INFECTION AND IMMUNITY, Dec. 2003, p. 6820–6829 Vol. 71, No. 12
0019-9567/03/$08.00+0 DOI: 10.1128/IAI.71.12.6820–6829.2003

Resistance and Susceptibility to Filarial Infection with *Litomosoides sigmodontis* Are Associated with Early Differences in Parasite Development and in Localized Immune Reactions

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Received 30 May 2003/Returned for modification 26 July 2003/Accepted 22 September 2003

In order to understand natural resistance to filariasis, we compared *Litomosoides sigmodontis* primary infection of C57BL/6 mice, which eliminate the worms before patency, and BALB/c mice, in which worms complete their development and produce microfilariae. Our analysis over the first month of infection monitored migration of the infective larvae from the lymph nodes to the pleural cavity, where the worms settle. Although immune responses from the mouse strains differed from the outset, the duration of lymphatic migration (4 days) and filarial recovery rates were similar, thus confirming that the proportion of larvae that develop in the host species upon infection is not influenced by host genetic variability. The majority of worms reached the adult stage in both mouse strains; however, worm growth and molting were retarded in resistant C57BL/6 mice. Surprisingly, the only immune responses detected at 60 h postinfection occurred in the susceptible mice and only upon stimulation of cells from lymph nodes draining the inoculation site with infective larva extract: massive production of interleukin-6 (IL-6) and IL-5 (the latter cytokine was previously suspected to have an effect on *L. sigmodontis* growth). However, between days 10 and 30 postinfection, extraordinarily high levels of type 1 and type 2 cytokines and expansion of pleural leukocyte infiltration were seen in the resistant C57BL/6 mice, explaining the destruction of worms later. Our results suggest that events early in the infection determine susceptibility or resistance to subsequent microfilarial production and a parasite strategy to use specific immune responses to its own benefit.

In areas where filariasis is endemic, a small proportion of the population (<5%) consistently shows neither microfilariae nor pathology. Characterization of the protective mechanisms operating in this putatively immune population is highly desirable and has consequently received great attention (18, 23, 41, 46, 47, 50). However, experimental investigations or field observations aiming to correlate parasitological, immunological, and genetic factors with resistance in humans are limited for evident ethical reasons. The filaria *Litomosoides sigmodontis* has proven to be a good murine model (1, 8, 42), since in various strains of mice it mimics the spectrum of parasitological outcome of human filariasis (42). Recently, genetically modified mice have been employed to rapidly identify key immune components; the absence of Th2 (28, 48, 49) or Th1 (gamma interferon [IFN-γ]) (44) cytokines as well as of B cells and antibodies (2, 35) has been shown to modify *L. sigmodontis* development or survival, as observed in the nonpermissive *Brugia* models (6, 14, 27, 43). However, the inherent physiological and immunological artifacts that can occur in deficient mice sometimes limit the interpretation of the results.

Two previous studies compared the splenic and antibody responses to *L. sigmodontis* in genetically intact susceptible BALB/c mice to those in resistant B10D2 (33) or C57BL/6 (28) mice. No clear differences were observed in the first study, and a late stronger Th2 bias characterized the susceptible mice in the second study.

We focused on local processes occurring during the first month of infection as *L. sigmodontis* infective larvae are inoculated in the host's subcutaneous tissues and then enter the lymphatic system and migrate to the pleural cavity, where they grow and sexually mature (34, 51). We used the permissive BALB/c mice, in which the *L. sigmodontis* patent phase begins 55 to 60 days postinoculation, and the C57BL/6 mice, in which filariae are progressively encysted and destroyed from day 40 postinfection (D40 p.i.) onwards, thus producing no microfilariae (28). We monitored parasitological and immunological features in three compartments: the draining lymph nodes, which until now have not been studied with this filaria; the pleural cavity; and the peripheral blood. The parasitological parameters included the duration of lymphatic migration; the number of recovered live filariae and their size, stage, and sex as assessed by morphological characters; and the presence of pleural granulomas. The immune reactions of the mice were assessed by characterizing the leukocyte populations and their

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proliferation and the production of type 1 and 2 cytokines, antibodies, and the chemokine eotaxin as the filarial development progresses.

MATERIALS AND METHODS

Filariae, mice, parasite extracts, and infection protocols. The filaria *L. sigmodontis* was maintained, and infective third-stage larvae (L3) were recovered by dissection of the mite vector *Ornithonyssus bacoti* as previously described (16). Water-soluble parasite extracts as a source of antigenic components were processed from homogenized and sonicated infective larvae or adults (Ad). After centrifugation, the supernatant was collected and frozen at −70°C until further use. Protein content was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA).

Six- to 7-week-old female BALB/cAnNHsd and C57BL/6NHsd mice 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, particularly gastrointestinal nematodes such as pinworms, which are known to share antigenic molecules with filariae (26). Mice were injected with 40 L3 in 200 μl of RPMI 1640 subcutaneously into the right lumbar area. All experiments and procedures conformed to the French Ministry of Agriculture regulations for animal experimentation (1987).

Parasitological status of mice: recovery rate and morphological analysis of the filariae. Mice were sacrificed at 60 h (six mice per group) and at 4, 5, and 7 days (two mice at each day) after inoculation. The parasitological and immunological statuses were assessed at 60 h (11 mice per group), 10 days (just after the third molt) (16 mice per group), or 30 days (during the fourth molt) (30 mice per group) after inoculation. These assays were performed according to a protocol described previously (34) with some modifications. In this study, mouse tissues were not dilacerated for recovering the worms, but filariae were recovered by flushing the pleural cavity. This technique does not give information on the migrating larvae but allows determination of when they enter the pleural cavity. The peritoneal cavity was also observed under a dissection microscope in order to detect filariae that might have been present, but none were recovered. The location, motility, and gross morphology of the filariae were recorded. Filariae were harvested, counted, and fixed in hot 70% ethanol (D30 p.i.) or 4% formaldehyde (≤D10 p.i.) for detailed morphological analysis. The filarial development was assessed by the following parameters: (i) percentage of mice with filarial worms, (ii) rate of recovery of filariae (number of worms recovered/number of larvae inoculated × 100), (iii) number of live worms partially surrounded by inflammatory cells (these worms were used to calculate the recovery rate), (iv) number of dead worms or pieces of worms in granulomas (these were not included in the recovery rate), (v) size of worms, (vi) stage of worms based on tail and buccal cavity characteristics (34), and (vii) sex ratio of recovered worms identified from the end of the L3 (34) (number of females/total worm burden).

