Loss of Complement Activation and Leukocyte Adherence as *Nippostrongylus brasiliensis* Develops within the Murine Host

Paul R. Giacomin,1 Hui Wang,1 David L. Gordon,2 Marina Botto,3 and Lindsay A. Dent1*

School of Molecular and Biomedical Science, University of Adelaide, Adelaide, Australia;1 Department of Microbiology and Infectious Diseases, Flinders Medical Centre, Adelaide, Australia;2 and Rheumatology Section, Imperial College, Hammersmith Campus, London, United Kingdom3

Received 19 April 2005/Returned for modification 27 May 2005/Accepted 10 August 2005

Complement activation and C3 deposition on the surface of parasitic helminths may be important for recruitment of leukocytes and for damage to the target organism via cell-mediated mechanisms. Inhibition of complement activation would therefore be advantageous to parasites, minimizing damage and enhancing migration through tissues. The aim of this study was to determine ex vivo if complement activation by, and leukocyte adherence to, the nematode *Nippostrongylus brasiliensis* change as the parasite matures and migrates through the murine host. Pathways of activation of complement and the mechanism of adherence of leukocytes were also defined using sera from mice genetically deficient in either C1q, factor B, C1q and factor B, C3, or C4. Substantive deposition of C3 and adherence of eosinophil-rich leukocytes were seen with infective-stage (L3) but not with lung-stage (L4) larvae. Adult intestinal worms had low to intermediate levels of both C3 and leukocyte binding. For L3 and adult worms, complement deposition was principally dependent on the alternative pathway. For lung-stage larvae, the small amount of C3 detected was dependent to similar degrees on both the lectin and alternative pathways. The classical pathway was not involved for any of the life stages of the parasite. These results suggest that in primary infections, the infective stage of *N. brasiliensis* is vulnerable to complement-dependent attack by leukocytes. However, within the first 24 h of infection, *N. brasiliensis* acquires the ability to largely avoid complement-dependent immune responses.

The rapid recruitment of leukocytes to infected tissues is a crucial early step in resistance to parasitic helminths. In order to damage the target organism, many effector cells make close contact with the parasite and this can occur via complement-and antibody-dependent mechanisms (16, 29, 47). Although other mediators may also play minor roles (4, 37, 45), in the absence of parasite-specific antibody and especially in the early stages of primary infection, complement is likely to be the most important factor facilitating leukocyte recruitment and attachment to the parasite. C3b and its cleavage products facilitate attachment of leukocytes to the helminth, while C3a and C5a may act as early chemotactic factors. Eosinophils are major effector cells in host resistance to infection with a range of parasitic helminth species (14, 38, 44, 47) and adherent eosinophils release products that can damage, immobilize, and/or kill the parasite (6, 10).

Using physical or chemical strategies to deplete complement activity in vitro, parasites have been shown to activate complement by either the alternative or classical pathway (19, 28, 31, 36, 42). Although sera from humans and animals with spontaneous mutations in genes encoding individual complement proteins have also been used in studies with parasites (32, 40, 50), it has not previously been possible to comprehensively analyze each complement pathway in a single host species.

A number of pathogenic species of bacteria, viruses, and fungi have evolved strategies to evade activation of comple-

---

* Corresponding author. Mailing address: School of Molecular and Biomedical Science, University of Adelaide, North Terrace, Adelaide, South Australia, Australia 5005. Phone: 61 8 8303 4155. Fax: 61 8 8303 7532. E-mail: lindsay.dent@adelaide.edu.au.
large numbers and within 2 hours of infection, many are strongly adherent to the larvae (9). Larvae are trapped in the skin for an extended period and, relative to wild-type mice, few reach the lungs (9). However, those L3 that escape the skin and migrate to the lungs to develop into L4, do not elicit a strong inflammatory response, and for at least 24 to 48 h postinfection, few eosinophils are either recruited or adhere to lung-stage larvae (9).

