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Complement-dependent killing of *Nippostrongylus brasiliensis* infective larvae by rat alveolar macrophages

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SUMMARY

Histopathological studies have provided circumstantial evidence that helminth parasite destruction occurs in the lung; however controlled *in vitro* studies on the helminthocidal activity of lung cells have not been reported. This study presents evidence that *Nippostrongylus brasiliensis* infection in the rat induces alterations in broncho-alveolar lavage (BAL) cell numbers, differential counts, and *in vitro* helminthocidal activity. Normal, uninfected rats yielded $3{\cdot}3 \pm 0{\cdot}6 \times 10^6$ BAL cells/rat, consisting predominantly of alveolar macrophages (>90%). However on days 2–8 post-infection there was a 1·5—2·4-fold increase in BAL cell numbers with a significant neutrophilia on day 2 and a significant increase in the absolute number of all cell types on day 8. On day 32 post-infection, BAL cell numbers had returned to control levels. Normal BAL cells neither adhered to nor killed *N. brasiliensis* infective larvae (L3) in the presence of rat complement. By contrast BAL cells recovered from infected rats on days, 2, 8 or 32 post-infection (D2, D8 and D32 BAL cells, respectively) adhered under similar conditions. However, only D8 and D32 BAL cells killed L3. This complement-dependent killing correlated with significantly increased numbers of C3 receptor bearing alveolar macrophages in D8 and D32 BAL cells. Complement-dependent alveolar macrophage helminthocidal activity may therefore play an important role in lung resistance against resident or migrating helminths.

Keywords helminthocidal alveolar macrophages complement receptors *Nippostrongylus brasiliensis*

INTRODUCTION

There is a paucity of information on lung helminthocidal mechanisms despite the fact that various helminths confront the lung in many host-parasite systems. Circumstantial evidence from histopathological studies suggests that larvae of *Nippostrongylus brasiliensis* (Taliaferro & Sarles, 1939), *Dictyocaulus viviparus* (Jarrett & Sharp, 1963), *Trichinella spiralis* (Boyer *et al.*, 1971) and schistosomules of *Schistosoma japonicum* (Davis, Hsu & Hsu, 1963) and *S. mansoni* (von Lichtenberg, Sher & McIntyre, 1977) are killed in the lungs of infected hosts under certain conditions. Recently, Urban & Tromba (1980) reported that *Ascaris suum* second stage larvae incubated with swine alveolar macrophages were delayed in their development.

While considerable data have been accumulated on the helminthocidal activities of various effector cells from the peripheral blood and peritoneal cavity (Capron *et al.*, 1975; Dean, Wistar & Murrell, 1974; McLaren & Ramalho-Pinto, 1979; Mackenzie *et al.*, 1980; Ellner *et al.*, 1982), the relevance of such data to the events in the lung is not known. This is a significant point given

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considerable evidence that leucocytes from different anatomical compartments are functionally distinct (Walker, 1976; Ryning, Krahenbuhl & Remington, 1981). Using the rat–*N. brasiliensis* model, it has been shown that rat peritoneal macrophages adhere to and kill *N. brasiliensis* infective larvae (L3) in the presence of complement (Mackenzie *et al.*, 1980). We have used broncho-alveolar lavage (BAL) to investigate whether leucocytes from the rat lung air spaces are also helminthocidal to *N. brasiliensis* L3, since these cells may come into direct contact with the larvae during parasite migration through the lung.

MATERIALS AND METHODS

Animals. Outbred female Sprague-Dawley rats weighing 175–200 g (Charles River Breeding Laboratories, Montreal, Canada) were used as source of sera and cells, and for maintenance of *N. brasiliensis*. Inbred female CBA/J mice from Jackson Laboratories, Bar Harbor, Maine, USA were used in experiments to assess the infectivity of larvae treated with cells and sera.

