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ORIGINAL PAPER

Trichinella spiralis: killing of newborn larvae by lung cells

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Abstract The migratory stage of *Trichinella spiralis*, the newborn larva (NBL), travels along the pulmonary microvascular system on its way to the skeletal muscle cells. The present work studies the capability of lung cells to kill NBL. For this purpose, in vitro cytotoxicity assays were performed using NBL, lung cell suspensions from Wistar rats, rat anti-NBL surface sera, and fresh serum as complement source. The cytotoxic activity of lung cells from rats infected on day 6 p.i. was compared with that from noninfected rats. Two and 20 h-old NBL (NBL_2 and NBL_{20}) were used as they had shown to exhibit different surface antigens altering their biological activity. Sera antibodies were analyzed by indirect immunofluorescence assay, and cell populations used in each assay were characterized by histological staining. The role of IgE in the cytotoxic attack against NBL was analyzed using heated serum. The FcεRI expression on cell suspensions was examined by flow cytometry. Results showed that lung cells were capable of killing NBL by antibody-dependent cell-mediated cytotoxicity (ADCC). Lung cells from infected animals yielded the highest mortality percentages of NBL, with NBL_{20} being the most susceptible to such attack. IgE yielded a critical role in the cytotoxic attack. Regarding the analysis of cell suspensions, cells from infected rats showed an increase in the percentage of eosinophils, neutrophils, and the number of cells expressing the FcεRI receptor. We conclude that lung cells are capable of killing NBL in the presence of specific antibodies, supporting the idea that the lung is one of the sites where the NBL death occurs due to ADCC.

Keywords *Trichinella spiralis* · Newborn larvae · Antibody-dependent cell-mediated cytotoxicity · Lung cells

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Introduction

Different helminth species pass through the lung developing immunological disorders (Silveira et al. 2002; Reece et al. 2006; Burke et al. 2011). It is known that migrant *Trichinella spiralis* larvae or newborn larvae (NBL) go through the bloodstream of different organs, including the lung, during their migration to the skeletal muscle (Despommier et al. 2005). Given the lung architecture and the size of capillaries being similar to the size of NBL, this organ provides a suitable environment for the interaction with the effector components of the immune system. Considering the above characteristics, it has been proposed by different research groups that the lung is one of the main organs for the retention and destruction of NBL (Binaghi et al. 1981; Wang and Bell 1986; Bruschi et al. 1992).

The latest results obtained in our laboratory demonstrated a significant inflammatory reaction of lung parenchyma developing hyperplasia of the bronchus-associated lymphoid tissue (BALT) before and during the NBL passage through the lung. This inflammation is characterized by the presence of mast cells and eosinophils scattered throughout the lung parenchyma, the goblet cell hyperplasia, and the local production of cytokines, chemokines, and antibodies (Venturiello et al. 2007; Gentilini et al. 2011).

In vitro and in vivo studies revealed the importance of leukocytes, from peritoneal exudate and peripheral blood, as effector cells, and specific antibodies against larval surface to

promote its death through antibody-dependent cell-mediated cytotoxicity (ADCC), the major mechanism of the NBL death (Kazura and Grove 1978; Gansmuller et al. 1987; Wang and Bell 1988; Venturiello et al. 1993).

This research work was aimed to study the cytotoxic capacity of lung cells against *T. spiralis* NBL by means of an in vitro assay. Cells from noninfected animals and from animals infected on day 6 p.i. were used. At this time-point, the highest passage of NBL through the lung is recorded (Harley and Gallicchio 1971), and cell populations derived from the inflammatory process are present. In this assay, 2 and 20 h-old NBL (NBL_2 and NBL_{20}) were used because, as described previously, they showed that during the first hours of life, antigenic variations occur in the NBL cuticle (Jungery et al. 1983) altering its biological activity and its resistance against cytotoxic attacks (Gansmuller et al. 1987; Venturiello et al. 1993; Moskwa 1999).