Characterization of pleural exudate leukocytes. Pleural wash fluid was obtained sequentially from each mouse. First, the pleural cavity was washed with 2 ml of cold phosphate-buffered saline (PBS), and filariae were pelleted by sedimentation at 1 × *g*. The supernatant was collected and centrifuged at 1,900 rpm for 6 min at 4°C. The supernatant was then collected and stored at −20°C for later use in cytokine assays. The remaining pellet containing pleural exudate cells (PleC) was kept on ice. Second, the pleural cavity was further washed with 8 ml of cold PBS. Filariae were recovered from the pellet of this washed fluid after sedimentation at 1 × *g* and were pooled with those obtained from the pellet of the first 2 ml of wash fluid. The 8 ml of supernatant was added to the 2-ml pellet containing the first PleC preparation. This solution was homogenized by flipping the tubes and centrifuged at 1,900 × *g* for 6 min at 4°C. Five milliliters of PBS–0.2% (wt/vol) NaCl was first added to the pellet, and after resuspension of the cells, 5 ml of PBS–1.6% (wt/vol) NaCl was added to lyse erythrocytes by osmotic shock. After centrifugation at 1,900 × *g* for 6 min at 4°C, PleC were resuspended in 1 ml of RPMI 1640 and counted in PBS–0.04% trypan blue (Sigma-Aldrich, St. Quentin Fallavier, France) by using a Malassez cell.

Proportions of polynuclear eosinophils, polynuclear neutrophils, and mast cells in the pleural exudate were determined with cytospin cell preparations obtained by centrifuging (500 × *g*, 3 min) a suspension of 100,000 PleC in 150 μl of PBS–1% fetal calf serum against slides and staining with May-Grünwald-Giemsa stain. Lymph node cells (LyNC) and PleC were further phenotyped by cytofluorimetry. Data were collected with a FACScalibur flow cytometer and analyzed with Cell Quest software (Becton Dickinson-PharMingen, Le Pont de Claix, France). The following rat anti-mouse antibodies were used: anti-CD19 (clone ID3) conjugated to fluorescein isothiocyanate, as a marker of B cells; anti-CD5 (clone 53-7.3), a marker found on B1 cells and not B2 cells, conjugated to phycoerythrin; anti-CD4 (clone RM 4-5) conjugated to fluorescein isothiocyanate; and anti-CD8 (clone 58-6.7) conjugated to phycoerythrin (all antibodies from Becton Dickinson-PharMingen) and anti-F4/80 (clone C1:A3-1), a marker of macrophages, conjugated to phycoerythrin (Tebu-Caltag, Le Perray-en-Yvelines, France). It should be noted that we may have underestimated the number of macrophages at D30 p.i. because we observed a lower mean fluorescence intensity after staining with the F4/80 monoclonal antibody (MAb). This is probably due to the downregulation of this marker by IFN-γ (19), which is present at high concentrations in the pleural cavity at that time.

LyNC and PleC stimulation in vitro. The draining right inguinal lymph node and the iliac and lumbar lymph nodes were collected and studied separately (infected mice) or pooled (naive mice). The lymph nodes were crushed between sterile glass slides, and the resulting cell suspension was passed through a 100-μm nylon cell strainer (Becton Dickinson).

LyNC and PleC were cultured in duplicate in 96-well plates at 3×10^5 cells/well in RPMI 1640 medium supplemented with 10% newborn calf serum, 2 mM L-glutamine (Sigma Aldrich), 100 U of penicillin per ml, and 100 μg of streptomycin per ml (Life Technologies-Gibco BRL, Cergy-Pontoise, France) and stimulated for 24, 48, or 72 h (LyNC) or for 72 h only (PleC) with L3 and Ad filarial extract (10 μg/ml) or concanavalin A (ConA) (5 μg/ml; Sigma) at 37°C in 5% CO_2-enriched atmosphere.

DNA synthesis was assessed by incorporation of [^{3}H]thymidine (Amersham) at 1 μCi/well into the 24-, 48-, and 72-h-stimulated cells and incubation for another 16 h. This was expressed as a stimulation index (counts per minute of stimulated culture wells/mean counts per minute of nonstimulated culture wells).

Cytokine ELISA of culture supernatants, pleural wash fluids, and sera. Twenty-four-, 48-, and 72-h-stimulated LyNC and 72-h-stimulated PleC culture supernatants (diluted 1:3 for interleukin-4 [IL-4], IL-5, and IL-6 and 1:6 for IL-10 and IFN-γ), pleural wash fluids (not diluted), and sera (diluted 1:5 for IL-4 and IL-5 and 1:10 for IL-10 and IFN-γ) collected at 60 h p.i. (LyNC only) or at D10 or D30 p.i. from individual mice were assayed for cytokine content by enzyme-linked immunosorbent assay (ELISA) in triplicate. This assay was performed as recommended by the manufacturer, using the following purified and biotinylated MAb pairs and corresponding recombinant cytokines: R4-6A2 and XMG1.2 for IFN-γ, BVD4-1D11 and BVD6-24G2 for IL-4, TRFK5 and TRFK4 for IL-5, MP5-20F3 and MP5-32C11 for IL-6, and JES5-2A5 and SXC-1 for IL-10 (Becton Dickinson-PharMingen). The reaction was revealed with Amdex streptavidin-horseradish peroxidase conjugate (Amersham, Orsay, France) followed by the addition of tetramethylbenzidine and H_2O_2 reagents (KPL Laboratories, Gaithersburg, Mass.), and the optical density was measured at 450 nm. Dilutions of known quantities of recombinant cytokines were used for the standard curve. Results are expressed in picograms per milliliter. Detection limits were 2 pg/ml for IL-4, 20 pg/ml for IL-5, 90 pg/ml for IL-6, and 10 pg/ml for IL-10 and IFN-γ.

IgG1, IgG2a, and IgG2c ELISA. The amounts of immunoglobulin G1 (IgG1), IgG2a, and IgG2c antibodies reactive to filarial Ad extract in the sera of mice were assessed on D10 and D30 p.i. The plates were coated overnight at 4°C with Ad extract diluted at a final concentration of 10 μg/ml in coating buffer. Rat anti-mouse IgG1 MAbs were used for detection. IgG1 was measured by using the A85-1 clone. IgG2a antibodies for BALB/c mice and IgG2c antibodies for C57BL/6 were measured by using the clone R19-15 directed to pooled BALB/c and C57BL/6 mouse Ig (Becton Dickinson-PharMingen). The reaction was revealed by the same method as in the cytokine assays. Results are expressed as optical densities. The lowest detection levels were 0.007 and 0.016 ELISA units (optical density) for IgG1 for IgG2a and IgG2c, respectively, as defined previously (12).