Serum. Serum was obtained from cardiac blood of normal rats. When necessary, normal rat serum (NRS) was treated in one of the following ways: heated at 56°C for 1 h; incubated with 15 mg/ml zymosan (Sigma Chemical Co., St Louis, Missouri, USA) at 37°C for 1 h after which it was separated from zymosan by centrifugation at 1,500*g* for 5 min at 4°C; incubated with 20 u/ml *Naja naja kaouthia* cobra venom factor (CoVF) (Cordis Laboratories, Inc., Miami, Florida, USA) at 37°C for 1 h; mixed with EDTA or EGTA with or without Mg^{2+} ions to a final chelator and divalent cation concentration of 10mM.

Media. Phosphate-buffered saline (PBS) was used for washing the parasites and for BAL. Hanks' balanced salt solution with 25mM HEPES, pH 7·4, 295 ± 5 mOsm/kg H_2O, and containing 10% heat-inactivated agammaglobulinaemic horse serum (AHS), 200 u/ml penicillin and 200 μg/ml streptomycin, was used for peritoneal lavage, RPMI 1640 (GIBCO Ltd., Grand Island, New York, USA) similarly buffered and supplemented, was used for cell-parasite cultures.

Parasite maintenance and preparation for infection and in vitro *assays. N. brasiliensis* L3 were cultured from the faeces of infected rats as described previously (Love & Ogilvie, 1975). Eight to fourteen day old L3 collected with a Baermann apparatus were washed three times in sterile PBS, once with 6·7 mM sodium hypochlorite for 15 min and three times with sterile PBS containing 200 u/ml penicillin and 200 μg/ml streptomycin. The parasite suspension was adjusted to 500 L3/ml for cell–parasite cultures and 3,000 L3/ml for infection of rats; each rat received 3,000 L3 subcutaneously as described by Love & Ogilvie (1975).

BAL. Broncho-alveolar cells were obtained under sterile conditions by a modification of the technique described by Brain & Frank (1973). Briefly, rats were anaesthetized by intraperitoneal sodium pentobarbital and exsanguinated. The trachea was cannulated with polyethylene tubing and the lungs were washed with 10 5 ml aliquots of PBS (37°C). The recovered lavage fluid was then transferred into sterile 50 ml polypropylene tubes and centrifuged at 200*g* at 4°C for 10 min. The pellet of broncho-alveolar cells from individual rats were resuspended and washed twice in RPMI medium. Contaminating erythrocytes, if present, were lysed by 154 mM ammonium chloride. Cell counts and viabilities were determined using 0·4% trypan blue.

Peritoneal lavage. Resident peritoneal cells were obtained from non-infected rats as already described (Mackenzie *et al.*, 1980); the only modification was that 50 ml of ice cold Hanks' medium prepared as described above was used. The peritoneal exudate cells were washed twice in RPMI medium and incubated in 60 × 15 mm plastic tissue culture Petri dishes (Falcon Plastics, Oxnard, California, USA) for 2 h at 37°C in a 95% air/5% CO_2 humidified incubator to remove adherent cells. The non-adherent cell population was washed twice in RPMI medium and 1 ml (10×10^6 cells/ml) was overlaid onto 2 ml of 17% metrizamide in Hanks' medium. After centrifugation at 350*g* at 4°C for 15 min the interface band, consisting of 90–96% mononuclear cells (NPM), was recovered and washed twice in RPMI medium.

Definition of cell types. Cytocentrifuge smears were stained with Diff-Quik (Dade Diagnostic, Inc., Aguada, Puerto Rico) and differential counts made on the basis of morphology.

Cell–parasite cultures. The BAL cells from normal, uninfected rats and from 2, 8 and 32 day

post-infection rats (D2, D8 and D32 BAL cells, respectively) and the non-adherent peritoneal mononuclear macrophages (NPM) were adjusted to 8×10^6/ml. Into the well of a flat bottomed, 96 well Micro Test II tissue culture plate (Falcon Plastics) were added 100 μl of intact or treated NRS, 100 μl of extensively washed L3 (500/ml) and 100 μl of the appropriate effector cells. Control wells contained L3 and sera alone, or L3 and medium alone. The cell–parasite cultures were placed in a 37°C humidified 95% air/5% CO_2 incubator for 18 h. Cell adherence was scored by counting all the L3 in each well and recording the proportion that had 10 or more adherent cells. Parasite death was scored by counting all the L3 in each well and recording the proportion of straight and immobile L3 which in our hands correlated well with reduced infectivity *in vivo* as described (Mackenzie *et al.*, 1980).