In the first stage of this study we determined that N. brasilienensis develops a strategy to resist complement-dependent immunity as it matures from L3 to L4. C3 deposition and eosinophil-rich leukocyte adherence were compared on infective-stage L3 collected from in vitro cultures with parasites recovered from the skin, the lungs, and the small intestine. In the second stage of this study, we used sera from mice genetically deficient in either C1q, factor B, C1q and factor B (dou-

**TABLE 1. Dimensions and surface area of N. brasiliensis L3, L4, and adult worms**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Infective-stage L3</th>
<th>Lung-stage L4</th>
<th>Adult worms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (mm)</td>
<td>0.78 ± 0.02</td>
<td>1.14 ± 0.02</td>
<td>3.92 ± 0.14</td>
</tr>
<tr>
<td>Diameter (mm)</td>
<td>0.04 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>Surface area (mm²)</td>
<td>0.10 ± 0.01</td>
<td>0.20 ± 0.01</td>
<td>1.36 ± 0.05</td>
</tr>
</tbody>
</table>

Approximate total surface area was estimated using parasite length and width measurements to calculate the area of the curved surface of a cylinder (i.e., surface area = π × length × diameter). Data are means ± SEM for 27 (L3), 22 (L4), or 23 (worms) samples.

(18) were used as indicated.

Preparation of larvae and worms. Infective-stage N. brasiliensis L3 were obtained from fecal culture after passage through female Hooded Wistar rats aged 6 to 8 weeks as previously described (9). L3 were used between 9 and 28 days after establishment of fecal cultures. L3 were washed three times in phosphate-buffered saline (PBS), pH 7.4, and concentrated by centrifugation at 50 × g for 5 min. Larvae were then resuspended in PBS (immunofluorescence studies) or in culture medium (leukocyte coculture). Skin air pouches were generated as previously described (9). Aliquots (30 l) of approximately 120 L3, 60 L4, or 8 worms in 10-ml plastic test tubes. Unbound serum components were then removed by 10 washes with 200 l of PBS/0.05% vol/vol Tween 20 (PBST) in 96-well plates using a multichannel pipettor, or by two washes in 5 ml PBS where test tubes were used. Parasites were allowed to settle under unit gravity (96-well plates) or were centrifuged at 18 × g for 5 min (test tubes) between each wash. Parasites were then incubated with 50 l of a 1:50 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse C3 antibody (ICN/Cappel, catalog no. 55510) for 1 h at room temperature in the dark. Unbound antibody was removed by washing as described above. Finally, parasites were resuspended in 200 l of 25% vol/vol glycerol in PBS in 96-well plates, or where parasites were incubated in test tubes, aliquots of 200 l/well of parasite suspensions were added to the wells of a flat-bottomed, solid black 96-well plate (Costar, catalog no. # 3915, Corning Inc.).

**Measurement of fluorescence intensity.** Fluorescence in individual wells of solid black or clear 96-well microtiter plates was quantitated in arbitrary units with a Molecular Imager FX phosphorimager (Bio-Rad USA, catalog no. 170-7800) as described previously (19). In some experiments, fluorescence intensity per parasite was calculated to reduce variation due to differences in the exact number of parasites per well.

**CFSE labeling of leukocytes.** Peritoneal cells from male or female heterozygous interleukin-5 (IL-5) transgenic C57BL6 or CBA/Ca mice (15), aged 7 to 10 weeks, were used as an eosinophil-rich (55 to 75%) source of leukocytes. Mice were killed by carbon dioxide asphyxiation and peritoneal cells were harvested with lavage with mouse-mossmaly PBS (MPBS) (43). Cells were washed twice in MPBS, twice in RPMI 1640 containing 0.1% vol/vol fetal calf serum and then resuspended in RPMI 1640/0.1% fetal calf serum at 10⁶ cells/ml. Cells were incubated in 2.5 l M CFSE for 10 min at 37°C in 5% CO₂ and excess CFSE was removed by washing in culture medium.

