Interaction of leptospires with murine microglial cells

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SUMMARY

Leptospires, the agents of leptospirosis, exert tropism for the central nervous system, in the course of mammal infection. We evaluated the interaction between murine microglial cells and strains of pathogenic *L. interrogans* leptospires and non-pathogenic *L. biflexa* leptospires, mainly by flow cytometric assays. In the absence of opsonic conditions microglia are capable of ingesting - even quite slowly - the spirochetes and killing the non-pathogenic strain. The adhesion to microglia, which is quick and relevant for all the strains, does not involve the CR3 integrin receptor. These findings suggest that the murine microglia - in non opsonic conditions in vitro - do not effectively clear the pathogenic leptospires.

KEY WORDS: leptospires, microglia, phagocytosis

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INTRODUCTION

Leptospirosis is an acute febrile illness characterised by extensive vasculitis caused by a Spirochete belonging to the genus *Leptospira* (Faine *et al.,* 2000). Clinical symptoms of leptospirosis vary from subclinical to severe forms with renal and hepatic dysfunction. Like other members of the pathogenic spirochetes, such as *Treponema* and *Borrelia*, Leptospires show a predilection for the central nervous system (CNS). During early infection leptospires exert a tropism for the CNS, though the organs much affected by the outcome of infection in humans are liver and kidneys. Up to 25% of patients experience mild symptoms, such as neck stiffness and eye pain; in these patients spirochete may be cultured from the CSF. Later in the infection, CNS manifestations are common with pleocytosis; 50% of ill subjects develop aseptic meningitis *(*Edwards GA, Domm BM,1960) with sudden and severe headache. This picture, known as "swineherds' meningitis", is usually ascribed to the serovar Pomona *(*Gsell OR, 1983). Late encephalitis and myelitis are rare and regard up to 7% of the patients. In the presence of severe icteric leptospirosis, subarachinoid haemorrhage and intracerebral bleeding can be seen.

The outcome of *Leptospira* infection depends on the capacity of these micro-organisms to escape innate defence systems: one of these is phagocytosis. A number of studies have been carried on Leptospires and macrophages of different species and tissues such as guinea pig peritoneal

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macrophages (Cinco *et al.*,1981; Cinco *et al.*,1982) and rat Kupffer cells (Marangoni *et al.,* 2000). All these studies agreed in finding that leptospires are scarcely phagocytosed and killing occurs only in the presence of specific antibodies.
Microglia are the resident phagocyte populations of the CNS where they play an important role as innate immune responses in the brain. As such microglia are uniquely poised to provide an initial line of defence against micro-organisms invading the CNS prior to leukocyte infiltration. Further, microglia exert many of the immune effector functions associated with macrophages, which can be responsible for the toxic effects on the CNS. In fact, they become activated by microbial invasion and can elaborate a wide array of proinflammatory mediators, including IL-1, TNF-alpha, NO, IL-6 and chemochines (Rock *et al.,* 2004; Nakamura, 2002).
The present study investigated *Leptospira* susceptibility to phagocytosis and killing by microglial cells in non-opsonic conditions. Different leptospires with different levels of pathogenicity were used: non-pathogenic, pathogenic high passage and low passage, virulent strains. A murine microglial cell line immortalised with a *v-raf/v-myc* oncogene carrying retrovirus was employed as a prototype of CNS effector cells. Since we have demonstrated (Cinco *et al.,* 2002) that leptospires adhere to phagocytes via the CR3 (CD11b/CD18), the main receptor of complement protein iC3b, the involvement of this receptor in adhesion by glial cells was also investigated.

MATERIALS AND METHODS

Leptospira strains, culture and labelling
The leptospira strains used were: pathogenic strains Wijnberg (serovar Copenhageni), 142, serovar Icterohaemorrhagiae, low-passage virulent strain Lutar serovar Sejroe and the non-pathogenic strain Patoc 1, serovar Patoc. They were grown in EMJH medium and labelled with fluorescein isothiocyanate (FITC) as already described (Cinco *et al.,* 2002).