Materials and methods

Animals and infection

Two-month-old female Wistar rats were orally infected through a gastric canula with 2000 muscle larvae (ML) per rat. ML were obtained from muscle tissue of Swiss mice by the artificial digestion method (Nöckler and Kapel 2007). Noninfected rats were used as controls. During all the experiments, animals were provided with water and food ad libitum and exposed to 12-h light–dark cycles; room temperature was kept at 21±1 °C. All experimental protocols were approved by the Institutional Review Board of the Instituto de Estudios de la Inmunidad Humoral (IDEHU) and conducted in compliance with the guidelines established by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

Collection of newborn larvae

NBL were obtained as described previously (Dennis et al. 1970). Briefly, adult worms were recovered from the intestine of rats 5–6 days after oral infection with 7000 ML. Worms were cultured in RPMI medium (Gibco, GrandIsland, NY, USA) supplemented with antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin, Gibco) and 5 % fetal calf serum (FCS) (Natocor, Villa Carlos Paz, Córdoba, Argentina) at 37 °C in a humidified atmosphere with 5 % CO_2. NBL were collected 2 h later and used immediately (NBL_2) or maintained overnight in culture medium under the same conditions (NBL_{20}). Larvae were alive and in good condition as judged by their motility.

Sera

Sera from rats were collected at 6 (n=5) or 45 days p.i. by cardiac puncture. As a negative control, sera were collected from noninfected rats (noninfected rat sera (NIRS)). All sera were heat-inactivated at 56 °C for 30 min. Fresh NIRS were used as a complement source.

The 45-day p.i. serum, reference cytotoxic serum (RCS), is a pool of sera from three rats infected with 5000 ML/animal presenting anti-NBL surface antibodies, and its helminthocytotoxic capacity against NBL was assessed using rat peritoneal leukocytes.

Aliquots of RCS heated (RCSh) at 56 °C for 3 h were used to check the loss of IgE cytotoxic effect (Ishizaka et al. 1967).

Preparation of lung cell suspensions

Rats were anaesthetized and bled by cardiac puncture in order to obtain lung cell suspensions. Tissues were perfused by injecting 40 ml of PBS (pH 7.4)–heparin (10 IU/ml, Sigma) into the heart right ventricle until lungs turned white. Lungs were removed, cut into small pieces, and digested in RPMI medium (Gibco) containing collagenase A (0.5 mg/ml, Roche Diagnostics, Mannheim, Germany), DNase (0.1 mg/ml, Roche Diagnostics), L-glutamine (1.46 g/100 ml, Gibco), penicillin (100 IU/ml, Gibco), and streptomycin (100 μg/ml, Gibco) for 40 min at 37 °C with occasional shaking. Then, tissues were homogenized and cell suspensions were filtered through a nylon mesh to remove tissue debris. The remaining erythrocytes were lysed using ammonium chloride buffer solution. Cell suspensions were suspended and washed twice with PBS plus EDTA 5 mM and 3 % FCS (Natocor). Lung cells were counted on a hemocytometer using trypan blue stain (Gibco), and cell viability was invariably higher than 95 %. Lung cell suspensions presented between 84–87 % of leukocytes and 13–16 % of nonleukocyte lung cells.

Finally, cell suspensions were properly resuspended in RPMI medium containing 100 μg/ml streptomycin, 100 IU/ml penicillin, and 5 % FCS.

Leukocyte formula

Lung cell suspensions from noninfected (n=5) and infected rats (n=5) were concentrated by cytocentrifugation at 400 rpm for 10 min (Shandom Cytospin III, USA) and stained with Giemsa (Merck, Darmstadt, Germany). The leukocyte formula was established by identifying 200 cells in an optical microscope.

Antibody-dependent cell-mediated cytotoxicity assay

In order to study the cytotoxic properties of lung cells, the assay was performed in flat-bottomed microwell modules

(Nunc, Roskilde, Denmark). A volume of 30 μl of NBL suspension in RPMI (Gibco) containing approximately 50 NBL was placed into each well, and the exact number of larvae was counted. One hundred microliters of lung cell suspension from infected (n=5) and noninfected (n=5) rats, 20 μl of serum (RCS, NIRS, 6 days p.i., RCSh), and 10 μl of fresh NIRS were added to each well. Lung cell suspensions were resuspended at a ratio of 6000 leukocytes/NBL.