**Analysis of leukocyte adherence to parasites.** Adherence of CFSE-labeled leukocytes to parasites was quantitated using an adaptation of methods described previously (19). Aliquots (30 l) of approximately 120 L3, 60 L4, or 8 worms in

---

**MATERIALS AND METHODS**

**Animals.** Mice were bred in-house under clean barrier conditions at the University of Adelaide and Imperial College, London, and handled according to institutional animal ethics committee guidelines.

**Culture medium and sera.** Leukocytes were cultured with parasites in RPMI 1640 culture medium (Institute of Medical and Veterinary Science, Adelaide, Australia) supplemented with 2 mM l-glutamine, 12 μg/ml gentamicin, 16 μg/ml penicillin, and 10% heat-inactivated (56°C for 30 min) fetal calf serum (MULTISER, Trace Biosciences, Sydney, Australia). Serum was collected from mice by cardiac puncture under pentobarbitone sodium anesthesia. Where indicated, serum complement activity was selectively inhibited by heating for 30 min at 56°C (heat-inactivated mouse serum [HMS]). Sera from wild-type CBA/Ca and C57BL/6 mice (normal mouse serum [NMS]) and from C57BL/6 mice deficient in either C1q, factor B, C1q and factor B (double mutant), C3, or C4 to determine that the pathways of complement activation differ as the parasite matures and migrates through the murine host.

Infective-stage L3 larvae (9). However, those L3 that escape the skin and migrate to the lungs to develop into L4, do not elicit a strong inflammatory response, and for at least 24 to 48 h postinfection, few eosinophils are either recruited or adhere to lung-stage larvae (9).

In the first stage of this study we determined that N. brasilienensis develops a strategy to resist complement-dependent immunity as it matures from L3 to L4. C3 deposition and eosinophil-rich leukocyte adherence were compared on infective-stage L3 collected from in vitro cultures with parasites recovered from the skin, the lungs, and the small intestine. In the second stage of this study, we used sera from mice genetically deficient in either C1q, factor B, C1q and factor B (double mutant), C3, or C4 to determine that the pathways of complement activation differ as the parasite matures and migrates through the murine host.

In the first stage of this study we determined that N. brasilienensis develops a strategy to resist complement-dependent immunity as it matures from L3 to L4. C3 deposition and eosinophil-rich leukocyte adherence were compared on infective-stage L3 collected from in vitro cultures with parasites recovered from the skin, the lungs, and the small intestine. In the second stage of this study, we used sera from mice genetically deficient in either C1q, factor B, C1q and factor B (double mutant), C3, or C4 to determine that the pathways of complement activation differ as the parasite matures and migrates through the murine host.
culture medium were mixed with 100 μl of CFSE-labeled leukocytes (10⁶ total) and 70 μl of either culture medium, NMS, ΔMS, or complement deficient serum (7% final concentration) was then added. After incubation at 37°C in 5% CO₂ for 2 h, unbound cells were removed by nine washes in MPBS and fluorescence was measured using the phosphorimagery, as described above. UV microscopy was used to visualize fluorescent cells attached to parasites.

Statistical analysis. Data for individual experiments, with each sample assayed in duplicate or triplicate are presented as mean ± standard error of the mean and were analyzed using Student’s unpaired t test and GraphPad Prism software (version 3.03, GraphPad Software Inc.).

RESULTS

Detection of C3 on N. brasiliensis. The extent of C3 deposition on L3 collected from fecal culture plates was compared to that for L4 recovered from the lungs of CBA/Ca mice either 24 or 48 h postinfection and to adult worms recovered 7 days postinfection from the small intestine. Using immunofluorescence microscopy, it was apparent that C3 deposition in the presence of NMS from CBA/Ca mice was much greater on L3 than on L4 collected either 24 or 48 h after infection (Fig. 1a [i to iii]). C3 was deposited at very high levels over the entire surface of L3. In contrast, the small proportion of L4 that did exhibit some labeling had a very different and irregular pattern of deposition (Fig. 1a [ii and iii]). Intermediate but evenly distributed C3 deposition was observed on intestinal worms (Fig. 1a [iv]).

