Immunology 1994 **83** 302–307

Neutrophils as effector cells of T-cell-mediated, acquired immunity in murine listeriosis

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SUMMARY

The control of the infections caused by *Listeria monocytogenes*, considered an example of an intracellular parasite, is thought to involve co-operation between antigen-specific T cells and activated macrophages. Here we investigated the participation of polymorphonuclear leucocytes in the mechanisms of resistance during the immune phase of the antimicrobial response to *L. monocytogenes* infection. We found that BALB/c mice were unable to express T-cell-mediated (acquired) immunity to this pathogen in the absence of granulocytes. We propose that neutrophils should be included in the concept of cell-mediated immunity and that their antimicrobial role is not exclusively expressed during the early phases of a primary infection.

INTRODUCTION

Listeria monocytogenes infection in mice has been used as a model to study intracellular parasitism and the role of T-cell-mediated immunity to infection. Until now, this infectious model has been considered to represent a typical example of interaction between T cells and macrophages in the control of an infection by a micro-organism with an intracellular habit of replication.[1] Numerous investigators have described the $CD8^+$ subset of T cells as the cells able to confer acquired immunity to listeriosis, using either adoptive transfer assays,[2–6] selective *in vivo* T-lymphocyte subset depletion[7–9] or, more recently, gene knock-out technology.[10,11] Natural killer cells have also been implicated in the early resistance to listeriosis.[12] Several cytokines, including tumour necrosis factor[13–15] and interferon-γ,[16–19] play an important role in resistance to infection by *L. monocytogenes*.

Regarding the effector arm of immunity less is known. Macrophages have always been considered the main effector cells of immunity although neutrophils, or neutrophils plus exudative monocytes,[20–22] have been shown to be important mediators of early resistance in primary infections. Recent work by Conlan & North has raised the hypothesis that neutrophils may mediate resistance to *L. monocytogenes* residing inside the hepatocyte through a novel pathway, namely being directly cytolytic of infected hepatic parenchymal cells.[23] We decided to evaluate whether T-cell-mediated immunity in listeriosis might involve the participation of neutrophils. We show here that those granulocytes are necessary not only for the early resistance in a primary infection but also for the immune phase of acquired resistance.

Received 11 April 1994; revised 9 June 1994; accepted 12 June 1994.

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MATERIALS AND METHODS

Animals

Female BALB/c mice were purchased from the Gulbenkian Institute (Oeiras, Portugal) and kept under standard hygiene conditions in our facilities. They were studied when they were 6–8 weeks old.

Reagents and antibodies

Thioglycollate broth (Difco, Detroit, MO) was prepared in saline as specified, autoclaved and injected intraperitoneally into mice (0·5 ml/animal). The RB6-8C5 cell line was provided by Dr R. L. Coffman (DNAX, Palo Alto, CA). This hybridoma was grown in ascites fluid in HSD nude mice and the antibody was purified using either an EconoPac Serum IgG column (BioRad, Richmond, CA) or by precipitation with 50% saturated $(NH_4)SO_4$ (the results were similar in both cases). This antibody has been extensively characterized and has been shown to bind and lyse neutrophils plus eosinophils *in vivo* and *in vitro*.[21,22,24–26] RPMI-1640 tissue culture medium and fetal calf serum (FCS) were from Gibco (Paisley, UK) and rabbit complement from Serotec (Oxford, UK).

Bacterial infection

Listeria monocytogenes strain EGD was grown in Antibiotic 3 Broth (Difco), split into aliquots and kept frozen at −70° until use. Mice were intravenously infected with the specified number of viable bacteria by injecting the bacterial suspensions through one of the lateral veins of the tail. In some experiments, mice were intravenously immunized with 1×10^4 CFU of *L. monocytogenes* 30 days before challenge.

Study of the peritoneal cell population

Peritoneal cells were collected from mice by washing the peritoneal cavity with 5 ml of phosphate-buffered saline (PBS)

and aspirating a fraction of the washout fluid. The cellular concentration was calculated by using a haemocytometer and cytospin preparations were obtained using a Shandon II cytocentrifuge. The slides were stained with the Hemacolor kit (Merck, Darmstadt, Germany) and differential cell counts were then made. The total number of different cell types per peritoneal cavity was calculated from the data above (% of cell × cell concentration × 5 ml). Blood cells were also counted in a haemocytometer and the differential cell counts performed in Hemacolor-stained blood smears.