BV-2 cells and culture conditions
Infecting primary microglia cells with a v-raf/v-myc carrying retrovirus (J2), (Blasi *et al.,* 1999), generated the microglial cell line BV/2. This cell line, that retains the morphological, phenotypic and functional properties of freshly isolated microglial cells, was maintained in vitro in RPMI 1640 medium (Gibco, Grand Island, NY), supplemented with 10% heat-inactivate FBS (Defined Hyclone, Logan, Utah), gentamycin (50 µg/ml) and L-glutamine (2 mM). Biweekly, cells were detached by vigorous shaking and fresh cultures were started at a cell concentration of 5x 10^4/ml. The clone was checked for expression of CR3 receptor by flow cytometry: the cells were stained with phycoerythrin-conjugated Mab 2LPM19c for 30 min on ice, washed twice with phosphate-buffered saline (PBS: pH=7.4) and resuspended in PBS-0.5% paraformaldehyde for cytometric reading. Viability of the cells after 6 hours coincubation with leptospires was evaluated by the Live/Dead Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR), according to the manufacturer's directions.

Reagents and Immunologicals
Monoclonal antibodies (Mabs) employed to stain the CR3 receptor were: anti-human CD11b clone CR3 Bear-1 (Caltag), Monoclonal M1/70 and isotype-matched control IgG1 (Caltag); for staining leptospires human serum from a leptospirosis patient recognizing both the serovars Icterohaemorrhagiae and Copenhageni as well as strain Patoc1 and the anti-human IgG FITC conjugate (Sigma) were used.

Measurement of adhesion and phagocytosis
Phagocytosis and adhesion of leptospires by BV-2 cells were evaluated by flow cytometric readings. FITC labelled leptospires were suspended in MEM (Minimal Essential Medium, Sigma) at 10^8 bacteria/ml and added to BV/2 cells resuspended in RPMI 1640 medium antibiotic depleted, at 1/40 cell to bacteria ratios. The suspensions were incubated at 37°C under shaking for 90 and 120 min. At the end of incubation time 100 µl of the sample were added to 500 µl of cold PBS and read by flow cytometry. To measure leptospire adhesion, immediately before analysis 100 µl of Crystal Violet (working solution 0.02%) were added to an aliquot of sample to quench the extracellular fluorescent leptospires, as described (Cinco *et al.*, 2002). Results were expressed as Mean Fluorescence Intensity (MFI) or as % of positive cells referred to a negative control con-

stituted by untreated microglia cells. MFI from Crystal Violet quenched tubes was considered as adhesion, and was subtracted from MFI tubes without Crystal Violet, resulting as true phagocytosis. The possible inhibition of adhesion via the CR3 receptor was performed by adding to BV/2 as agonist Mab anti-CD11b or Mab isotype-matched IgG1 for control both 3 µg/ml and incubating 30 min at 4°C. Leptospires were added at ratio 1/40 cell/bacteria and incubated at 37°C for 1 h under shaking.

Phagocytosis was also evaluated on adherent BV-2 cells by fluorescence microscopy as described (Cinco *et al.,* 2002, Marangoni *et al.,* 2003) briefly: a 0.3 ml portion of BV-2 cell suspension (150.000 cells/ml) was added to 8-wells culture plate (LAB-TEK, Nalge-Nunc International, Naperville, Ill.) and incubated overnight in RPMI 1640. Spirochetes added ($5 \cdot l0^6$/well) were incubated at 37°C with BV-2 cells (final ratio 100 bacteria per macrophage) in RPMI 1640, for 30, 60 and 120 min. After incubation, BV-2 cells were washed in phosphate buffered saline (PBS, pH 7.4), fixed in methanol and incubated for 1 h with 1:100 diluted human anti-leptospires immune serum. Bacterial cells were visualized with anti-human fluorescein-conjugated antibody (Dako, Copenhagen, Denmark), diluted 1:30 in PBS. After washing in PBS, counterstaining was performed with Evan's Blue solution 1% (w/v) for 3 min. The slides were finally washed with water, air dried and coverslipped. The results were read under a Nikon Eclipse E600W UV light microscope (Nikon, Instech Co., Ltd., Japan) at a magnification of 400. Images were recorded with a Nikon Digital still Camera DXM1200 (Nikon, Instech Co., Ltd., Japan). A mean of 100 BV-2 cells were counted in each experiment. Experiments were performed in triplicate.