Preparation of EAC reagents and EAC rosettes. For complement receptor assays, reagents and solutions of veronal-buffered saline containing gelatin and EDTA (GVBE) or Ca^{2+} and Mg^{2+} ions (GVBS) were prepared according to Ross (1981). Sheep erythrocytes (E) (Qualicum Scientific Co., Ottawa, Canada) were washed and resuspended to 5% vol./vol. in GVBE. Antibody and complement sensitized erythrocytes (EAC) were prepared using a subagglutinating dilution of rabbit IgM anti-E antibody (Cappel Laboratories Inc., Cochranville, Pennsylvania, USA) in GVBE and NRS optimally diluted in GVBS. One hundred microlitres of 1% vol./vol. EAC in GVBS was added to 100 μl of NPM, normal, D2, D8 or D32 BAL cells (4×10^6/ml) in 75 × 15 mm polypropylene tubes, and the mixture centrifuged at 70*g* for 10 min at 4°C. The pellets were incubated at 37°C for 15 min. After resuspension, the cells were evaluated for EAC rosettes using the new methylene blue stain (J. T. Baker Chemical Co., Phillipsburg, New Jersey, USA). Two hundred NPM or alveolar macrophages were counted and the number of cells with three or more erythrocytes attached was recorded. Specificity controls included cells mixed with E, EA, or EA incubated with NRS previously treated with zymosan, CoVF, or heat.

Statistics. The Student's *t*-test for unpaired observations was used in the statistical analysis of the results.

RESULTS

Broncho-alveolar cell recoveries

Upon lavage, normal uninfected rats yielded $3{\cdot}3 \pm 0{\cdot}6 \times 10^6$ BAL cells, over 90% of which were alveolar macrophages; neutrophils and lymphocytes constituted less than 10% of the BAL cells (Table 1). Mast cells and eosinophils were rarely encountered and constituted < 1% of BAL cells from uninfected rats. *N. brasiliensis* infection resulted in alterations in recoverable BAL cell numbers and differential counts. On day 2 post-infection, there was a 1·5-fold increase in the number of recoverable BAL cells, with a significant neutrophilia. On day 8 of infection, there was a 2·4-fold increase in recoverable BAL cells; all cell types were significantly increased in number as

Table 1. Total broncho-alveolar cell recoveries and differential counts from rats given a primary *N. brasiliensis* infection

Days post-infection	BAL cells/rat ($\times 10^6$)†	Differential (%)†			
		Macrophages	Neutrophils	Lymphocytes	Eosinophils
0*	3·3±0·6	95±2	3±0·8	2±1	<1
2	5·1±0·4§	72±4¶	22±4¶	3±0·7	3±1
8	7·9±1·2‡	92±1	4±1	3±0·7	2±0·5
32	4·0±0·7	88±2§	3±0·7	7±2	2±0·3

* Control uninfected rats.

† Mean ± s.e. of five to 20 experiments with 15–60 rats.

‡ $P < 0{\cdot}01$ *vs* controls; § $P < 0{\cdot}05$ *vs* controls; ¶ $P < 0{\cdot}001$ *vs* controls.

compared with those in uninfected controls. On day 32 of infection, the number of recoverable BAL cells had returned to normal levels. Mast cells from infected rats represented $< 1\%$ of the cells recovered on all days studied.