Eotaxin assay. The concentration of murine eotaxin in pleural wash fluid was determined by a two site-ELISA. The antibodies used for capture and detection of eotaxin were, respectively, MAb AF420NA (0.4 μg/ml) and MAb BAF420 (0.05 μg/ml) from R&D Systems (Abingdon, United Kingdom). The reaction was revealed with a neutravidin-horseradish peroxidase system. The detection limit was 10 pM.

Statistical analysis. The nonparametric Mann-Whitney U test was used for all comparisons between the two mouse groups ($n > 5$). When comparing filarial lengths and widths ($n > 20$), Student's *t* test for unpaired groups was used. A *P* value of <0.05 was considered significant.

RESULTS

Only a defect in filarial growth differentiates resistant C57BL/6 mice from susceptible BALB/c mice from D10 p.i.

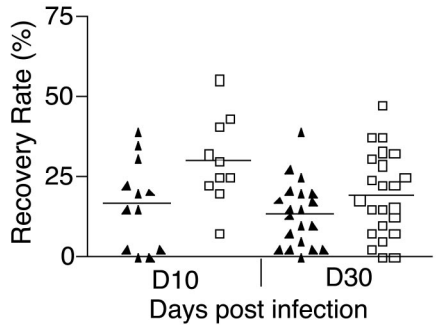

FIG. 1. Rates of recovery of *L. sigmodontis* from mice during the first month of infection. Data shown are the individual values per mouse at D10 p.i. and D30 p.i. in C57BL/6 (triangles) and BALB/c (squares) mice. Horizontal lines represent means.

Among several relevant parasitological parameters, only a defect in filarial growth differentiated resistant C57BL/6 from susceptible BALB/c mice from D10 p.i. No larvae were found in the pleural cavity before D4 p.i. in mice of both strains. The recovery rate was analyzed at D10 p.i. in two experiments and at D30 p.i. in three experiments. During this infection time lapse, the recovery rate did not significantly differ between C57BL/6 and BALB/c mice, although in each experiment the recovery rate tended to be lower in the C57BL/6 mice at both time points (Fig. 1). At D30 p.i., the prevalence, but not intensity, of live worms partially surrounded by inflammatory cells was slightly higher in C57BL/6 mice than in BALB/c mice (13 versus 4 mice [out of 30], respectively, with a mean of 1.4 versus 2 worms in these mice). Granulomas were negligible in both strains at D10 and D30 p.i.

Filariae of both sexes were smaller in the resistant C57BL/6 mice at D10 p.i. ($P = 0.0001$ for female worms and $P < 0.0001$ for male worms) (Fig. 2), but all larvae were at the fourth stage. On D30 p.i. a lower proportion of worms in C57BL/6 mice had molted into Ad stages as assessed by qualitative morphological characteristics (66% ± 7.9% in C57BL/6 mice versus 87.3% ± 4.9% in BALB/c mice [$P = 0.02$]), and they were smaller ($P < 0.0001$ and $P = 0.0125$ for male and female worms, respectively) (Fig. 2). In both strains worms that were still at the fourth stage were smaller than Ad (Fig. 2), suggesting that molt 4 could not occur before the worms had reached a given size.

The early and transient activation of the lymph node draining the inoculation point was much lower in C57BL/6 mice. Cytokine production by LyNC draining the site of inoculation was measured after 72 h of culture with parasite extracts or mitogen. At 60 h p.i, L3 extracts had no detectable effect on cytokine production in infected C57BL/6 mice, whereas in infected BALB/c mice, they led to a high level of production of IL-5 and IL-6 (Fig. 3). In both strains, the response of the infected mice to Ad extracts was low. When cultured with ConA, infected C57BL/6 LyNC produced IL-4, IL-6, and predominantly IL-5 and IFN-γ (Fig. 3). In infected BALB/c mice, all cytokines were produced at high levels with ConA (Fig. 3). IL-10 could be detected only in the LyNC of the BALB/c strain and only when stimulated with ConA (4,250 ± 3,250 pg/ml).

[^{3}H]thymidine incorporation was low in the presence of L3 or Ad worm extracts in lymph nodes of naive and infected mice

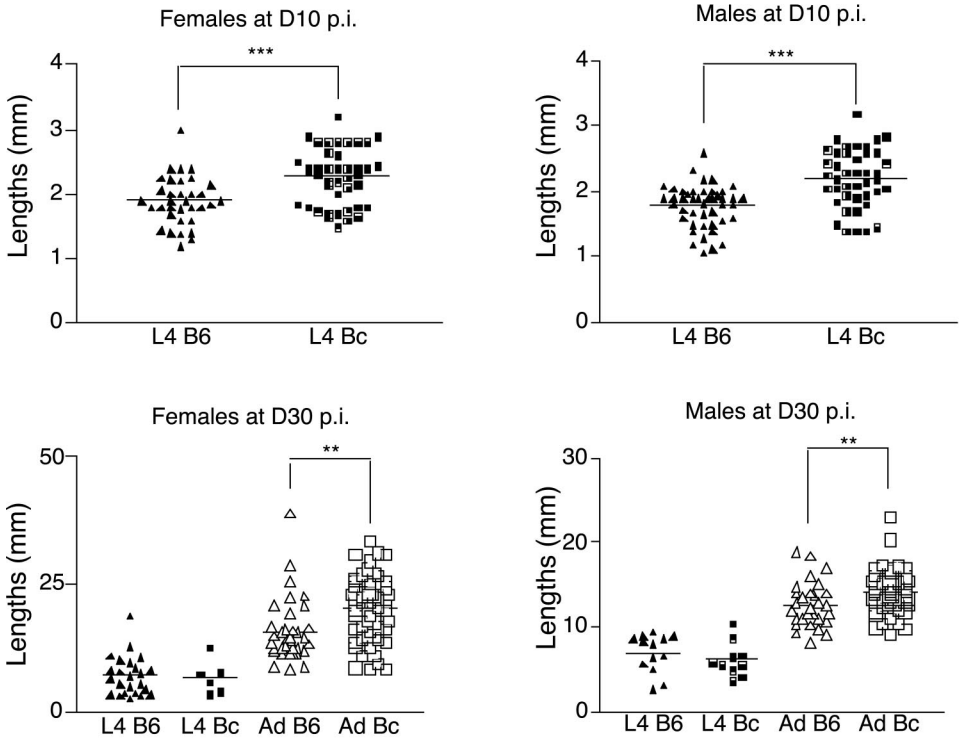

FIG. 2. Lengths of *L. sigmodontis* during the first month of infection. Data shown are the individual values for each filaria recovered at D10 p.i. and D30 p.i. in C57BL/6 (B6) and BALB/c (Bc) mice. Horizontal lines represent means. **, $P < 0.01$; ***, $P < 0.001$ (Student *t* test).