Evaluation of killing by microglial cells

Leptospires washed twice in PBS were added to BV-2 cells ($2x10^6$) at a ratio of 100/1 in a 1ml sample containing RPM1 antibiotic depleted medium. The mixtures were incubated at 37°C under shaking and at appropriate time intervals 100 µl of each sample were added to 900 µl of 0.25% saponin in ice to lyse the cells. After 15 min of treatment the number of surviving leptospires adherent to or released from the macrophages were detected by viable counting with the method of limiting dilution as already described (Blasi *et al.*, 1999). Briefly 20 microl of each saponin treated sample were placed on microtiter plates filled with 180 µl EMJH medium/well and thereafter diluted in a titration series (1/2, 1/4, 1/8). The plates were sealed with a sterile acetate film and incubated at 30°C for 5 days. Bacterial growth was evaluated by counting the number of leptospires grown in each well by a Thoma counting chamber under dark-field microscopy. The percentage of non viable leptospires was calculated by comparing the count samples containing BV/2 with those without the cells (100% growth). All killing tests were performed in triplicate.

Flow cytometry and statistics

Readings of fluorescence were performed with a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San JosÈ, Calif.) equipped with an air-cooled argon ion laser fitted at 488 nm and with a filter setting for FITC 530 nm (green fluorescence FL1) and PE 585-nm (red fluorescence FL2). 10.000 events were acquired in list mode and analysed with CellQuest software (Becton Dickinson). Single green fluorescence emission of FITC-labelled spirochetes bound to BV/2 cells was measured and expressed as a percentage of positive cells or Mean Fluorescence Intensity. The expression of CR3 antigen on the cells was quantified as red fluorescence signal. For each experiment, unstained cells or cells stained with isotype-matched monoclonal antibodies were used as negative controls to set threshold positivity. Data from repeated experiments were analyzed by the unpaired Student *t*-test, using GraphPad Prism (San Diego, CA).

RESULTS

Adhesion to and phagocytosis by BV-2 cells

After exposure of BV-2 cells to Spirochetes for 24 hours at 1/40 or 1/100 ratio, microglial cells remained viable (data not shown), indicating that the cells were not killed by the *Leptospira* strains employed in this study. FITC labelled leptospires were co-incubated with the BV/2 without opsonisation: figure 1 shows the FACS profile of the microglial cell fraction becoming flu-

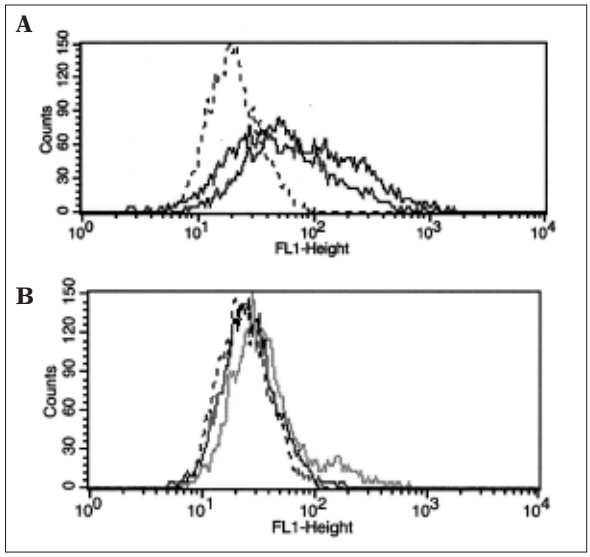
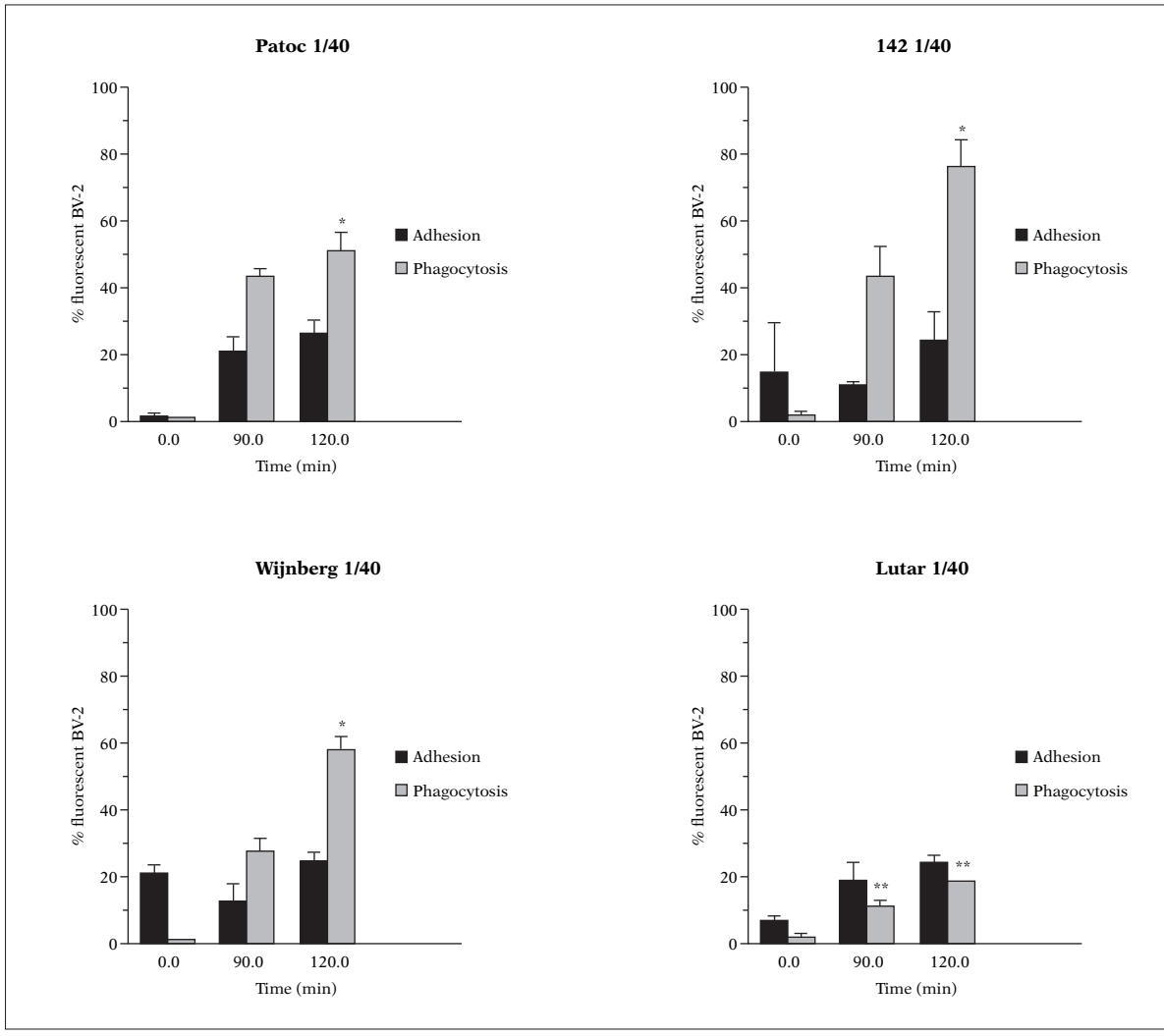