The helminthocytotoxicity effect was studied by modifying the leukocyte–NBL ratio using lung cells from infected animals and RCS. In these assays, the ratios studied were 1000, 2000, 4000, and 6000 leukocytes/NBL. Reactions were maintained at 37 °C for 20 h in a humidified atmosphere with 5 % CO_2.

The NBL death was assessed using direct microscopy by two independent observers in a blind fashion, and the mortality percentage was calculated according to the following formula:

% Mortality=[(NBLi−NBLf)/NBLi]×100, where NBLi and NBLf are the numbers of living NBL, judged by their motility, counted at the beginning and at the end of the reaction respectively.

FcεRI expression

The FcεRI expression was examined by flow cytometry. Five hundred thousand lung cells were incubated with the working dilution of purified mouse anti-rat high affinity IgE receptor (BD Biosciences, San Diego, CA, USA) or purified mouse IgG1, κ isotype control (BD Biosciences) in a final volume of 100 μl at 4 °C for 30 min in PBS containing 3 % FCS and 5 mM EDTA. After performing the corresponding washes, cells were incubated under the conditions mentioned previously with the working dilution of R-PE-conjugated Goat $F(ab')_2$ anti-mouse IgG (H+L) (Caltag Laboratories, Burlingame, CA, USA). Samples were processed on a FACSCalibur flow cytometer (BD Biosciences), and the Cyflogic software version 1.2.1 was used for data analysis.

Determination of anti-NBL surface total serum Ig (IgGAM), IgA, IgE, IgG1, and IgG2a

Titration of specific IgGAM, IgA, IgE, IgG1, and IgG2a anti-NBL in rat sera was calculated using the indirect immunofluorescence assay (IFA) on slides containing methanol-fixed NBL by the method described previously (Nuñez et al. 2002). Slides were incubated with 20 μl of different serum dilutions, and the reaction was continued by adding 20 μl of working dilution of fluorescein anti-rat IgG (H+L) for IgGAM detection (Vector, Burlingame, CA, USA), goat anti-rat IgA, IgE, IgG1, and IgG2a (Bethyl Laboratories, Inc., Montgomery, TX, USA). Fluorescein anti-goat IgG (Sigma, St. Louis, MO, USA) was added. Finally, slides were mounted using buffered glycerin and analyzed in a fluorescence microscope (Olympus, Japan). All steps were carried out at 37 °C with three washes with PBS-Tween 0.1 % in between.

Statistical analysis

Data were analyzed using the one-way ANOVA test, and the unpaired t test was used for the statistical analysis of FcεRI lung cell expression. A $p<0.05$ was considered significant. Data were analyzed using the GraphPad Prism 6 software.

Results

Determination of anti-NBL surface total serum Ig (IgGAM), IgA, IgE, IgG1, and IgG2a

The following titers of anti-NBL surface antibodies in RCS were obtained using IFA: IgGAM (2048), IgA (256), IgE (128), IgG1 (512), and IgG2a (512).

The five sera collected at day 6 p.i. provided the following titers: IgGAM (2), IgA (negative), IgE (4), and IgG1 (negative), and remarkably, IgG2a was found in one animal only (4).

Killing of NBL using lung cells and reference cytotoxic serum or 6 days p.i. sera

In these in vitro assays, rat lung cells were able to attach to and kill the parasite only in the presence of anti-NBL surface antibodies (Fig. 1). The cell adhesion to larvae in the presence of antibodies occurred in a few minutes. In the absence of specific antibodies, neither significant cell adhesion nor parasitic death was observed (NBL_2 7 %, and NBL_{20} 12 %).

By using RCS, the mortality percentage of cells obtained from infected animals was higher than that from noninfected animals. This was observed in NBL_2 (53±3.5 vs. 30±2 %; $p<0.001$) and NBL_{20} (83±6 vs. 61±6 %; $p<0.001$) (Fig. 1a, b).