Complement-dependent killing of L3 by a peritoneal mononuclear cell population

Mackenzie *et al.* (1980) reported that *N. brasiliensis* L3 activated complement by the alternative pathway and that a non-adherent peritoneal mononuclear cell population (NPM) killed L3 by a complement-dependent mechanism. We have confirmed, using EDTA and EGTA treatments of NRS (Fine *et al.*, 1972) and immunofluorescence studies that *N. brasiliensis* L3 activates complement by the alternative pathway and that NPM adhered to and killed L3 in the presence of complement (data not presented). Of the NPM population $61 \pm 7\%$ ($n = 18$) expressed EAC (C3) receptors.

Complement-dependent adherence to, and killing of L3 by alveolar leucocytes

BAL cells were employed throughout these studies without prior separation. We scored both the adherence of BAL cells to L3 and L3 mortality at 18 h after incubation with NRS. While normal BAL cells did not adhere to L3 after 18 h, D2, D8, and D32 BAL cells adhered to L3 (Fig. 1). Treatment of the NRS with heat virtually abolished cell adherence (Fig. 1) and zymosan and CoVF had a similar effect. Immunofluorescence studies further demonstrated that adherence occurred only under conditions in which rat C3 was fixed on the surface of L3 (data not shown). When cytocentrifuge smears of L3 and the adherent cells were stained, it was found that in D2 BAL cells, the adherent cells were 72 ± 5 and $28 \pm 5\%$ macrophages and neutrophils respectively while in D8 and D32 BAL cells, the adherent cells were predominantly ($> 90\%$) macrophages.

When the helminthocidal activity of BAL cells was studied, it was established that normal and even D2 BAL cells were not helminthocidal in the presence of NRS. By contrast, D8 and D32 BAL cells killed *N. brasiliensis* L3 (Fig. 2). As with the adherence reaction, treatment of NRS which inactivated or depleted complement abolished the helminthocidal activity of D8 and D32 BAL cells. Since cell adherence at 18 h correlated with L3 mortality, and it was macrophages that predominated in the adherence reaction of D8 and D32 BAL cells, these results implicate the alveolar macrophage in complement-dependent *in vitro* killing of L3 by D8 and D32 BAL cells.

Complement receptor expression by alveolar macrophages

The adherence and helminthocidal activity of D8 and D32 BAL cells occurred only under

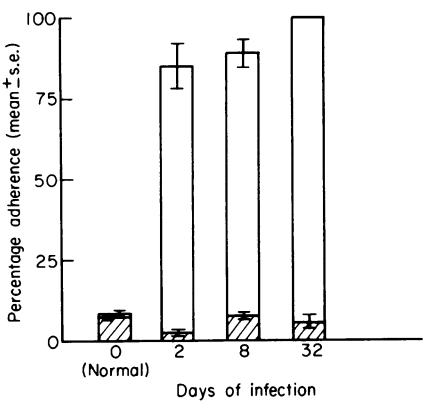

Fig. 1. Complement requirement for the adherence of BAL cells to *N. brasiliensis* L3. The results of adherence, scored as percentage L3 with ≥ 10 adherent cells, represent the mean $\pm$ s.e. of three to eight experiments carried out in triplicate at a cell to target ratio of 16,000:1 and at a final serum dilution of 1:3. Adherence for D8 and D32 BAL cells in the presence of zymosan or CoVF treated NRS was comparable to that in the presence of heated NRS. Adherence for D32 BAL cells in the presence of untreated NRS was consistently 100% in three experiments. □ = NRS; ■ = heated NRS.

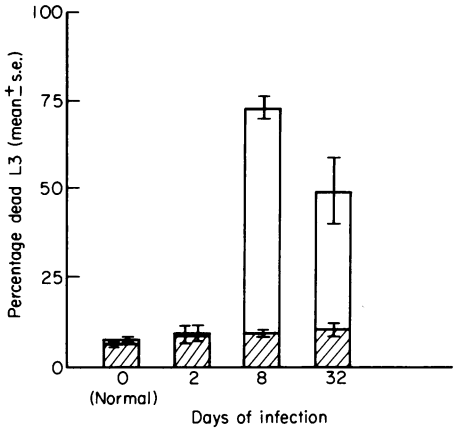

Fig. 2. Helminthocidal activity of BAL cells from rats given a primary *N. brasiliensis* infection. The results represent the mean ± s.e. of three to 10 experiments carried out in triplicate at a cell to target ratio of 16,000:1 and at a final serum dilution of 1:3. Helminthocidal activity of D8 BAL cells in the presence of zymosan or CoVF treated NRS was comparable to that in the presence of heated NRS. □ = NRS; ■ = heated NRS.

