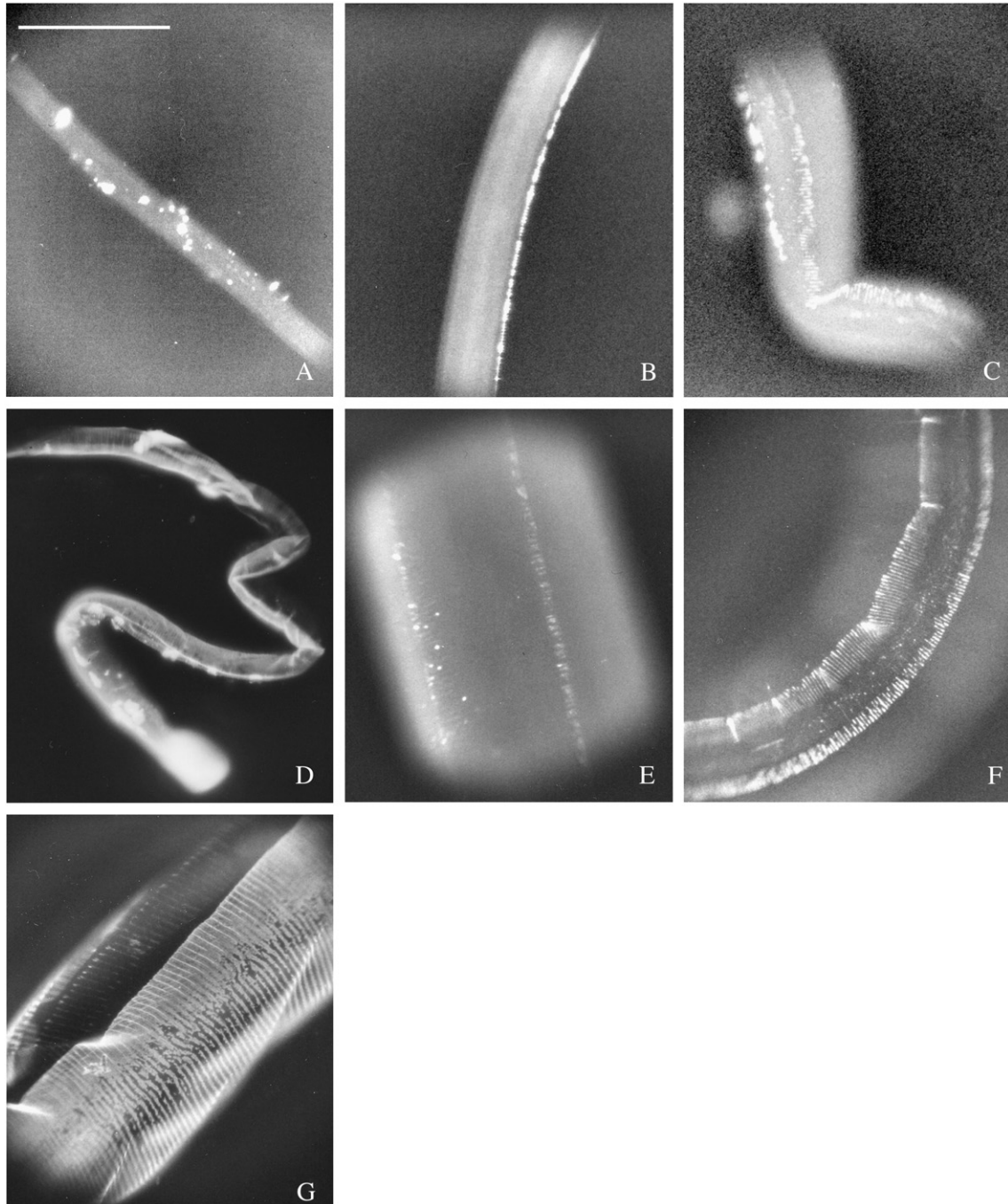


Fig. 3. Progression of carbohydrates on *L. sigmodontis* cuticle through the first month in host. A. Pattern of staining observed in all infective third stage larva ($n=20$) recovered from the mites. B. Third stage larva at D5 p.i. ($n=25$): thin median line of fluorescent spots. C. Beginning of the third exuviation at D8 p.i. ($n=13$): longitudinal bands of transverse striae, on median and lateral fields. D. Exuvium of the third moult ($n=4$): intense staining. E. Fourth stage larva at D21 p.i. ($n=6$): thin median and lateral lines of fluorescent spots. F. Beginning of the fourth exuviation at D21 p.i. ($n=9$): longitudinal bands of transverse striae on lateral fields. G. Exuvium of fourth moult ($n=5$): intense and regularly distributed staining. Worms were collected from jirds. Exuviae of 3rd and 4th moults were obtained *in vitro*. Cuticles were stained with FITC-labelled lectin HPA. The number of studied specimens is indicated in bracket. Scale bars in μm : A–G: 50.