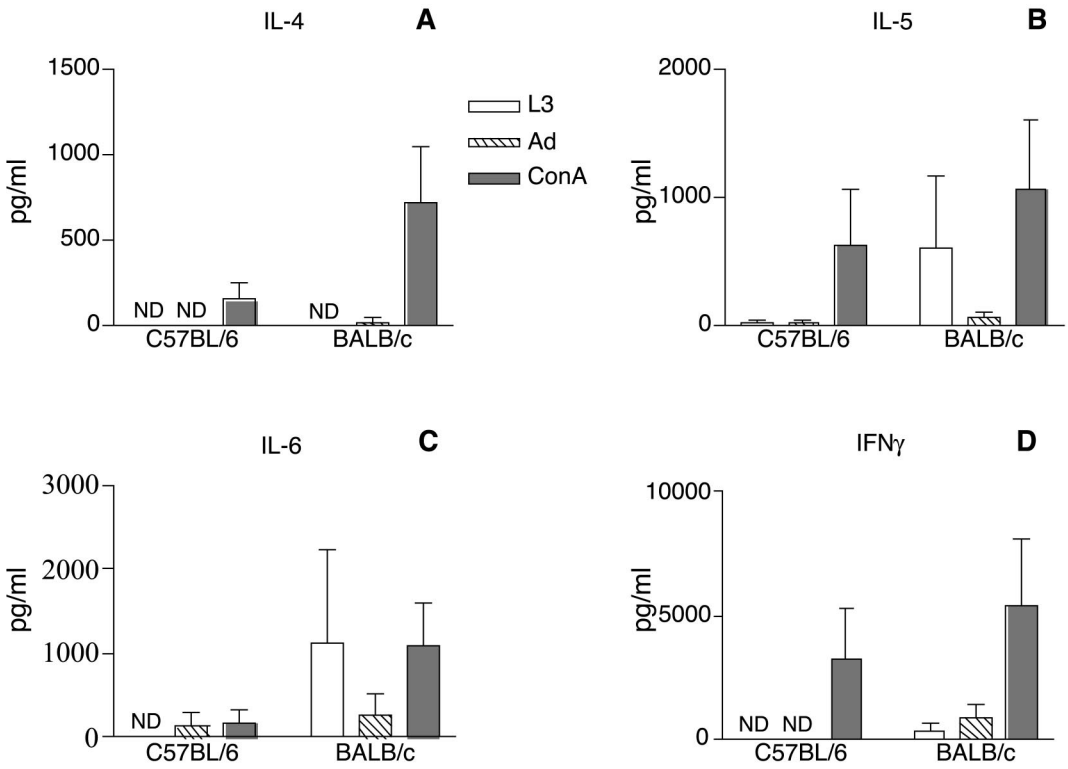

FIG. 3. In vitro cytokine production in C57BL/6 and BALB/c mice by LyNC at 60 h p.i. Levels of IL-4, IL-5, IL-6, and IFN-γ in culture supernatant after 72 h of stimulation with L3 larval extract, Ad extract, or ConA are shown. The values shown are representative of those from three independent experiments and are those found after subtracting values from the control mice. Values are means ± standard errors of the means for six mice per group. ND, not detected.

of both groups (data not shown). As shown in Fig. 4, the inguinal LyNC proliferated with a maximum stimulation index after 48 h of culture but at a lower level in C57BL/6 mice (stimulation index of 100 versus 400). Stimulation indices of naive LyNC were similar to those in the infected mice for each strain, but the maximum incorporation appeared later (at 72 h of culture), likely due to fewer T lymphocytes in the lymph nodes of these mice. These results, together with those above, suggest that the infective larvae induced a local immune reaction in mice of both strains (Fig. 4).

In both strains of mice, the immune reactivity as assessed by LyNC cytokine production did not persist, as it was consistently much lower at D10 and D30 p.i. than at 60 h p.i.; IL-4, IL-5, IL-6, and IL-10 were below detection limits. Nonetheless,

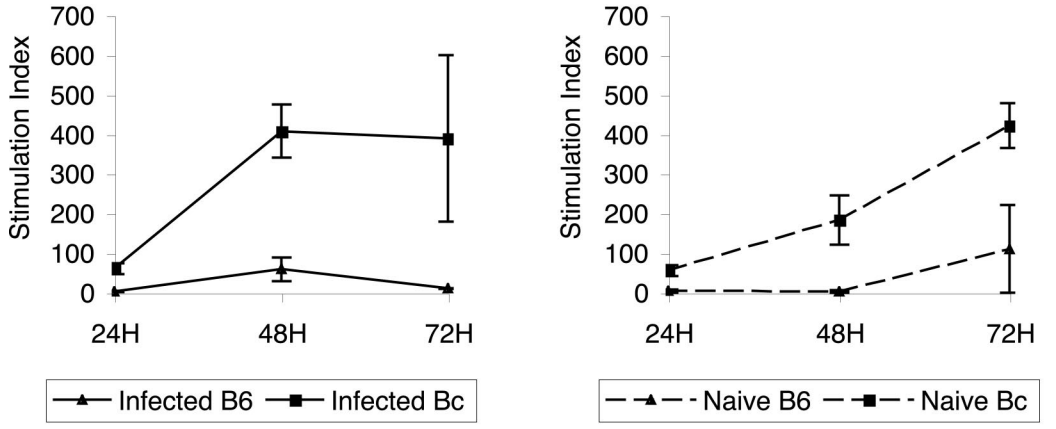

FIG. 4. LyNC stimulation indices after ConA stimulation at 60 h p.i. LyNC from primary infected or naive C57BL/6 mice and BALB/c mice were stimulated with 5 μg of ConA per ml for 24, 48, or 72 h. DNA synthesis was assessed by [^{3}H]thymidine incorporation over 16 h as described in Materials and Methods. Counts per minute ranged from approximately 50 in unstimulated wells to 20,000 in stimulated wells. Data are expressed as the mean stimulation index ± standard deviation.