Passive transfer of immune spleen cells[5]

Spleen cells from uninfected mice or mice that had been immunized 6 days earlier with 1×10^4 CFU of *L. monocytogenes* were aseptically collected, teased in RPMI-1640 medium containing 2% FCS and sequentially treated with NH_4Cl to lyse erythrocytes, J11d.2 hybridoma (American Type Culture Collection's TIB183 cell line) supernatant (1:1 in RPMI medium) with rabbit complement for 45 min to lyse B cells and granulocytes, and allowed to stand for 2 hr in a plastic Petri dish at 37° to remove adherent cells. The remaining cells, enriched in T cells, were resuspended in 0·5 ml and inoculated into naive BALB/c mice that had been infected 2 hr earlier with 2×10^5 CFU of *L. monocytogenes*. Each mouse was transfused with one spleen equivalent.

Statistical analysis

The data were compared using the Student's *t*-test.

RESULTS

The RB6-8C5 rat monoclonal IgG2b is specific for neutrophils and eosinophils and is able to lyse specifically these cells when administered *in vivo* in mice.[21,22,24–26] In a first set of experiments we evaluated the efficacy and specificity of action of the RB6-8C5 antibody. The *in vivo* activity and specificity of this antibody in BALB/c mice was evaluated by inoculating doses of 0·05, 0·1, 0·2, and 0·5 mg intravenously and looking at the resident cell population in the peritoneal cavity and at the ability to recruit cells 6 hr after the intraperitoneal challenge with 0·5 ml of thioglycollate broth. Eosinophil numbers were reduced in the undisturbed peritoneal cavity from

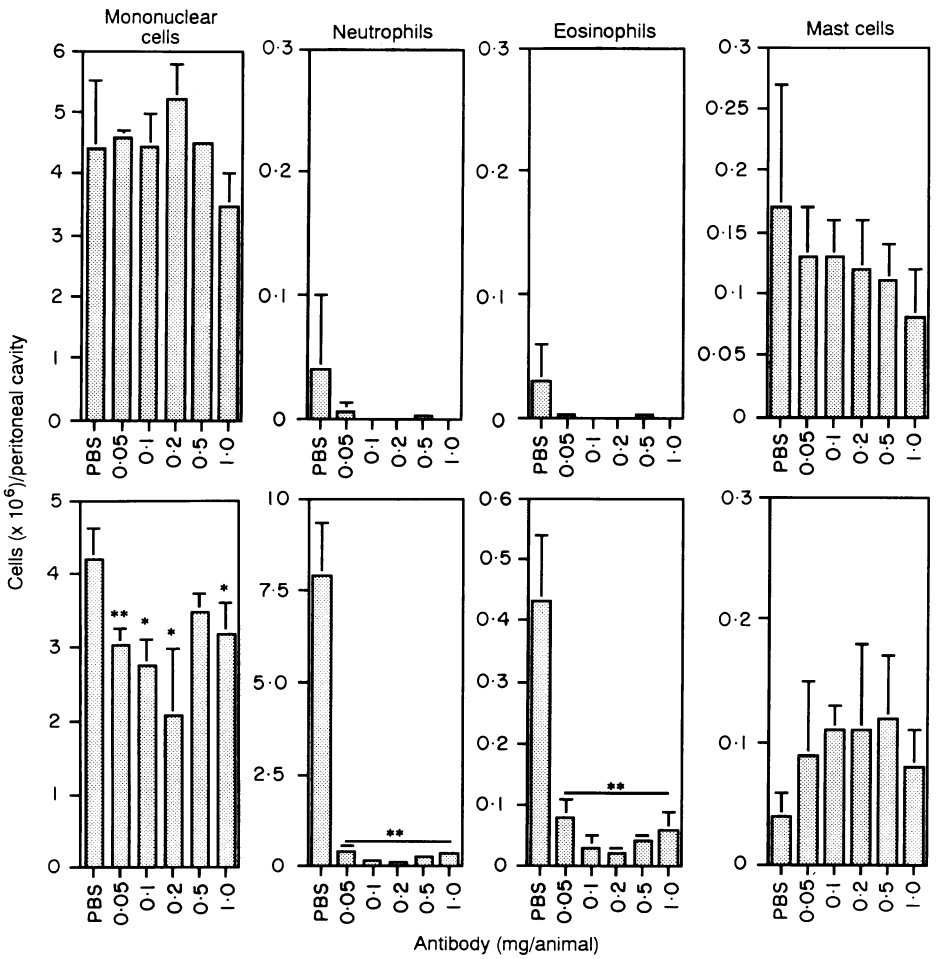

Figure 1. Effects of RB6-8C5 antibody administration on the resident peritoneal cell population (top row) and on the cells recruited 6 hr after the administration of 0·5 ml of thioglycollate broth (lower row). Each value represents the mean of four animals. Statistical differences between antibody-treated and controls (PBS) are marked $*P < 0{\cdot}05$ and $**P < 0{\cdot}01$. The antibody was administered intravenously 24 hr before collection of resident cells and 24 hr before the inoculation of thioglycollate.