oesophageal internal cuticular lining. The formation of the exuvial granuloma was initiated by the attachment of leukocytes on this anterior part of the shed cuticle. This led to the formation of incomplete monolayers of leukocytes, the posterior extremity remaining free of cells. The newly *in vivo* moulted filariae, although in close proximity with their exuviae, never presented attached leukocytes.

At the end of the first month/beginning of the second month of infection, the second type of granulomas was observed around very young adults but only in 20% of C57BL/6 mice (Fig. 1); these granulomas were sometimes in close contact with the exuvial granulomas of the fourth moults. This type of granuloma was difficult to observe as it appeared during a short window of time and only in one host species.

Finally, during the second month of infection and later on, granulomas contained developed adult worms as previously described [6,7,34]. These worms were often still motile (Fig. 1).

3.2. In exuvial granulomas the neutrophil/eosinophil balance depended on the host species

Cell composition of exuvial granulomas was quantitatively assessed on May Gründwald-Giemsa stained thick films made from dissected granulomas, and by specific staining of granuloma sections for neutrophils and eosinophils. As expected, neutrophils were absent or present in very low numbers in the pleural exudates of the rodents during the first month (Fig. 2A). Neutrophils were not observed or were rare in the exuvial granulomas of the third moult (Fig. 2A) of both C57BL/6 and BALB/c mice. Their proportion slightly increased in the exuvial granulomas of the fourth moult ($4.9 \pm 1\%$ and $9.5 \pm 2.1\%$, respectively). However, in jirds, neutrophils represented $18.9 \pm 4.8\%$ and $25.5 \pm 4.7\%$ of leukocytes exuvial in granuloma of the third and fourth moult respectively, suggesting an intense inflammatory process in this host at these time points. The percentage of eosinophils in PleCs