Quantitative analysis of C3 deposition (i.e., immunofluorescence-total parasite surface area) confirmed the large differences in C3 binding between L3 and L4 opsonized with NMS (Fig. 1b). Larvae typically showed relatively high autofluorescence. Nevertheless, the level of fluorescence detected on L4 incubated with NMS was no greater than that seen with L4 opsonized with serum heat-treated to inactivate C3 (i.e., ΔMS). There were no qualitative or quantitative differences between 24- and 48-h lung larvae. Adult worms showed significant levels of C3 deposition after opsonization with NMS (Fig. 1b), but not at the same intensity as seen on L3. Host-derived C3 was not detected on either L4 or intestinal worms, as fluorescence of PBS-opsonized parasites exposed to anti-C3 antibody was no greater than background (i.e., where anti-C3 antibody was omitted).

To determine if inhibition of C3 deposition occurred soon after infection and at a time when many larvae are migrating to other tissues, we assessed fixation of endogenous and exogenous C3 on parasites recovered from skin air pouches 30 to 150 min after injection. It was difficult to accurately count and dispense larvae recovered from the skin because they tended to coalesce and so quantitative analysis was not performed. However, when assessed visually, skin larvae recovered 30 min postinjection demonstrated clear evidence of in vivo C3 deposition (Fig. 1c [iv]). When subsequently opsonized with NMS, further increases in C3 deposition were evident (Fig. 1c [v]) similar to that seen on L3 from cultures (Fig. 1c [iii]). Skin larvae recovered 150 min postinfection were comparable to those retrieved after only 30 min (data not shown).

Adherence of leukocytes to N. brasiliensis. The adherence of eosinophil-rich CFSE-labeled peritoneal leukocytes to L3, L4 and intestinal worms opsonized with serum was determined. Large numbers of leukocytes adhered to the surface of NMS-opsonized L3 (Fig. 2a [i]), however, few if any cells bound to similarly treated lung larvae (Fig. 2a [ii and iii]). In samples stained with Vital New Red and Alcian Blue, 92% of leukocytes in the first layer of cells adhering to L3 were eosinophils, with some macrophages (6%) and the occasional mast cell also present. Quantitative analysis of leukocyte adherence using a phosphorimagery clearly demonstrated the disparity between leukocyte adherence on L3 and L4 (Fig. 2b).

Large numbers of CFSE-labeled leukocytes bind to L3 and, as reported previously (19), visual assessment revealed that this was reduced to negligible levels when ΔMS or culture medium were substituted for NMS (Fig. 2b and data not shown), indicating that leukocyte adherence was complement mediated. In contrast, few leukocytes adhered to either 24- or 48-hour L4, regardless of the opsonization conditions (Fig. 2b). Some leukocytes adhered to intestinal worms, but unlike L3, this was largely restricted to the posterior end (Fig. 2a [iv]). When assessed quantitatively, leukocyte binding to adult worms was at most only modest (Fig. 2b) but decreased significantly if ΔMS was used instead of NMS, indicating that it was also complement-mediated.

C3 deposition and leukocyte adherence on N. brasiliensis L3 are largely dependent on the alternative complement pathway. Until recently, it has only been possible to use physical or chemical methods of depleting complement activity and these are sometimes only partially effective or may not be specific for complement proteins. It is now possible to use sera from mice that have been genetically manipulated to be deficient in one or more proteins of the complement pathway. We performed a comprehensive analysis of C3 deposition and leukocyte adherence on infective-stage larvae in the presence of NMS and ΔMS from wild-type C57BL/6 mice and most particularly with sera from syngeneic mice genetically deficient in either C1q, factor B, Clq and factor B (double mutant), C3, or C4 (Fig. 3).

There was no significant difference in C3 deposition on L3 exposed to NMS or Clq-deficient serum (Fig. 3a), indicating that C1q and the classical pathway play no role in mediating C3 deposition on N. brasiliensis L3. In contrast, C3 deposition was almost completely abrogated in the absence of factor B (with and without Clq), indicating that the alternative pathway is paramount in this experimental system. A small amount of factor B-independent C3 binding was evident, since C3 deposition was significantly higher in the absence of factor B than it was when larvae were exposed to C3-deficient sera (C3⁻/⁻ and ΔMS). This is consistent with the observation that C3 deposition was slightly reduced when C4 was ablated, indicating a possible minor role for the C4-dependent lectin pathway.