C57BL/6 mice had a tendency to produce more IFN-γ in response to Ad antigen than BALB/c mice at D10 p.i. (respectively, 148.7 ± 14.2 pg/ml and 33.9 ± 8.8 pg/ml [$P = 0.09$]). [^{3}H]thymidine incorporation upon ConA stimulation at D30 p.i. was similar in infected and naive mice, although it was lower in C57BL/6 than in BALB/c mice.

Pleural leukocyte populations show contrasting kinetics in C57BL/6 and BALB/c mice, characterized by a delayed but greater increase in the resistant strain. The number of pleural leukocytes increased over time in the inoculated mice. The total numbers of cells recovered in C57BL/6 and BALB/c mice, respectively, were as follows: $1.7 \times 10^6 \pm 0.3 \times 10^6$ and $1.9 \times 10^6 \pm 0.5 \times 10^6$ at D0 p.i., $4 \times 10^6 \pm 1.2 \times 10^6$ and $6.6 \times 10^6 \pm 2.2 \times 10^6$ at D10 p.i., and $30 \times 10^6 \pm 7.3 \times 10^6$ and $18 \times 10^6 \pm 7 \times 10^6$ at D30 p.i. There was no statistical difference between the PleC numbers at D0 or D10 p.i. Although the numbers of PleC appeared to be higher at D30 p.i. in C57BL/6 mice, the difference did not achieve statistical significance.

However, regarding the kinetics of PleC accumulation, significant differences were observed between the two strains when different time points were compared. At D10 p.i. in C57BL/6 mice, only B2 cells and $CD4^+$ T cells had significantly increased in numbers since D0, whereas in BALB/c mice, total PleC and B2, $CD4^+$, and $CD8^+$ T cells had increased between D0 and D10 p.i. (Fig. 5). At D30 p.i. in C57BL/6 mice, each cell population had strongly increased in number compared to at D10 p.i. ($P < 0.001$), in contrast to the case for BALB/c mice, in which no such increase was observed between D10 p.i. and D30 p.i. except for B1 cells (Fig. 5). The steeper increase in cell numbers in C57BL/6 mice was particularly obvious with $CD4^+$ and $CD8^+$ T cells: compared to those in naive mice they increased by 30- and 45-fold, respectively, whereas they increased by only 8- and 7-fold, respectively, in BALB/c mice.

Interestingly, no neutrophils were observed in cytospin preparations from both strains, and mast cells did not exceed 0.5% at any time point.

Pleural cell proliferation was measured at D30 p.i. In the presence of filarial extracts (L3 and Ad) and ConA, no cells proliferated, whereas those obtained from naive mice proliferated under ConA stimulation, although weakly (stimulation index of 20 ± 12.5 in both strains). The absence of a mitogenic reaction to ConA suggests an active T-cell proliferation suppression. The mechanism proposed by Loke et al., through contact with alternatively activated macrophages (31), is consistent with our infection model in which macrophages and IL-4 are abundant in the pleural cavity (Fig. 5). In our studies, as for other filarial models, no lymphoproliferation could be measured by stimulation with Ad or larval worm extracts (13, 20, 21, 40).

Pleural cytokines and eotaxin reach higher concentrations in C57BL/6 mice than in BALB/c mice. We measured the cytokine content in the pleural fluid and the production of antigen-specific cytokines by PleC in vitro at D10 and D30 p.i. In the pleural wash fluid at D10 p.i., all cytokines were below their detection limit in both strains. However, in vitro cytokine production gave evidence of activation of some cell lines. Although at low levels, C57BL/6 PleC produced more IL-5 than those of BALB/c mice after Ad antigen stimulation (respectively, 18.6 ± 6.2 pg/ml versus 1.5 ± 1.4 pg/ml [$P = 0.004$]) and after ConA stimulation (58.8 ± 4.2 pg/ml versus 34 ± 6.2 pg/ml [$P = 0.026$]). Low levels of IFN-γ were detected sporadically from PleC stimulated with Ad antigen from both strains of mice (data not shown).

At D30 p.i., much higher pleural cytokine concentrations were observed in C57BL/6 mice than in BALB/c mice ($P < 0.01$ for IL-4, IL-5, and IL-10 and $P = 0.016$ for IFN-γ) (Table 1). However, in vitro cytokine production hardly differed in C57BL/6 and BALB/c mice. A low specific production of IFN-γ was detected only in BALB/c PleC after ConA stimulation, whereas similar production of Th2-type cytokines was observed in both strains: IL-10 was produced after L3 (lowest level), Ad, and ConA stimulation; IL-5 was produced after Ad and ConA stimulation; and IL-4 was produced after ConA stimulation.

Eotaxin, a chemokine known to attract eosinophils (24), was also found in significant larger quantities in the pleural fluid of infected C57BL/6 mice (60.1 ± 7.5 pM, compared to 31.7 ± 5.8 pM in BALB/c mice [$P = 0.04$]).

Higher cytokine levels and a steeper increase of antibody levels in the peripheral blood of C57BL/6 mice compared to BALB/c mice. In the sera of naive mice, only IL-5 was above the detection limit in C57BL/6 mice (32 ± 1.3 pg/ml). In infected mice, on D10 p.i., IL-4 was undetectable and levels of IL-5, IL-10, and IFN-γ in serum were similar in both groups: IL-5 was measured at 1.2 ± 0.4 pg/ml in the sera of C57BL/6 mice and at 2.4 ± 0.5 pg/ml those that of BALB/c mice; IL-10 was at 24 ± 5 and 19 ± 10 pg/ml, respectively; and IFN-γ was at 11 ± 1.7 and 8 ± 1.4 pg/ml, respectively. At D30 p.i., cytokine levels increased dramatically in the sera of C57BL/6 mice, in contrast to BALB/c mice ($P < 0.0001$ for IL-4, IL-5, and IFN-γ and $P = 0.0002$ for IL-10) (Table 1).

IgG1 and IgG2c or IgG2a (for C57BL/6 and BALB/c mice, respectively) at D10 p.i. were barely detectable in the sera of C57BL/6 mice but were detected in BALB/c mice (Fig. 6) ($P = 0.002$). The comparison between IgG2a and IgG1 titers indicated that at D10 p.i. BALB/c mice had a predominant Th1 polarization. Antibody titers were too low to reach a conclusion for the C57BL/6 mice. At D30 p.i., the antibody concentrations increased in both mouse strains. IgG1 levels were similar in C57BL/6 and in BALB/c mice, whereas the IgG2c concentration in C57BL/6 mice was higher than the IgG2a concentration in BALB/c mice ($P = 0.0047$). At this time of infection, both strains exhibited a mixed T-helper type response (Fig. 6).