The assays performed with RCS revealed that NBL_{20} were more susceptible to immune attacks by both types of lung cell suspensions than NBL_2, showing higher mortality percentages (infected, 83±6 vs. 53±3.5 %, $p<0.001$; noninfected, 61±6 vs. 30±2 %, $p<0.001$).

No significant differences were observed when preparing ADCC reactions using fresh serum either at different dilutions or heat-inactivated serum. This indicates that in these working conditions, the complement is not essential for cytotoxic attack.

The use of RCSh caused a marked decrease in the mortality percentages of both types of NBL (Table 1).

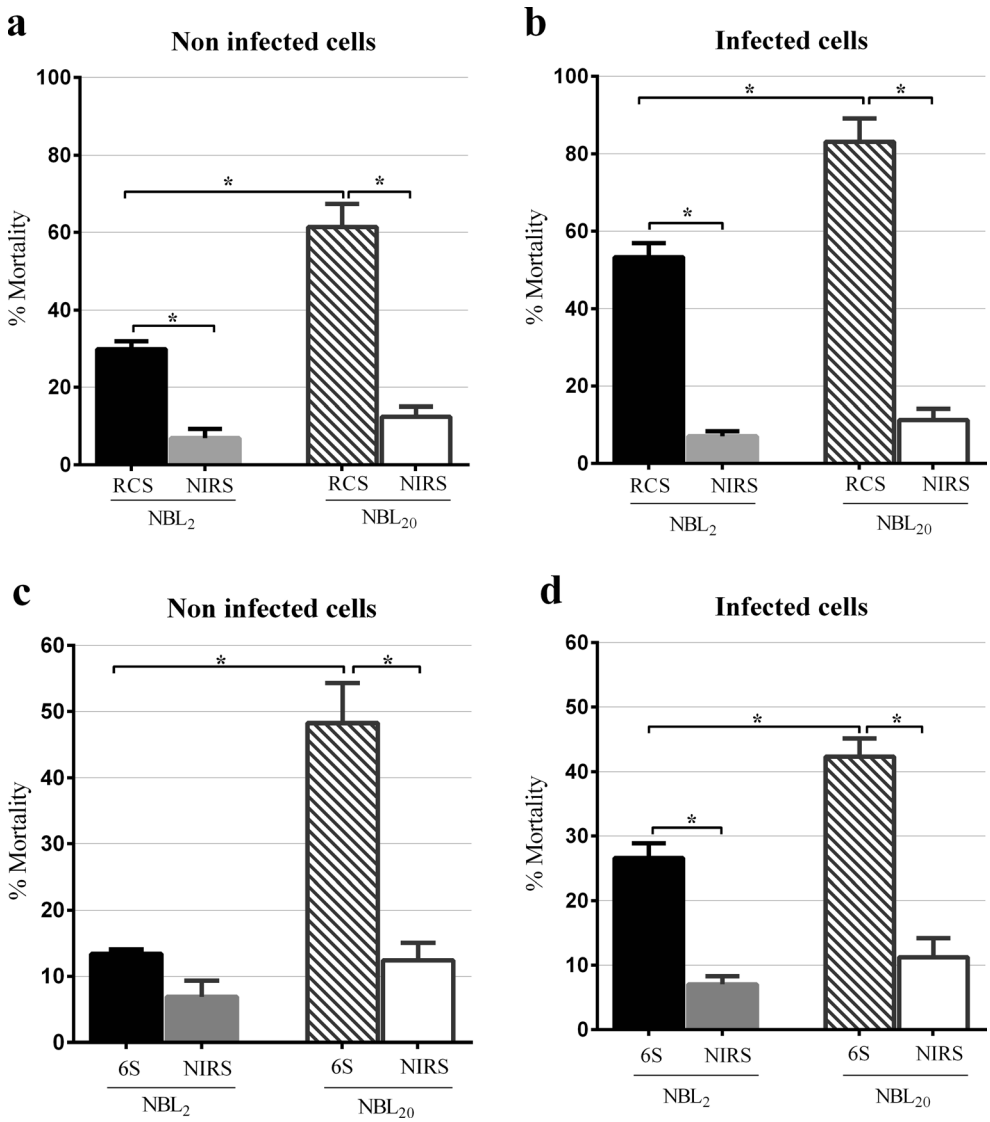

Fig. 1 Cytotoxic activity of lung cells in the presence of reference cytotoxic serum (RCS) or sera from rats infected on day 6 p.i. (6S). ADCC assays were performed with cells obtained from noninfected rats (**a**, **c**) or rats infected on day 6 p.i. (**b**, **d**) and newborn larvae 2 h (NBL_2) or 20 h (NBL_{20}). The results are expressed as the mean of mortality percentages of NBL±S.E.M. obtained from five independent experiments performed in duplicate. Data were analyzed using one-way ANOVA. *Asterisks* (*) indicate significant differences ($p<0.001$). *NIRS* noninfected rat sera

The use of sera from day 6 p.i., which had lower titers of anti-NBL surface total antibodies and isotypes than RCS (see Determination of anti-NBL surface total serum Ig (IgGAM), IgA, IgE, IgG1, IgG2a), revealed that lung cells from infected animals were able to adhere and kill NBL_2 (27±2 %) and NBL_{20} (42±3 %), while cells from noninfected animals could only cause the death of NBL_{20} (48±6 %) (Fig. 1c, d). The mortality percentages observed using the five sera from day 6 p.i were similar to each other.

Table 1 Role of serum IgE in the cytotoxic activity of lung cells

Cells	NBL_2		NBL_{20}	
	RCS	RCSh	RCS	RCSh
Infected	48±1	14±3	66±2	35±1
Noninfected	30±1	1±1	44±1	19±4

NBL mortality percentage with noninfected rat sera and cells from noninfected (NBL_2 7±2; NBL_{20} 12±3) and infected rats (NBL_2: 7±1; NBL_{20}: 11±3). ADCC assays were performed with cells obtained from rats infected on day 6 p.i. or noninfected, newborn larvae 2 h (NBL_2) or 20 h (NBL_{20}). Reference cytotoxic serum (RCS) and RCS heated at 56 °C for 3 h (RCSh) were used. Results are expressed as the mean of the mortality percentage of NBL±S.E.M. obtained from two independent experiments performed in duplicate