The adherence of peritoneal leukocytes was similarly assessed in the presence of culture medium, NMS, ΔMS or sera from complement-deficient mice. C3 was required for adherence, which was not affected by an absence of C1q (Fig. 3b). However, adherence was significantly reduced in the absence of factor B, again highlighting the importance of the alternative pathway. There were no significant differences in leukocyte adherence with factor B-deficient serum when compared to sera deficient in C3 (C3⁻/⁻ and ΔMS) and in the presence of culture medium only. The absence of C4 had no effect on cell adherence, indicating that while the lectin pathway may contribute slightly to complement deposition on L3, for complement-mediated leukocyte adherence, the alternative pathway is sufficient.
Pathway of complement activation changes as *N. brasiliensis* matures. We next compared C3 deposition on L3, L4 (48 h postinfection), and adult worms 7 days postinfection (iv), opsonized with 20% NMS (47× magnification). C3 was detected using FITC-conjugated goat anti-mouse C3 antibody. (b) Quantitation of C3 on parasites using a phosphorimager. L3, L4 or adult worms were opsonized with PBS, ΔMS or NMS and then incubated with or without (no anti-C3) fluorescein isothiocyanate-conjugated anti-C3 antibody. C3 binding is expressed as mean fluorescence well ± standard error of the mean (n = 2 or 3). *, significantly greater than ΔMS control; #, significantly greater than L4 and adult worm plus NMS (P < 0.05), as determined by Student’s unpaired t test. (c) Representative fluorescence photomicrographs (47× magnification) demonstrating mouse C3 binding to infective-stage L3 directly from culture plates (i to iii) or skin air-pouch-derived larvae 30 min postinjection (iv to vi). Larvae were treated with NMS, ΔMS, or PBS as indicated.
iment (Fig. 4a), the minor contribution from the lectin pathway was not evident. When comparing sera from CBA/Ca and C57BL/6 mice, we noticed a higher level of C3 deposition on L4 treated with NMS from the latter (mean fluorescence/well ± standard error of the mean, CBA/Ca versus C57BL/6, 3.29 ± 0.09 × 10^3 versus 6.72 ± 0.38 × 10^3 respectively, P < 0.001, n = 3). Interestingly, this difference was not evident when screening L3 (mean fluorescence/well ± standard error of the mean, CBA/Ca versus C57BL/6, 1.25 ± 0.08 × 10^4 and 1.29 ± 0.02 × 10^4 respectively, not significant, n = 3). Although there was a measurable difference in the level of complement activation on L4 treated with sera from these two strains, this was not associated with differences in the numbers of either lung larvae (day 2) or worms (day 7) recovered (Mean no. of parasites ± standard error of the mean for C57BL/6 and CBA mice [n = 3 to 4] respectively. L4: 323 ± 47 versus 289 ± 44, P > 0.05. Intestinal worms: 254 ± 99 versus 164 ± 33, P > 0.05.).

Although still modest compared to C3 fixation on L3 analyzed in the same experiment (Fig. 4a), the complement deposition on L4 treated with NMS from wild-type C57BL/6 mice was greatly diminished when sera from mice deficient in either factor B or C4 were used (Fig. 4b), indicating that both the alternative and lectin pathways contribute to C3 activation on lung-stage larvae. In three separate experiments, C3 deposition on L4 was significantly less with serum from C4−/− than from factor B−/− mice (Fig. 4b and data not shown) and so for lung-stage larvae, the lectin pathway may be at least marginally more important. The pathway of complement activation for adult worms was similar to that for L3, since deposition of C3 was in both instances significantly reduced in sera from factor B−/− mice (Fig. 4c). For adult worms, some C3 deposition would appear to be independent of the alternative pathway. For all of the life stages assessed, the classical pathway (C1q) was not involved.