DISCUSSION

Our observations underline the local and stage-specific character of the immune responses that accompany filarial development. These responses are observed early (at 60 p.i.) (Fig. 3 and 4), although briefly, in the lymph nodes that drain the site of inoculation, and they displayed a marked L3 specificity as assessed in BALB/c mice (Fig. 3). The responses are then found in the pleural compartment (Fig. 5; Table 1), as observed in several previous studies (2, 4, 37, 44, 48, 49).

It was expected that a stronger immune reaction in the C57BL/6 mice than in the BALB/c mice would account for the major filarial destruction that occurs starting after the first month of infection in the resistant strain (28, 29). In fact, the immune responses in the resistant mice were weak early in the

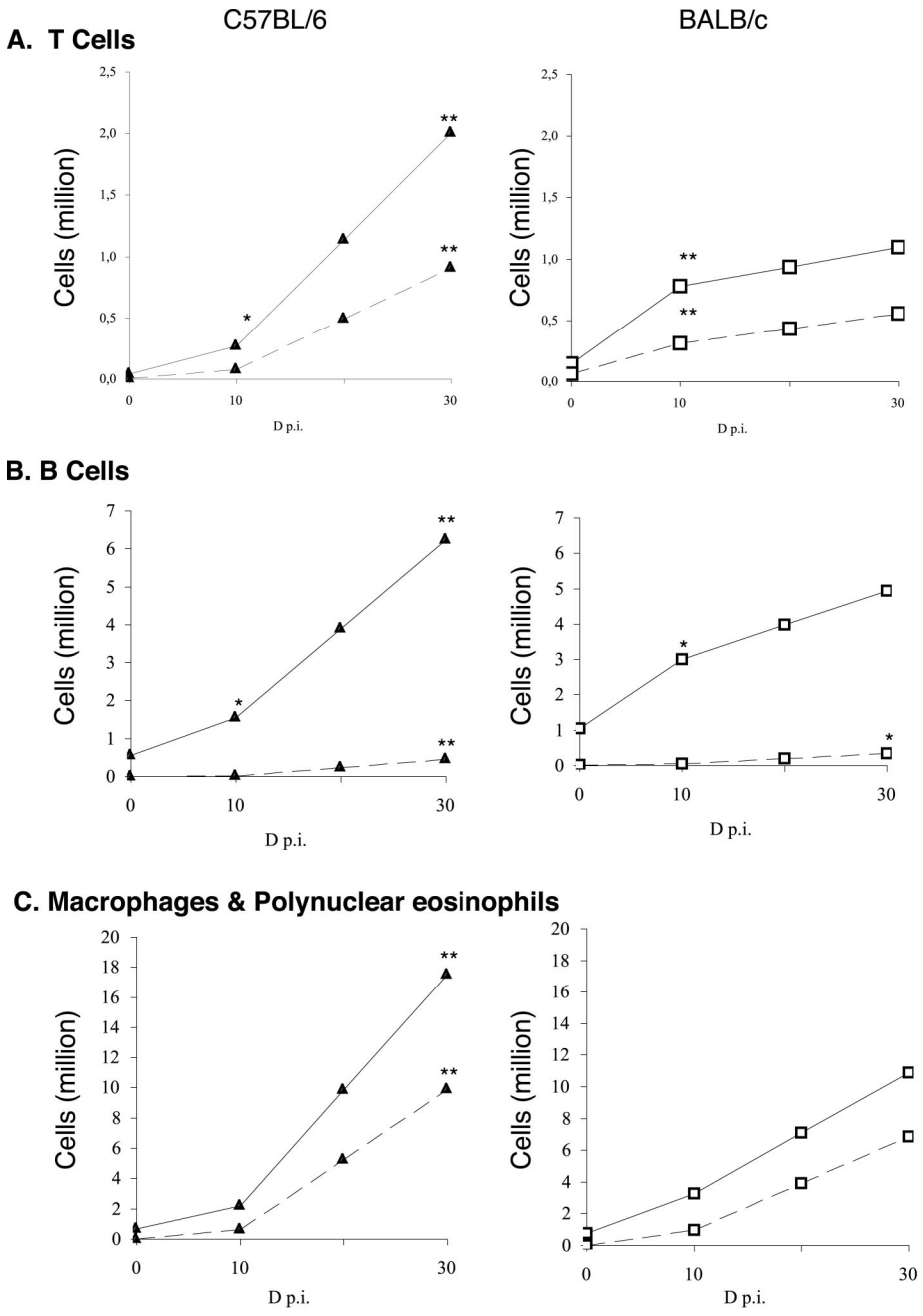

FIG. 5. Kinetics of pleural leukocyte accumulation during the course of infection. Cell numbers in C57BL/6 and BALB/c mice were assessed before infection (D0 p.i.) and at D10 p.i. and D30 p.i. (A) $CD4^+$ T cells (solid lines) and $CD8^+$ T cells (dashed lines); (B) B2 cells ($CD19^+$ $CD5^-$) (solid lines) and B1 cells ($CD19^+$ $CD5^+$) (dashed lines); (C) macrophages ($F4/80^+$) (solid lines) and eosinophils (dashed lines). For naive mice,C57BL/6 $n = 8$ and BALB/c $n = 14$; at D10 p.i., C57BL/6 $n = 6$ and BALB/c $n = 7$, and at D30 p.i., C57BL/6 $n = 6$ and BALB/c $n = 9$. Differences between consecutive time points were assessed. *, $P < 0.05$; **, $P < 0.01$ (Mann-Whitney U test).

infection and reached high intensities only on D30 p.i. (Fig. 3 to 5).

One month following inoculation, a strong mixed Th1-Th2 immune response was mounted against the worm in the resistant mouse strain, as indicated by elevated concentrations of cytokines in the pleural exudate and serum (Table 1), as well as specific antibodies (Fig. 6). Our results are in agreement with those of Le Goff et al. (28) in that the Th2 bias is less pronounced in the resistant C57BL/6 mice than in the susceptible BALB/c mice. Although the primary infection that we analyzed differs from the more complex situation in residents of areas of endemicity, who are repeatedly subjected to inoculation with infective larvae, a strong mixed response is one of the characteristic features observed in individuals putatively immune to onchocerciasis (23).