$0{\cdot}3 \times 10^6 \pm 0{\cdot}1 \times 10^6$ to undetectable levels (Fig. 1, top row). There was an almost complete depletion of neutrophils and a partial depletion of eosinophils in thioglycollate-treated animals (control mice receiving PBS had $7{\cdot}88 \times 10^6 \pm 1{\cdot}43 \times 10^6$ neutrophils and $0{\cdot}43 \times 10^6 \pm 0{\cdot}11 \times 10^6$ eosinophils whereas neutrophil numbers varied from $0{\cdot}37 \times 10^6 \pm 0{\cdot}17 \times 10^6$ to $0{\cdot}10 \times 10^6 \pm 0{\cdot}04 \times 10^6$ and eosinophils from $0{\cdot}08 \times 10^6 \pm 0{\cdot}03 \times 10^6$ to $0{\cdot}02 \times 10^6 \pm 0{\cdot}01 \times 10^6$ in mice treated with 0·05–1·0 mg of RB6-8C5) (Fig. 1, lower row). The reduction in mononuclear cells was only partial as compared to the controls (Fig. 1, lower row).

The depletion of granulocytes was studied kinetically in mice receiving thioglycollate 24, 48 and 72 hr after the intravenous inoculation with PBS or 0·1 or 0·5 mg of RB6-8C5. Almost complete depletion of neutrophils was observed with the two doses of antibody at 24 and 48 hr post-administration (96·7% to 99·2% depletion) but there was a considerable recovery in the ability to recruit neutrophils 72 hr after the inoculation of either dose of antibody (42·0% and 16·6% depletion with 0·5 and 0·1 mg, respectively) (Fig. 2).

Neutrophils were the only cell type depleted from the blood after treatment with 0·1 mg of antibody (87·3% depletion 24 hr after the administration of 0·1 mg of antibody) (results not shown).

These experiments led us to choose the administration of 0·1 mg every other day as the protocol able to confer continued depletion of neutrophils for up to 4 days.

The effect of granulocyte depletion on *L. monocytogenes* infection was then evaluated. In a first experiment, BALB/c mice were given 0·1 mg of RB6-8C5 antibody 1 day before infection to one group of animals whereas the other group received PBS. The infection was then followed after the intravenous (i.v.) inoculation of 10^4 CFU of *L. monocytogenes* strain EGD. Mice receiving PBS showed an initial proliferation of the bacteria in the spleens and livers but were able to clear the infection after 4 days of infection (Fig. 3a). In contrast, mice receiving RB6-8C5 antibody at days −1 and +1 of infection showed progressive bacterial multiplication (Fig. 3a) and died at day 3 of infection. In a second experiment, mice were infected i.v. with 3×10^3 *L. monocytogenes* and depleted of granulocytes at different time-points of infection. Again, depletion of granulocytes before the infection caused the rapid multiplication of the bacteria (Fig. 3b). If the animals received only one dose of antibody 24 hr before the infection, they were able to start controlling the infection at day 1 even though at higher bacterial loads (Fig. 3b). The continued administration of antibody promoted, as before, progressive bacterial proliferation (Fig. 3b) that was not followed beyond day 3. The late administration of the RB6-8C5 antibody, at a time when control mice had begun controlling the infection caused a recrudescence of the infection in the liver (Fig. 3b). In the spleen, *L. monocytogenes* continued to be eliminated even though at a slower rate (Fig. 3b).

Mice that had been immunized with 10^4 *L. monocytogenes* one month earlier were challenged i.v. with 10^6 organisms and the bacterial multiplication monitored and compared to that observed in non-immune animals. Unlike the latter that died within 2 days of infection with massive numbers of bacteria in the organs, immunized animals were able to control the infection soon after challenge (Fig. 3c). However, immune mice that were depleted of granulocytes from the beginning of the infection showed an even more rapid bacterial proliferation resulting in the death of the animals in 2 days (Fig. 3c). The late administration of antibody caused a recrudescence of the infection in the liver and a slight impairment of bacterial elimination in the spleen (Fig. 3c).