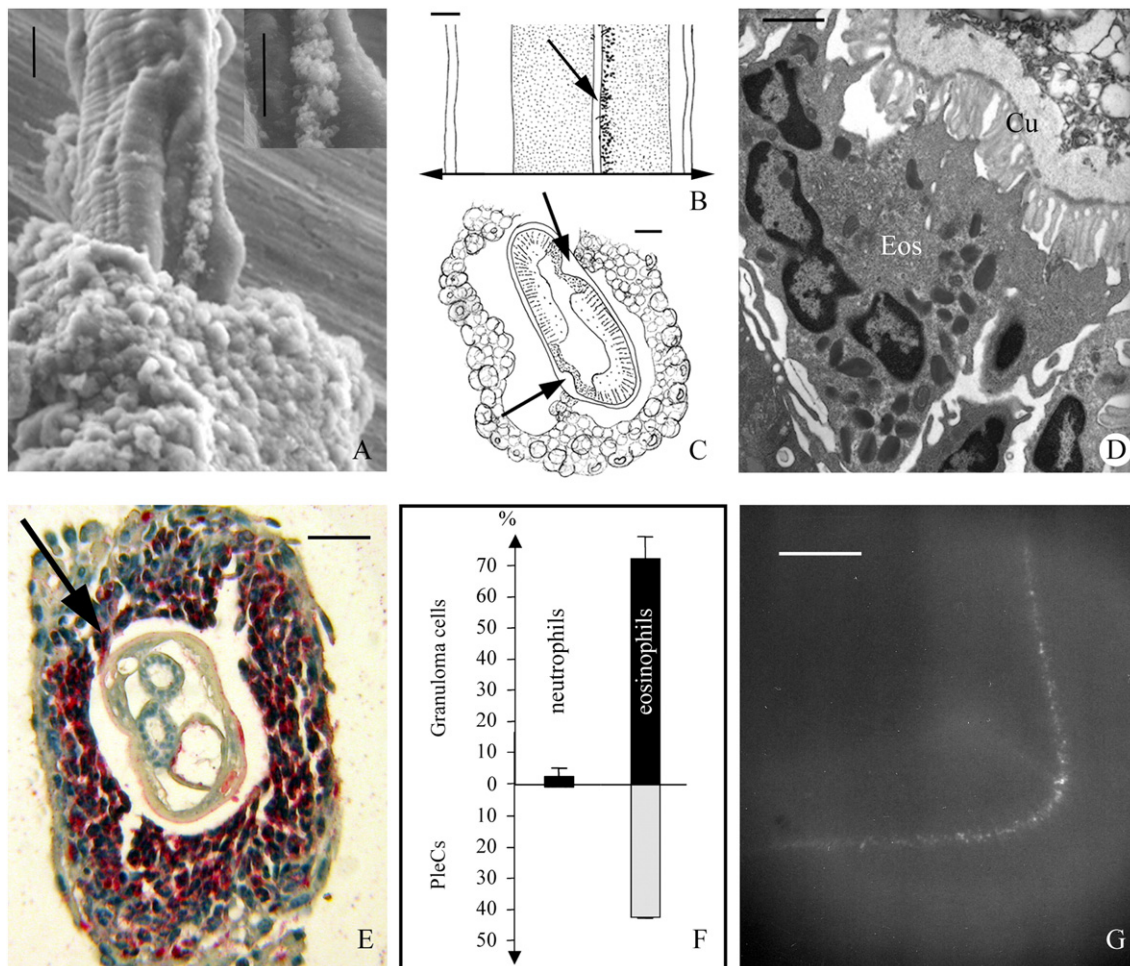


Fig. 4. Young adult granuloma from C57BL/6 mice. A. Leukocyte coat around a filaria showing a particular pattern of leukocyte alignment; detail in upper right corner (Environmental Microscope). B. Worm lateral view enlightening the leukocyte alignment in the filarial lateral plan (dotted area: lateral hypodermal chord; arrow: lateral line). C. Transverse section of a granuloma showing the specific attachment of cells on the lateral lines (arrows: lateral thickenings of *L. sigmodontis* cuticle). D. Young adult with altered hypodermis in a granuloma, specimen with abnormal thin cuticle (Cu). One eosinophil is identified with crystalloid granules (Eos). E. Transverse section of a young adult granuloma revealing the massive presence of eosinophils (MBP staining) and their binding to the worm lateral lines (arrow: lateral line). F. Percentages of neutrophils and eosinophils in granuloma cells and pleural exudate cells (PleCs). G. Carbohydrates are present on the lateral line of non-encapsulated young adult filaria (D31 p.i.) only from C57BL/6 mice (FITC-labelled lectin HPA). Scale bars in μm : A–C: 10; D: 1; E and G: 25. Twenty young adult granulomas were isolated from 7 individual experiments, in 9 out of 47 C57BL/6 mice.

was very low in C57BL/6 mice (5–10%) whereas it reached 20–30% in BALB/c PleCs by D30 p.i. However the percentage of eosinophils in exuvial granulomas was high in C57BL/6 mice (C57BL/6 versus BALB/c mice: $52.3 \pm 6.2\%$ and $32.6 \pm 3.4\%$). Conversely, the percentages of eosinophils in jirds were low both in PleCs and exuvial granulomas (Fig. 2B). Macrophage percentages were close to 50% in all hosts. Metaphases of dividing macrophages were frequent. Mastocytes were absent or rare in exuvial granulomas from all rodents, never exceeding 1% and the same proportions were found in the pleural cavity.

3.3. Carbohydrate residues were absent from newly moulted filariae and progressively covered the cuticle during the third and fourth moulting processes

To examine the ability of cells to bind to the cuticle, we analysed the surface expression of carbohydrates (GlcNAc/NeuNAc and GalNAc, respectively) by using FITC-labelled WGA and HPA lectins. Worms were stained immediately after their recovery from the rodents. Cell-free exuviae of third and fourth moults were obtained after overnight *in vitro* culture of moulting filariae. Changes of carbohydrates over time were studied by analysing surface GalNAc. Surface GlcNAc/NeuNAc were only studied during the fourth stage and moulting period, and presented patterns and developments similar to those of GalNAc. The infective larvae recovered from the vector, used as a reference, displayed a very constant pattern of fluorescent patches irregularly distributed on the body (Fig. 3A). During the third stage in the host, between D5 and D8 p.i., almost half of the larvae presented no evidence of GalNAc whereas the others showed thin median lines of small fluorescent spots (Fig. 3B). At the beginning of the third exuviation, staining had greatly