conditions in which C3 was fixed to the surface of L3 (Figs 1 & 2). Therefore because the alveolar macrophages appeared to be the effector cells killing *N. brasiliensis* L3, we studied C3 complement receptor expression (EAC rosettes) on alveolar macrophages. Normal and D2 alveolar macrophages contained 11 ± 5 and 22 ± 3% C3 receptor positive macrophages respectively (Fig. 3); this difference in the proportion of C3 receptor positive macrophages in normal and D2 alveolar macrophages was statistically significant ($P < 0{\cdot}05$). By contrast, D8 and D32 alveolar macrophages contained 46 ± 7 and 31 ± 6% C3 receptor positive macrophages, respectively; these proportions of receptor positive macrophages were significantly higher than that in normal alveolar macrophages ($P < 0{\cdot}001$ and $P < 0{\cdot}05$, respectively).

To correlate the proportion of C3 receptor positive macrophages with BAL cell adherence and helminthocidal activity, the absolute numbers of C3 receptor bearing macrophages per 10^6 BAL cells were calculated. Normal, D2, D8, and D32 BAL cells contained a mean of $1{\cdot}1 \times 10^5$, $1{\cdot}6 \times 10^5$,

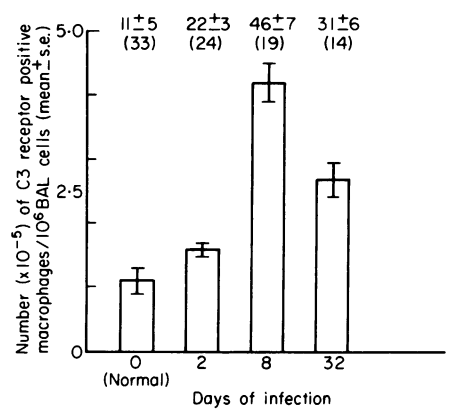

Fig. 3. Alterations in absolute numbers of C3 receptor positive alveolar macrophages in BAL cells of rats undergoing a primary *N. brasiliensis* infection. The bars represent the mean ± s.e. absolute numbers of C3 receptor positive alveolar macrophages per 10^6 BAL cells. These were calculated from the mean % C3 receptor positive alveolar macrophages, mean % alveolar macrophages in differential counts, and mean number of BAL cells/rat. The figures above the bars represent the mean ± s.e. percentage EAC rosettes of normal, D2, D8 and D32 BAL cells; the number of rats individually assayed is indicated in parenthesis.

$4{\cdot}2 \times 10^5$ and $2{\cdot}7 \times 10^5$ C3 receptor positive alveolar macrophages per 10^6 BAL cells respectively (Fig. 3). These results suggest that the adherence and helminthocidal activity of D8 and D32 BAL cells were related to their content of C3 receptor bearing alveolar macrophages and that the lack of killing by D2 BAL reflected insufficient numbers of these cells.

DISCUSSION

We confirmed the observations of Mackenzie *et al.* (1980) that *N. brasiliensis* L3 activate complement by the alternative pathway and that a non-adherent mononuclear cell population from the normal rat peritoneal cavity adheres to and kills L3 by a complement-dependent mechanism. We further extended these observations by showing that $61 \pm 7\%$ of the rat peritoneal mononuclear cells express C3 receptors, which probably accounts for the complement-dependent killing of L3. We then utilized this *in vitro* system to investigate the helminthocidal activity of BAL cells.