The dependence on granulocytes of the ability of immune spleen cells to confer protection was evaluated in adoptive transfer experiments whereby control or immune spleen cells were administered to groups of four mice infected with *L. monocytogenes* 2 hr earlier. Mice receiving immune spleen cells exhibited low numbers of bacteria 48 hr after challenge whereas mice receiving non-immune cells had 1000-fold higher bacterial numbers in the spleen and 100-fold higher in the liver (Fig. 4). However, mice depleted of granulocytes showed massive bacterial proliferation in the organs whether they had received

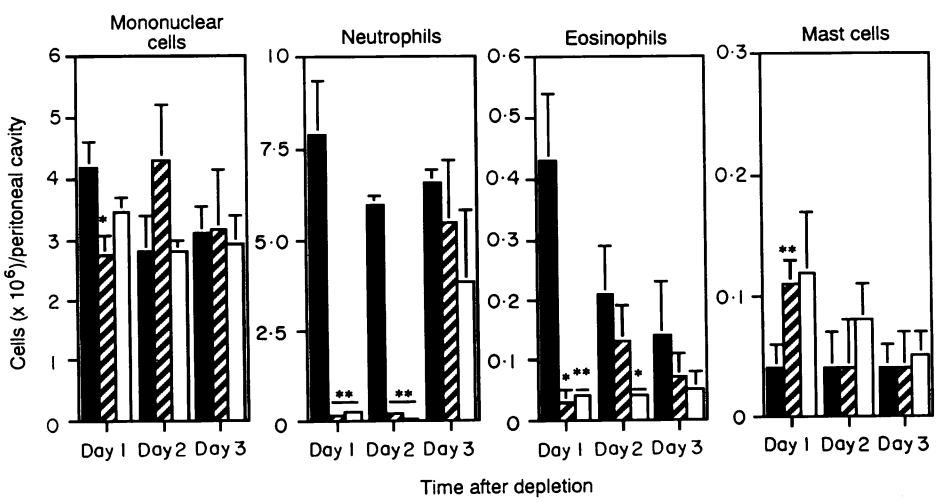

Figure 2. Number of cells 6 hr after an intraperitoneal administration of 0·5 ml of thioglycollate in animals treated 24, 48 or 72 hr earlier with 0·1 (▨) or 0·5 (□) mg of RB6-8C5 antibody. Results were compared with those from control animals receiving PBS at the same time-points (■). Statistics as in the previous figure.