increased on all larvae, forming longitudinal bands of transverse striae, on median and lateral fields (Fig. 3C). Then, the binding area was spread and intense on the shed exuviae (Fig. 3D). On the contrary, no newly emerged L4 displayed positive staining. A similar evolution of the HPA binding was observed on the old fourth stage, fourth moulting process, fourth exuvium (Fig. 3E–G) and young adults. Phasmids, amphids and buccal cavity of third and fourth stages larvae were positively stained with both FITC-labelled HPA and FITC-labelled WGA. Lectins did not bind on exuviae directly extracted from the granulomas.

3.4. Young adult granulomas isolated from C57BL/6 mice showed a distinct pattern of leukocyte attachment on worms and revealed a specific cell composition

In almost 20% of the C57BL/6 mice, very young adult filariae were embedded in granulomas between D27 and D39 p.i. Such young adult granulomas were observed just after the fourth moult, and concerned 10% of the worms recovered. These granulomas were the only ones to present a pattern of leukocyte attachment on worm lateral lines (Fig. 4A–D). The cells attached to the lateral line were eosinophils as evidenced by MBP staining (Fig. 4E). Although lectins did not bind to worms extracted from those granulomas, non-encapsulated young adult worms recovered at the same time showed a particular pattern of small fluorescent patches on the worm lateral lines (Fig. 4G). They were absent or faint on worms from the BALB/c mice. These young adult granulomas in C57BL/6 mice showed a very particular cell composition. Eosinophils were massively present (70%), correlating with their high percentage in the pleural exudates. At that time, neutrophils were almost absent from both granulomas and PleCs (Fig. 4F). The worms in these young adult