The mortality percentages of NBL_{20} observed with cells from infected vs. noninfected animals were not significant (42±3 vs. 48±6 %, respectively; $p>0.05$). This suggests that greater amounts of antibodies and/or presence of other isotypes would be necessary for the infected cells to show their highest activity against the larvae.

In these ADCC assays, the NBL_{20} were more susceptible to the immune attack exerted by both types of lung cell suspensions than NBL_2 and showed higher mortality percentages (infected, 42±3 vs. 27±2 %, $p<0.001$; noninfected, 48±6 vs. 13±1 %, $p<0.001$).

Killing of NBL_2 vs. NBL_{20} according to the ratio infected lung leukocytes per larva

Considering that lung cells from infected animals showed higher cytotoxic activity against NBL_2 and NBL_{20} in the presence of RCS, the mortality percentage was assessed in terms of the ratio leukocytes per larva (Fig. 2).

The results show that the mortality percentage relies on the ratio leukocytes per NBL. From 4000 leukocytes/NBL, a significant increase was observed in the mortality percentage of NBL_{20} with significant differences ($p<0.001$) in relation to the mortality percentage of NBL_2. The NBL_2 presented its maximum mortality (55 %) at a ratio of 6000 leukocytes/larva but without significant differences compared to the mortality percentages obtained with 2000 and 4000 leukocytes/NBL_2.

No ratios greater than 6000 leukocytes/NBL were used because working with larger amounts of cells may cause technical difficulties in reading the assays.

These results show that the mortality of NBL relies on the ratio lung leukocytes per larva and confirm that NBL_{20} are more sensitive to the attack exerted by lung cells from infected rats in the presence of RCS.

Analysis of cell suspensions

Leukocytes were counted on the cell suspensions used in the in vitro cell cytotoxicity assays, and the leukocyte formula was established (Table 2). The cell composition in infected animals registered some changes revealing an increase in eosinophils and neutrophils. These cells act as effector cells in the ADCC mechanism against NBL.