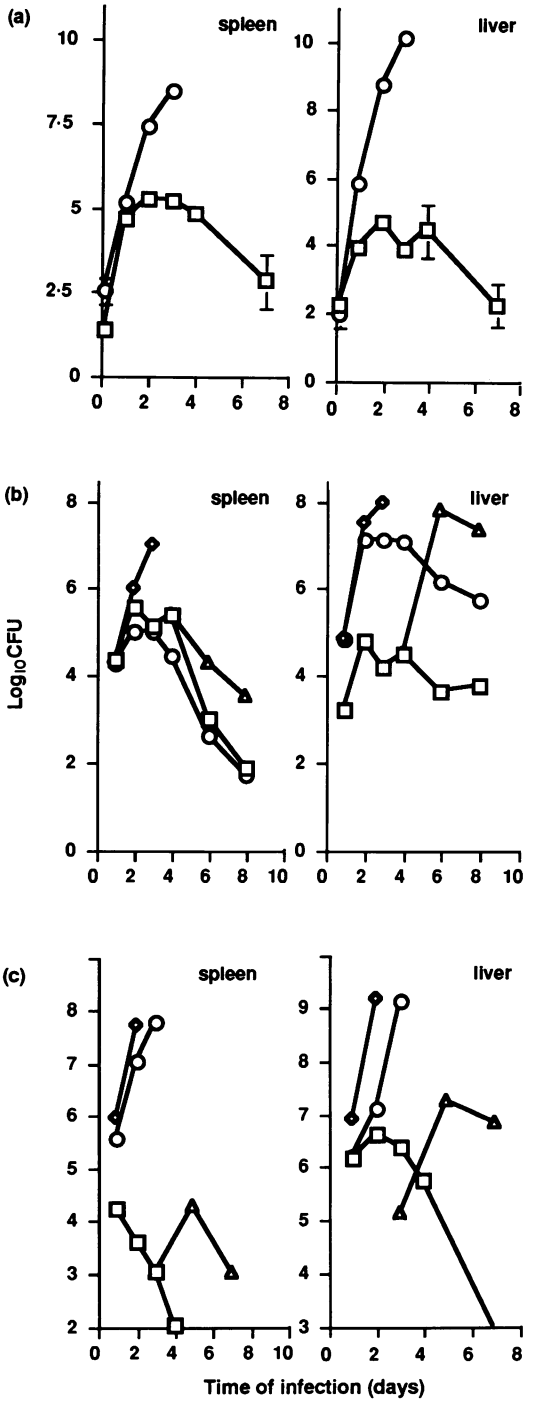

Figure 3. Proliferation of *L. monocytogenes* in the spleens and livers of infected BALB/c animals depleted or not of neutrophils. (a) Course of the infection following the i.v. inoculation of 1×10^4 CFU into non-immune untreated (□) or RB6-8C5-treated (○) (0·1 mg on days −1 and +1 of infection) mice. (b) Course of the infection following the i.v. inoculation of 3×10^3 CFU into non-immune untreated mice (□) or non-immune mice given 0·1 mg RB6-8C5 on day −1 (○), on days −1 and +1 (◇), or on days +4 and +6 (△) of infection. (c) Course of infection after the inoculation of 1×10^6 CFU into non-immune/untreated mice (○), immune/untreated mice (□), immune mice treated with 0·1 mg of RB6-8C5 at either days −1 and +1 (◇) or at days +3 and +5 (△) of infection. Standard deviation plots were omitted from (b) and (c) for clarity; they did not exceed 10% of the mean.

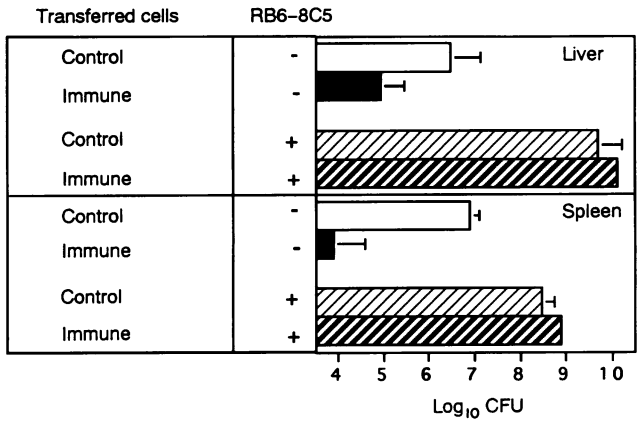

Figure 4. Growth of *L. monocytogenes* in the spleens and livers of groups of four BALB/c mice 48 hr after inoculation with 2×10^5 CFU of the bacteria. Mice received either non-immune or immune spleen cells 2 hr after the infection and either PBS or 0·1 mg of RB6-8C5 antibody at the time of the infection. The number of viable bacteria in mice receiving immune spleen cells was statistically lower than in mice receiving control cells ($P < 0{\cdot}01$ in both organs, Student's *t*-test). In the RB6-8C5-treated animals, two mice in the non-immune group and three in the immune group were dead at 48 hr.

immune or non-immune spleen cells before challenge (Fig. 4). Of these two latter groups, only two mice survived in the group that received non-immune cells and only one in the group that received immune cells. In an independent experiment, immune cells were still able to transfer the same degree of protection whether they were treated *in vitro* with RB6-8C5 and complement before transfer or not. Thus, the spleens of challenged mice had (at 48 hr post-challenge) 4·59 ± 0·29 $\log_{10}$ CFU in the control group receiving non-immune T cells, and 2·51 ± 0·74 and 2·51 ± 0·62 $\log_{10}$ CFU in the groups receiving immune T cells that were not treated or treated with RB6-8C5, respectively.

DISCUSSION

A defined dichotomy in the type of mechanisms mobilized by the host against infectious agents has been accepted for the last three decades.[1] According to those views, infected hosts depend on the interplay between polymorphonuclear leucocytes, antibody and complement to control the proliferation of extracellularly growing microbes. On the other hand, micro-organisms proliferating inside host cells would be controlled by T lymphocytes. Among the latter, those micro-organisms infecting mononuclear phagocytes are thought to be killed by the co-operation between specifically sensitized T cells and activated macrophages. *Listeria monocytogenes* has been considered to be an intracellular bacterium controlled by T cells through the activation of the host cell, the macrophage.