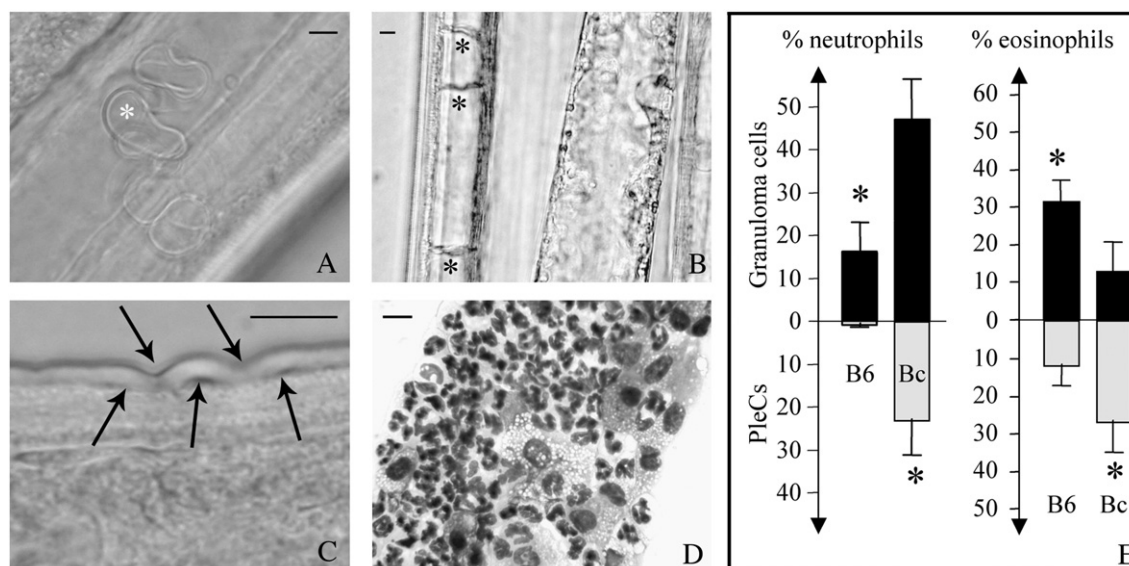


Fig. 5. Alteration of non-encapsulated developed adult worms and developed adult granuloma cell composition. A and B. First signs of alteration in hypodermis, presenting different aspects such as tortuous lines or strands (*) (C57BL/6, D48 p.i.). C. Loosening of hypodermis and corrugated cuticular worm surface (C57BL/6; D48 p.i.). D. High density of neutrophils and macrophages in a developed adult granuloma (BALB/c, D74 p.i.). E. Percentages of neutrophils and eosinophils in developed adult granulomas and in pleural exudate cells (PleCs) recovered from C57BL/6 mice between D45 and 48 p.i. and from BALB/c mice between D68 and 74 p.i. Results are expressed as mean \pm SEM; *, $P < 0.05$ (Kruskall Wallis *H*-test). Scale bars in μm : A–D, 10. Eighteen worms were studied (A–C), 8 granulomas isolated from 4 BALB/c mice and 8 from 4 C57BL/6 mice were analysed (D and E), 1 individual experiment.

granulomas were all altered, often severely. In addition to damaged internal tissue, 15% of worms had an abnormally thin cuticle, 0.4 μm instead of 3 μm , indicating that the new adult cuticle formation was impaired. Furthermore the exsheathment was not complete in 10% of the worms: although the anterior part had moulted, as assessed by the thick adult buccal capsule, the posterior part was still inside the exuvium.

3.5. *Filariae* showed structural alterations initiated in the hypodermis before they were enclosed in developed adult granulomas

Unhealthy filariae were identified by several abnormal morphological characters in C57BL/6 mice at D45–48 p.i., in

BALB/c mice at D68–74 p.i and in jirds between 12 and 15 months p.i. The early signs of alteration were not evenly distributed and the entire worm had to be observed. The alteration was initiated in the hypodermis of the lateral chords, evidenced by tiny folded membranes in the syncytium (Fig. 5A), loosening of hypodermis with transverse strands still adhering to the cuticle (Fig. 5B), resulting in a corrugated cuticle surface (Fig. 5C) and shrinking of muscle fibres, thickening and darkening of the syncytium, loss of refringency and general grey aspect. In female adult worms, refringent spherical inclusions were abundant in genital duct walls; all eggs were aborted, and finally lesions of ovules and ovaries appeared. However, altered worms presented abundant and apparently healthy spermatozoa