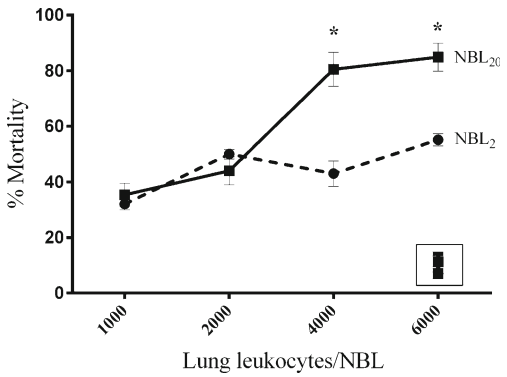

Fig. 2 Newborn larvae (*NBL*) mortality of different ages according to the ratio lung leukocytes per larva. These ADCC assays were performed using lung cells from infected rats on day 6 p.i., rat reference cytotoxic serum, and newborn larvae 2 h (*NBL_2*) or 20 h (*NBL_{20}*). Values using noninfected rat sera are shown in the *box*. Results are expressed as the mean of the mortality percentage of NBL±S.E.M. obtained from five independent experiments performed in duplicate. Data were analyzed using one-way ANOVA. *Asterisks* (*) indicate significant differences between NBL_2 and NBL_{20} ($p<0.001$)

Table 2 Leukocyte differential count of lung cell suspensions of noninfected and infected rats on day 6 p.i.

	Lung cells suspension	
	Noninfected (%) ($n=5$)	Infected (%) ($n=5$)
Lymphocytes	37–42	27–36
Macrophages	48–50	31–45
Neutrophils	8–12	15–30
Eosinophils	0–2	5–14

Data are expressed as the percentage range obtained for each leukocyte population

The analysis of the FcεRI expression revealed a significant increase ($p<0.05$) in the percentage of events in cell suspensions from infected vs. noninfected animals (Fig. 3).

Discussion

An important phase of the immune attack exerted by the host against *T. spiralis* NBL occurs through the ADCC mechanism (Kazura and Grove 1978; Gansmuller et al. 1987; Wang and Bell 1988; Venturiello et al. 1993). The exact site where this mechanism takes place is unknown. We have recently shown that NBL are retained and killed in the lungs of infected rats during the early stage of infection (Venturiello et al. 2007; Gentilini et al. 2011). However, the mechanism involved in this death is yet unknown. To date, there are no studies based on the cytotoxic capacity of tissue lung cells against NBL. Although we have previously studied the cytotoxic capacity of BAL cells (Venturiello et al. 2007), the cellular composition of BAL does not represent the whole lung (Pabst et al. 2008; Siracusa et al. 2008).

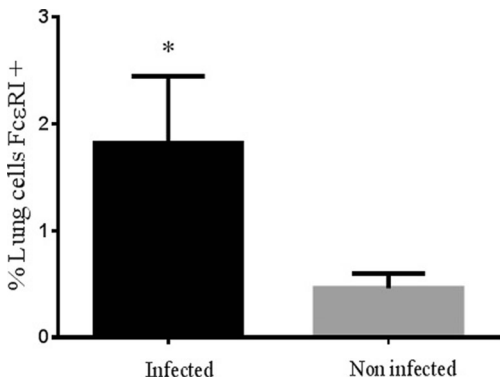

Fig. 3 Surface expression of FcεRI in lung cell suspensions. Percentage of cells expressing the receptor in infected (day 6 p.i.) and noninfected rats. Results are expressed as the mean±S.E.M. of the percentages of positive events in the total cell population in three independent experiments. The *asterisk* (*) indicates a significant difference ($p<0.05$)

The results show that lung cells are able to kill NBL through a mechanism that relies on the anti-NBL surface antibodies. Antigenic variations of the NBL cuticle and the type of cell suspension used, infected vs. noninfected animals, proved to have an impact on the larval death caused by the ADCC mechanism.