Here we present evidence that granulocytes are required for the expression of T-cell-mediated immunity. The antibody used was not able to affect T-cell function as immune T cells remained as competent to transfer protection after treatment with RB6-8C5 and complement as without this treatment. Although the RB6-8C5 antibody recognizes and lyses eosinophils *in vivo*, the observed effects of this antibody on the resistance to listeriosis most probably depend on the depletion

of neutrophils. In fact, *in vivo* depletion of eosinophils was most often only partial. In addition, we have not found any increase in peritoneal eosinophils during intraperitoneal infections with *L. monocytogenes* and flow cytometric analysis of spleen cells from infected mice has shown that the resident RB6-8C5^{dull} populations (which correspond to eosinophils[21]) disappear after the inoculation of the bacteria, suggesting that these cells are not recruited to infectious foci (R. Appelberg, unpublished observations). Finally, depletion of eosinophils by anti-interleukin 5 monoclonal antibody only marginally affected resistance to listeriosis lacking the dramatic effect observed with the depletion with the RB6-8C5 antibody (A. G. Castro & R. Appelberg, unpublished observations).

It is still not clear how T cells protect through the activity of neutrophils and, possibly, eosinophils. It is known that T cells may be involved in the recruitment of neutrophils[27–32] and that they may also activate them, namely to perform enhanced antimicrobial functions.[33–38] Thus, neutrophils could be ingesting and killing the bacteria directly. However, except for the initial period after inoculation, *L. monocytogenes* are found inside other cells. Thus, to access the bacteria and kill them, lysis of the infected cells should occur. This suggests that neutrophils can dissolve infected hepatic parenchymal cells[23] and ingest intracellular *L. monocytogenes*. Alternatively, neutrophils could be co-operating with cytolytic T cells able to free the *L. monocytogenes* from host cells devoid of an effective antimicrobial machinery. The neutrophils could, finally, co-operate with the macrophage either by tranferring some of their antimicrobial molecules to the mononuclear cells, as suggested in mycobacterial infections,[39] or by affecting their migration to the lesions. Neutrophils are known to possess unique microbicidal systems and are, in general, more potent in performing their toxic antimicrobial activities than mononuclear phagocytes although just for a short time. Those unique molecules may be the key feature that make neutrophils so indispensable for the control of listeriosis and, probably, of other intracellular bacterial infections. An effect on macrophage migration, although possible, is unlikely to account for the observed importance of neutrophils in cell-mediated resistance to *L. monocytogenes* as it has been shown that such resistance can be expressed in the absence of granuloma formation.[40]

Different protective effector mechanisms appear to be acting in different organs. In the spleen, unlike the liver, the late administration of granulocyte-depleting antibodies did not significantly affect resistance. In this case, splenic macrophages were probably able to acquire neutrophil molecules from the early influx of these granulocytes. When granulocytes were prevented from reaching the spleen early during the infection, macrophages alone were unable to control the infection even when activated by T cells as expected during a secondary infection. We thus speculate that, in the spleen, the co-operation between neutrophils and macrophages is of prime importance during the immune phase of resistance to listeriosis (but not before macrophage activation by T cells or even natural killer cells) since neutrophils act only as accessory cells, supplying molecules to the effector cell, the macrophage. In agreement with our results, Conlan & North[21] have recently reported that during the early phases of primary infections, neutrophils do not add any significant protection in the spleen. In contrast, in organs where *L. monocytogenes* are able to infect parenchymal cells, such as the liver, neutrophils were required to control the infection both before and after the acquisition of cell-mediated immunity and monocytes were not able to substitute for neutrophils as initially suggested by Rosen *et al.*[22] This fact may depend on the necessity of lysing infected hepatocytes that may appear in any phase of the infection.[23] Moreover, the same type of neutrophil/macrophage co-operation may also occur in the immune phase of resistance to *L. monocytogenes* in the liver.

In summary, we present evidence that adds to the concept that T cells and macrophages interact with granulocytes in the control of intracellular microbes, such an interaction constituting a neglected part of the cell mediated immunity mechanisms.

ACKNOWLEDGMENTS

This work was supported by fundings from the Junta Nacional de Investigação Científica e Tecnológica. The authors are indebted to Dr R. Coffman for supplying the RB6-8C5 hybridoma.

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