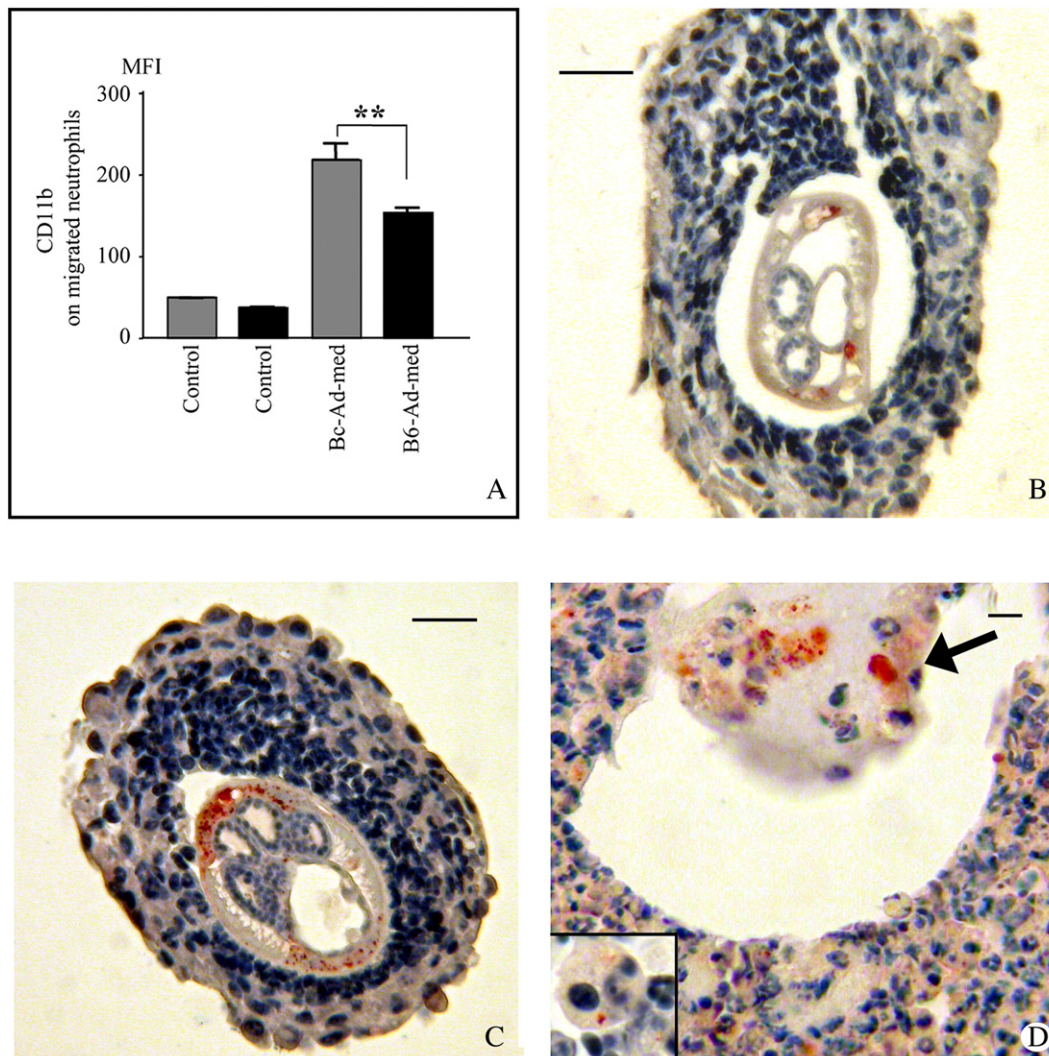


Fig. 6. Neutrophils recruitment in murine filarial granulomas. A. CD11b expression of migrated neutrophils. 27–30 days old adult filariae were isolated from BALB/c and C57BL/6 mouse pleural cavity respectively. In each strain of mice, groups of 4 filariae were incubated in culture medium. After overnight incubation, filariae were removed and the medium was named Bc-Ad-med (culture medium of adult filariae isolated from BALB/c mice) and B6-Ad-med (culture medium of adult filariae isolated from C57BL/6 mice) respectively. BALB/c or C57BL/6 mice neutrophils from bone marrow cells were allowed to migrate to supernatants in a Transwell chemotaxis assay for 1 h at 37 °C. Migrated neutrophils were collected and the geometric mean fluorescence intensity of CD11b expression was established by FACS. No chemokinesis was observed when the filarial medium (respectively Bc-Ad-med and the B6-Ad-med) was added to both the upper and lower chambers. Results are expressed as mean \pm SEM ($n=6-8$ separate experiments performed in triplicate); **, $P<0.01$ (Kruskall Wallis H -test). B and C. *Wolbachia* was present in young adult filariae and not in granuloma cells recovered from C57BL/6 mice at early stage (B) and at later stage when granuloma cells have collapsed (C). D. *Wolbachia* (WSP staining) was identified in cells of developed adult granulomas (arrow: very altered worm with infiltrated cells); lower corner: detail of *Wolbachia* and apoptotic neutrophil phagocytised by a macrophage. Scale bars in μm : B and C: 25; D: 50.

in the ejaculatory duct and in the uteri, as well as microfilariae that, when present, were often undamaged and motile. The proportion of non-encapsulated worms which were altered increased over time and reached 100% at D48 p.i. and D74 p.i. in C57BL/6 and BALB/c mice, respectively. Cell composition of granulomas recovered during the second and third months was studied in mice. These granulomas contained altered motile or dead adult worms and were observed from D45 p.i. onward in C57BL/6 mice and from D60 p.i. in BALB/c mice.

These granulomas were composed of eosinophils, macrophages and neutrophils. In both mouse strains neutrophils from developed adult granulomas were more abundant than from exuvial granulomas (Fig. 5D). However the proportion of neutrophils reached higher levels in BALB/c mice (47% versus 17.8% in C57BL/6 mice), correlating with their higher level in the pleural exudate (Fig. 5E). The percentages of eosinophils in developed adult granulomas were lower than in exuvial granulomas. They were higher in C57BL/6 mice than in BALB/c mice and it was the reverse in PleCs (Fig. 5E). Apoptotic neutrophils were frequent as well as phagocytic macrophages (Fig. 6D) and giant cells. Mature adults did not show any positive HPA binding as evidenced on filariae recovered from BALB/c mice at D68–74 p.i.