FIGURE 1 - *Flow cytometric analysis of of FITC labelled leptospire adhesion and phagocytosis, with BV-2 cells after 0* ***A*** *and 120 min.* ***B****: Dotted line indicates BV-2 baseline. Grey line phagocytosis plus adhesion and black line indicates phagocytosis only.*

FIGURE 2 - *Adhesion and phagocytosis of different strains of Leptospires to BV-2. Values are expressed as percentage of BV-2 cells becoming fluorescent after binding and uptake of FITC labelled leptospires. Bars express the means ± SD of three independent determinations. * = P ≤0.05 (difference among strains); **P = 0.0069 (difference between strain Lutar and other strains).*



orescent after the addition of fluorescent leptospires, at time 0 and 120 min. Clearly visible is the presence of fluorescent cells increasing with time; the black line in the graph indicates phagocytosis, the grey line refers to adhesion plus phagocytosis. This distinguishes between phagocytosis and adhesion. As reported in figure 2, each of three *Leptospira* strains coincubated with BV-2 (40/1 ratio) for 120 min were able to progressively associate with the microglial cells; adhesion was followed by phagocytosis which increased slowly with time. A significant difference in uptake among strains ($p < 0.05$) was observed: after 120 min about 50% of BV-2 internalised Patoc1 strain, 62% internalised strain Wijnberg, 80% strain 142 and only 18% engulfed strain Lutar. The differences in phagocytosis between the non-pathogenic Patoc1 and the virulent strain Lutar ($p = 0.0069$) were highly significant. Compared to the others this strain was both poorly adherent and scarcely internalised.