The parasitological development of the filarial worm can be

TABLE 1. Cytokine concentrations in pleural wash fluids and sera from C57BL/6 (B6) and BALB/c (Bc) mice at D30 p.i.[a]

Sample	Expt[a]	No. of mice (B6/Bc)	Concn (pg/ml)[b]							
			IL-4		IL-5		IL-10		IFNγ	
			B6	Bc	B6	Bc	B6	Bc	B6	Bc
Pleural wash fluid[c]	1	6/6	2.5	0.8	24.8	1.2	296.3	7.9	90.2	22.9
	2	6/6	1.9	0	216.9	2	18,142.6	0	309.6	36.2
Serum[d]	2	6/6	0.1	0	159	28	18,290	0	4,084	0
	3	8/8	8	0.2	102	36	15,165	525	2,660	265
	4	6/3	2.95	0	347	4.7	5,412	130	5,253	844

[a] Four experiments were performed, two for the pleural wash fluid and three for the serum; experiment 2 included both.
[b] Concentrations, measured by two-site ELISA, in infected mice, values for naive mice were subtracted.
[c] The combined results from the two experiments were statistically different ($P < 0.01$ for IL-4, IL-5, and IL-10 and $P = 0.016$ for IFN-γ) as calculated by the Mann-Whitney U test.
[d] The combined results from the three experiments were statistically different ($P < 0.0001$ for IL-4, IL-5, and IFN-γ and $P = 0.0002$ for IL-10) as calculated by the Mann-Whitney U test.

described in terms of a number of distinct parameters (34, 42). For those that we measured, we observed little variation between the two mouse strains: prevalence and average number of worms per mouse (Fig. 1), arrival of the first larvae in the pleural cavity on D4 p.i., all the larvae being at stage 4 on D10 p.i., and predominantly Ad worms on D30 p.i. (Fig. 2). However, the worms collected from the resistant C57BL/6 mice were significantly smaller whether at the larval or Ad stages (Fig. 2). In these mice the delay in development was also accompanied by a delay in molting; 33% of the worms were still at stage 4 on D30 p.i., compared to 13% in the susceptible BALB/c mice.

The kinetic analysis of the natural course of infection of *L. sigmodontis* and, concurrently, of the immune responses in the two mouse strains with contrasting susceptibilities provides insights into factors which influence important parameters of filarial development.

The fact that immune responses differ very early in the infection, being significantly weaker until D10 p.i. in C57BL/6 mice, although recovery rates are similar for the first month in the two mouse strains (Fig. 1) can seem paradoxical. Indeed, for lymph nodes specific cytokine production is very weak (Fig. 3) and lymphocytes display weaker proliferation in C57BL/6 mice (stimulation index of 100, compared to 400 in BALB/c

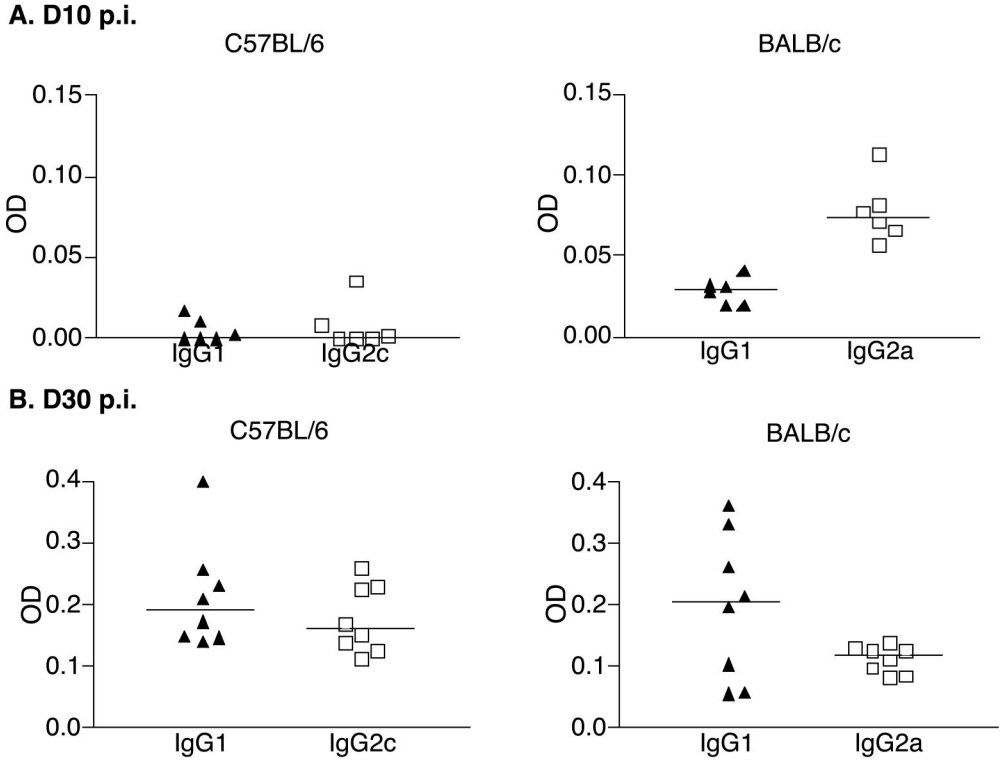

FIG. 6. Specific antibody isotype levels. Levels of IgG1, IgG2a (BALB/c), and IgG2c (C57BL/6) in C57BL/6 mice and BALB/c mice were determined at D10 p.i. and D30 p.i. Values are expressed in arbitrary units of optical density (OD). Horizontal lines represent medians.

mice) (Fig. 4), and in the pleural cavity leukocyte infiltration and in vivo cytokine production are close to the levels measured in naive mice (Fig. 5; Table 1). Studies with several other mouse strains, i.e., resistant B10D2 mice (34), wild-type and IL-5-overexpressing CBA/Ca mice (36), C57BL/6 IL-4 knockout mice (28) and IL-5 knockout mice (29), BALB/c μMt mice (in which B1 lymphocytes do not mature) (37), and finally anti-IL-5-treated BALB/c mice (35), showed that the early recovery rate of *L. sigmodontis* does not vary. Thus, at the onset of the primary infection, mouse responses have no major effect on the recovery rate. Previous investigations have shown that this recovery is essentially determined by the inflammatory events that are immediately triggered at the inoculation site (10, 34). These events are accompanied by a very marked subcutaneous infiltration by neutrophils (30, 37). We have previously proposed that the proportion of larvae that develop corresponds to the proportion of those that escape the inflammatory process by penetrating in the lymphatic vessels (7, 10). In humans the parasite is inaccessible to investigation, particularly at the onset of the infection. From our observations, which suggest that the immunological or genetic status of the host does not significantly affect early recovery, we predict that a similar proportion of larvae will develop in individuals residing in areas where filariae are endemic, including those considered putatively immune. Therefore, a stage older than the infective larvae may be the main target of protective immune responses in humans, given the rapid molting (2 to 3 days) of the filarial L3 larvae of the genus *Onchocerca* to stage 4 (9, 11).