3.6. Neutrophil recruitment to murine filarial granulomas was linked to CD11b expression and to the exposure of *Wolbachia* endosymbionts

Neutrophil recruitment increased between the third exuvial granulomas and the developed adult granulomas in mice: 2 to 15% of the cells in C57BL/6 mice, 2 to 45% in BALB/c mice. In order to explain this difference between C57BL/6 and BALB/c mice, we analysed the expression of the β 2-integrin CD11b on migrated neutrophils. The β 2-integrins are important components of the neutrophil trafficking pathway. Notably, Mac-1 (CD11b/CD18) is rapidly upregulated on the neutrophil cell surface following appropriate activation. In an *in vitro* chemotaxis assay using Transwell filters, both C57BL/6 and BALB/c murine bone marrow derived neutrophils migrated similarly to filarial culture media (data not shown). However the migrated neutrophils harboured differential expression of CD11b: it was higher on BALB/c murine neutrophils than on C57BL/6 murine neutrophils, reflecting the high difference observed in developed adult granuloma (Fig. 6A).

In addition to host strain differences, *Wolbachia* was also analysed because it is a potent neutrophil chemoattractant due the release of its LPS-like components [14]. *Wolbachia* were observed in the lateral chords (Fig. 6B and C) as well as in the ovaries, the eggs and the uterine microfilariae. However, WSP staining was negative in all three types of granulomas except at the late stage of adult granulomas, when filariae were massively altered. In this case WSP was detected in a few macrophages within the granulomas (Fig. 6D).

4. Discussion

In this study we focused on granulomas that form during the larval and adult development of the filariae *L. sigmodontis* in

the pleural cavity of mice and jirds. Besides granulomas forming around fully developed adult worms, we described two new types: granulomas encapsulating exuviae and those around young adult in resistant mice only (Fig. 1). We have found that the granulomas are associated with a succession of distinct inflammatory reactions that are linked both to the moulting processes of the filaria and to the host susceptibility.

The granulomas in all cases were composed of granulocytes and macrophages that did not strictly reflect the pleural exudate cell populations. This suggests that the mechanisms of recruitment to the pleural cavity and those that determine granuloma composition are different.

During the larval phase of *L. sigmodontis* no neutrophils were found in the pleural exudates as described previously [3,26,28]. However, they were found in the exuvial granulomas, in different numbers depending on the host. Massive recruitment of neutrophils was noted in the permissive jird, where they constituted 18.9% and 25.5% of the granulomas around the moult 3 and the moult 4 respectively, indicating that exuviation induced an acute inflammatory reaction in the most permissive host. On the other hand, exuvial granulomas in mice contained very few neutrophils and no detectable *Wolbachia* as assessed by staining for the *Wolbachia* Surface Protein (WSP). They often contained numerous shed cuticles, likely due to the natural aggregation of the filariae in the pleural cavity (Attout et al., in preparation), but never larvae, showing that third stage and fourth stage larvae did not induce granuloma formation, as observed in the natural host [35]. Adhesion of cells to exuviae was immediate and no cell-free exuviae were ever found *in vivo* during both moults 3 and 4.

By contrast, neutrophils were the major component and formed an inner circle around the worm in granulomas that formed around the senescent adult worms in BALB/c mice, as described previously [6,7]. This feature correlates with the current concept that the intracellular symbionts *Wolbachia* are necessary to attract neutrophils [7,9–11,36]. However, in young adult C57BL/6 granulomas neutrophil numbers were low and remained low in granulomas around developed adult filariae. This difference in the proportion of neutrophils between granulomas recovered in BALB/c mice and those in C57BL/6 mice could be due to (i) microfilariae, which were only released in BALB/c mice and harbour *Wolbachia* [37], contributing to the extended recruitment of neutrophils; (ii) CD11b as evidenced by a higher upregulation in BALB/c murine neutrophils than in those of C57BL/6 murine neutrophils (Fig. 6A).

Indeed Mac-1 (CD11b/CD18) is rapidly upregulated on the neutrophil cell surface following appropriate activation and has been implicated in the regulation of diapedesis, respiratory burst, phagocytosis and chemotaxis of neutrophils [38]. Thus upregulation of CD11b expression by neutrophils could be one mechanism by which neutrophils adhere to the mesothelium during their transmigration into the inflamed pleural cavity, and aggregate around filariae in this cavity. Such a mechanism would be less efficient in C57BL/6 mice than in BALB/c mice and could contribute to the different cell composition in young adult granuloma, regarding the mouse strain.