There are at least two compartments of macrophages in the lungs, namely fixed interstitial macrophages and luminal intra-alveolar macrophages. The BAL technique employed in our studies recovers cells that are representative of the intra-alveolar cellular milieu (Hunninghake *et al.*, 1979, 1981). Our investigation appears to be the first detailed analysis of the potential role of broncho-alveolar leucocytes in host resistance to a metazoan parasite which passes through the lung. We have shown that a primary *N. brasiliensis* infection in the rat induces helminthocidal activity of alveolar macrophages which is expressed *in vitro* in the presence of a component of NRS which appears to be complement. BAL cells from normal uninfected rats and from D2 infected rats lacked this parasiticidal activity, but BAL cells from rats infected for 8 and 32 days possessed the complement-dependent helminthocidal activity. Furthermore, the adherence to and killing of C3 bearing larvae correlated with significantly increased numbers of C3 receptor bearing alveolar macrophages in D8 and D32 BAL cells.

Our explanations for the lack of killing of L3 by the adherent D2 BAL cells are speculative at this stage. Firstly it is possible that the C3–C3 receptor interaction which activates helminthocidal mechanisms is a threshold phenomenon and this threshold is not attained by D2 alveolar macrophages which are markedly activated at this time as assessed by various criteria (Gauldie, Richards & Lamontagne, 1983). Fig. 3 clearly shows that the C3 receptor positive macrophages are fewer in number in D2 than in D8 and D32 BAL cells. Secondly, Gauldie, Lamontagne & Befus (unpublished observations) have observed increased intracellular levels of alpha-1-protease inhibitor (A1Pi) in D2 alveolar macrophages. There is evidence that A1Pi not only inhibits antibody-dependent cellular cytotoxicity and natural killer activity against tumour cells (Ades *et al.*, 1982) but also complement-dependent phagocytosis and EAC rosette formation (Mod *et al.*, 1981; Dierich, Landen & Schmitt, 1979). Thus, A1Pi in D2 alveolar macrophages may inhibit their helminthocidal potential.

The mechanism by which D8 and D32 alveolar macrophages kill *N. brasiliensis* L3 remains unknown. A role for oxygen metabolites (Bass & Szejda, 1979; Kazura *et al.*, 1981; Joseph, 1982) and arginase (Mahmoud *et al.*, 1979; Olds *et al.* 1980) has been postulated in the *in vitro* killing of *Trichinella spiralis* newborn larvae and *Schistosoma mansoni* schistosomules. However, preliminary investigations in this laboratory suggest that neither oxygen metabolites nor arginase are involved in the helminthocidal activity of alveolar macrophages reported in this paper. Incorporation of large, non-cytotoxic amounts of superoxide dismutase, catalase or L-arginine into the cell–parasite cultures did not abrogate the killing of L3. It has been postulated that host cell derived collagenase might be involved in killing *N. brasiliensis* L3 *in vivo* (Lee, 1976). As activated macrophages release collagenase (Werb & Gordon, 1975), the role of this enzyme in the *in vitro* helminthocidal activity of alveolar macrophages still remains to be determined.

N. brasiliensis L3 reach the lungs of normal rats within 24 h after skin penetration or subcutaneous injection. Between days 2 and 4 most of the parasites, now L4, leave the lung. The biological significance of helminthocidal activity appearing in the lungs of rats 1 week after a primary infection and lasting for at least 3 weeks may therefore be a protective mechanism for the effective handling of larvae passing through the lungs in subsequent infections. Thus during a

primary infection, most of the parasites probably pass through the lungs unharmed; however in the lungs of a previously infected rat, larvae encounter an immunologically hostile environment consisting of increased numbers of intra-alveolar macrophages bearing C3 receptors. The studies of Taliaferro & Sarles (1939) provide *in vivo* information that is consistent with the model. Thus, under certain conditions *N. brasiliensis* L3 and other nematodes that migrate through the broncho-alveolar spaces (Sarles & Taliaferro, 1936; Taliaferro & Sarles, 1939; Jarrett & Sharp, 1963) may be killed in the lungs. Our studies suggest that the alveolar macrophage plays a significant role in this killing and that the BAL technique is a useful tool for studying the effector mechanisms involved in lung resistance against helminths.

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