In contrast to the recovery rate, the filarial growth parameter proved to be highly sensitive to the distinct immune responses which are linked to genetic diversity in the host species. The major histocompatibility complex background does not play an important role in the control of parasite development, since similar levels of *L. sigmodontis* growth retardation are observed in B10D2 (H2d haplotype as for BALB/c) mice (34) and in C57BL/6 (H2b haplotype) mice. However, a significant impact on the size of the filarial worm has been noted in infections of mice under precisely defined cytokine conditions: compared to controls, *L. sigmodontis* worms were larger on D10 p.i. in transgenic CBA/Ca mice that overexpress IL-5, whereas the filariae were smaller on D20 p.i. in anti-IL-5-treated BALB/c mice (35, 36). That cytokines may influence parasite development has precedence, as the absence of IL-7 has been shown to lead to *Schistosoma mansoni* dwarfism (45, 52). Thus, the results obtained for IL-5 in our *L. sigmodontis* infections are of particular interest in light of the contrasting responses in the BALB/c and C57BL/6 lymph nodes (Fig. 3). High-level L3-specific IL-5 production at the onset of the infection was associated with more rapid development to adulthood, suggesting that IL-5 or related immune responses may be advantageous to the worms. These data combined with the data above strongly implicate IL-5 as an immune signal for the parasite development. The lack of influence of an IL-5 knockout in C57BL/6 mice on filarial development lends support to this hypothesis (29). The production of IL-6 is also noteworthy. It is also produced in large quantities (1,200 pg/ml) solely by BALB/c LyNC following larval extract stimulation (Fig. 3), and the absence of this cytokine, which is associated with the inflammatory process, from similarly stimulated C57BL/6 LyNC is surprising. Whether IL-6 has an effect similar to that of IL-5 on the growth and development of *L. sigmodontis* remains to be determined. IL-4 could not be detected in our experiments, although in the *Brugia pahangi* model, the corresponding mRNA was found in the lymph nodes draining the inoculation site (15).

The expression of patency appears to be critically dependent on the survival of worms in the pleural cavity between D30 p.i. and D40 p.i. of the infection. At D30 p.i., and in both mouse strains, the leukocyte populations recovered from the pleural cavity have a similar composition (Fig. 5): macrophages, eosinophils, B1 and B2 cells, and CD4 and CD8 T cells. Production of cytokines from these cells is similar when they are stimulated in vitro, but paradoxically, when pleural exudates are analyzed, cytokine levels and, interestingly, that of eotaxin are significantly more elevated in C57BL/6 mice than in BALB/c mice (Table 1). Chemokines and cytokines might originate from cell types other than leukocytes, e.g., pleural mesoendothelial cells (5, 25, 38). However, the steep increase of the PleC between D10 p.i. and D30 p.i. in C57BL/6 mice (Fig. 5) might be sufficient to explain the high in vivo concentrations. In C57BL/6 mice, there are interesting analogies between the responses to the filarial infection that we observed on D30 p.i. and those to ovalbumin sensitization (39): IL-4, IL-5, and eosinophil infiltration levels in the pulmonary tissues are found to be higher in C57BL/6 mice than in BALB/c mice, whereas the in vitro splenic production of IL-4 and IL-5 is weaker than that in BALB/c mice. In the filarial infection, the initial factors that are responsible for the intense accumulation of leukocytes in C57BL/6 mice have not been identified. The molting of the filarial worm is considered to stimulate a protective response (17). In our case, the fourth molting could not be incriminated in the extraordinarily high cytokine levels in the C57BL/6 mice, since the proportion of worms that had molted at D30 p.i. was actually lower in this mouse strain. On the other hand, the duration of stage 4 was longer than that in BALB/c mice, and thus stage 4 could be implicated. The deletion of the IL-4 gene in C57BL/6 mice profoundly modifies both immune responses and filarial development, namely, polarization to Th1 and *L. sigmodontis* achievement of patency (28), whereas a deletion of the IL-5 gene did not improve survival (29), in contrast to observations for BALB/c mice (32, 48, 49). Thus, an effector role for eosinophils in worm destruction during primary infection seems to be better established in BALB/c mice than in C57BL/6 mice.

Although the role of neutrophils has been demonstrated, albeit at later stages of the infection (3, 44, 49), this cell type was absent from the pleural cavity during the first month of the infection in both mouse strains. It is this population which responds fastest to intrusions into the subcutaneous tissues (e.g., inoculation of L3 or RPMI [30, 35]) and into the peritoneal cavity (e.g., inoculation of L3, RPMI, or PBS [unpublished observations]). The absence of neutrophils in the pleural cavity thus suggests that under natural conditions where the larvae migrate through the lymphatic vessels (10, 51), access to the pleural cavity is achieved in such a way as to avoid an early recruitment of neutrophils.

In conclusion, we have demonstrated that parasitological and immune differences distinguish the early courses of *L. sigmodontis* infection in refractory and susceptible mice. We propose that these are at the basis of major differences in the

immune destruction of worms, observed later in the infection, which lead to the absence of microfilariae in the circulation. The association of susceptibility and the strong production of IL-5 in response to larvae very early suggest a parasite strategy to use a specific immune response to its own profit. On the other hand, the key events which determine resistance appear to be those which underlie the lack of detectable immune responses 60 h following inoculation and/or those which lead to the steep late increase of specific immune responses. Further studies can now focus on the identification of specific L3 larval antigens, such as those encoded by the abundant larval transcripts alt-1 and alt-2 (22), and L4 larval antigens, which might be implicated in these two events in resistant and susceptible mice, respectively.

ACKNOWLEDGMENTS

This work was supported by European Community grant ICA4-CT-1999-10002. M. Korenaga was supported by the overseas research program and a grant-in-aid (no. 14570216) of the Japanese Ministry of Education, Science, Technology, Culture and Sport and by grants from the President of Kochi Medical School.

We thank Geneviève Milon (Pasteur Institute) for her comments and suggestions on this work, T. J. Williams (Imperial College School of Medicine, London, United Kingdom) for providing the eotaxin assay to C.M., and an anonymous referee for constructive comments.

S. Babayan and M.-N. Ungeheuer contributed equally to this work.

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Editor: W. A. Petri, Jr.