**DISCUSSION**

Evasion of potentially damaging host immune responses is essential for helminths to be successful parasites. In one sense, the parasite is likely to be at its most vulnerable upon entry into the host, principally because it must quickly change from a form suited either to a free-living stage or to residence in another host. Conversely, a naïve host is particularly vulnerable in the first few days of infection, since it is totally or largely dependent on innate mechanisms of resistance. In this and our previous studies (9, 14, 19), we have shown the infective-stage L3 of *N. brasiliensis* to be extremely vulnerable to complement-dependent attack by eosinophils. Complement activation on *N. brasiliensis* L3 occurs primarily via the alternative pathway. However within the first 24 h of infection, during maturation
from L3 to lung-stage L4, *N. brasiliensis* acquires the ability to evade complement-dependent immune responses. This may explain why the inflammatory response at the site of initial infection, the skin, is rapid and substantive, whereas that in the lungs is slow to develop. The eosinophil chemotactic factors C3a and C5a may be responsible for rapid recruitment of eosinophils into the skin, but are less likely to be available when the lungs become infected. Interestingly, there are major changes in the pathways of complement activation as *N. brasiliensis* matures, with the lectin pathway becoming more important for the limited levels of complement activation on lung-stage larvae.

Innate resistance of wild-type mice to primary infection with *N. brasiliensis* is typically very low for most strains, with a large proportion of the infecting dose (>50%) migrating through the lungs and colonizing the small intestine (14). In addition, since the parasite has a very short transit time through the host (approximately 9 to 14 days), the life cycle is completed before an effective adaptive immune response can be generated. Our studies suggest that provided sufficient and appropriate effector cells can be recruited, innate resistance against *N. brasiliensis* is very effective (9, 14). Eosinophils appear to be a potent force in trapping larvae in the skin in primary infections (9) and this study indicates that C3 is almost exclusively responsible for facilitating stable attachment of these and other leukocytes to the target. Our data also suggest that complement may be important for recruitment of leukocytes, since in the absence of C3 deposition on L4 (Fig. 1), few leukocytes are to be found in the lungs until after the majority of larvae have migrated to the gut (7, 9, 48). Although chemokines such as the...
is purely speculative. However, complement inhibitors (host or parasite derived) may be more active with CBA serum and/or in vivo in both strains. C57BL/6 serum may have more available C3, but this seems unlikely, since these differences were not evident when L3 were tested. Importantly, we and others have not detected major differences in parasite burdens when infections in CBA and C57 strains are directly compared (12, 46).

In conclusion, we have demonstrated that infective-stage larvae of *N. brasiliensis* activate the alternative pathway of complement, mediating a high level of leukocyte adherence to these larvae. Complement fixation at this stage of the parasite's life cycle may also be important for recruitment of eosinophils and other leukocytes into the initial site of infection, the skin.Entrapment of larvae may then follow, provided that sufficient effector cells are present. However, within 24 h of infection, the parasite develops the capacity to inhibit deposition of C3, and this could explain the relative absence of an early inflammatory response to L4 in the lungs. In acquiring resistance at this level, larvae may more readily migrate through tissues to undergo further maturation. Interestingly, there is a shift in pathways through which the small amount of complement is activated by lung-stage L4, with the lectin pathway now being a relatively major contributor. As the parasite enters the gut and undergoes further maturation to the adult worm stage, the alternative pathway again becomes more important for complement activation. However, both innate and early adaptive immune responses may be of less consequence to a parasite that resides in the lumen of the gut.

Although we have established in vitro that complement is at least transiently important, a critical role in innate resistance to parasitic helminths in vivo has yet to be conclusively determined. More definitive proof will require investigations of the kinetics of infections in the various complement-deficient mice described here.

**ACKNOWLEDGMENTS**

This work was supported in part by the National Health and Medical Research Council (Australia) and School and Faculty Strategic Research Funds from the University of Adelaide.

We thank Bruce Lyons for assistance in establishing CFSE labeling techniques.

**REFERENCES**