Eosinophils were the major component of granulomas in the absence of a patent phase. Thus granulomas in resistant C57BL/

6 mice as well as granulomas with exuviae in BALB/c mice were rich in eosinophils (>30%) with hardly any neutrophils detected except a few in granulomas of adult filariae in C57BL/6 mice. Eosinophils were even massively present (>70%) in the granulomas engulfing young adults in C57BL/6 mice. This could be linked to the higher release of the eosinophil chemoattractant CCL3 in C57BL/6 mice: at D30p.i. the pleural fluid concentration of eotaxin was 60.1 ± 7.5 pM in C57BL/6 mice as opposed to 31.7 ± 5.8 pM in BALB/c mice [3].

At the early stage of any type of granuloma, the cell coat surrounding the parasite or its exuvium was not tightly attached to the filarial cuticle. This was particularly obvious in granulomas from C57BL/6 mice containing young adults where there was a space between the cell coat and the worm surface (Fig. 4C). However, in old granulomas the cell coat seemed to have collapsed and was in tight contact with the parasite.

To investigate the attachment of the granulomas to the filariae, we sought to correlate the adhesion of the host's cells to the filarial carbohydrates of the surface coat through lectin binding [17]. Lectin binding to the worm surface was increased in both third and fourth stages, from areas located along the median lines to longitudinal bands of transverse striae on both median and lateral positions. This suggests that the hypodermic secretory activity by seam cells increased as the cuticle matured [20,39–41]. Lectins did not bind to the newly moulted worms. However, an abnormal expression of carbohydrates was observed on young adult filariae from C57BL/6 mice, whereas none was detected on filariae from BALB/c mice and jirds. This could reflect a malfunctioning of the hypodermic area of the worms in resistant mice. In resistant hosts, the carbohydrates on the young adult's lateral line correlated strongly with the adhesion of eosinophils (Fig. 4G). In all other cases, the presence of carbohydrates was not sufficient to induce cell adhesion processes, since leukocytes did not bind to larvae. Indeed third stage and fourth stage larvae induced neither granuloma formation nor lysis, as had been observed in the natural host [35]. In *T. canis*, mucins, which are highly glycosylated proteins with oligosaccharides linked via GalNac or GlcNac, have been shown to prevent cell adhesion to the worm [18], which could explain why no cell adhesion was evidenced despite the presence of carbohydrates.

Thus, the origin of the different granulomas was diverse: (i) in the case of granulomas mainly composed of neutrophils e.g. around adult worms, a recruitment of these granulocytes is likely to occur by a LPS-like dependent mechanism [7,9–11,36]. (ii) In the case of granulomas mainly composed of eosinophils, a glycan-dependent process could be implicated. Indeed it was shown that a mouse sialic acid-binding immunoglobulin, lectin (Siglec)-F (equivalent to human Siglec-8) was selectively expressed on murine eosinophils and recognised 6'-sulfo-sialyl Lewis X as a preferred glycan ligand [42]. The activation of Siglec-8 induced the apoptosis of eosinophils, leading to the release of the toxic eosinophilic granules that may have a helminthicide effect beneficial to the host [43]. Such a positive role of the siglecs was shown in the resolution of infection by the bacteria *Neisseria meningitidis* [44].

In the three types of rodents used in this study, it was observed in the late time points of infection that (i) granulomas contained

only altered worms, (ii) altered though cell-free worms were also present, and (iii) the proportion of cell-free altered worms decreased with time while the proportion of cell-coated altered filariae increased. These observations suggest that the alteration of the filarial worm was required for cell recruitment and granuloma formation. Indeed, early signs of alteration in adult worms were detectable mainly in the hypodermis and, as a result, in the muscle attachment to the cuticle (Fig. 5A–C).

To conclude, both neutrophils and eosinophils are active components of *L. sigmodontis* granulomas, but their involvement depends on many factors related to the influence of host susceptibility to filarial development (patent phase or not, filarial hypodermal physiology...), and to the host's immune response (chemokine and cytokine production, cell adhesion properties...). All together this study suggests that granulomas represent complex phenomena and they seem to be a consequence rather than a cause of parasite degeneration.

Acknowledgements

We would like to thank Dr SM Rankin, Leukocyte Biology Section, Imperial College London for her assistance and Dr Georges Snounou, USM307, MNHN Paris for his valuable comments.

This work was supported by the European Community grant ICA4-CT-1999-10002.

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