Cells from animals infected on day 6 p.i., the time-point where the highest passage of NBL through the lung was recorded (Harley and Gallicchio 1971), had a higher cytotoxic capacity than those from noninfected animals. Noteworthy, cell suspensions on day 6 p.i. derived from lungs exhibited a significant inflammatory response with an increase in eosinophils and neutrophils (Table 2), two major effector cells of the death of NBL by ADCC (Kazura and Grove 1978; Bass and Szejda 1979; Venturiello et al. 1993, 1995). This result, together with the increase in cells expressing FcεRI on its surface, an important receptor involved in the ADCC mechanism against helminths (Gounni et al. 1994; Dombrowicz et al. 2000), provides a better understanding of the high cytotoxic capacity of lung cells from infected animals presented in vitro.

The highest mortality percentages obtained by RCS were due to a higher titer and presence of other isotype-specific antibodies against NBL surface than sera obtained on day 6 p.i. The IgE appears to be the main isotype involved in its death according to the results observed using sera from day 6 p.i., because it was the only isotype detected. The importance of IgE was confirmed when carrying out experiments using RCSh, which exhibited the decrease in the cytotoxic effect (Table 1). This is in line with the results reported elsewhere in relation to other helminths (Mehta et al. 1980; Capron et al. 1981).

In previous studies, when we analyzed the cytotoxic capacity of BAL cells, we demonstrated that these cells are capable of inducing the death of NBL in the presence or absence of specific antibodies against NBL surface (Venturiello et al. 2007). This differential behavior among BAL cells and those used in the present study possibly occurs due to the fact that the leukocyte composition and activation of these cell populations are different (Pabst et al. 2008; Siracusa et al. 2008).

It is well known that the NBL cuticle is a dynamic organ that undergoes significant changes during its maturation (Jungery et al. 1983). For this reason, this study was focused on the effect of these changes in the attack exerted by the lung cells. Our results proved that these larvae cuticle antigenic variations have an important role in this ADCC mechanism. NBL_{20} were more susceptible to this attack than NBL_2 both with RCS and sera from day 6 p.i., regardless of the cell type used (Fig. 1). This phenomenon was strengthened by studying the mortality of NBL while varying the ratio of lung leukocytes per larva. NBL_{20} showed a significant increase in the mortality percentage, and the NBL_2 mortality percentage remained constant, without reaching the NBL_{20} levels (Fig. 2). Previous studies (Gansmuller et al. 1987; Moskwa 1999) demonstrated that NBL_{20} are more resistant to cytotoxic attack mediated by antibodies and murine peritoneal exudate cells. On the other hand, Venturiello et al. (1993) showed that human peripheral blood eosinophils are capable of attacking NBL_{20} but not NBL_2. Although these results seem to be controversial, it is quite clear that the antigenic changes observed in the NBL cuticle modify their own biological behavior. These differences appear to depend on the cell populations used in the in vitro assays as well as the species from which they derive.

When using cells and sera from day 6 p.i., it was observed that the NBL is attacked immunologically by lung cells and antibodies (Fig. 1d). This result is very important because these assays partly reveal the situation that occurs in vivo on the day with the highest passage of NBL through the lung of the infected host. Of note, significant differences between the mortality percentages of NBL_2 (27±2 %) and NBL_{20} (42± 3 %) were observed under these conditions.

Regarding the potential involvement of the complement, the research lines carried out have shown no differences in mortality percentages in the presence or absence of fresh serum. It appears that under the conditions of the in vitro system used, complement components are not essential in the ADCC mechanism, in line with the observations provided by other research groups (Kazura and Grove 1978; Bass and Szejda 1979).

The results shown in this study support the hypothesis that the lung may be one of the main site of retention and death of the NBL by ADCC (Binaghi et al. 1981; Wang and Bell 1986; Bruschi et al. 1992). It can be postulated that the NBL during its migration to the skeletal muscle through the lymphatic and blood circulation systems can be opsonized by serum and tissue antibodies. Those antibodies activate effector cells, present in the early inflammatory process of the lung, that mediate the death of NBL. Only those larvae able to evade this immune effector mechanism will reach its final ecotope.

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