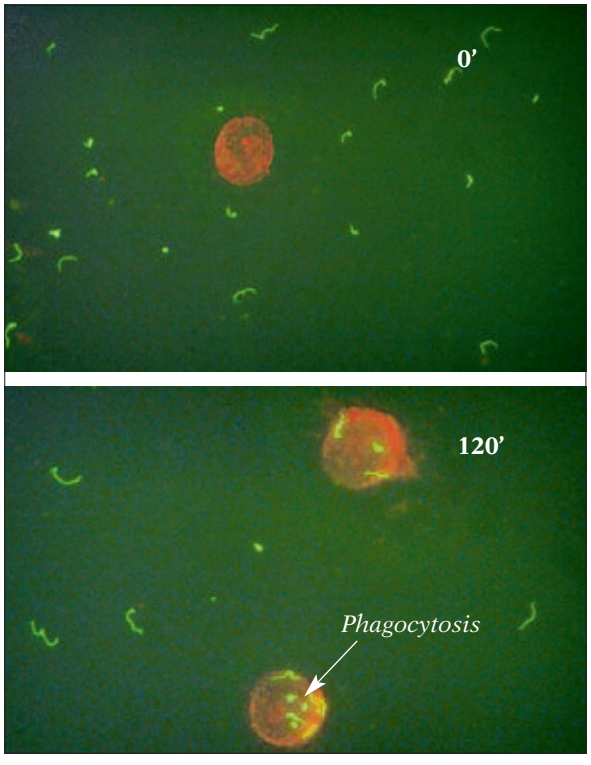


FIGURE 3 - *BV-2 cells with Leptospira strain Patoc at ratio 1/40 at time 0 and 120'. Spirochetes were stained with an immune serum antileptospire and a FITC anti-human IgG conjugate. Cells stained in red with Evans's blue.*

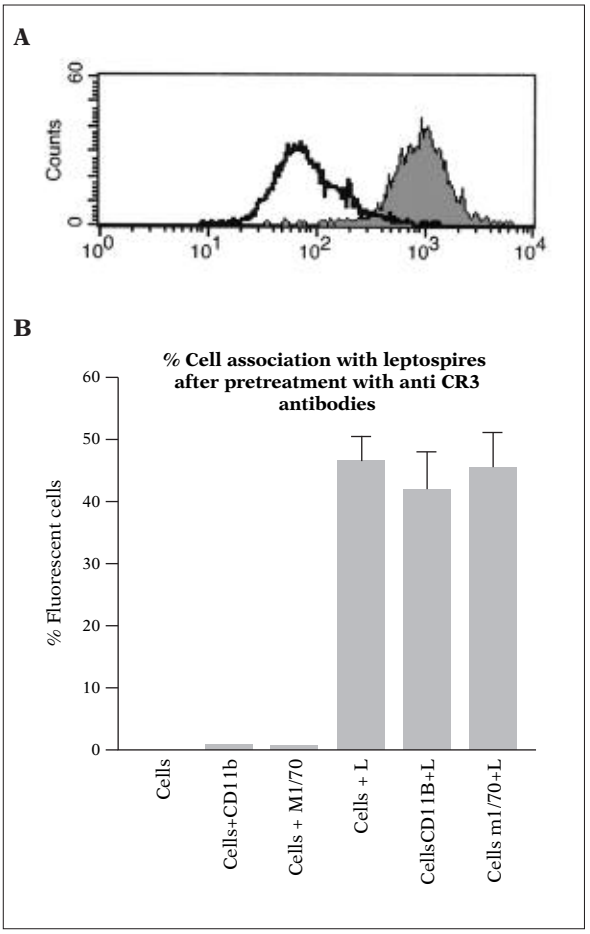


FIGURE 4 - ***A*** *Expression of CR3 receptor on BV-2. The second peak (arrow) is revealed by reaction with fluorescent antibody anti CD11b and indicates the presence of the receptor.* ***B*** *Adhesion of Leptospira strain 142 to BV-2 cells preincubated with monoclonal antibodies to CR3, after 1-hour co-incubation. Graphs express the means of ± SD of three independent determinations.*

Concerning adhesion, there were no significant differences among strains, suggesting the phenomenon is not strain specific.

Adhesion and phagocytosis events were confirmed in experiments performed with BV-2 grown on tissue cultures slides. As an example figure 3 shows the adhesion and uptake of strain Patoc 1: at time 0 min no spirochetes were associated with the cells, while after 30 min leptospires were associated with BV/2. Interestingly the micro-organisms retained their spiral morphology, meaning adhesion and fluorescent spheric bodies, which indicate phagocytosis (Marangoni *et al.*, 2003).

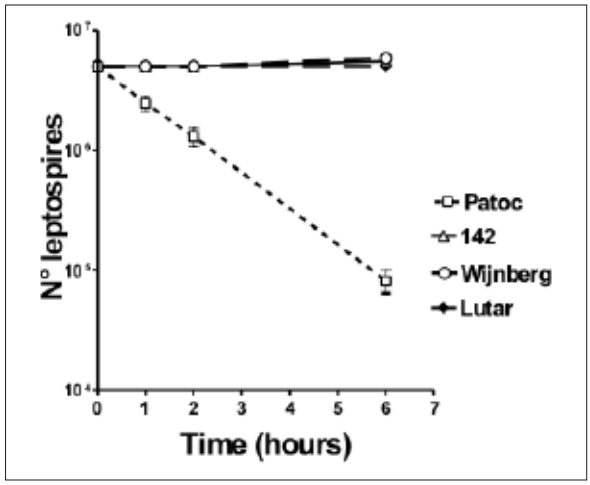


FIGURE 5 - *Time killing of Leptospira strains coincubated with BV-2 for 6 hours. Graphs express the means of ± SD of three independent determinations.*

As reported in Fig. 4, A BV-2 cells express the CR3 receptor; when co-incubated with leptospires in presence of anti-CD11b or monoclonal antibody M1/70 as agonists, no significant decrease of adhesion was observed, (Fig. 4, B). This finding indicates that in non opsonic conditions, the CR3 receptor is not involved in *Leptospira* adhesion to microglia cells.

Leptospira killing by BV-2 cells

In further experiments BV-2 cells were tested for killing leptospires; as reported in figure 4, only the non pathogenic strain Patoc1 was sensitive, only 20% of the bacteria remaining viable after two hours of contact. The pathogenic strains fully retained their viability throughout the six hours of co-incubation with cells.

DISCUSSION

The murine microglia used in this study were not affected by exposure to leptospires and were effective in binding and ingesting non-opsonized spirochetes. After prompt adhesion to BV-2 leptospires were taken up and phagocytosis proceeded quite slowly compared with phagocytosis observed with other types of monocytes. According to previous findings (Cinco *et al.,* 1994), 65% of peripheral blood mononuclear cells would internalise leptospires in 30 min; microglial cells might perhaps need more time to evolve from a resting to an activated macrophage state. Both pathogenic and non-pathogenic strains are uptaken at an equivalent rate. Only strain Lutar, a first passage strain belonging to serovar Sejroe, is scarcely internalized. At present we have no explanation for this behaviour: still being virulent it may retain the capacity of avoiding - or limiting- microglia uptake.

Adhesion to BV-2 cells occurred rapidly without significant differences between pathogenic and the non-pathogenic strain Patoc1, suggesting the involvement of a generalised non-strain specific mechanism/s of binding. Regarding the possible role of CR3 integrin as adhesion receptor, our data show that though microglia BV-2 expresses the CD11b/CD18 receptor, it does not participate in the binding of leptospires. This finding is quite unexpected as CR3 is involved in the adhesion of Leptospires and also *B. burgdorferi* to other monocytes and mammalian cells in non-opsonic conditions (Cinco *et al.,* 2002; Cinco *et al.*, 1997). Conversely we would argue that one possible adhesion to BV-2 mechanism is fibronectin mediated. In fact it has been proven (Cinco *et al.*, 2002, Merien *et al.,* 2000) that the binding of leptospires to mammalian cells arises in fibronectin primed cells, and there is increasing evidence of leptospira ligands to this protein (Merien *et al.,* 2000, Choy H, personal communication). Though internalised, the leptospires retained their viability throughout the observation; only the non-pathogenic strain Patoc1 is killed by the microglia. To kill the pathogenic leptospires might instead require a longer incubation time of contact. In the light of the results of this study we can infer that microglia do not rely on complement or Fc receptor mediated uptake of spirochetes. Moreover the murine microglia cells seem to actively remove pathogenic leptospires, but not to affect their viability as occurs with other neurotropic spirochetes such as *B. burgdorferi* which is killed within one hour of contact (Kuhlow *et al.,* 2005). Though the microglia cells act in the brain parenchyma which is relatively deficient in immunoglobulin and complement, it cannot be excluded that the clearance capacity of microglia towards leptospires is greater *in vivo*. Besides phagocytosis the microglia play a key role ampli-

fiyng the inflammatory response which is triggered by adhesion and phagocytosis of microorganisms: preliminary experiments in our hands show that this is due to co-incubation of leptospires or Leptospira extracts with BV-2 cells.

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